Original Article

EFFECT OF ETHANOLIC PROPOLIS EXTRACT ON GUT MICROBIOTA OF HONEY BEES (*APIS MELLIFERA*) INFECTED WITH *NOSEMA* SPP.

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Abstract

Honey bees (*Apis mellifera*) are essential pollinators but are facing numerous threats from such pathogens as *Nosema* spp., which disrupt gut function and colony health. The honey bee gut microbiota contributes to digestion, immunity, and defense; imbalances in this community can intensify disease outcomes. This study used a caged bee model to evaluate how propolis, a natural resinous compound produced by bees, impacts gut microbial composition, microbiota dynamics, *Nosema* infection severity, and survival. Propolis supplementation promoted the growth of beneficial bacterial genera, *Bifidobacterium* and *Commensalibacter*, while reducing the prevalence of opportunistic taxa. It also alleviated the negative influence of ethanol exposure on microbiota stability. Importantly, microbial diversity and structure were maintained more effectively in propolis-treated bees, even under infection stress. These findings highlight the potential of propolis as a natural therapeutic agent that supports microbial homeostasis and mitigates disease impacts in honey bees. By demonstrating the dual role of propolis in protecting both microbiota composition and host resilience, this research offers insights into sustainable strategies for enhancing bee health and combating colony losses.

Keywords: *Apis mellifera*, gut microbiota, *Nosema* infection, propolis, 16S rRNA

INTRODUCTION

Honey bees are essential for ecosystem balance and crop pollination, aiding nearly 80% of flowering plants (Carreck & Williams, 1998). Recently, global declines have been driven by malnutrition, pesticides, habitat loss, and disease, especially in vulnerable individuals (Insolia et al., 2022). One major biological challenge is *Nosema ceranae,* a microsporidian parasite that has largely replaced *Nosema apis* and exhibits higher virulence, especially under warmer conditions (Gisder et al., 2017). Infections damage the midgut epithelium, impairing digestion and nutrient absorption (Panek et al., 2018), and are linked to reduced lifespan, behavioral changes, and compromised foraging and flight performance (Dussaubat et al., 2013; Dosselli et al., 2016).

The gut microbiota, essential to bee health, supports digestion, metabolism, immunity, and pathogen resistance (Li et al., 2017). Disruptions caused by *Nosema* spp. may exacerbate susceptibility to other stressors. Encouragingly, microbiome-supporting agents have shown potential in restoring balance and combating infection (Borges et al., 2021).

Propolis, a natural resinous substance produced by bees, possesses notable antimicrobial and immunomodulatory properties. Despite variability in its composition, it has been associated with improved bee immunity and reduced pathogen load (Huang et al., 2014;

Socha et al., 2015; Mura et al., 2020; Naree et al., 2021). However, its role in restoring gut microbiota under *Nosema* infection is not well understood. Additionally, as ethanol is commonly used to deliver propolis, its potential influence on microbial communities warrants closer examination (Miłek et al., 2024).

This study explores the effects of ethanol-based propolis supplementation on gut microbiota in bees infected with *N. ceranae*, with the aim of identifying strategies to support microbial balance and host resilience.

MATERIALS AND METHODS

Caged bee experimental setup

Using the caged bee model from Trytek et al. (2022) at the same Lublin apiary, colonies were screened each spring for *Nosema* spp. via microscopy and PCR. In June, three brood combs from a healthy colony were incubated (35°C, 60% RH) and newly emerged *Apis mellifera* workers remained on combs for two days fed bee bread and honey to develop microbiota. Emerging *A. mellifera* workers were screened for *Nosema* by light microscopy (ECLIPSE E200, Nikon) and PCR (Martín-Hernández et al., 2007). *Nosema*-free bees were then allocated to six groups (40 bees per group, 10 replicates; 60 cages total).

