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The effect of *Scleranthus perennis* and *Hottonia palustris* extracts on honey bee lifespan and nosemosis development

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Abstract

Introduction: Nosemosis, which is caused by microsporidian parasites belonging to the genus *Vairimorpha* (formerly *Nosema*), is one of the main reasons for the decline in honey bee (*Apis mellifera*) colonies worldwide. Following the withdrawal of fumagillin from the European Union, there is an urgent need to identify effective, natural management of this disease. This study investigated the effects of *Scleranthus perennis* L. (Caryophyllaceae) and *Hottonia palustris* L. (Primulaceae) extracts on honey bee survival, *Vairimorpha* spp. infection development, and gut microbiota composition. **Material and Methods:** Two independent cage experiments were conducted using newly emerged bees kept uninfected and provided with either the water (group suffix 3) or the butanol (group suffix 6) fraction of *Scleranthus perennis* (SP) and counterparts provided analogously with *Hottonia palustris* (HP), and newly emerged bees infected with *Vairimorpha* (*Nosema*) spp. and subsequently provided with either the water or the butanol fractions of the respective plant extracts. Each cohort had 240 bees and was investigated over 22 d. Bacterial community composition was assessed using 16S rRNA gene metabarcoding (Illumina MiSeq). **Results:** Compared to untreated infected controls, both *S. perennis* and *H. palustris* extracts significantly improved the survival of infected honey bees, and the HP6 additionally enhanced survival in uninfected controls. A significant reduction in *Vairimorpha* (*Nosema*) spp. spore loads was observed in all supplemented groups. In particular, supplementation with *H. palustris* extracts reduced nosemosis development by ≈22-fold (HP3) and ≈55-fold (HP6), while *S. perennis* extracts reduced it by ≈56-fold (SP3) and ≈4.6-fold (SP6) compared to infected untreated controls. Metabarcoding showed that *Vairimorpha* (*Nosema*) infection was the primary factor shaping the bee microbiota, with infected bees clearly differing from uninfected controls in α- and β-diversity, whereas plant-extract supplementation had no consistent

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effect on the overall microbiota in either healthy or infected bees. **Conclusion:** Overall, *S. perennis* and *H. palustris* extracts (notably HP6) appear to be promising natural agents for supporting honey bee health and mitigating nosemosis, as they were associated with reduced spore loads. The aqueous fractions (SP3 and HP3) showed no evidence of a community-wide alteration of the gut microbiota, while the effect of the more bioactive butanol fractions on gut microbiota composition remains to be directly established.

Keywords: *Apis mellifera*, dysbiosis, nosemosis, *Hottonia palustris*, *Scleranthus perennis*.

Introduction

The honey bee (*A. mellifera*) is one of the most important pollinators globally. In recent years, losses in overwintering colonies have been observed, with negative effects on the economy and the environment. The decline in the bee population worldwide is attributed to various diseases affecting these insects, such as nosemosis, varroosis, and American foulbrood. This situation is further exacerbated by nutritional stress, environmental pollution caused by pesticides used in agriculture, and climate change (4, 17). One of the main reasons for the decline in bee colonies is nosemosis, a disease caused by microsporidia of the genus *Vairimorpha* (*Nosema*). Infection occurs *via* the faecal–oral route when bees consume food or faeces containing *Vairimorpha* (*Nosema*) spores. Furthermore, bees can become infected with *Vairimorpha* (*Nosema*) *ceranae* spores when cleaning combs that become contaminated within the hive (37, 47). The infection damages the epithelial cells lining the bee's midgut, leading to necrosis of the intestinal tissue and impairing the bee's ability to digest food and absorb nutrients (16, 46, 49). One of the most widely used substances for treating nosemosis is the antibiotic fumagillin, which is derived from the fungus *Aspergillus fumigatus*. Fumagillin inhibits the enzyme methionine aminopeptidase (7, 61). However, because of fumagillin's penetration into bee products, posing a risk to consumers, its use has been banned in the EU (14).

Because the risk exists of synthetic substances contaminating bee products, researchers are investigating natural compounds that are effective against nosemosis. Numerous plant-derived natural products have been tested in recent years, including extracts from more than 20 botanical species (3, 5, 7, 12, 18, 30, 32, 38, 41, 45, 46, 48, 50). Notably, amongst the tested species, only two members of the Asteraceae family, *Artemisia dubia* (Wall.) and *Aster scaber* Thunb. demonstrated anti-nosemosis activity at concentrations that were non-toxic to honey bees (7, 18). In a previous study, an extract of *Eleutherococcus senticosus* also showed very promising results, prolonging honey bee lifespan and effectively combatting nosemosis (51).

Several other studies have shown that essential oils derived from mint (*Mentha piperita* L.), lemon balm (*Melissa officinalis* L.), coriander (*Coriandrum sativum* L.) and summer savory (*Satureja hortensis* L.), as well as various phytochemicals including caffeine, clove oil, gallic acid, kaempferol, p-coumaric acid, and propolis, can increase honey bee longevity while reducing levels of *Vairimorpha* (*Nosema*) infection to varying degrees (3, 5, 7, 12, 18, 30, 38, 41, 48). Certain phytochemicals, such as clove oil, laurel extract and sulforaphane, also exhibited moderate efficacy in reducing *Vairimorpha (Nosema)* infection. However, these compounds were also associated with a significant reduction in honey bee lifespan (32, 38, 45). Several other phytochemicals tested, including amygdalin, anabasine, aucubin, catalpol, nicotine and thymol, had no observable effect on *Vairimorpha* (*Nosema*) infection (32, 38, 45). Overall, despite the growing body of work on plant-derived compounds against nosemosis, only a limited subset shows clear efficacy without adverse effects on honey bee survival, and potential impacts on the gut microbiota remain insufficiently characterised.

