



Research Article

## Protective effects of natural products and gut bacteria to reduce pesticide-induced mortality in honeybees

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### ABSTRACT

The population and diversity of honeybees face significant threats from agricultural pesticides. This study evaluated the protective effects of natural products, bee propolis, flaxseed oil, black seed oil, and gut bacteria isolated from the alimentary tracts of local honeybee (*Apis mellifera jemenitica*) of Saudi Arabia against four insecticides: acetamiprid, deltamethrin, imidacloprid, and alpha-cypermethrin. Thirteen gut bacterial strains, including *Bacillus australimaris*, *Peribacillus frigiditolerans*, and *B. siamensis* were isolated and assessed for their potential to enhance honeybee survival. Diets containing propolis or gut bacteria significantly extended the lifespan of bees exposed to pesticides. Propolis-fed bees survived 17.36, 20.14, 20.47, and 19.63 days under deltamethrin, acetamiprid, imidacloprid, and alpha-cypermethrin, respectively, compared to 15.63 to 18.29 days for gut bacteria-fed bees. Diets with flaxseed or black seed oil offered moderate protection (10.84–14.38 days). The untreated control group had the highest survival (21.54 days), while pesticide-only groups showed the lowest survival (5.39–9.11 days). Daily consumption varied significantly across treatments. Propolis had the highest mean consumption (12.64 µL/bee/day), similar to the untreated control (14.51 µL/bee/day). Flaxseed and black seed oil showed moderate consumption rates (8.15–8.57 µL/bee/day), while gut bacteria diets reached 10.45 µL/bee/day. Pesticides reduced consumption significantly, with acetamiprid, deltamethrin, and imidacloprid yielding 3.16–4.34 µL/bee/day, and alpha-cypermethrin slightly higher at 6.10 µL/bee/day. These findings underscore the potential of natural products and gut bacteria in mitigating pesticide toxicity in honeybees, improving survival, feeding behavior, and overall health. The diversity of isolated gut bacteria highlights promising applications in apiculture and pollination services.

### 1. Introduction

Honeybees hold a crucial position among pollinators owing to their considerable economic and ecological importance. In addition to producing honey, wax, venom, and propolis (Havard *et al.*, 2020), honeybees provide pollination services that support biodiversity and significantly enhance crop quality and yield (Khan and Ghramh, 2021). However, honeybee populations and diversity are under threat from multiple factors, including various pathogens, parasites, and the excessive use of chemical pesticides in agriculture (Oldroyd, 2007; Barbosa *et al.*, 2015; Sun *et al.*, 2024). Pathogens such as *Nosema* spp. present significant challenges to honeybee health by reducing their productivity and survival (Ghramh and Khan, 2023). Moreover,

honeybees are highly susceptible to pesticides partly due to a shortage of genes encoding detoxification enzymes (Claudianos *et al.*, 2006). The resulting global decline in these critical pollinators is alarming, as it may threaten food security and biodiversity (Potts *et al.*, 2010).

Insecticides are widely used for crop pest management and to control desert locust outbreaks in many regions (including Saudi Arabia), but their indiscriminate application can have numerous negative consequences. Over-reliance on synthetic insecticides can lead to pest resistance and resurgence, elimination of natural predators, pollinators, and decomposers, leaving harmful residues on crops and in the environment, and ultimately decreasing biodiversity and weakening the ecological resilience of agroecosystems (Hafeez *et al.*, 2022; Paula *et al.* 2023). Deltamethrin, for example, is a broad-spectrum, fast-acting

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Type II pyrethroid insecticide (alpha-cyano pyrethroid) commonly used against locusts and lepidopteran insects (Elliott et al., 1978; Hafeez et al., 2019). While effective against target pests, deltamethrin is highly toxic to honeybees and can cause both acute and chronic health effects. Similarly, imidacloprid, a systemic neonicotinoid, exhibits chronic toxicity in pollinators (Cresswell, 2011), and pyrethroids such as alpha-cypermethrin (another widely used insecticide) pose significant risks to non-target species like honeybees (Almeida et al., 2017). Even sublethal exposures to these insecticides can impair honeybee survival and performance by reducing foraging activity, learning ability, and colony growth (Bartling et al., 2024). Insecticide stress can also disrupt bees' physiology, behavior, immunity, and gut microbiota (Lin et al., 2023; Siddiqui et al., 2022), ultimately affecting the health of the entire colony and potentially contributing to colony collapse.

Given the dangers of pesticides, recent research has turned to natural mitigative strategies. Some natural products derived from bees themselves have shown protective effects; for instance, bee propolis and royal jelly can help shield honeybees from insecticide toxicity by modulating their detoxification pathways and enhancing antioxidant defenses (Simone-Finstrom et al., 2017). In parallel, beneficial microbes within the bee gut microbiome, crucial for digestion, nutrient provisioning, and immune support (Khan et al., 2020), may also counteract pesticide stress. Certain gut bacteria can metabolize or degrade insecticides and thereby improve bee survival. For example, *Lactobacillus kunkeei* and *Bifidobacterium asteroides* have been found to break down neonicotinoid and organophosphate insecticides both *in vivo* and *in vitro* (Almeida et al., 2017). These insecticide-degrading gut bacteria also enhance honeybee detoxification gene expression and immune responses, while reducing oxidative stress and inflammation, thus promoting gut barrier integrity and overall homeostasis under insecticide exposure (Almeida et al., 2017). As a result, both natural products and symbiotic gut bacteria have been proposed as promising tools to improve honeybee health and resilience in the face of insecticide stress.

In addition to bee-derived substances, various plant essential oils and extracts possess properties that could benefit honeybee health. Some natural plant products have notable antimicrobial, antioxidant, and anti-inflammatory components that might aid in managing harmful gut microbes and improving bee resilience. Flaxseed (*Linum usitatissimum*) contains 35%–45% oil, of which more than 70% is alpha-linolenic acid (ALA), a polyunsaturated omega-3 fatty acid known for health-promoting effects (Al-Madhagy et al., 2023). Blackseed (*Nigella sativa*) oil is similarly rich in unsaturated fatty acids (e.g., linoleic, linolenic) and includes bioactive compounds like thymoquinone, which exhibits antioxidant, anti-inflammatory, and antimicrobial properties (Forouzanfa et al., 2014). Propolis, typically composed of beeswax, pollen, plant resins, and essential oils, also demonstrates strong antimicrobial and immunomodulatory properties (Zhang et al., 2021). Together, these natural substances may support honeybee gut health by suppressing the proliferation of harmful gut bacteria and could work synergistically with beneficial gut microbes to enhance the bees' overall tolerance to stressors. While previous studies have examined the impacts of insecticides and tested various individual interventions (such as essential oils or probiotic bacteria), these approaches have largely been investigated in isolation. For example, Simone-Finstrom et al. (2017) showed that propolis can counteract pesticide-related toxicity by boosting honeybee immunity and detoxification pathways, and Almeida et al. (2017) demonstrated that introducing certain gut bacteria improves insecticide tolerance in bees. More recent studies have reinforced the critical role of the gut microbiota in mediating pesticide detoxification. Wu et al. (2020) found that honeybee gut symbionts upregulate cytochrome P450 gene expression, enhancing detoxification capacity. El Khoury et al. (2022) showed that specific gut bacteria can degrade neonicotinoids like clothianidin *in vitro*, and El Khoury et al. (2024) demonstrated that supplementing honeybees with such probiotic strains improved survival and preserved gut microbial balance under pesticide exposure. However, to our knowledge, no study has simultaneously evaluated multiple natural products alongside beneficial gut microbes in honeybees under insecticide stress. The current work addresses this gap by jointly assessing propolis, flaxseed oil, blackseed oil, and a gut bacterial supplement as a combined strategy

