In vitro acaricidal activity of several natural products against ibex-derived *Sarcoptes scabiei*.

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ABSTRACT

In this study we analysed the effect of the temperature, diverse strains of *Bacillus thuringiensis*, *Lysinibacillus sphaericus* and nanoformulations with essential plant oils (EONP) on the survival of *Sarcoptes scabiei* mites derived from naturally-infested Iberian ibex (*Capra pyrenaica*). In general, mites maintained at 12ºC survived more than those maintained at 35ºC (40.7 hr and 31.2 hr, respectively). Mites with no treatment survived 27.6 h on average. Mites treated with *B. thuringiensis* serovar. *konkukian* and geranium EONP showed significant reduction in their survival. Despite the fact that these agents seem to be promising candidates for controlling sarcoptic mange in the field, further research is still needed to get stable, efficient and eco-friendly acaricides.

Keywords: *Bacillus thuringiensis*, essential plant oils, nanoparticles, *Sarcoptes scabiei*, survival

1. Introduction

Sarcoptes scabiei is an astigmatid mite causing a dermal disease, namely sarcoptic mange, in domestic and wild mammalian hosts, including man, worldwide, reaching high morbidity and mortality rates (Bornstein et al., 2001; Pence &

Ueckermann, 2002; Arlian & Morgan, 2017). Transmission of this mite between susceptible hosts may be direct, indirect, or a combination of both (Browne et al., 2022).

Control of this disease in wild populations is a challenging task. Although multiple doses of subcutaneous ivermectin (200-400 µg/kg) is the treatment most commonly used (Rowe et al., 2019), its implementation with free-ranging animals is very difficult from a logistic viewpoint. Moreover, this approach may have undesirable impact on non-target organisms and favours the development of resistance by the mite (Walton et al., 2000), among other "secondary" effects (Moroni et al., 2020).

The development of resistance of *S. scabiei* against acaricidal compounds is increasing (Currie et al., 2004; Mounsey et al., 2010; Andriantsoanirina et al., 2014). Therefore, it is crucial to develop new drugs for treating scabies (Walton et al., 2004). *Bacillus thuringiensis* is a Gram-positive bacterium which produces one or several crystalline proteins referred to as δ-endotoxins (Hill & Pinnock, 1998). After being ingested by susceptible arthropods, the *B. thuringiensis* δ-endotoxin crystals are dissolved in the midgut with a consequent production of activated toxic polypeptides commonly known as δ-endotoxin crystal proteins (Cry proteins) (Höfte & Whiteley, 1989), which may belong to a number of distinct structural families (Crickmore et al., 2021). These toxins seem to disrupt the selective permeability of the cell membrane, which ultimately causes the arthropod death from starvation and/or septicemia (Knowles & Dow, 1993). Like *B. thuringiensis*, *Lysinibacillus sphaericus* is another gram positive bacterium able to produce a range of insecticidal proteins and which exerts its effects in a similar manner (Berry, 2012).

Essential oils (EO) are mixtures of diverse volatile compounds synthesized by plants to protect themselves and are considered as new ecofriendly insecticides, since they may show good biological activity against a number of insect pests, low toxicity to

humans and rapid degradation in the environment (Jesser et al., 2020a). However, EO show some disadvantages such as instability, volatility, and low solubility in water, which may limit their applications (Jesser et al., 2020b).

In recent years, the integration of nanotechnology in the field of biopesticides has garnered significant attention. An innovative method, the nanoformulation of EO, has emerged as a solution to shield active compounds from environmental conditions and prevent the gradual loss of EO (Kumar et al., 2020). Several materials, including proteins, synthetic emulsifiers, polysaccharides (such as starch and chitosan), and polyethers (like polyethylene glycol and poly-ε-caprolactone), have been explored for their effectiveness in nanoformulating EO or its constituents (Athanassiou et al., 2018; De Luca, et al., 2021). Polyethylene glycol 6000 (PEG 6000) has been extensively investigated over the past few decades for medical, food industry, and pest control applications. This material showed a broad range of solubility, lacks antigenicity and immunotoxicity, and is easily excreted from living organisms without toxicity concerns. PEG 6000 polymeric nanoparticles loaded with EO (EONP) are considered as one of the most important emerging trends in insect pest control (Campolo et al. 2018; Werdin et al. 2014; 2017).