There is evidence suggesting that essential oils and other plant natural products can affect the gut microbiota of honey bees, primarily through their antimicrobial properties and potential toxicity at certain dosages, although reports are limited and effects appear variable, with core gut taxa often remaining largely unchanged (15). Adult honey bees harbour a specialised gut microbiota of relatively low complexity, comprising fewer than ten core species, including *Lactobacillus*, *Bifidobacterium*, *Snodgrassella*, *Gilliamella* and *Neisseria* (1, 21, 23, 24, 29, 31, 33). These gut symbionts confer numerous beneficial effects: they facilitate nutrient digestion and absorption, provide protection against pathogenic microorganisms such as *Paenibacillus larvae* and *Vairimorpha* (*Nosema*) *ceranae*, and exert immunomodulatory functions that strengthen colony health and extend individual lifespan (40). Although the core microbiota composition remains broadly consistent across geographic and genetic backgrounds, it is sensitive to dietary changes, malnutrition, infection, pesticide exposure and anthropogenic land-use alterations (28, 50, 58). Diet appears to be a major determinant of bacterial community structure, with forager bees maintaining a contingent

microbiota shaped largely by the floral resources they exploit (28, 53). Disruption of adequate forage availability, particularly in anthropogenic landscapes dominated by monocultures or urban environments, may therefore compromise microbiome integrity and, consequently, disease resistance in honey bee colonies (11, 19).

Two rare plant species, *Scleranthus perennis* L. (Caryophyllaceae) and *Hottonia palustris* L. (Primulaceae), were selected for this study because both are rich in flavonoids as the main bioactive secondary metabolites. These compounds have known antimicrobial and antiparasitic potential, but neither has been studied for honey bee health, making them promising but understudied candidates as natural remedies for *Vairimorpha* (*Nosema*) infection. *Scleranthus perennis* is a perennial herb found in Western Asia, Europe and North Africa. A phytochemical analysis of this plant isolated flavone glycosides (27). The second species, *H. palustris*, is a perennial aquatic plant mainly found in oxbow lakes in temperate regions. Three flavone derivatives have been isolated from its aerial parts (56). The rich phytochemical composition of these two plants suggested value in investigation of whether extracts of them could reduce nosemosis while maintaining host fitness and gut microbiome stability. This study evaluated the effects of their extract fractions on honey bee survival, *Vairimorpha* (*Nosema*) infection and gut microbiota composition.

Material and Methods

Plant material and extraction. The collection and preparation of plant materials for this research were described in detail in earlier publications. A detailed procedure for preparing individual extracts was presented in those articles, and was the one adopted for this study (27, 56). The *Scleranthus perennis* water extract was designated SP3 and the butanol extract SP6; the *Hottonia palustris* extracts were analogously designated HP3 and HP6.

Identification by LC-MS of secondary metabolites in SP3, SP6, HP3 and HP6 extracts. First, chromatographic separation was optimised, and analytical methods for qualitative and quantitative determinations were validated. Also, the phytochemical composition of selected SP3, SP6, HP3, and HP6 plant extracts was assessed using spectrophotometric and specialised LC-MS methods. The results were reported previously (27, 56).

Experimental honey bees. Healthy honey bees (*Apis mellifera*) were obtained in June and July 2022 from an apiary located in the Lubelskie voivodeship in Poland. The brood frames were placed in an incubator at 34.5°C and 85% relative humidity until emergence. One-day-old bees were placed in special wooden cages with dimensions of 12 × 12 × 4 cm (Fig. 1). The front wall of the cage was made of glass, and the cage contained an air vent and an opening for feeding *via* syringe. In each cage 40 honey bees were placed. The bees were kept under these standardised cage conditions from the day of emergence onward, with no access to field-collected nectar or pollen; therefore, no seasonal differences in forage availability between June and July could influence bee physiology in this study.

Experimental design for survival and nosemosis assays in *A. mellifera*. Experimental groups for the study of the bees' lifespan were as follows: uninfected bees given *S. perennis* were SP3 (120 mg/120 mL sugar syrup) and SP6 (120 mg/120 mL sugar syrup); uninfected bees given *H. palustris* were HP3 (120 mg/120 mL sugar syrup) and HP6 (120 mg/120 mL sugar syrup). The control group was a single group for comparison with both the *S. perennis*- and *H. palustris*-supplemented groups, and it received sugar syrup only. Each group comprised 240 honey bees. This concentration was selected based on earlier preliminary experiments with both plant extracts, in which they improved honey bee survival without signs of phytotoxicity (26, 57).

Two independent experiments were conducted: the first on honey bees that emerged in June, and the second on those that emerged in July. Both trials employed an identical panel of extracts/fractions (SP3, SP6, HP3, and HP6) and control groups, and both followed a standardised *in vitro* cage protocol (16, 60); therefore, they represent two independent biological replicates of the same experimental design rather than separate experiments testing different treatments, which is why their results were treated as repetitions in the statistical analysis. In both experiments, dead bees were removed, and food was replenished every two days. On the third day of each experiment, prior to the start of the extract treatment, a half of the bees in the SP3 and SP6 groups, as well as a half of the HP3 and HP6 groups, were infected with *Vairimorpha* (*Nosema*) spp., becoming groups InSP3, InSP6, InHP3 and InHP6. The bees which were not infected were controls and formed groups CSP3, CSP6, CHP3 and CHP6. In accordance with standard methods for *Vairimorpha* (*Nosema*) research (16), bees were group-fed an infectious suspension containing approximately 30,000 spores per bee *via* sugar syrup, and infection success was subsequently confirmed by microscopic spore counting using a haemocytometer.

On day 6, the bees were given the extracts at specific concentrations. Until day 15, the bees were sustained with the extracts. From day 15 onwards, the bees were fed a sugar-syrup solution until the end of the experiment. Two control groups were included in both experiments: a negative control consisting of infected bees fed solely with sugar syrup and a positive control consisting of healthy (uninfected) bees administered extracts following the same schedule as the experimental groups. An additional baseline control group comprised healthy bees that were fed only 1 : 1 (w/v) sugar syrup. Dead bees were counted and removed every other day until the end of both experiments. Survival was recorded daily in each cage. For analysis, counts of surviving bees were pooled across the six cages in each group (total n = 240 bees per group) to generate a single group-level survival curve (survival percentage over time).

The experimental layout for assessing *Vairimorpha* (*Nosema*) infection intensity was identical to that used for the survival assay (six cages per group, 40 bees per cage for a total 240 bees per group); however, the level of *Vairimorpha* (*Nosema*) infection was quantified at a single time point (day 22) and analysed at the cage level, using cage means derived from all 40 individually measured bees per cage (n = 6 cages per group). *Vairimorpha* (*Nosema*) spp. spore counts were determined according to the methodology given in earlier publications (9, 16, 22). Day 22 was selected as the assessment point based on preliminary experiments with these extracts, which indicated that inter-group differences in spore load increased progressively over the course of infection and were most pronounced by the end of the precedential experimental periods (26, 57).