to protect caged honeybees from the adverse effects of insecticides. This study will help in developing novel and effective approaches to mitigate the negative effects of insecticides on honeybee health and productivity. Our investigation provides new insights into the interactions between natural products, gut microbiota, and insecticides in honeybees, which will contribute to the conservation and sustainability of honeybees and other pollinators as essential ecosystem service providers.

## 2. Materials and Methods

### 2.1 Chemicals and reagents

All insecticides (Deltamethrin, Acetamiprid, Imidacloprid, and Alpha-cypermethrin); oils (Black seed oil, Flaxseed oil) were purchased from local market. While, NaCl (Merck, Darmstadt, Germany); Tween 80 (Merck, Darmstadt, Germany); Peptone (Thermo Fisher Scientific, Waltham, MA, USA); Sodium hypochlorite; Phosphate-buffered saline; Sucrose; Nutrient agar plates; Lactic acid, Ethanol ; Whatman filter paper No. 1; QIAamp DNA Mini Kit (Qiagen, Hilden-Germany); Taq PCR Master Mix (Qiagen, Hilden-Germany), and Ethidium bromide were supplied by local suppliers.

### 2.2 Propolis collection

The propolis collection was carried out in Al-Souda Abha, Saudi Arabia, from the beehives of local honeybee (*A. m. jemenitica*) colonies using the methodology of ALaerjani et al. (2022) to ensure consistency. A spatula (stainless-steel) was utilized for the collection of the propolis, then placed in 50mL Falcon tubes, and frozen for further analysis. Raw propolis (30 g) was first cleaned and broken into small pieces to increase the surface area for solvent contact. The fragmented propolis was transferred into a 1,000 mL glass beaker (Paul Marienfeld GmbH & Co. KG, Germany), and 70% (v/v) ethanol was added as the extraction solvent until the total volume reached approximately 1 L. The beaker was covered tightly with a polythene film to minimize solvent evaporation and contamination. The mixture was placed on a magnetic stirrer with a hot plate (Daihan Laabtech Co., Ltd., South Korea) and stirred vigorously at room temperature to ensure uniform mixing and dissolution of resinous components. After stirring, the beaker was left undisturbed for 24 h at room temperature. At the end of the extraction period, the mixture separated into two distinct layers: a lower waxy sediment and an upper clear ethanolic extract. The supernatant (upper layer) containing the ethanolic extract of propolis was carefully decanted and filtered through Whatman No. 1 filter paper to remove residual debris. To achieve maximum extraction efficiency, an additional volume of 70% ethanol was added to the remaining waxy residue, and the extraction procedure (stirring, settling, and separation) was repeated three consecutive times. All filtrates obtained from the three extraction cycles were combined and transferred into sterile Petri dishes for solvent evaporation at room temperature until complete removal of ethanol. The resulting dry crude ethanolic propolis extract was collected, weighed, and stored in an airtight amber glass vial at 4°C until further.

### 2.3 Collection of honeybee specimens to isolate gut bacteria

In November 2022, honeybee specimens were collected from the local honeybee colonies at the King Khalid University (Center of Bee Research and its Products), Abha, Saudi Arabia. Sterile forceps were used to collect incoming workers of *A. m. jemenitica* at hive entrances. Three healthy bee colonies were selected to ensure randomness, and 20 worker bees were collected from each colony (60 workers/apiary). The collected bees were then placed in separate sterile centrifuge tubes (50 mL) containing 35 mL of sterile physiological saline (Khan et al., 2017).

### 2.4 Isolation of gut bacteria from the local honeybees

#### 2.4.1 Gut bacteria Isolation

Before gut dissection, the collected bees underwent a disinfection process to eliminate external microorganisms by immersing the

specimens in a 1% aqueous solution of sodium hypochlorite for 10 s, followed by three rinses in sterile purified water (Engel et al., 2013). Dissection of the whole gut of ten randomly selected worker bees/colonies in a sterile environment using sterile forceps and a laminar flow hood, starting from the ventriculus to the rectum (Moran et al., 2012). The dissected guts (n = 10) were then homogenized in 10 mL of phosphate-buffered saline (PBS) in a pestle. To culture the gut bacteria from the homogenized guts, the method of Anderson et al., 2013 and Khan et al., 2017 was utilized with some modifications. Specifically, a sterilized wooden cotton applicator was used to plate the homogenate on five nutrient agar plates (Neogen® Culture Media (NCM)). The plates were then incubated microaerobically at 36 °C and 80% relative humidity for two days. Pure isolated bacterial colonies on agar plates were designated based on their size (small, medium, and large), color (white, cream, opaque, and yellow), and morphology (circular, irregular, filamentous). The selected colonies were streaked multiple times on fresh agar plates individually to obtain a pure bacterial culture.

#### 2.4.2 DNA extraction

As per the instructions, the method used to extract bacterial DNA from pure colonies involved the QIAamp DNA Mini Kit (Qiagen, Hilden-Germany). Initially, a 1.5 mL tube containing 180 µL of Buffer ATL was used to transfer bacteria collected from the culture plates with an inoculation loop. Subsequently, proteinase K (20 µL) was put into the tube, which was vortexed, and simmered at 56°C to lyse the bacterial cells. Then, Buffer AL (200 µL) was added, followed by 99% ethanol (200 µL). This blend was then reassigned to the QIAamp mini spin column positioned in a 2 mL assembly tube and centrifuged at 6,000 rpm for a period of 1 min. After discarding the filtrate, buffer AW1 (500 µL) was supplementary to the QIAamp mini spin column, and centrifuged. The process was repeated with 500 µL of buffer AW2 and centrifuged (14,000 rpm) for 3 min. Subsequently, the mini spin column was transferred to a new 1.5 mL microcentrifuge tube, and 200 µL of Buffer AE was added, which was simmered for 1 min at normal room temperature. Finally, the sample was centrifuged at 8,000 rpm for 1 min to extract the DNA.