Bees were kept in darkness under controlled lab conditions (25°C, 65-70% RH) for three days (EPPO, 2010) in sterilized, evenly spaced cages, fed 50% sucrose *ad libitum*. Thereafter, bees were split into infected and control groups. Infected bees received sucrose with *N. ceranae* spores (10^4 spores/mL, ~1.8 ± 0.2 mL/bee) for two days via modified 5 mL luer-tip syringes. Spores came from infected bees (Sinpoo et al., 2018); controls received spore-free filtrate. Infection was confirmed by 16S rRNA PCR (PCR Mix Plus Red, A&A Biotechnology, Poland).

Two days post-infection, bees were grouped (10 cages each): (1) non-infected control (NIC), (2) non-infected + alcohol (NIA), (3) non-infected + propolis (NIP), (4) infected control (IC), (5) infected + alcohol (IA), (6) infected + propolis (IP).

Treatments (ethanol, propolis extract, or plain syrup) were given in 10 mL/L sucrose. Propolis extract (Bartpol, Poland) was made by dissolving 50 g of crude propolis in 400 mL of 70% ethanol for three weeks (refrigerated, decanted, and passed through a 0.45 µm filter). The volume was readjusted to 400 mL. Concentration is reported as 0.125 g/mL crude propolis equivalents, not corrected for insoluble material. At 10 mL/L, the final ethanol concentration was ~0.7% (v/v) in both the ethanol-only and propolis syrups; control syrups contained no ethanol. Bees had daily access to 5 mL of treatment via modified syringes.

Mortality was recorded every 2-3 days. Feeders were replaced as needed, and sucrose intake monitored. Dead bees were pooled per group, homogenized in 10 mL distilled water, and spore loads counted via hemocytometer (Mortensen et al., 2016).

Sample collection

On day 18, when bees typically shift from in-hive tasks to foraging, four bees per group were randomly selected for microbiota analysis. One bee per cage was selected for sequencing in each group to ensure independence at the cage level. Bees were frozen at -80°C for 20 min, and intestines extracted via the anus. Each was placed in a sterile 1.5 mL vial for DNA extraction. All steps occurred under sterile conditions in a laminar flow chamber.

DNA Isolation, 16S rRNA sequencing, and microbiota profiling

Total DNA was extracted from *A. mellifera* gut samples using the Tissue & Bacterial DNA Purification Kit (EURx, Gdańsk, Poland), following the manufacturer's protocol with minor modifications. Samples were homogenized in Lyse T solution with the use of BeadTubeDry (EURx, Gdańsk, Poland) and the FastPrep-24 5G homogenizer (MP Biomedicals, LLC, Irvine, CA, USA) at 6 m/s for 40 s, followed by a 1-hour lysis at 56°C. DNA quantity and quality were assessed with a Synergy H1 reader, Qubit 2.0 Fluorometer, and agarose gel electrophoresis. The V3-V4 regions of the bacterial 16S rRNA gene were amplified using primers 341F and 806R

with sample-specific barcodes (online Suppl. Tab. S1). PCR products of the correct size were selected on 2% agarose gels, pooled equally, end-repaired, A-tailed, and ligated with Illumina adapters. Libraries were sequenced on the Illumina NovaSeq X platform to obtain 250 bp paired-end reads. Data were processed with the DADA2 (v1.26) pipeline (Callahan et al., 2016): reads were demultiplexed, trimmed, quality-filtered (allowing two errors per 250 bp), dereplicated, merged, and denoised to obtain amplicon sequence variants (ASVs), with chimeras subsequently removed. Processing results are summarized in online Suppl. Table S1. Sequence data are available under NCBI BioProject accession PRJNA1115052.

Bioinformatic and statistical analysis

Sequencing data were analyzed in R (v4.2.3). Contaminants were filtered using the frequency method in the "decontam" package (Davis et al., 2018). ASVs with ≤2 reads in <20% of samples were removed to limit false positives. Taxonomy was assigned via BEExact database (v 2023.01.30) (Daisley & Reid, 2021), and curated with the NCBI databases.