Statistical analysis of survival and spore loads. The number of cages per treatment group (six) was deliberately chosen to limit the total number of honey bees used in the experiment, in line with the principle of using the minimum number of animals necessary to obtain meaningful results. Survival data were pooled and analysed at the level of the whole experimental group (240 bees per group) rather than modelling cage as a random effect, as this group-level approach provided a stable and interpretable estimate of the overall treatment effect without requiring a larger number of replicate cages.

Survival curves were analysed using Kaplan–Meier estimates and compared with the log-rank test using the survival package in R. Spore counts were log10-transformed prior to analysis. For analysis of spore loads, groups were compared against infected untreated controls within each experiment, using Welch's *t*-test with Holm correction using the base R (stats). A two-sided P-value < 0.05 was considered significant.

Experimental design for metabarcoding-based analysis of bee microbiota. For metabarcoding of the bee gut microbiota, specimens were selected from both healthy and infected groups, with experimental groups as follows: healthy, unsupplemented bees; healthy bees supplemented with *S. perennis* extract; healthy bees supplemented with *H. palustris* extract; infected, unsupplemented bees; infected bees supplemented with *S. perennis* extract; and infected bees supplemented with *H. palustris* extract. Because microbiome profiling through next-generation sequencing is highly resource intensive, metabarcoding analyses were limited to the water fractions (SP3 and HP3), which showed beneficial outcomes in the infection and survival assays. A total of 47 honey bee specimens were profiled, 4 specimens per group from the June experiment and 4 specimens per group from July experiment, except for the positive-control group, from which 1 June specimen was lost and only 3 were analysed.

DNA extraction. Metagenomic DNA was extracted from whole bees without gut dissection. Prior to DNA extraction, whole bees were surface-sterilised by immersion in 70–97% ethanol and rinsed in sterile water to remove surface-associated and environmental DNA. Metagenomic DNA was then extracted using the PureLink Microbiome DNA Purification Kit (Thermo Fisher Scientific, Bleiswijk, the Netherlands) according to the manufacturer's instructions for stool samples, with a minor modification involving the addition of a preliminary homogenisation step. For this purpose, one sterile stainless-steel bead with a diameter of 5 mm (Qiagen, Hilden, Germany) and one thawed insect were placed into a 2 mL microcentrifuge tube using sterile tweezers, and 700 µL of S1 lysis buffer was added. The mixture was homogenised three times for 30 s at 30 Hz using a TissueLyser II homogeniser (Qiagen), after which the steel bead was removed using a magnet. Subsequently, the homogenised samples were thoroughly mixed with 100 µL of S1 lysis enhancer solution, and the glass beads provided with the kit were added. The samples were incubated at 65°C for 10 min, and DNA isolation was continued according to the manufacturer's instructions. The concentration of the isolated DNA was measured using a Qubit 2 Fluorometer (Life Technologies Holdings, Singapore).

DNA sequencing. Metabarcoding sequencing was performed by Genomed S.A. (Warsaw, Poland). Libraries were prepared by amplifying the hypervariable V3–V4 region of the 16S rRNA gene using primers 341F and 785R and Q5 Hot Start High-Fidelity Master Mix, following the

manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Amplicon libraries were sequenced on an Illumina MiSeq platform (San Diego, CA, USA) using paired-end technology (2 × 300 nucleotides) with a MiSeq Reagent Kit v. 3. Initial data processing using MiSeq Reporter v. 2.6 included automatic sample demultiplexing and the generation of fastq files. Adapter sequences and low-quality reads were removed using Cutadapt software (34). Amplicon sequence variants were inferred using the DADA2 pipeline (8). Taxonomic assignment was performed in QIIME2 using the SILVA v. 138 reference database (6). Sequencing data were deposited in the NCBI Sequence Read Archive under the accession No. PRJNA1416302.

Exploratory analyses of metabarcoding data. Sequencing depth was assessed by constructing rarefaction curves, and reads were rarefied to an even depth using the phyloseq package in R (35). The α-diversity indices (Sobs, Shannon and evenness) were calculated using the vegan package in R (39). Differences in α-diversity metrics between the experimental groups were evaluated using ANOVA implemented in PAST v. 3.08 (20). The same software was used to perform an analysis of similarity (ANOSIM) to assess the statistical significance of differences in microbiota composition between the sample groups at the genus level. Non-metric multidimensional scaling (NMDS) based on the Bray–Curtis distance matrix was applied to visualise between-group differences in microbiota composition (β-diversity) using the vegan package in R. The same package was used to test differences in bacterial community composition between the experimental groups at the genus level using non-parametric multivariate analysis of variance (PERMANOVA). In addition, DESeq2 implemented within phyloseq in R was used to conduct differential abundance analysis between groups based on the raw count data (35, 36).



Fig. 1. Experimental honey bee cage

Results

Analysis of biologically active ingredients in selected extracts (HP3, HP6, SP3 and SP6). The aqueous HP3 matrix was highly polar and had a relatively low overall phenolic load, with 12.44 ± 0.20 mg pyrogallol equivalents per gram of extract (Peq/g extract). Conversely, the butanol HP6 matrix was a more concentrated intermediate-polarity preparation (64.52 ± 1.87 mg Peq/g extract) that was enriched in glycosylated flavonoids, including apigenin 7-O-(2''-O-β-glucuronide)-β-glucopyranoside and related flavone and flavonol derivatives. The differentiation of the extracts of *S. perennis* was also observed to be polarity-dependent. The aqueous type, SP3, was a broad hydrophilic preparation with a moderate total phenolic content (99.20 ± 0.40 mg Peq/g extract). On the other hand, the butanol fraction, SP6, exhibited a more selective flavonoid profile and a higher phenolic load (161.32 ± 2.91 mg Peq/g extract). This profile included diagnostically relevant C-glycosylflavones, such as lucenin-1, vicenin-2

and chrysoeriol-6-C-β-D-glucopyranoside-8-C-α-arabinopyranoside.