#### 2.4.3 PCR and DNA sequencing

For the amplification (16S rRNA gene), PCR primers 27F 5'-AGAGTTTGATCMTGGCTCAG-3' (Mattila et al., 2012) and 1492R 5'-TACGGYTACCTTGTACGACTT-3' were utilized (Moran et al., 2012). A 50 µL reaction mixture was used to perform PCR on a Gene Amp PCR System 9700 (Applied Biosystems), which consists of 25 µL of Taq PCR Master Mix (Qiagen), 2 µL of the forward primer (10 µM), 2 µL of the reverse primer (10 µM), 11 µL of genomic DNA (concentration > 30 ng/µL), and 10 µL of nuclease-free water. Using a GENIUS system, a PCR stuffs were then evaluated via gel electrophoresis on a 1% w/v agarose gel in 1x TBE buffer (100 V, 45 min). Ethidium bromide was used to color the gel and visualized by a gel system from Cleaver Scientific Ltd., U.K.

The bacterial isolates underwent PCR amplification. Their resulting products were sequenced at both ends using universal primers 27F and 1492R by Macrogen10F, 254, Beotkkot-ro, Geumcheon-gu, and Seoul (Gasan-dong, World Meridian I).

The DNA sequence chromatograms were visually inspected and were edited using Bio Edit (Hall, 1999). To align the sequences, MEGA X (Kumar et al., 2018) was employed. Furthermore, the comparison of these sequences with those in the NCBI GenBank database was performed using BLAST-N (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The resultant sequences were consigned at the National Center for Biotechnology Information (NCBI) by the accession numbers as shown in Table 1.

#### 2.4.4 Phylogenetic analysis

A phylogenetic analysis was conducted to study the diversity of gut bacteria and their evolutionary context. To obtain closely related partial 16S rRNA sequences, BLAST-N was used to search the NCBI database (<https://blast.ncbi.nlm.nih.gov>). These sequences and those

**Table 1.**

Application of different treatments, including positive and negative control treatments.

Treatment	Feeder-1	Feeder-2
Black seed oil (0.1% v/v)	0.250 mL black seed oil + 0.125 mL Tween 80 to 250 mL (w/v) of 50% sucrose solution.	Sublethal insecticide in 50% sucrose solution (e.g., Acetamiprid 2 ppm, Deltamethrin 1 ppm, Alpha-cypermethrin 2 ppm, or Imidacloprid 1 ppm)
Flax seed oil (0.5% v/v)	0.250 mL Flax seed oil + 0.125 mL Tween 80 to 250 mL (w/v) of 50% sucrose solution	Sublethal insecticide in 50% sucrose solution (e.g., Acetamiprid 2 ppm, Deltamethrin 1 ppm, Alpha-cypermethrin 2 ppm, or Imidacloprid 1 ppm)
Propolis extract (0.04 mg/mL)	Propolis solution was prepared by adding 0.5 mL of 2% (w/v) propolis extract (dissolved in 49 mL lactic acid) to 250 mL of 50% (w/v) sucrose solution and mixing thoroughly	Sublethal insecticide in 50% sucrose solution (e.g., Acetamiprid 2 ppm, Deltamethrin 1 ppm, Alpha-cypermethrin 2 ppm, or Imidacloprid 1 ppm)
Gut bacteria (1 × 10 <sup>7</sup> CFU/mL)	Mixed stock of 12 bacterial strains (1 × 10 <sup>9</sup> CFU/mL each) by dilution in sterile 50% (w/v) sucrose solution, following Pachla et al. (2021) with minor modifications	Sublethal insecticide in 50% sucrose solution (e.g., Acetamiprid 2 ppm, Deltamethrin 1 ppm, Alpha-cypermethrin 2 ppm, or Imidacloprid 1 ppm)
Positive control	Sublethal insecticide (e.g., Acetamiprid 2 ppm, Deltamethrin 1 ppm, Alpha-cypermethrin 2 ppm, or Imidacloprid 1 ppm)	-
Negative control	50% (w/v) sucrose solution	-

retrieved from Genbank were aligned and manually edited using Bio Edit (Hall, 1999). MEGA X was used for phylogenetic and molecular evolutionary analyses with 500 bootstrap replicates using the neighbor-joining method and BioNJ algorithm to a matrix of pairwise distance estimated using the Maximum Composite Likelihood (MCL) approach.

### 2.5. Preliminary bioassay and measuring the sublethal dose of insecticide

#### 2.5.1 Experimental cages

Specially designed wooden cages were used for these experiments. The dimensions of these cages were 15 × 15 × 5 cm. The cage was fitted with glass on one side, while the other side had an iron grid that allowed for the circulation of air. Additionally, two holes were located on the top surface of the enclosure and equipped with a 20 mL syringe containing sucrose solution with a concentration of 50% (w/v), and another hole was provided with a treatment (Khan and Ghranh, 2022).

#### 2.5.2 Collection of honeybees

The research was performed at the Center of Bee Research and its Products (CBRP) at King Khalid University, Abha, Saudi Arabia. Closed brood frames from both sides were obtained from the local honeybees (*A. m. jemenitica*) hives. These frames were placed in an incubator at 34°C and 70% to 80% R.H. Freshly emerged honeybees were collected and placed in cages. Each cage contained ≈ 30 bees.

#### 2.5.3 Insecticides

Four insecticides commonly used in agriculture and urban pest control were selected. These included Deltamethrin (2.5%, K-Othrine SC25; Bayer AG, Germany), Acetamiprid (20%, Garastin 200 SL; Gerasa, Jordan), Imidacloprid (25%, Acochem 25% WP), and Alpha-cypermethrin (10%, Alpha Power 100 SC; Atiaf Al Tabyah Trading Est., Saudi Arabia). They represent the major insecticide classes, pyrethroids and neonicotinoids, commonly encountered by honeybees in agricultural and urban environments, enabling realistic evaluation of pesticide toxicity and protective treatments.

### 2.5.4 Preliminary bioassay

Sublethal doses of insecticides (Deltamethrin, Acetamiprid, Imidacloprid, and Alpha-Cypermethrin) were estimated by following Laurino et al. (2011) method, with a few modifications. For each insecticide, seven different concentrations causing >0% and <100% mortality were prepared. The concentrations used in the experiment were 10, 6, 4, 2, 1, 0.5, and 0.1 ppm. All these concentrations were prepared in a 50% sugar solution. Approximately 30 newly emerged bees were placed in a cage (each equipped with a 20 mL syringe) containing the tested insecticide concentrations in 50% (w/v) sucrose solution. These cages, along with the bees, were placed in an incubator set at 34°C and maintained at 70% to 80% relative humidity (R.H.). Data were recorded at 12, 24, 36, and 48 h after initial exposure. Dead bees were removed from the cages and counted regularly, while bees showing no movement after being touched with a needle were considered dead. This revised methodology allows for the evaluation of the sublethal effects of multiple insecticides (Deltamethrin, Acetamiprid, Imidacloprid, and Alpha-Cypermethrin) under uniform experimental conditions.