Data were normalized with "rarefy_even_depth" function in the "phyloseq" package. α-diversity was calculated using "phyloseq" and "vegan" packages; β-diversity via NMDS based on Bray-Curtis dissimilarity, and visualized with "phyloseq" and "ggplot2". Group structure was tested by PERMANOVA (9999 permutations) with the "adonis2" function.

To assess the effects of *Nosema* infection and treatments on gut microbiota, log-transformed, rarefied genus-level counts were analyzed. Assumptions for parametric testing were evaluated with the use of the Shapiro-Wilk (normality) and Levene's (variance homogeneity) tests. Depending on results, either two-way ANOVA ("stats" package) or non-parametric Aligned Rank Transform (ART) ANOVA ("ARTool" package) was applied to test the main effects of infection status and treatment, as well as their interaction.

Mortality differences among treatments were analyzed with the use of Kaplan-Meier curves via the "survival" package. Curves were compared with the "survdiff" function with the use of the log-rank (Mantel-Cox) post hoc test to identify significant differences between them.

The p-values obtained in the statistical tests were adjusted to control for the false discovery rate. Results were considered statistically significant if the p-value was less than 0.05 and the q-value (FDR-adjusted p-value) was less than 0.1.

RESULTS

Effects of alcohol and propolis supplementation on honey bee survival, *Nosema* infection intensity, and feeding behavior

Survival analysis revealed that *Nosema* infection significantly increased honey bee mortality compared to the non-infected control group (χ^2=5.73, p=0.017, q=0.023; Fig. 1A, B). Alcohol supplementation further intensified mortality in both healthy (χ^2=16.49, p<0.001, q<0.001) and infected bees (χ^2=50.18, p<0.001, q<0.001), with the highest mortality observed in *Nosema*-infected individuals receiving alcohol (~91% by the end of the experiment). Significant differences were also found between bees given propolis and those receiving alcohol in both non-infected (χ^2=30.71, p<0.001, q<0.001) and infected groups (χ^2=79.55, p<0.001, q<0.001). In contrast, propolis had no significant effect on survival in either healthy (χ^2=1.71, p=0.192) or infected bees (χ^2=1.73, p=0.189), suggesting a protective role against alcohol-induced mortality (Fig. 1A, B). Besides survival, propolis supplementation led to a significant reduction in *Nosema* spp. spore counts (p=0.032, n=10, day 18; Fig. 1C). Average food intake did not differ significantly among groups, although infected bees tended to consume slightly more, possibly due to infection-induced energetic stress (Fig. 1D).

Bacterial community structure, composition, and differential taxa

Sequencing of *A. mellifera* gut 16S rRNA yielded 1,923,599 high-quality merged reads (73.84% of input), with 56,709-90,539 reads per library