Honey bee survival. Across the two independent cage trials, honey bee survival differed between treatments and infection status (Figs 2 and 3). Supplementation of uninfected bees with SP3 or SP6 extract fractions (Fig. 2A) did not significantly alter survival compared with the sugar-syrup control (log-rank: control *vs* CSP3, P-value > 0.05; control *vs* CSP6, P-value > 0.05). In contrast, in *Vairimorpha* (*Nosema*)-infected bees (Fig. 2B), the SP3 and the SP6 fractions both significantly improved survival relative to the infected untreated control (log-rank: infected *vs* InSP3, P-value < 0.001; infected *vs* InSP6, P-value < 0.001), indicating a protective effect of the supplementation with *S. perennis* extracts on bees with infection. Survival data by group are presented in Tables 1, 2, 3, and 4.

Supplementation with HP3 did not affect survival in uninfected bees (control *vs* CHP3, P-value > 0.05), whereas supplementation with HP6 resulted in a modest but significant increase in survival (control *vs* CHP6, P-value = 0.032). In infected bees (Fig. 3B), both fractions improved survival compared with the infected untreated control (log-rank: infected control *vs* InHP3, P-value < 0.01; infected control *vs* InHP6, P-value < 0.001).

End-point survival at day 22 was consistent with the Kaplan–Meier curves and log-rank results, showing the same group-level survival patterns. By day 22, survival in uninfected SP groups was 30.8% in control bees (the group neither supplemented with SP nor with HP), 35.8% in CSP3, and 32.5% in CSP6, whereas in the infected control group it had declined to 16.7% (in the group neither supplemented with SP nor with HP) compared with 31.3% in InSP3 and 27.9% in InSP6 (Fig. 2). A more pronounced effect was observed for the supplementation with *H. palustris* extract fractions: in uninfected groups survival reached 47.1% in CHP6 *vs* 30.8% in the unsupplemented control group and 32.5% in CHP3. In infected groups, survival was 26.3% in InHP3 and 31.3% in InHP6 compared with 16.7% in the infected unsupplemented control group (Fig. 3). Overall, supplementation with the HP6 fraction yielded the most consistent survival benefit across infection conditions, improving it in both uninfected and infected groups.

***Vairimorpha* (*Nosema*) spp. infection development.** All tested extract fractions significantly reduced spore loads compared with the infected untreated control (Fig. 4). For *S. perennis* extract supplementation, mean ±SD spore loads across cages decreased from (1.13 ± 0.08) $\times 10^6$ spores/bee in the infected unsupplemented controls to (2.00 ± 0.18) $\times 10^4$ in InSP3 and (2.45 ± 0.22) $\times 10^5$ in InSP6 groups, corresponding to approximately 56-fold and 4.6-fold reductions, respectively (Fig. 4A). Compared with the control group, both fractions reduced spore loads significantly (adjusted P-value < 0.0001 for both InSP3 and InSP6 groups).

For *H. palustris*, supplementation also markedly lowered infection intensity: mean ±SD spore loads decreased from (8.76 ± 0.96) × 10^7 spores/bee in the infected control to (4.00 ± 0.18) × 10^6 in InHP3 and (1.60 ± 0.06) × 10^6 in InHP6 groups (approximately 22-fold and 55-fold reductions) (Fig. 4B). These differences were significant in comparisons with the control group (adjusted P-value **<** 0.0001 for both InHP3 and InHP6 groups).

Although the absolute spore loads in the infected control groups differed between the two trials (1.13 × 10^6 *vs* 8.76 × 10^7 spores/bee for the *S. perennis* and *H. palustris* trials, respectively), reflecting natural batch-to-batch variability in colony-derived inoculum, the relative reduction in spore load induced by each extract fraction, calculated against its own trial-matched infected control, was consistently strong and statistically significant across both trials, indicating that this measure of efficacy is robust to differences in baseline infection intensity between trials.

Metabarcoding of bee microbiota. The number of obtained high-quality reads per sample ranged from 59.628 to 121.225 (median 80.414). The rarefaction curves for all samples converged to a horizontal asymptote (Supplementary Fig. S1), indicating that the sequencing depth was sufficient to capture a substantial proportion of bacterial diversity.

Analysis of α-diversity indices (Sobs, Shannon and evenness) using ANOVA revealed statistically significant differences between non-infected control bees and infected bees across all supplementation variants (Table 5). Differences in bacterial community composition between the control and infected groups were further supported by ANOSIM (R = 0.458, P-value < 0.001), confirming that infection status was the essential factor influencing microbial diversity.

In contrast, no statistically significant differences in α-diversity were observed between sample groups differing in plant extract supplementation, either among healthy or infected bees, suggesting that supplementation did not affect microbial diversity within the sample. Ordination by NMDS showed a clear separation between uninfected and infected bees across supplementation variants, indicating infection-associated shifts in community composition. In contrast, no distinct clustering was observed among groups differing in supplementation within either infection status

(Fig. 5). The absence of differences in bacterial community composition between plant extract supplemented groups and non-supplemented control groups in both healthy and infected bees was also confirmed by the PERMANOVA test (Supplementary Table S1).

Differential abundance analysis performed using DESeq2 confirmed infection-associated shifts in the bee microbiota across all experimental groups (Fig. 6). Comparisons between non-infected and infected bees identified several genera with significantly altered abundances after false-discovery-rate correction (adjusted P-value < 0.05). For example, *Pantoea*, *Orbus*, *Lonsdalea*, *Serratia*, and *Snodgrassella* exhibited significantly lower abundance in infected bees, whereas *Raoultella*, *Morganella*, *Lachnoclostridium*, *Sedimentibacter*, *Ruminococcaceae incertae sedis*, and the *Eubacterium fissicatena* group were consistently enriched following infection. In addition, members of the family Enterobacteriaceae, including *Escherichia-Shigella*, *Klebsiella* and *Enterobacter*, were frequently overrepresented in infected bees.

In contrast, comparisons between plant extract–supplemented and unsupplemented groups within the same infection status showed no significantly differentially abundant taxa, suggesting that supplementation had a minor effect on bacterial abundance. The detailed results of pairwise comparisons between infected and uninfected bees are presented in Supplementary Tables S2–S4.