### 2.6 Application of treatments for honeybee survival analysis

The experiment utilized 20 mL syringes to administer treatments to newly emerged honeybees ( $n \approx 30$ ). Each cage contained two syringes: one syringe was filled with a sublethal dose of an independent insecticide, which included Acetamiprid (2 ppm), Deltamethrin (1 ppm), Alpha-cypermethrin (2 ppm), or Imidacloprid (1 ppm), all dissolved in 50% sucrose solution. The second syringe contained the specific treatment solutions. The oil feeding solution of each flaxseed oil and black seed oil was prepared by adding 0.250 mL of flaxseed oil (or black seed oil) and 0.125 mL of Tween-80 (0.05% v/v final concentration) to 250 mL of 50% (w/v) sucrose solution. The mixture was vortexed (or briefly sonicated) to form a uniform emulsion and mixed thoroughly before feeding. The feeding solution of gut bacteria was prepared by following Pachla et al. (2021) with some modifications. Each strain was grown overnight in the nutrient broth, pelleted ( $4,000 \times g$ , 10 min, 4 °C), washed twice with 0.85% NaCl, and resuspended in 50% (w/v) sucrose solution. Cell densities were adjusted by OD–CFU calibration and plate counts to  $1 \times 10^9$  CFU/mL per strain. Equal CFU of each of the 12 strains were combined to form a stock solution (total  $\approx 1 \times 10^9$  CFU/mL). Working feeding solution was prepared fresh by diluting the stock solution with sterile 50% sucrose solution to the target concentration ( $1 \times 10^7$  CFU/mL) by adding 2.5 mL of  $1 \times 10^9$  CFU/mL stock solution to 247.5 mL of sucrose solution. Propolis ethanol extract (0.04 mg/mL) was prepared by adding 0.500 mL of 2% (w/v) ethanolic propolis extract (dissolved in 49 mL lactic acid) to 250 mL of 50% (w/v) sucrose solution and mixing thoroughly. The negative control consisted of one syringe containing a 50% sucrose solution and another syringe containing water only. In the positive control, one syringe contained the sublethal insecticide concentrations

(Acetamiprid 2 ppm, Deltamethrin 1 ppm, Alpha-cypermethrin 2 ppm, or Imidacloprid 1 ppm), while the second syringe contained only water (Table 1). Solutions were freshly prepared before administration, and the cages were maintained under controlled conditions. To prevent bees from avoiding the sublethal insecticide syrup, the two syringes were offered on an alternate-day schedule: on one day, bees were provided with only the insecticide solution to ensure exposure, and on the following day, only the treatment solution (propolis, oil, or gut bacteria) was offered. This alternating feeding regime ensured both effective insecticide exposure and subsequent treatment intake. The bees' consumption rates and mortality were recorded daily to ensure precise delivery and monitoring of the treatments.

### 2.7 Data analysis

All statistical analyses and visualizations were conducted using SPSS-23 statistical software (Chicago, USA). Probit analysis was performed to calculate the median lethal concentrations ( $LC_{50}$ ) and the lethal concentrations for 90% mortality ( $LC_{90}$ ), with mortality data adjusted using Abbott's formula. The dose-response curve steepness was analyzed to assess the relative potency of the insecticides. Kaplan-Meier survival analysis was used to evaluate the survival probabilities under various treatment conditions. Survival curves were compared to identify statistically significant differences among treatments with a significance level set at  $p < 0.05$ . Boxplot visualizations were constructed to analyze daily honeybee consumption data, displaying the interquartile ranges (IQR) and medians for groups exposed to Deltamethrin, Acetamiprid, Imidacloprid, and Alpha-cypermethrin across the various treatments. Comparative statistical tests were applied to detect significant variations in consumption patterns, with statistical significance also set at  $p < 0.05$ .

## 3. Results

### 3.1 Isolation and characterization of gut bacteria

Molecular characterization based on 16S rRNA partial sequence led to a total of 12 gut bacterial strains, including *Bacillus australimaris*, *Peribacillus frigiditolerans*, *B. siamensis*, *B. altitudinis*, and *Priestia endophytica*. The details information from NCBI blast has been stated in Table 2.