Nanoformulations of *B. thuringiensis* Cry proteins and EO contribute to increase the activity period of such active compounds in the environment and allow reductions in the amount to be used (De Oliveira et al., 2014; De Oliveira et al., 2021).

The aim of this study was to test the potential acaricide effect of several treatments with *B. thuringiensis* and *Lysinibacillus sphaericus* Cry proteins and essential oils nanoparticles formulations.

2. Materials and methods

Preparation of Bacillus thuringiensis *and* Lysinibacillus sphaericus

Bacillus thuringiensis and *Lysinibacillus sphaericus* spore/crystal preparations were produced using a method previously described (Jones et al., 2008), by growing the strains in Embrapa medium (Monnerat et al., 2007) until approximately 95% sporulation (judged by phase contrast microscopy) after which spores and crystals were harvested by centrifugation and washed in distilled water before lyophilisation and storage at 4˚C. Bacterial strains used were obtained as follows: *Lysinibacillus sphaericus* strain IAB59, *Bacillus thuringiensis* serovar. *higo* strain T44001, *Bacillus thuringiensis* serovar. *israelensis* strain 4Q7, *Bacillus thuringiensis* serovar. *kurstaki* strain HD1 from the *Bacillus* Genetic Stock Center: *Bacillus thuringiensis* GP 138, a kind gift from Prof Alejandra Bravo, UNAM, Mexico; and *Bacillus thuringiensis* serovar. *konkukian* strain 97-27 and *Lysinibacillus sphaericus* strain 2362 from the collection of the Pasteur institute, France.

Preparation of nanoparticles

The EONP was synthesized using the melt-dispersion method, a procedure previously outlined by Werdin González et al. (2014). Initially, 20g of PEG 6000 were heated to 65°C on a hotplate stirrer. Subsequently, 2g of geranium (*Geranium maculatum*) or peppermint (*Mentha piperita*) EO were added to the molten PEG 6000. Geranium and peppermint oils were purchased from Swiss-Just (Switzerland). PEG 6000 was acquired from Merck, Germany. Concurrently, the mixture of PEG 6000 and EO was stirred with a D-500 Handheld Homogenizer (D-lab instrument limited) for 15 minutes at 15,000 rpm. The EONP spontaneously formed when the mixture was cooled to -4°C. After 45 minutes at that temperature, the resulting mixture was thoroughly

ground in a refrigerated mortar box at 0°C, and the product was sifted through a stainless-steel sieve with a mesh size of 230. The EONP were stored in airtight polyethylene pouches at 27 ± 2°C within desiccators containing calcium chloride for seven days before further experimentation.

EO and EOPN composition

According to Yeguerman et al. (2022), the chemical composition of both pre- and post-formulation essential oils (EOs) was analysed using gas chromatography-mass spectrometry (GC-MS) with an Agilent 7890B gas chromatograph coupled to an Agilent 5977A mass spectrometer. A HP-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) was utilized, with helium serving as the carrier gas at a flow rate of 1.0 mL min−1. The oven temperature was initially set at 50 °C for 2 minutes, then ramped at 5 °C min−1 to 200 °C and held for 15 minutes. Injection block temperature was maintained at 280 °C, and 1 μL aliquots of samples were injected. Ionization energy of 70 eV was used for mass spectrometry, scanning from 35 to 550 m/z. Retention indices (RI) of components were determined using a series of n-alkanes (C8-C20). Further identification was accomplished using NIST 2.0 database. Relative percentages of individual components were calculated by averaging gas chromatography with flame ionization detection (GC-FID) peak areas obtained on a DB-5 column under similar conditions. Key components like α-pinene, limonene, menthol, pulegone, and geraniol were confirmed by comparing with their standard samples (Sigma-Aldrich) via co-injection. Essential oils were extracted from polymeric nanoparticles (EOPN) by dissolving 0.5 g of each sample in 5 mL of distilled water, heating at 65 °C for 30 minutes with magnetic stirring. Upon melting of PEG 6000, 4 mL of petroleum ether was added, and the mixture was stirred for 2 hours. Afterward, the ether phase containing the extracted EOs was collected, diluted to a concentration of