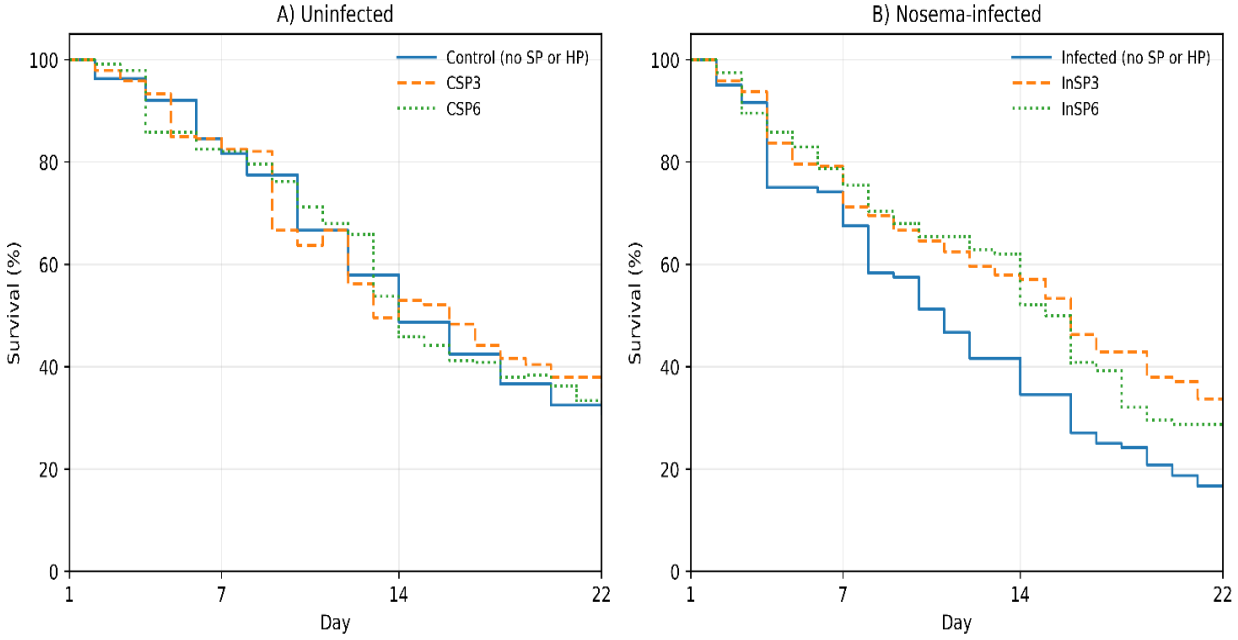


Fig. 2. Twenty-one day survival of honey bees (*Apis mellifera*), some supplemented with *Scleranthus perennis* (SP) extracts and others not supplemented. A) Uninfected groups: Control (no SP or HP) – bees given only sugar syrup; CSP3 – bees given SP3 water fraction in sugar syrup; CSP6 – bees given SP6 butanol fraction in sugar syrup. B) Infected groups: Infected (no SP or HP) – control bees given sugar syrup; InSP3 – bees given SP3 water fraction in sugar syrup; InSP6 – bees given SP6 butanol fraction in sugar syrup. Total n = 240

Table 1. Survival of uninfected *Apis mellifera*, some supplemented with *Scleranthus perennis* (SP) extracts and others not supplemented

Group	Survivors on day 1	Survivors on day 7	Survivors on day 14	Survivors on day 22
Control (no SP or HP)	240	196	117	74
CSP3	240	198	127	86
CSP6	240	197	110	78

Control – bees given only sugar syrup; HP – *Hottonia palustris*; CSP3 – bees given SP3 water fraction in sugar syrup; CSP6 – bees given SP6 butanol fraction in sugar syrup

Table 2. Survival of *Vairimorpha*-infected *Apis mellifera*, some supplemented with *Scleranthus perennis* (SP) extract and others not supplemented

Group	Survivors on day 1	Survivors on day 7	Survivors on day 14	Survivors on day 22
Infected (no SP or HP)	240	162	83	40
InSP3	240	171	137	75
InSP6	240	181	125	67

Infected – control bees given only sugar syrup; HP – *Hottonia palustris*; InSP3 – bees given water-fraction extract SP3 in sugar syrup; InSP6 – bees given butanol-fraction extract SP6 in sugar syrup

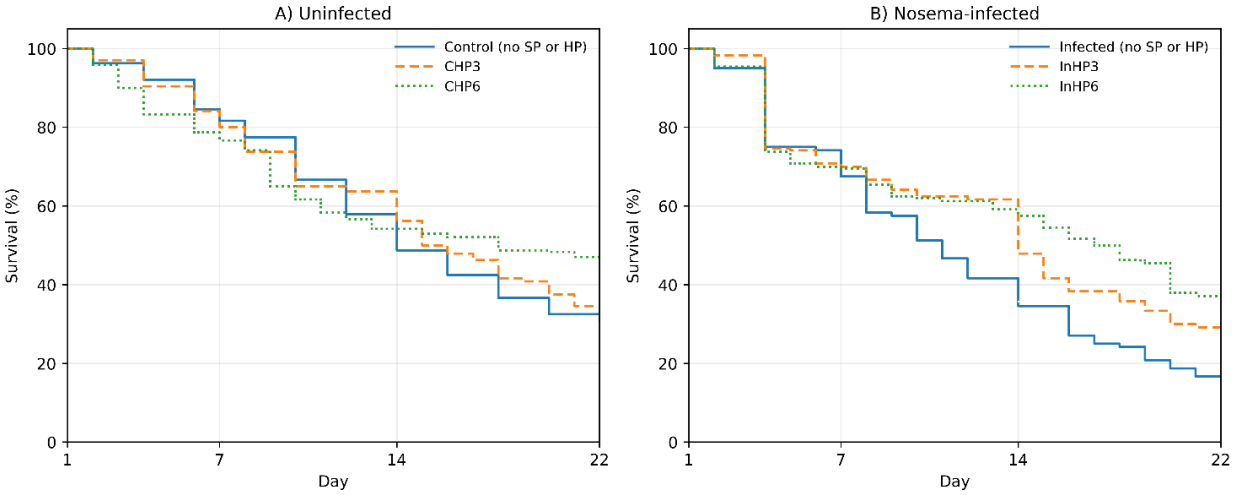


Fig. 3. Twenty-one day survival data for honey bees (*Apis mellifera*), some supplemented with *Hottonia palustris* (HP) extracts and others not supplemented. A) Uninfected groups: Control (no SP or HP) – bees given only sugar syrup; CHP3 – bees given HP3 water fraction in sugar syrup; CHP6 – bees given HP6 butanol fraction in sugar syrup. B) Infected groups: Infected (no SP or HP) – control bees given sugar syrup; InHP3 – bees given HP3 water fraction in sugar syrup; InHP6 – bees given HP6 butanol fraction in sugar syrup. Total n = 240