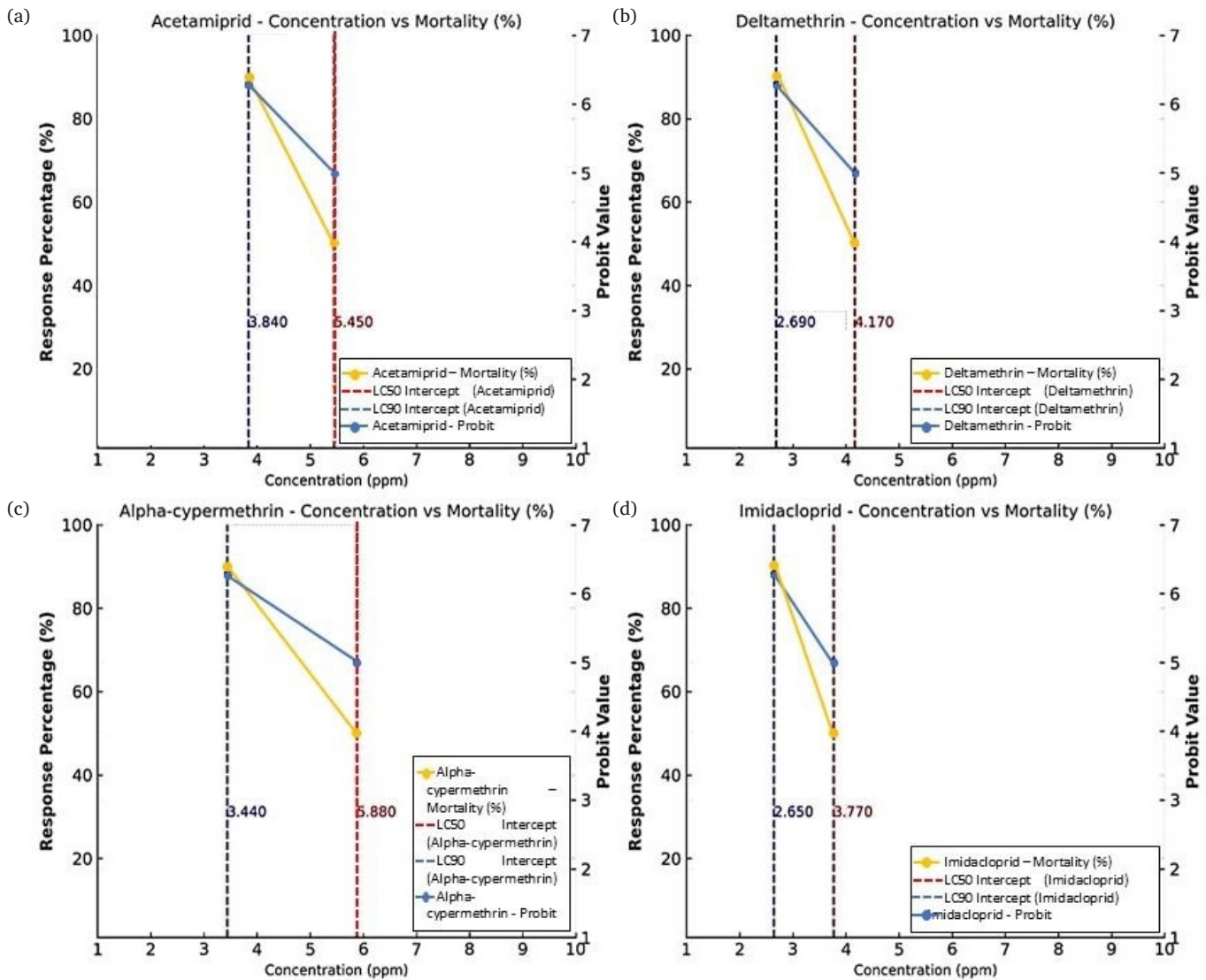
### 3.2 Phylogenetic analysis

The 16S rRNA gene phylogeny (Fig. 1) revealed that the 12 gut bacterial isolates from *A. m. jemenitica* span multiple well-supported clades within the genera *Bacillus*, *Peribacillus*, and *Priestia*. All Saudi honeybee isolates (highlighted in the tree) are interspersed among reference strains from diverse geographic origins, with high bootstrap values confirming the robustness of these groupings. The three *B. subtilis*

**Table 2.** Identification of gut bacteria isolated honeybee (*Apis mellifera jemenitica*) from Abha Saudi Arabia.

Organism	Strain	Accession No.	Phylum	Class	Order	Family	Genus	Accession No. Blast match	Identity (%)
<i>Bacillus australimaris</i>	JMGB-4	PQ596421	Bacillota	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	ON955422	99.49
<i>Peribacillus frigiditolerans</i>	JMGB-5	PQ596422	Bacillota	Bacilli	Bacillales	Bacillaceae	<i>Peribacillus</i>	OL839962	99.93
<i>Bacillus siamensis</i>	JMGB-6	PQ596423	Bacillota	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	MW287259	99.93
<i>Bacillus altitudinis</i>	JMGB-7	PQ596424	Bacillota	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	NR_042337	99.73
<i>Bacillus subtilis</i>	JMGB-8	PQ596425	Bacillota	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	MH424579	99.87
<i>Priestia endophytica</i>	GB_1_SAUDI	PP346328	Bacillota	Bacilli	Bacillales	Bacillaceae	<i>Priestia</i>	OR485175	99.51
<i>Bacillus subtilis</i>	GB_2_SAUDI	PP346329	Bacillota	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	MT114596	100.00
<i>Bacillus pumilus</i>	GB_4_SAUDI	PP346330	Bacillota	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	JQ798393	99.65
<i>Peribacillus frigiditolerans</i>	GB_5_SAUDI	PP346331	Bacillota	Bacilli	Bacillales	Bacillaceae	<i>Peribacillus</i>	MT081073	100.00
<i>Bacillus subtilis</i>	GB_6_SAUDI	PP346332	Bacillota	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	MT081073	100.00
<i>Bacillus pumilus</i>	GB_7_SAUDI	PP346333	Bacillota	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	OP364521	100
<i>Bacillus tequilensis</i>	GB_8_SAUDI	PP346334	Bacillota	Bacilli	Bacillales	Bacillaceae	<i>Bacillus</i>	JF41129	99.93





**Fig. 2.** Dose-response relationship and Probit analysis for four insecticides: (a) Acetamidrid, (b) Deltamethrin, (c) Alpha-cypermethrin, and (d) Imidacloprid, showing mortality percentages (%) at varying concentrations (ppm). LC<sub>50</sub> and LC<sub>90</sub> values are indicated by blue and red dashed lines, respectively, along with corresponding Probit intercepts. The steepness of the dose-response curves reflects the potency and efficacy of each insecticide. Each point represents the mean of three independent replicates ( $n = 30$  bees per concentration). Data were analyzed using Probit regression and goodness-of-fit  $\chi^2$  tests ( $p < 0.05$ ) to determine LC<sub>50</sub> and LC<sub>90</sub> values.

to achieve mortality, with an LC<sub>50</sub> of 3.44 ppm and an LC<sub>90</sub> of 5.88 ppm. The broader range between LC<sub>50</sub> and LC<sub>90</sub> reflected a slower dose-response relationship, indicating the need for significantly higher concentrations to achieve near-total lethality. Imidacloprid emerged as the most potent insecticide in the study, with the lowest LC<sub>50</sub> (2.65 ppm) and LC<sub>90</sub> (3.77 ppm). Its steep dose-response curve indicated a sharp increase in mortality with minimal concentration increases, further underscoring its efficacy. Among the tested insecticides, Imidacloprid and Deltamethrin were the most effective, achieving higher mortality rates at lower concentrations, whereas Acetamidrid and Alpha-cypermethrin required higher doses to achieve similar effects. These findings suggest that the choice of insecticide should be guided by the desired level of efficacy and the required concentration for achieving target mortality rates, with Imidacloprid being the most suitable option for applications demanding high potency.

### 3.4 Impact of some natural products on the longevity of bees

The results depicted in Fig. 3 illustrate the survival of honeybees (in days) under various treatments when exposed to four insecticides: Acetamidrid, Deltamethrin, Imidacloprid, and Alpha-Cypermethrin. The negative control group demonstrated the highest survival duration

of  $21.54 \pm 2.33$  days, representing the natural life span of honeybees without external stressors.

Propolis-treated honeybees showed significant protective effects, with survival durations ranging from  $17.36 \pm 2.84$  days (Deltamethrin) to  $20.14 \pm 2.27$  days (Acetamidrid), highlighting its ability to mitigate the toxicity of these insecticides. Similarly, the gut bacteria treatment improved survival, with durations ranging from  $15.63 \pm 1.91$  days (Acetamidrid) to  $18.29 \pm 2.35$  days (Alpha-Cypermethrin), showcasing its potential to counteract pesticide-induced stress. Flaxseed oil offered moderate benefits, with survival durations between  $12.23 \pm 1.87$  days (Alpha-Cypermethrin) and  $14.38 \pm 2.44$  days (Imidacloprid). Black seed oil exhibited a comparable trend, with survival durations ranging from  $10.84 \pm 4.31$  days (Deltamethrin) to  $12.38 \pm 3.87$  days (Alpha-Cypermethrin), indicating some level of protective efficacy. In contrast, the positive control groups exhibited the lowest survival durations, ranging from  $5.39 \pm 3.12$  days (Deltamethrin) to  $9.11 \pm 2.29$  days (Alpha-Cypermethrin), underscoring the detrimental effects of insecticide exposure without protective interventions. These results emphasize the superior effectiveness of propolis and gut bacteria in enhancing honeybee survival compared to flaxseed and black seed oils, which provide moderate protection.