0.001 mg mL−1 (0.1% v/v), and subjected to GC-MS and GC analysis for component identification.

EONP Size Measurement

A Malvern Nano ZS90 instrument was used to determine the size of the EONP. The Polydispersity Index (PDI) was calculated as the square of the standard deviation divided by the square of the mean size, serving as an indicator of the homogeneity or heterogeneity in the size distribution of the particles, following the method described by Pascoli et al. (2018). Each sample, consisting of 0.2 g of EONP, was suspended in 10 mL of distilled water for 30 minutes. Subsequently, the dispersion was filtered using Whatman N° 1 filter paper and allowed to equilibrate for 2 hours. Data were statistically compared using one-way analysis of variance (ANOVA), followed by the LSD test (N = 4).

EONP Encapsulation Efficiency

As outlined by Werdin González et al. (2014) the encapsulation efficiency was assessed using spectrophotometric methods. For this, 0.1 g of EONP were individually dissolved in 2 mL of an absolute ethanol-water solution (75:25). The resulting mixture was then centrifuged at 9000 rpm for 10 minutes. The supernatant was carefully collected and subjected to analysis via UV–vis spectrophotometry, employing a Shimadzu UV-1203 photometer with the Kinetics-2-Program Pack (P/N 206-62029-10; Shimadzu Corp., Kyoto, Japan) at a wavelength of 290 nm. This process was repeated for four samples, and the quantity of EO was determined by referring to an appropriate calibration curve for free EO in ethanol.

Encapsulation efficiency (EE) was determined from:

166 $$\text{EE (\%)} = \frac{\text{weight of loaded EO}}{\text{weight of initial EO}} \times 100$$

167 One way analysis of variance (ANOVA) and LSD were used in order to compare
168 the data (N = 4). The mean physical and chemical characteristics of the EONP
169 formulations are included in tables 2 and 3, respectively.

170 *Mite collection*

171 Ibex (*Capra pyrenaica*) with severe mange in the consolidation and chronic
172 stages (Espinosa et al., 2017), with lesions affecting ≥ 50% of the host skin surface were
173 selected as mite donors (Fig. 1a). The ibex were chemically immobilized with a mixture
174 of ketamine (3 mg/kg) and xylazine (3 mg/kg) (Casas-Díaz et al., 2011), and then
175 euthanized with T-61 Intervet® (combination of embutramide and mebezonium iodide)
176 at a dose of 1ml/1.5 kg.

177 For mite extraction we painted glass Petri dishes black (14 cm diameter), except
178 in a central circle at the bottom (5.5. cm diameter). Then, a 25 W lamp was placed 7-8
179 cm below the central circle and several skin pieces from the donor ibex were placed
180 around this circle (Figure 1b). In this way, a temperature gradient was created allowing
181 the mites to concentrate in the central area of the dish (Andrews, 1981) after overnight
182 exposure to the lamp (Fig. 1c). Once the mites left the host skin, the skin pieces were
183 removed (Fig. 1d). The aim of this method was to obtain live mites without
184 manipulating them, therefore, avoiding mechanical damage to mites which could affect
185 their survival.