Table 3. Survival of uninfected *Apis mellifera*, some supplemented with *Hottonia palustris* (HP) extract and others not supplemented

Group	Survivors on day 1	Survivors on day 7	Survivors on day 14	Survivors on day 22
Control (no SP or HP)	240	196	117	74
CHP3	240	192	135	78
CHP6	240	184	130	113

Control – bees given only sugar syrup; SP – *Scleranthus perennis*; CHP3 – bees given water-fraction extract HP3 in sugar syrup; CHP6 – bees given butanol-fraction extract HP6 in sugar syrup

Table 4. Survival of *Vairimorpha*-infected *Apis mellifera*, some supplemented with *Hottonia palustris* (HP) extract and others not supplemented

Group	Survivors on day 1	Survivors on day 7	Survivors on day 14	Survivors on day 22
Infected (no SP or HP)	240	162	83	40
InHP3	240	168	115	63
InHP6	240	167	138	75

Infected – control bees given only sugar syrup; SP – *Scleranthus perennis*; InHP3 – bees given water-fraction extract HP3 in sugar syrup; InHP6 – bees given butanol-fraction extract HP6 in sugar syrup

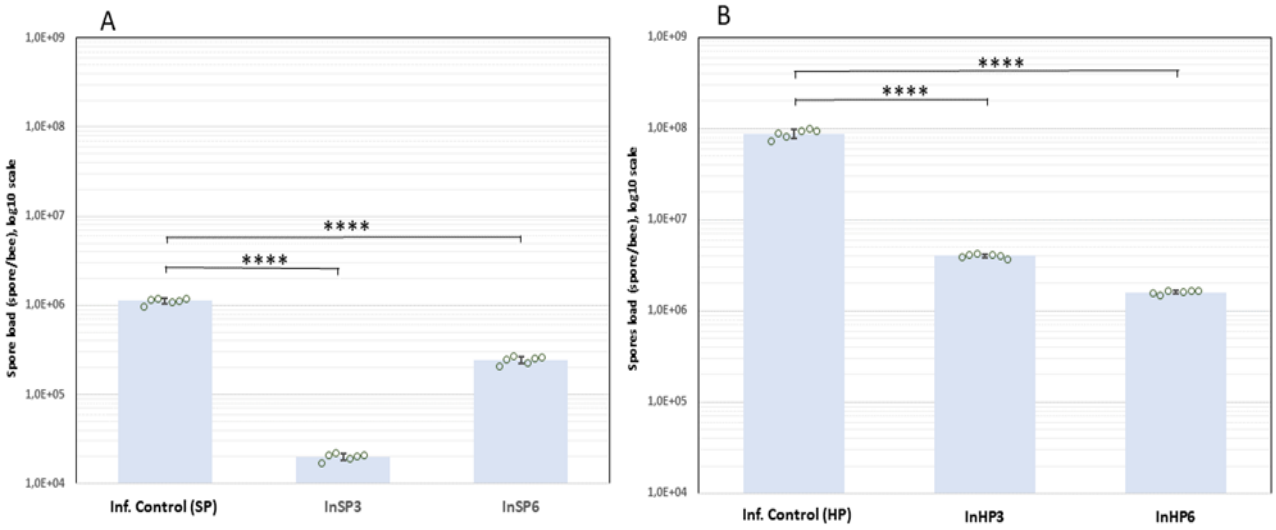


Fig. 4. Intensity of *Vairimorpha* (*Nosema*) spp. infection in infected honey bees, some supplemented with *Scleranthus perennis* (SP) and *Hottonia palustris* (HP) extracts and others not supplemented. Spores/bee were quantified on day 22. Each bar represents the mean ±SD across cages. A) Infected – untreated infected control bees given only sugar syrup (*S. perennis* trial); InSP3 – infected bees supplemented with water extract; InSP6 – infected bees supplemented with butanol extract. B) Infected – untreated infected control bees given only sugar syrup (*H. palustris* trial); InHP3 – infected bees supplemented with water extract; InHP6 – infected bees supplemented with butanol extract. The infected control groups shown in panels A and B were independent controls corresponding to the *S. perennis* and *H. palustris* experiments, respectively. Statistical comparisons were performed on log10-transformed cage means using Welch's *t*-test with Holm correction. Whiskers represent SD; **** indicates an adjusted P-value < 0.0001; individual points represent cage-level means

Table 5. Alpha diversity metrics of the microbiota of uninfected and infected bees

	Variant	Mean	SD	Variant	Mean	SD	P-value
Sobs	C	18[a]	±4	Inf	33[a]	±2	<0.001
Sobs	CSP	17[a]	±2	InfSP	31[a]	±5	<0.001
Sobs	CHP	20[a]	±2	InfHP	29[a]	±4	<0.001
Shannon	C	1.73[a]	±0.18	Inf	2.34[a]	±0.19	<0.001
Shannon	CSP	1.71[a]	±0.17	InfSP	2.31[a]	±0.24	<0.001
Shannon	CHP	1.90[a]	±0.15	InfHP	2.18[a]	±0.22	<0.05
Evenness	C	0.61[a]	±0.07	Inf	0.67[a]	±0.05	>0.05
Evenness	CSP	0.60[a]	±0.04	InfSP	0.67[a]	±0.04	>0.05
Evenness	CHP	0.64[a]	±0.04	InfHP	0.65[a]	±0.05	<0.05

C – uninfected bees; CSP – uninfected bees supplemented with *S. perennis* extract; CHP – uninfected bees supplemented with *H. palustris* extract; Inf – untreated infected bees given only sugar syrup; InfSP – infected bees supplemented with *S. perennis* extract; InfHP – infected bees supplemented with *H. palustris* extract. Values with the same letters indicate no significant differences (P-value > 0.05) between the means of variants within the same metric. The P-value depicts differences between the means of variants (uninfected *vs* infected