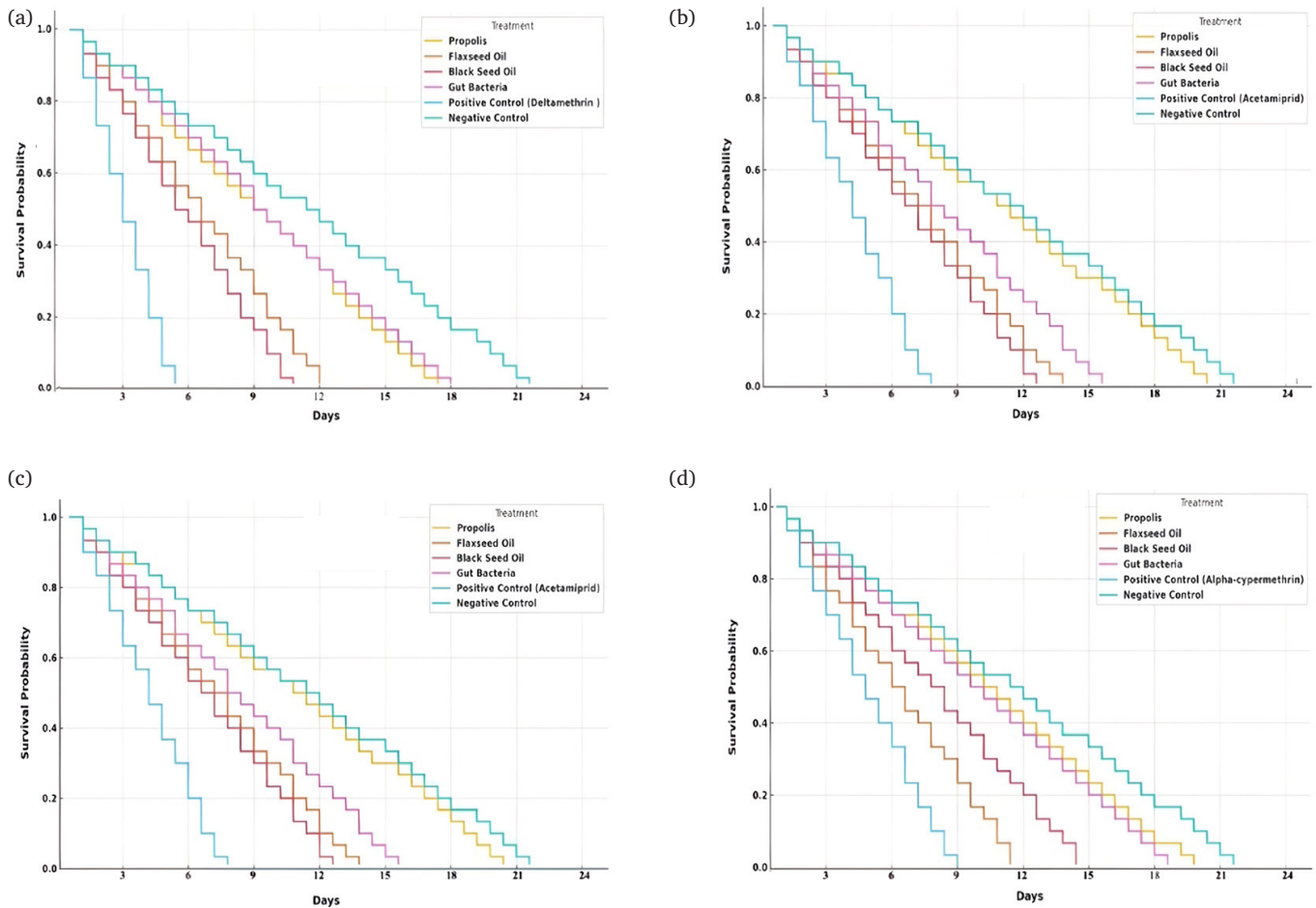


Fig. 3. Kaplan-Meier survival curves representing the effects of various natural treatments and pesticides on the longevity of *Apis mellifera jemenitica* bees. The y-axis indicates the Kaplan-Meier survival probabilities, and the x-axis shows the survival duration in days. Survival comparisons are presented with exposure of bees to (a) Deltamethrin, (b) Acetamiprid, (c) Imidacloprid, and (d) Alpha-cypermethrin all with treatments including propolis, flaxseed oil, black seed oil, gut bacteria, positive control, and negative control (sugar solution only). Each treatment was performed in three independent replicates ( $n = 30$  bees per replicate). Statistical differences in survival among treatments were analyzed using the Kaplan–Meier log-rank (Mantel–Cox) test with significance accepted at  $p < 0.05$ .

Natural treatments significantly improved the survival of nurse bees compared to the positive control group. Gut bacteria and propolis demonstrated the highest survival rates across pesticide exposures, enhancing the bees' life expectancy. The negative control (sugar solution only) consistently exhibited the highest survival across all experiments.

### 3.5 The daily consumption of honeybees across different treatments

The daily consumption of honeybees has been depicted in Fig. 4. Propolis exhibited the highest mean consumption,  $12.64 \pm 4.39$   $\mu\text{L}/\text{bee}/\text{day}$ , indicating a strong preference or tolerability for this treatment. Similarly, the Negative Control group showed a high mean consumption of  $14.51 \pm 5.37$   $\mu\text{L}/\text{bee}/\text{day}$ , slightly exceeding that of Propolis, suggesting uninhibited feeding in the absence of any treatment. Treatments with Black Seed Oil and Flaxseed Oil resulted in comparable mean consumptions of  $8.15 \pm 4.29$   $\mu\text{L}/\text{bee}/\text{day}$  and  $8.57 \pm 3.36$   $\mu\text{L}/\text{bee}/\text{day}$ , respectively. The gut bacteria treatment demonstrated a moderate mean consumption of  $10.45 \pm 4.76$   $\mu\text{L}/\text{bee}/\text{day}$ , suggesting a potentially beneficial effect of gut microbiota on feeding behavior and overall health.

In contrast, pesticides were associated with significantly lower consumption rates. Acetamiprid, Deltamethrin, and Imidacloprid had mean consumptions of  $4.34 \pm 2.59$   $\mu\text{L}/\text{bee}/\text{day}$ ,  $3.16 \pm 2.78$   $\mu\text{L}/\text{bee}/\text{day}$ , and  $3.49 \pm 3.12$   $\mu\text{L}/\text{bee}/\text{day}$ , respectively, reflecting reduced feeding behavior in response to these chemicals. Among pesticides, Alpha-cypermethrin exhibited slightly higher consumption  $6.10 \pm 2.41$   $\mu\text{L}/\text{bee}/\text{day}$  compared to others, though still below the untreated controls.