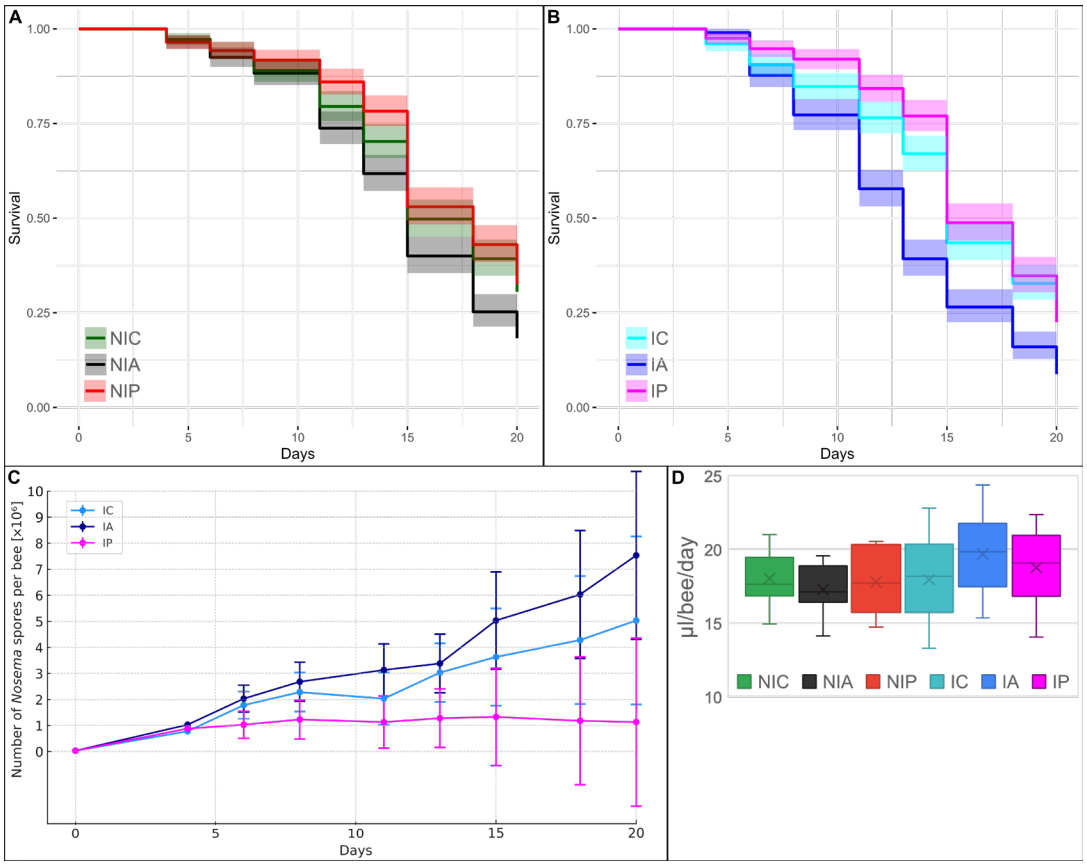

Fig. 1. Effects of alcohol and propolis supplementation on honey bee survival in non-infected (A) and *Nosema*-infected (B) groups, *Nosema* infection intensity (C), and feeding behavior (D).
Panels A and B displays Kaplan-Meier survival curves with shaded areas representing 95% confidence intervals.

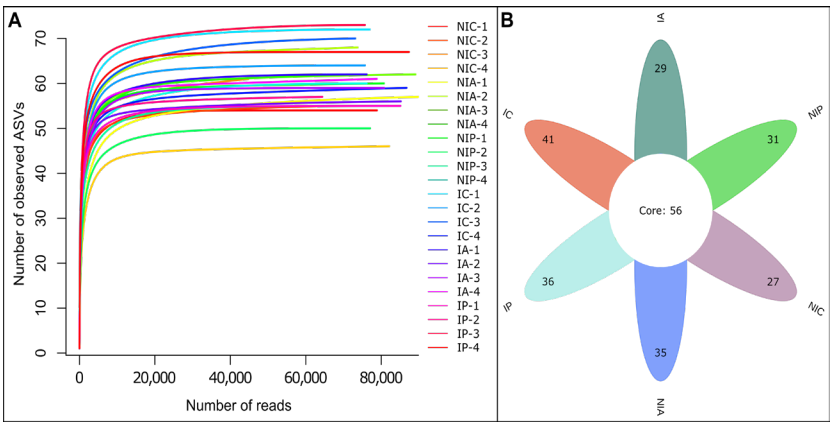

Fig. 2. Sequencing depth and distribution of ASVs across experimental groups. (A) Rarefaction curves of ASV richness for each sample. (B) Flower plot of core and non-core ASVs across experimental groups. The central circle indicates ASVs detected in all six groups. Each petal displays the count of ASVs present in the respective group but not shared across all groups.