186 The first assay (including mite extraction and following counts) was carried out
187 at 12ºC and 70% relative humidity (RH). During the remaining assays the plates were
188 maintained in an incubator at 35ºC and 45% RH. The number of control plates and
189 treatments of each assay are included in Table 1. After an initial count (including both

live and dead mites), live mites (those showing some kind of movement) were counted twice a day until the death of all the mites.

The acaricidal activity of geranium EONP and peppermint EONP was evaluated at 35 µg cm^{-2} and 70 µg cm^{-2}, respectively. The decision to use the concentration of peppermint for this nanoparticle was based on research by Jesser et al. (2020a), which indicated that the bioactivity of geranium EONP was higher than that of peppermint EONP against *Plodia interpunctella* (Lepidoptera: Pyralidae). The nanoparticles were dispersed in the central circle at the bottom base of glass Petri dish.

Statistical analysis

Survival of mites subjected to the different treatments was analysed using the non-parametric Kaplan-Meier estimate via survival curves (Kaplan and Meier, 1958). The Log-rank test (Kleinbaum and Klein, 2012) with Bonferroni correction allowed for multiple pairwise comparisons between the survival curves of each treatment in order to determine significant differences between them.

All statistical analyses were performed using R version 4.3.1 (R Core Team, 2023). We used the survfit() function to conduct the Kaplan-Meier estimations with the survival package (Therneau, 2023). The survival curves were drawn using ggsurvplot() function of the survminer package (Kassambara et al., 2020). The log-rank test was carried out using survdiff() function of the survival package. Multiple pairwise comparisons were conducted with the pairwise_survdiff() function of the survminer package. The statistical significance level set in all statistical analyses was 0.05.

3. Results and discussion

First, the chemical analysis of EOPN revealed that β-citronellol and geraniol were the predominant compounds in geranium EOPN (Table 3). Moreover, components such as linalool, menthone, citronellyl formate, and geranyl formate, which, in the pre-formulation sample, were between 8 and 11%, had a significant reduction after formulation (<1.7%). Additionally, minor components present in the original sample (<3%) were undetectable after formulation. In contrast, menthol emerged as the primary compound in peppermint oil and its nanoparticles, as indicated in Table 3. After formulation, a slight decrease was noted in the concentrations of isomenthone, p-menthen-3-one, and menthol acetate. Furthermore, minor components present in the initial sample (<6%) were not detected after formulation.

Mites in control plates survived, on average, 27.6 h. Mean and median survival times are shown in Table 3. We must take into account that this time measurement started with mite extraction, but, on average, ibex death to laboratory mite extraction took around 10.9 ± 6.1 h. As expected, average survival of mites maintained at low temperature (12 ºC) reached the highest values: 40.7 h, compared with that at 35ºC: 31.2 hr.

Kaplan-Meier survival analysis is depicted in figures 2 and 3. The overall survival function can be seen in Fig. 2a. Log-rank test (Fig. 2b) showed statistically significant differences in the mean survival time for most of the treatments (χ^2 = 38309, $p < 0.0001$). In particular, *B. thuringiensis* serovar. *konkukian* 97-27 and geranium EONP reduced mite survival significantly. Conversely, mites treated with *B. thuringiensis* GP 138, *B. thuringiensis higo* T44001, Ls IAB59 and peppermint EONP survived more than those maintained in the control plates (Table 4; Fig. 3). The remaining treatments did not show significant differences in mite survival compared with controls.