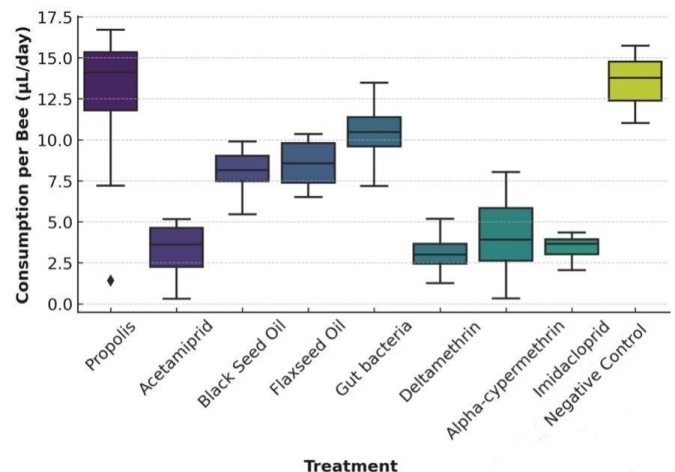


Fig. 4. Boxplot showing the distribution of honeybee consumption ( $\mu\text{L}/\text{bee}/\text{day}$ ). The bees were exposed to sublethal doses of deltamethrin, acetamiprid, imidacloprid, and alpha-cypermethrin across different treatments, including propolis, flaxseed oil, black seed oil, gut bacteria, and a negative control (50% sugar solution only). Each box represents the interquartile range (IQR), with the median indicated by the horizontal line inside the box. Data are based on three independent replicates ( $n = 30$  bees per replicate). Statistical comparisons among treatments were performed using one-way ANOVA followed by Tukey's HSD post hoc test ( $p < 0.05$ ).

These findings demonstrate the significant influence of treatment type on honeybee feeding behavior. While natural substances such as Propolis, plant oils, and Gut Bacteria enhanced or maintained normal feeding rates, pesticides reduced consumption, with notable variability between chemical types. This highlights the critical role of treatment choice in preserving honeybee health and behavior.

#### 4. Discussion

The isolation and molecular characterization of gut bacteria from *A. m. jemenitica* revealed 12 distinct bacterial strains, predominantly belonging to the genera *Bacillus*, *Peribacillus*, and *Priestia*. The phylogenetic analysis demonstrated well-supported clustering (high bootstrap values) and revealed clear evolutionary relationships between the Saudi Arabian isolates and their global counterparts.

For instance, *B. subtilis* isolates (PQ596425, PP346329, and PP346332) from Saudi Arabia formed multiple clades with *B. subtilis*, *B. siamensis* (PQ596423) and *B. tequilensis* (PP346334) strains from China, Japan, India, Argentina, Egypt, and the USA, indicating their broad evolutionary conservation. Similarly, *B. pumilus* (PP346330, PP346333) clustered closely with strains from Pakistan, Iran, and India, while *B. australimaris* (PQ596421) aligned with isolates from India and Argentina. The isolate *B. altitudinis* (PQ596424) grouped with strains from Iran and South Korea, reflecting its regional adaptation. *Peribacillus frigitolerans* (PP346331, PQQ596422) grouped with strains from Italy and Israel, and *Priestia endophytica* (PP346328) clustered alongside isolates from Mexico, Egypt, and the USA reflecting their wide geographic distribution. Overall, the phylogenetic topology highlights the genetic diversity and adaptability of Saudi Arabian honeybee gut bacteria, supporting the view that similar bacterial lineages occur across distant regions and ecological niches (Mattila et al., 2012). The phylogenetic analysis demonstrated a diverse evolutionary background of gut bacteria isolates, with robust clustering supported by high Bootstrap values. The presence of *B. subtilis* and *P. frigitolerans* among Saudi Arabian isolates reflects the adaptability of these species to diverse ecological niches. The distinct clustering of *Priestia endophytica* with isolates from the USA and Israel highlights the widespread distribution of certain gut bacterial strains. These findings align with Moran et al. (2012) and Engel et al. (2013), who reported the cosmopolitan nature of honeybee gut microbiota and its critical role in nutrient assimilation and detoxification. The evolutionary insights from this study provide a foundation for exploring the functional genomics of gut bacteria and their applications in apiculture. The identification of strains with potential biotechnological applications supports prior research suggesting the importance of gut bacteria in detoxification processes and overall bee health. The findings of Almeida et al. (2017) and Wu et al. (2020) are in accordance with our results, which highlight the critical role of gut microbiota in supporting detoxification processes and promoting host resilience against chemical stressors. The LC<sub>50</sub> and LC<sub>90</sub> values for acetamiprid, deltamethrin, alpha-cypermethrin, and imidacloprid revealed varying levels of toxicity, with imidacloprid and deltamethrin exhibiting the highest potency. The steep dose-response curves for these insecticides emphasize their acute toxicity, consistent with previous research highlighting the significant risks of neonicotinoids and pyrethroids to pollinators (Basu et al. 2024; Brittain and Potts, 2011). Interestingly, the broader LC<sub>50</sub>-LC<sub>90</sub> range for alpha-cypermethrin suggests a slower mortality response, potentially indicating delayed physiological impacts. The variation in LC<sub>50</sub> values among the four insecticides likely reflects differences in their chemical structure and mode of action, with neonicotinoids causing chronic neurotoxicity and pyrethroids inducing rapid knock-down effects. This aligns with findings by Laurino et al. (2011), who observed similar trends in honeybee responses to pyrethroids.

Propolis-treated bees demonstrated significantly extended survival under insecticide exposure, highlighting its potential as a protective agent. The survival durations observed for propolis-treated bees (17.36 to 20.47 days). The study by Simone-Finstrom et al. (2017) highlights the protective role of propolis in reducing microbial pathogens and modulating immune responses in honeybees. These mechanisms likely enhance overall resilience, suggesting that propolis could indirectly support survival under stressors like pesticide exposure through its

antimicrobial, antioxidant, and immune-boosting properties. The antimicrobial properties of propolis not only support gut health by reducing pathogen load but also indirectly enhance honeybee survival. Turcatto et al. (2018) demonstrated that propolis consumption activates antimicrobial peptide genes, bolstering immune responses and mitigating bacterial infections. These enhanced immune defenses likely contribute to improved resilience against environmental stressors, ultimately increasing the bees' chances of survival.