(online Suppl. Tab. S1). After filtering, 102 high-confidence ASVs were retained (online Suppl. Tab. S2). Rarefaction curves plateaued, which confirmed sufficient sequencing depth (Fig. 2A). Samples contained 46-73 ASVs (mean=60). Of the 102 ASVs, fifty-six (54.9%) were shared across all variants (Fig. 2B), and nineteen were found in every sample (online Suppl. Tab. S2). α-diversity metrics showed no major differences in species richness among groups. The Chao1 index aligned with observed ASVs (online Suppl. Tab. 1) indicated similar species richness across all groups (Tab. 1). This supports the conclusion that sequencing depth was sufficient to capture the majority of bacterial diversity in the samples, as also demonstrated by rarefaction curves (Fig. 2A). Although Shannon and Simpson indices trended lower in alcohol-fed groups (Tab. 1), differences were not statistically significant. Pooling by infection status revealed significantly higher Shannon and Simpson indices in

Table 1.

Comparison of alpha diversity indices in the gut microbiota of *A. mellifera*

Experimental variants	Alpha diversity indices*		
	Chao1	Shannon	Simpson
NIC	55.50±8.66	2.05±0.34	0.75±0.07
NIA	62.88±4.73	1.96±0.39	0.70±0.10
NIP	56.25±4.79	2.05±0.31	0.75±0.09
IC	66.50±5.51	2.51±0.54	0.83±0.12
IA	59.75±2.87	2.09±0.45	0.73±0.17
IP	63.00±8.49	2.43±0.05	0.83±0.03
Pooled by infection status			
Non-infected	58.21±6.69	2.02±0.32 (a)	0.73±0.08 (a)
Nosema-infected	63.08±6.20	2.34±0.41 (b)	0.80±0.12 (b)

*different lowercase letters in parentheses within the same column indicate statistically significant differences ($p<0.05$)

Table 2.

Effects of infection, treatment, and their interaction on bacterial genus abundance in the honey bee gut microbiota

Genus*	Effect	F-value	p-value	q-value
Commensalibacter	Treatment	11.08	<0.001	0.019
Bifidobacterium		7.80	0.004	0.047
Blautia	Infection	13.77	0.002	0.010
Enterococcus		14.00	0.001	0.010
Morganella		22.06	<0.001	0.005
Lacrimispora		9.25	0.007	0.025
Anaerocolumna		8.97	0.008	0.025
Tissierella		7.14	0.016	0.045
Hafnia-Obesumbacterium		12.28	0.003	0.013
Pseudomonas		11.71	0.003	0.013
	Infection × Treatment	11.07	<0.001	<0.001
Bartonella		6.05	0.010	0.085

*only genera with statistically significant effects are shown

Nosema-infected bees ($p<0.05$, n=12; Tab. 1), which suggested infection-driven shifts in community structure. To assess microbial community differences, β-diversity was analyzed via NMDS with the use of Bray-Curtis dissimilarity (Fig. 3). NMDS showed distinct clustering of infected vs. non-infected bees, with some overlap. PERMANOVA detected statistically significant but moderate effects of infection ($p=0.019$, $R^2=8.1\%$, $F=2.18$) and its interaction with supplementation ($p=0.005$, $R^2=15.3\%$, $F=2.06$). Plateauing rarefaction and stable richness (Fig. 2A, Tab. 1), a large shared ASV core across variants (Fig. 2B), and infection-associated shifts in evenness and community structure (Fig. 3, Tab. 2) together indicate that community changes primarily reflect shifts in the relative abundances of shared taxa rather than widespread ASV loss.

Out of the 102 unique ASVs identified in this study, 100% were taxonomically assigned at the phylum, class, order, and family levels, 98.04% (100 ASVs) at the genus level, and 35.29% (36 ASVs) at the species level. These ASVs were classified into three bacterial phyla, seven classes, eleven orders, fifteen families, and twenty-four genera (online Suppl. Tab. S3). Exact 16S rRNA matches allowed the identification of twenty-two species (online Suppl. Tabs. S2, S3). At the phylum level (Fig. 4A), *Pseudomonadota* dominated (average 80.23%,

ranging from 67.98% in the NIC group to 86.38% in the IA group), followed by *Bacillota* (18.73%) and *Actinomycetota* (1.04%). At the genus level (Fig. 4B), *Snodgrassella* (18.57-44.36%) was most abundant, then *Lactobacillus* (10.44-23.14%), *Bartonella, Pseudomonas*, and *Gilliamella*. Detailed abundances are in online Suppl. Table S3.