Figure 1a

Click here to access/download;Figure;VP Fig 1a.JPG

Figure 1b

Click here to access/download;Figure;VP Fig 1b.JPG

Figure 1c

Click here to access/download;Figure;VP Fig 1c.jpg

Figure 1d

Click here to access/download;Figure;VP Fig 1d.JPG

Figure 2

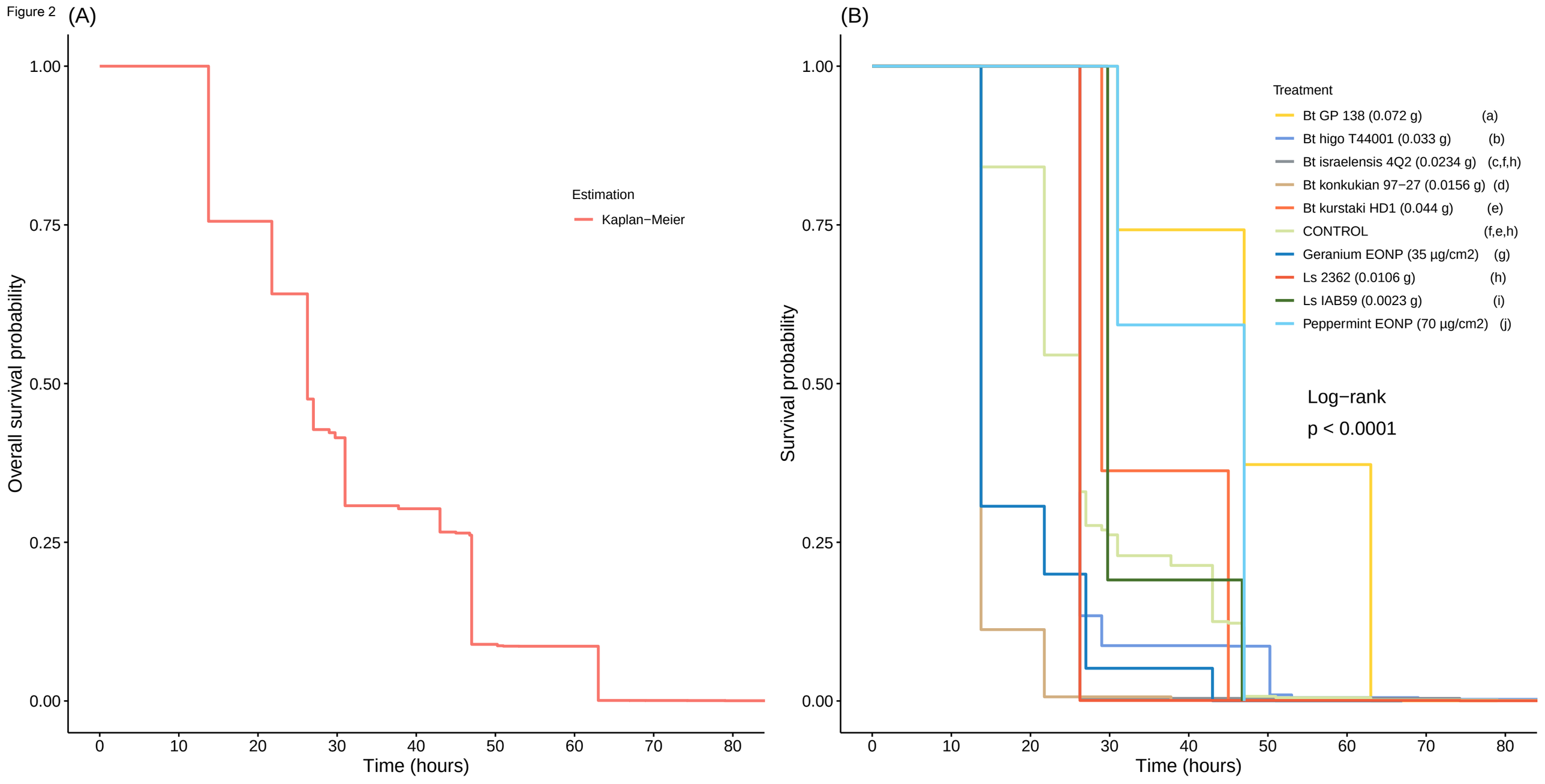
(A)
1.00
0.75
0.50
0.25
0.00
Overall survival probability
0
10
20
30
40
50
60
70
80
Time (hours)
Estimation
Kaplan−Meier
(B)
1.00
0.75
0.50
0.25
0.00
Survival probability
0
10
20
30
40
50
60
70
80
Time (hours)
Treatment
Bt GP 138 (0.072 g) (a)
Bt higo T44001 (0.033 g) (b)
Bt israelensis 4Q2 (0.0234 g) (c,f,h)
Bt konkukian 97−27 (0.0156 g) (d)
Bt kurstaki HD1 (0.044 g) (e)
CONTROL (f,e,h)
Geranium EONP (35 µg/cm2) (g)
Ls 2362 (0.0106 g) (h)
Ls IAB59 (0.0023 g) (i)
Peppermint EONP (70 µg/cm2) (j)
Log−rank
p < 0.0001