Gut bacteria treatments also enhanced survival (15.63 to 18.29 days). The increased survival could be a result of the role of gut microbiota in detoxifying harmful compounds. A study conducted by Almeida et al. (2017) underscored the critical role of gut microbiota in degrading insecticides, which could mitigate the toxic effects of these compounds. Similarly, our results demonstrate that treatments involving gut bacteria enhanced survival, likely by reducing the physiological burden of toxins. These findings collectively suggest that the detoxification capabilities of gut bacteria may play a pivotal role in improving resilience and extending the lifespan of honeybees exposed to environmental stressors. Similarly, Leska et al. (2022) demonstrated that lactic acid bacteria can bind and detoxify insecticides, reducing their harmful effects. This suggests that the detoxification capability of gut bacteria may play a crucial role in improving honeybee resilience and longevity (Leska et al., 2022). The role of gut microbiota in maintaining homeostasis and boosting immunity has been further validated by Engel and Moran (2013), who demonstrated that microbial communities in the bee gut significantly reduce oxidative stress and support detoxification processes. These findings align with studies by El Khoury et al. (2022) showing that endogenous gut microbiota metabolize pesticides like clothianidin, reducing their toxicity and extending bee survival. This improvement in survival may be attributed to the detoxification capabilities of the administered gut bacteria, which could reduce the harmful effects of pesticide exposure. The moderate protection offered by flaxseed and black seed oils (10.84 to 14.38 days) aligns with the study by Zakaria (2011), which highlights the antimicrobial and immunomodulatory properties of black seed oil (*N. sativa*) due to its phenolic compounds, such as thymoquinone. These compounds have been shown to enhance honeybee immunity and reduce pathogen loads, contributing to improved resilience against bacterial infections like American Foul Brood (AFB). Similarly, the bioactive components in black seed oil may mitigate the harmful effects of environmental stressors, thereby supporting the enhanced survival observed in our study. These findings underscore the potential of plant-derived oils as natural protective agents in honeybee health management. Similarly, Salem (2005) and Krist et al., (2006) suggest that the bioactive fatty acids in these oils, such as the omega-3 fatty acids in flaxseed oil and the polyphenolic compounds in black seed oil, are critical to their protective effects. Breed et al., (2012) highlighted in their study an additional potential benefit of flaxseed oil in honeybee colonies: its influence on nestmate recognition. By modulating the chemical profiles on honeybee cuticles, flaxseed oil was shown to enhance colony cohesion, reducing intra-colony aggression and improving social organization. Although, Breed et al., (2012) study focuses on behavioral dynamics rather than direct physiological effects, it suggests that flaxseed oil may indirectly contribute to colony stability and resilience under stress. The omega-3 fatty acids in flaxseed oil, known for their anti-inflammatory and antioxidant properties (Krist et al., 2006) likely reduce oxidative stress and improve overall health, which could synergize with the enhanced social cohesion described by Breed et al. (2012). Together, these effects may improve survival and colony robustness in challenging environments.

However, the findings of this study demonstrated that the positive control groups exposed to insecticides without any treatment exhibited the lowest survival rates (5.39 to 9.11 days), highlighting the severe impact of insecticide exposure on honeybee health. This aligns with the findings of Cresswell (2011), whose meta-analysis revealed that even field-realistic trace levels of the neonicotinoid imidacloprid could impair honeybee performance by 6% to 20%. Such sublethal effects, in combination with lethal toxicity, emphasize the overwhelming risks posed by insecticides to honeybee populations. Laurino et al. (2011) further support these observations, as their laboratory tests demonstrated the high toxicity of neonicotinoid insecticides, particularly

thiamethoxam and clothianidin to honeybees. Even at sublethal concentrations, these compounds induced significant mortality, highlighting their detrimental effects on honeybee populations.

The feeding behavior analysis revealed higher consumption rates in treated groups (8.15 to 12.64  $\mu\text{L}/\text{bee}/\text{day}$ ). In the negative control, the consumption rate was 14.51  $\mu\text{L}/\text{bee}/\text{day}$ , which is notably lower than the median daily intakes reported for other European subspecies [Retschnig et al., 2021](#). This difference may be partially attributed to inherent physiological traits of *A. m. jemenitica*, which is among the smallest-bodied honey bee subspecies. Body size is a critical determinant of metabolic demand and correlates with feeding rate, and thus, smaller bees are expected to consume less food relative to their larger counterparts. Additionally, *A. m. jemenitica* has evolved under arid and high-temperature environments like those of the Arabian Peninsula. Such environmental pressures may have selected for more efficient energy utilization and lower maintenance metabolic rates, both of which could contribute to reduced dietary intake. These physiological adaptations may also influence longevity patterns, as observed in our treatments, where, despite lower consumption rates, some diets, like propolis and gut bacteria, appeared to sustain or improve survival. These findings are consistent with the notion that not only diet quantity but also diet quality and microbial support systems influence honey bee health. Treated groups in our study not only mitigate toxicity accordingly but also seem to preserve the normal feeding behavior of honeybees, which is crucial for colony maintenance. Similar observations were reported by [Simone-Finstrom et al. \(2017\)](#), who found that propolis-treated colonies exhibited improved health metrics compared to untreated groups. The findings of [Simone-Finstrom et al. \(2017\)](#) suggest that propolis not only mitigates toxicity but also supports normal feeding behavior by improving the health and resilience of honeybees. By reducing microbial load and enhancing immune responses, propolis helps maintain homeostasis within the hive, which likely contributes to a stable consumption rate essential for colony maintenance. Conversely, bees exposed to pesticides exhibited significantly reduced consumption rates, with deltamethrin and imidacloprid showing the lowest values (3.16 and 3.49  $\mu\text{L}/\text{bee}/\text{day}$ , respectively). This reduction aligns with earlier findings by [Cresswell \(2011\)](#), suggesting that sublethal pesticide exposure impairs feeding behavior and energy balance. Interestingly, alpha-cypermethrin-treated bees showed slightly higher consumption (6.10  $\mu\text{L}/\text{bee}/\text{day}$ ), indicating potential differences in toxicity mechanisms that warrant further investigation.

## 5. Conclusions

This study demonstrates that natural products and gut bacteria enhance the resilience of *Apis mellifera jemenitica* to pesticide stress. Propolis and gut bacteria showed the strongest protective effects, improving survival and feeding activity, while flaxseed and black seed oils offered moderate benefits. The observed  $\text{LC}_{50}$  variations reflect differences in insecticide chemistry and mode of action, and the diversity of isolated gut bacteria (*Bacillus*, *Peribacillus*, *Priestia*) indicates their detoxification potential. Overall, integrating natural products with beneficial microbes provides a promising, eco-friendly strategy to safeguard honeybee health. Future studies should elucidate the molecular basis of these effects and validate their long-term impact under field conditions.

## CRedit authorship contribution statement

**Hamed A. Ghramh:** Conceptualization, funding acquisition, project administration, resources, visualization; **Badria M. Al-Shehri:** Conceptualization, data curation, formal analysis, software, supervision, validation, writing—original draft preparation, writing—review and editing; **Fatimah A. Al-Saeed:** Conceptualization, investigation, methodology, validation, visualization, writing—original draft preparation, writing—review and editing; **Mohammed Elimam Ahamed Mohammed:** Data curation, formal analysis, software, validation, visualization, and writing—review and editing; **Zubair Ahmad:** Data curation, formal analysis, investigation, methodology, software, validation, visualization; **Sultan Ahmad Alkahtani:**

Methodology, software, validation; **Naimah A. Alanazi:** Software, validation; **Khalid M. Alghamdi:** Software, validation; **Muhammad Hafeez:** Software, validation, writing—review and editing; **Khalid Ali Khan:** Conceptualization, data curation, formal analysis, funding acquisition, software, supervision, validation, visualization, writing—original draft preparation, writing—review and editing.

## Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have influenced the work presented in this paper.

## Declaration of generative AI and AI-assisted technologies in the writing process

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

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