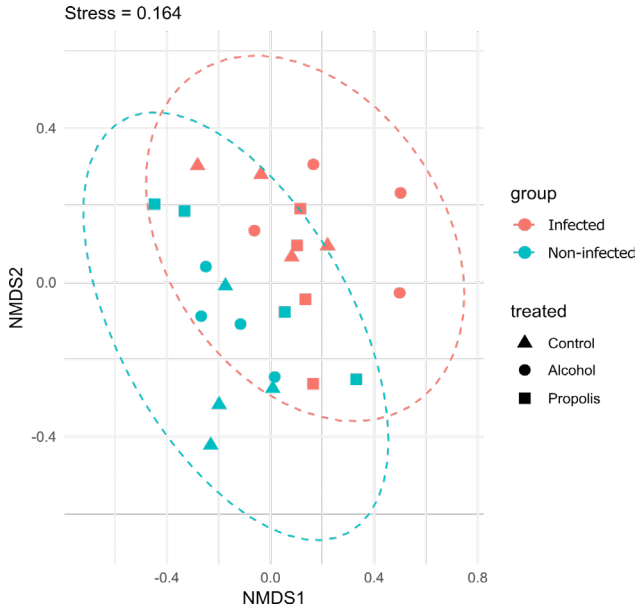

Fig. 3. NMDS ordination of gut microbial communities in honey bees based on Bray-Curtis dissimilarity. Each point represents a gut microbiota sample from an individual bee. Colors indicate infection status, and shapes indicate dietary treatment. Ellipses represent 95% confidence intervals around the centroids of the two infection groups.

The gut microbiota of *A. mellifera* included core bee-associated taxa - *Bifidobacterium*, *Gilliamella, Lactobacillus* (Firm-5), and *Snodgrassella,* and commonly reported non-core genera such as *Bartonella*, *Commensalibacter*, and *Frischella* (Motta & Moran, 2024). These taxa appeared across all groups (online Suppl. Tab. S3). Other consistently detected genera included *Pseudomonas*, *Bombella*, *Hafnia-Obesumbacterium*, *Apilactobacillus*, *Novisyntrophococcus*, *Blautia*, *Enterococcus*, *Apirhabdus*, *Pantoea*, and *Lacrimispora*, indicating a stable, diverse community shaped by host physiology and environment. Both *Nosema* infection and treatments (control, alcohol, propolis) significantly affected gut microbiota composition. To assess this, we analyzed genus-level abundances using log-transformed rarefied ASV counts. Genera with significant responses to infection, treatment, or their interaction (via two-way or aligned rank transform ANOVA) are shown in Table 2 and online Suppl. Fig. S1.

Treatment affected *Commensalibacter* and *Bifidobacterium*, regardless of infection. *Nosema* infection alone increased genera linked to dysbiosis: *Blautia*, *Enterococcus*, *Morganella*, *Lacrimispora*, *Anaerocolumna*, *Tissierella*, *Hafnia-Obesumbacterium*, and *Pseudomonas*. Significant interactions for *Pseudomonas* and *Bartonella*