Figure 3

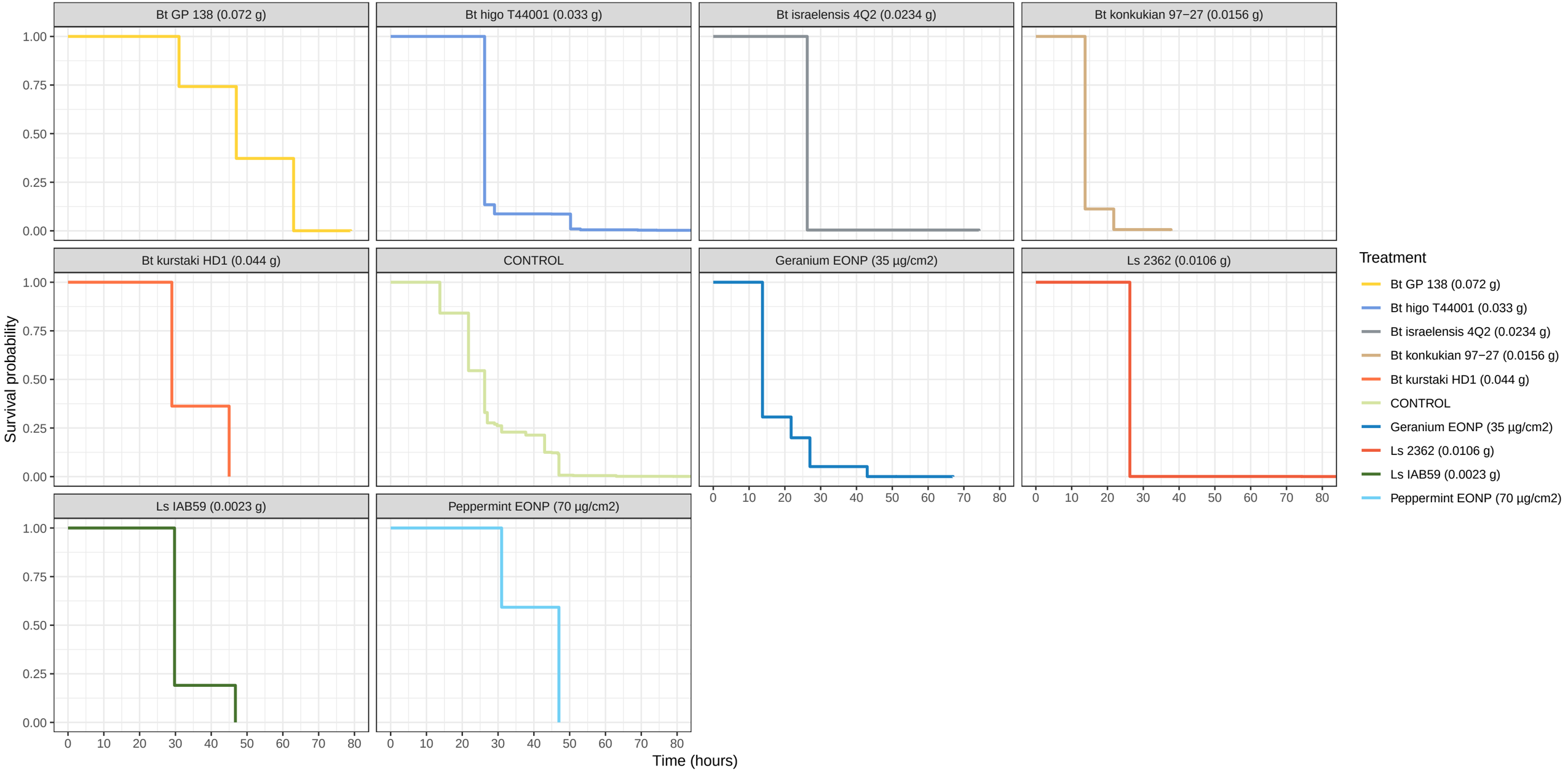
Bt GP 138 (0.072 g)
Bt higo T44001 (0.033 g)
Bt israelensis 4Q2 (0.0234 g)
Bt konkukian 97−27 (0.0156 g)
Bt kurstaki HD1 (0.044 g)
CONTROL
Geranium EONP (35 µg/cm2)
Ls 2362 (0.0106 g)
Ls IAB59 (0.0023 g)
Peppermint EONP (70 µg/cm2)
Survival probability
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
60
70
80
Time (hours)
Treatment
Bt GP 138 (0.072 g)
Bt higo T44001 (0.033 g)
Bt israelensis 4Q2 (0.0234 g)
Bt konkukian 97−27 (0.0156 g)
Bt kurstaki HD1 (0.044 g)
CONTROL
Geranium EONP (35 µg/cm2)
Ls 2362 (0.0106 g)
Ls IAB59 (0.0023 g)
Peppermint EONP (70 µg/cm2)