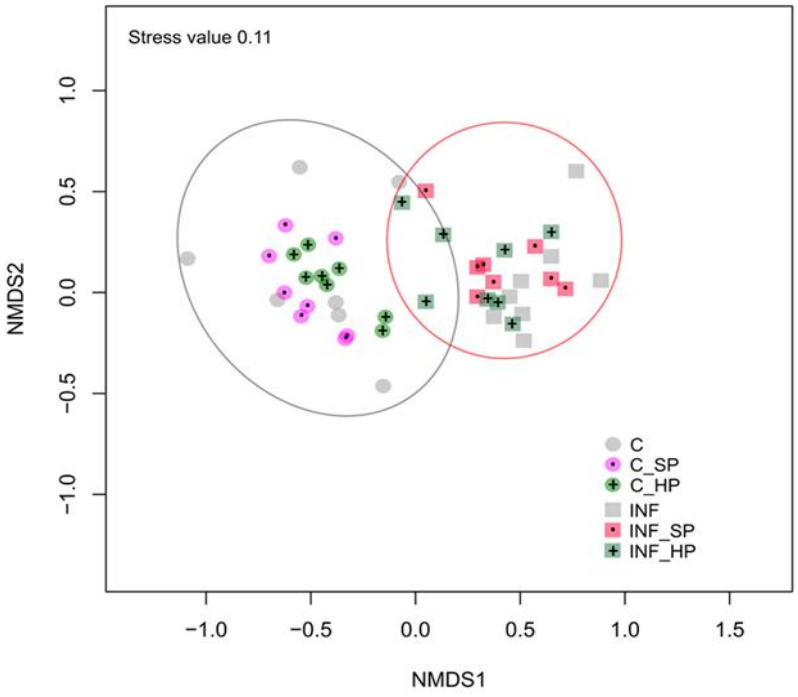


Fig. 5. Non-metric multidimensional scaling (NMDS) ordination of whole-bee-associated bacterial communities based on Bray–Curtis dissimilarities at the genus level. Each point represents an individual bee sample. C – uninfected non-supplemented control; C_SP – uninfected bees supplemented with the aqueous fraction SP3 of *Scleranthus perennis*; C_HP – uninfected bees supplemented with the aqueous fraction HP3 of *Hottonia palustris*; INF – infected non-supplemented control; INF_SP – infected bees supplemented with SP3; INF_HP – infected bees supplemented with HP3. Circles denote uninfected groups and squares denote infected groups; inner symbols distinguish the supplementation variants

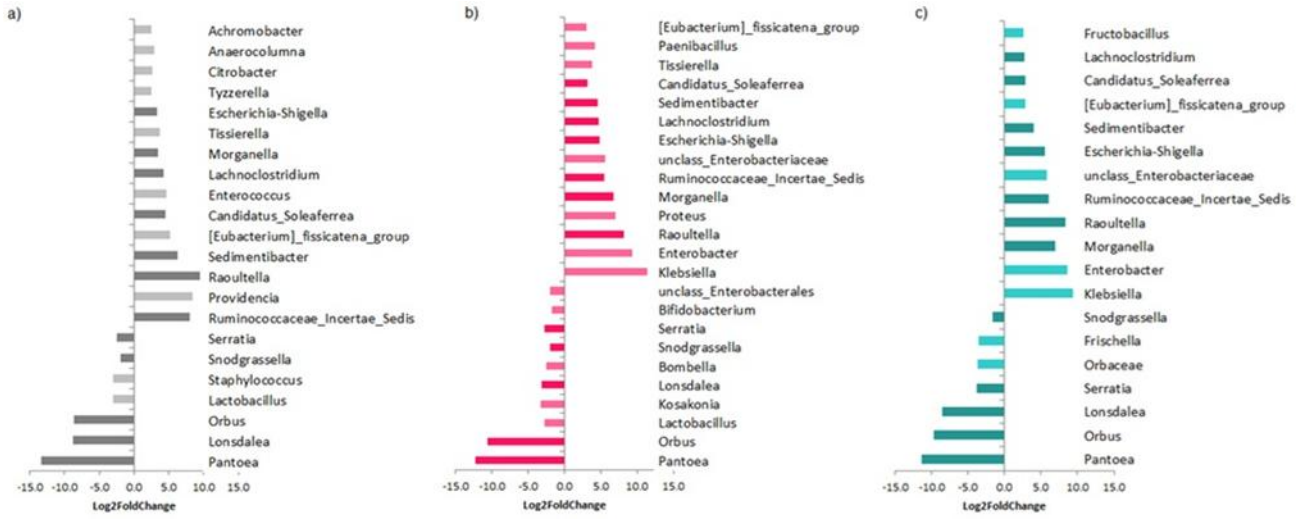


Fig. 6. Bacterial taxa at genus level showing significant differential abundance between infected and uninfected bees identified by DESeq2 analysis (adjusted P-value < 0.05) The results are presented separately for the three supplementation variants: a) no supplementation (infected control *vs* control as baseline); b) *Scleranthus perennis* (SP) supplementation (infected SP *vs* uninfected SP baseline); and c) *Hottonia palustris* (HP) supplementation (infected HP *vs* uninfected HP baseline). Darker colours and denser patterning indicate taxa that were consistently differentially abundant across all three supplementation variants

Discussion

The present study demonstrates that extracts from *S. perennis* and *H. palustris* can support honey bee health under cage conditions by improving survival of *Vairimorpha* (*Nosema*)-infected workers and markedly reducing infection intensity, as reflected by substantially lower spore loads compared with infected untreated controls. These findings align with the growing body of evidence showing that selected plant-derived preparations may limit the development of *Vairimorpha* (*Nosema*) spp. and reduce *Vairimorpha* (*Nosema*)-related health risks at the colony level, which is important given that effective synthetic treatment options are limited (10, 13, 30, 50).