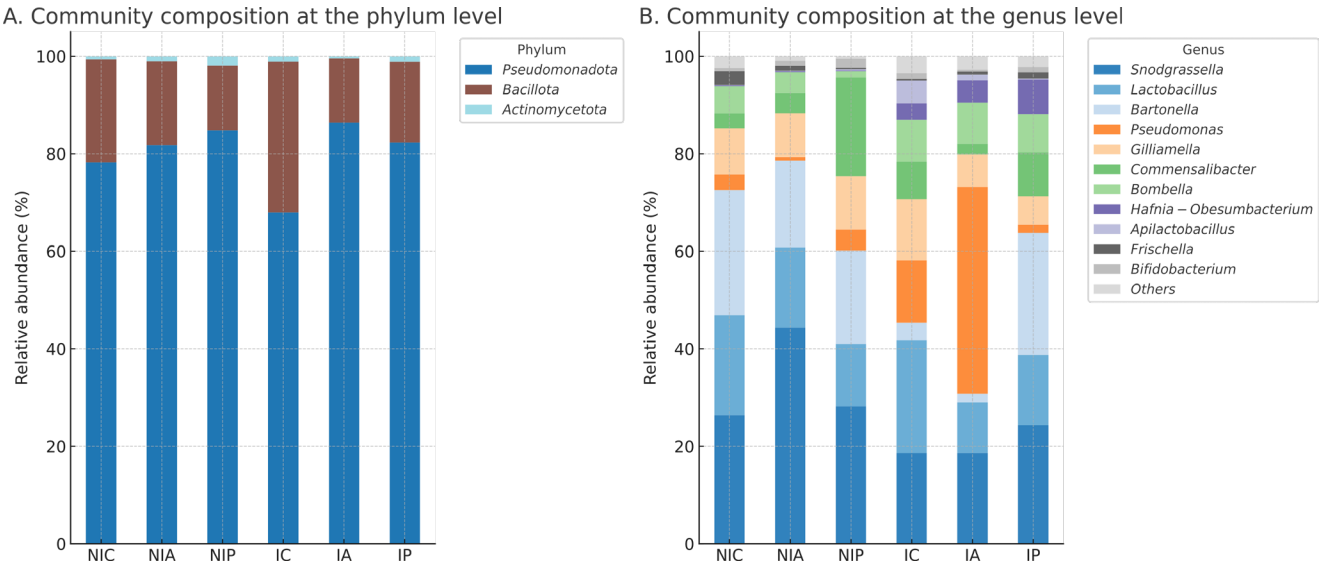

Fig. 4. Taxonomic classification of the honey bee gut microbiota at the phylum (A) and genus (B) levels. Panel (B) displays the 11 most abundant bacterial genera, with all other genera grouped as “Others”.

suggest infection-dependent effects, especially with propolis supplementation.

DISCUSSION

Propolis significantly reduces *Nosema* infection and supports honey bee health (Mura et al., 2020; Naree et al., 2021), likely due to its complex chemical makeup (Huang et al., 2014; Socha et al., 2015). Its bioactive compounds exhibit antibacterial, antioxidant, and anti-inflammatory effects (Sforcin, 2016), which may ease oxidative and energetic stress linked to *Nosema* infection. Our results align with reports of reduced bee longevity from *Nosema* infection (Trytek et al., 2022) and suggest that ethanol exposure exacerbates its effects. High mortality in ethanol-treated infected bees supports earlier findings of synergistic stress, where tissue acidification fosters parasite growth (Ptaszyńska et al., 2013). Infected bees often show increased syrup intake (Mayack & Naug, 2009), and although not statistically significant here, a similar trend was observed. Ethanol has also been shown to reduce syrup intake in both healthy and infected bees, likely due to its aversive properties (Ptaszyńska et al., 2013). In our study, while ethanol did not significantly affect feeding, it sharply increased mortality, especially in infected bees (~91%), suggesting additional toxic or metabolic stress.

Propolis lowered spore loads without affecting feeding, which indicates protective effects beyond intake. This aligns with Mura et al. (2020) and may result from immune stimulation, enhanced beneficial microbes, or direct antiparasitic action (Yemor et al., 2015). These results reinforce the potential dual function of propolis: suppressing pathogens and promoting host resilience, though the exact mechanisms remain to be elucidated.

Nosema infection increased α-diversity, whereas alcohol reduced it, which is consistent with stressor-linked microbiota shifts (Lau et al., 2024). Elevated diversity may reflect gut disruption or compensatory colonization in infected bees. Alcohol-driven dysbiosis reduced evenness and favored dominant taxa. PERMANOVA and ordination analyses confirmed that infection alters microbiota structure, echoing earlier findings (Lau et al., 2024). While alcohol or propolis alone had limited β-diversity effects, strong interaction terms suggest that treatment effects depend on infection status. These patterns were further reflected in significant shifts in key bacterial genera driven by both *Nosema* infection and treatment, particularly propolis, highlighting the microbiota's central role in host-pathogen interactions (Li et al., 2017).

Taxonomic profiling revealed a conserved gut structure despite compositional shifts. Of 102 ASVs, 98.04% were assigned to genus and over 35% to species. Core genera, *Lactobacillus, Gilliamella*, and *Snodgrassella*, were present in all groups (Kwong & Moran, 2016). *Bartonella*, though non-core, was also detected in all samples, and its seasonal presence, especially in winter, suggests potential metabolic roles in this context (Kešnerová et al., 2020; Li et al., 2022). This stable composition, regardless of infection or treatment, reflects a resilient microbiota with functional flexibility through shifts in abundance (Engel et al., 2016; Kwong & Moran, 2016; Motta & Moran, 2024).

Infected bees showed elevated dysbiosis-associated genera: *Blautia, Enterococcus, Morganella, Lacrimispora, Anaerocolumna, Tissierella, Hafnia-Obesumbacterium*, and *Pseudomonas*. The rise in *Hafnia* supports findings that *Hafnia alvei* may proliferate in immune-compromised bees (Lang et al., 2022). Similarly, *Morganella morganii*, known to interact with pathogens like *Paenibacillus larvae*, may act opportunistically (Al-Ghamdi et al., 2020).

Ethanol alone had limited impact on these genera, but combined with *Nosema* infection, it was associated with dysbiosis, mainly through *Pseudomonas* overgrowth, likely due to its metabolic plasticity under stress (Tsadila et al., 2023). Propolis counteracted this, reducing *Pseudomonas* in infected bees, consistent with its antimicrobial and microbiota-stabilizing properties (Sforcin, 2016; Mura et al., 2020; Naree et al., 2021).

A novel taxon, *Blautia liquoris*, was detected, which had not previously been associated with insects and was originally isolated from Baijiu pit mud (Lu et al., 2021). While *Lachnospiraceae* are common in bees (Lanh et al., 2022), *B. liquoris* was recently observed in overwintering, sugar-fed bees (Östervald, 2024), which suggests emergence under stress.

The increase in *Enterococcus* may reflect dual roles: some strains are pathogenic (Anjum et al., 2021), while others, like *Enterococcus faecium*, support immunity and gut development under stress (Du et al., 2021). Genomic data show that bee-derived strains lack human-associated virulence factors (Zaghloul & El Halfawy, 2022). Unlike core taxa, *Lacrimispora*, *Anaerocolumna*, and *Tissierella* are likely opportunists that appear mainly during *Nosema* infection, although it remains unclear whether their presence reflects dysbiosis or indicates a pathogenic role.

In uninfected bees, propolis increased *Commensalibacter* and *Bifidobacterium*, linked to immunity, nutrient processing, and pathogen exclusion (Chen et al., 2021). *Commensalibacter*, common in queens and winter bees, supports longevity (Kešnerová et al., 2020). Propolis also restored these taxa in infected bees, reversing ethanol's effect. *Bifidobacterium* aids homeostasis via polysaccharide breakdown and competition (Powell et al., 2021).

In sum, propolis showed dual functionality in our caged-bee model - antimicrobial activity and microbiota stabilization. It mitigated *Nosema*-associated dysbiosis, restored beneficial genera, and counteracted ethanol-linked imbalances. However, given the limited microbiome subsample and single time point, these results should be considered preliminary and validated with larger replication, multiple time points, field colonies, and propolis of diverse provenance.

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Supplementary online material:

Table S1. Sequencing data processing results

Table S2. Identified ASVs per individual sample
The table shows the number of reads after sequencing, quality control, bioinformatic processing, and taxonomic assignment according to the BEExact and NCBI databases. ASVs detected across all 24 sequencing runs are marked in yellow.

Table S3. Taxa relative abundance

Fig. S1. Interaction plots of bacterial gener.