Across two independent cage trials, extracts from both plant species improved the survival of infected bees; however, the HP6 fraction also extended lifespan in the uninfected controls, suggesting that its activity may extend beyond infection suppression and could enhance the bees' general physiological resilience under the experimental conditions. Notably, all supplemented infected groups showed a pronounced reduction in spore loads, indicating that the observed survival benefits were accompanied by reduced infection intensity, not just improved host condition. Taken together, the survival and spore-load outcomes support the potential of extracts from *S. perennis* and *H. palustris* as candidates for anti-nosemosis interventions, while also highlighting that efficacy and host response may vary between fractions, consistent with fraction-specific phytochemical composition.

Differences in bee responses among the tested fractions reflect variation in their phytochemical composition and, consequently, in the biological activity of their constituents. *Scleranthus perennis* contains flavone glycosides previously reported to exhibit bioactivity in other systems which may influence the overall health status of bees or contribute indirectly to improved tolerance of infection (27). Likewise, *H. palustris* has been reported to contain polymethoxyflavones and other flavone derivatives with documented bioactivity *in vitro* (56) which may contribute to the observed reduction in infection intensity; however, the active constituents and mechanisms against *Vairimorpha* (*Nosema*) require further confirmation. Future studies should therefore focus on chemical profiling and bioassay-guided fractionation to identify which phytoconstituents may be responsible for the anti-nosemosis activity.

Botanical interventions can have a narrow margin between benefit and adverse effects, and previous studies on other plant preparations have shown that some extracts may reduce *Vairimorpha* (*Nosema*) infection while negatively affecting bee survival, depending on dose, formulation or exposure time (44, 63). Although the present study identifies fractions that improved survival and reduced spore loads under the tested conditions, optimising dose and exposure time to balance efficacy and safety, and verifying effects under field conditions will be essential preparatory work to enable any practical application.

Metabarcoding analyses clearly indicate that infection was associated with a microbiota composition shift, with a reduction in the abundance of several taxa considered a part of the typical bee-associated microbiota (*Snodgrassella, Lactobacillus*, and *Bifidobacterium*) accompanied by an increase in the relative abundance of Enterobacteriaceae family members (*Escherichia-Shigella, Citrobacter, Enterobacter, Klebsiella,* and *Raoultella*) and selected gut-associated anaerobic bacteria (*Lachnoclostridium*, *Sedimentibacter,* and *Ruminococcaceae incertae sedis*). This pattern suggests infection-driven dysbiosis and a potential expansion of opportunistic bacteria (2, 42, 50, 62). *Vairimorpha* (*Nosema*) infection is well documented to cause severe damage to the midgut epithelium, including the formation of necrotic cell foci and interruption of intestinal continuity, which can result in intestinal leakage and increased host mortality (49). Such infection-driven necrotic damage represents a genuine threat to bee survival and provides a plausible route by which opportunistic taxa such as *Klebsiella* and *Pantoea* could proliferate beyond their normal ecological niche. These genera are commonly detected as low-abundance members of the honeybee gut community even under healthy conditions (50, 52), and disruption of gut integrity may facilitate their expansion. We therefore interpret the increased abundance of these taxa in infected bees as reflecting, at least in part, this infection-associated epithelial damage, rather than as a benign compositional shift within an otherwise intact gut community. Such shifts may also include increases in core bee gut taxa, such as *Gilliamella apicola* and *Snodgrassella alvi*, which have been suggested to play protective or compensatory roles in maintaining gut function under stress (25, 50, 54).

In contrast, comparisons between extract-supplemented and non-supplemented bees within the same infection status revealed no effect on α-diversity or consistent changes in taxa abundance, indicating that tested fractions had, at most, a limited impact on overall microbiota structure. Consequently, the beneficial effects of *S. perennis* and *H. palustris* observed here are

most plausibly explained by reduced infection intensity (lower spore loads) and improved host survival, rather than by broad microbiome modulation. Nevertheless, some studies show that plant extracts can modulate the structure of the gut microbiota, potentially improving its function. For example, tea extract has been reported to decrease potentially harmful taxa (*e.g. Escherichia/Shigella*) while enriching butyrate-producing bacteria such as *Faecalibacterium* (43). Moreover, interventions combining plant extracts with probiotics have been shown to reduce *Vairimorpha* (*Nosema) ceranae* levels while shifting the honey bee gut microbiota towards higher abundances of beneficial taxa, including *Lactobacillus* and *Bifidobacterium* (50, 59).

This study provides a controlled cage-based evaluation of the effects of *S. perennis* and *H. palustris* fractions on bee survival and *Vairimorpha* (*Nosema*) infection, with spore loads quantified at a defined endpoint. Survival was analysed at the group level and infection intensity at the cage level, consistent with the experimental design. Microbiome profiling focused on selected supplementation variants and used DNA extracted from whole bees, which offers a practical overview but may be less sensitive to subtle, gut-specific shifts. Based on these results, future studies could extend the approach to longitudinal sampling of *Vairimorpha* (*Nosema*) infection intensity, dose optimising, gut-targeted DNA extraction, and detailed phytochemical profiling of the tested extract fractions to identify the active compounds.

Conclusion

Scleranthus perennis and *H. palustris* extracts show promise as natural candidates to support honey bee health and mitigate nosemosis, as evidenced by improved survival of infected bees and strong reductions in spore loads. Microbiome analyses indicate that infection-driven dysbiosis is the dominant signal, while supplementation with the aqueous fractions (SP3 and HP3), the only fractions assessed by metabarcoding, does not induce a consistent, community-wide shift in gut bacterial composition. The effect of the butanol fractions on gut microbiota, despite their stronger biological activity, is an important direction for follow-up work (55). These results support further development and mechanistic investigation of these plant extracts as part of an integrated approach to nosemosis management. Given the ongoing challenge of colony losses and the limited availability of effective synthetic treatments such as fumagillin, advancing safe and practical natural alternatives remains an important goal for sustainable beekeeping and, ultimately, food security.

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Animal Rights Statement: All planned experiments were performed in accordance with good laboratory practice principles. Although insects are not subject to formal research permit requirements, the experimental design was carefully developed to minimise the number of insects required for the studies to the absolute minimum necessary for statistical validity and scientific rigour. The research protocol employed the smallest possible number of insects while ensuring adequate sample sizes for meaningful statistical analysis, and all procedures were designed to minimise any discomfort, stress or adverse conditions during experimentation.

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writing the article, final approval of the article. **Michał Tomczyk:** research concept and design, collection and assembly of data, data analysis and interpretation, writing the article, final approval of the article.

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