Table 1

	Assay 1 (19 Nov 2018)	Assay 2 (19 Apr 2021)	Assay 3 (28 Apr 2021)	Assay 4 (12 May 2021)	Assay 5 (19 May 2021)	Assay 6 (21 Jun 2021)
CONTROL	1	2	2	2	2	2
Ls 2362 (0.0106 g)	1					
Ls IAB59 (0.0023 g)				4		
Bt *israelensis* 4Q2 (0.0234 g)	1					
Bt *higo* T44001 (0.033 g)	1	2				
Bt *kurstaki* HD1 (0.044 g)		2				
Bt GP 138 (0.072 g)			2			
Bt *konkukian* 97-27 (0.0156 g)			2			
Geranium EONP (35 µg/cm^2)			2		2	
Peppermint EONP (70 µg/cm^2)						3

Table 2

	AS	PDI	EE (%)
GERANIUM EOPN	259 ± 12 **a**	0.228 ± 0.007 **a**	90.5 ± 2.32 **a**
PEPPERMINT EOPN	381 ± 29 **b**	0.532 ± 0.013 **b**	72.25 ± 1.6 **b**

[a] Different letters within the same row indicate statistical differences (LSD; $p < 0.05$).

Table 4

Treatment	**n**	**mean**	**se**	**median**
Bt GP 138 (0.072 g)	8829	48.8	0.1339	47
Bt *higo* T44001 (0.033 g)	1147	28.8	0.2713	26.2
Bt *israelensis* 4Q2 (0.0234 g)	488	26.4	0.1388	26.2
Bt *konkukian* 97-27 (0.0156 g)	2001	14.8	0.0690	13.8
Bt *kurstaki* HD1 (0.044 g)	91	34.8	0.8064	29
CONTROL	11211	27.6	0.1077	26.2
Ls 2362 (0.0106 g)	2564	26.3	0.0504	26.2
Ls IAB59 (0.0023 g)	278	33	0.4005	29.8
Geranium EONP (35 µg/cm2)	8603	18.1	0.0826	13.8
Peppermint EONP (70 µg/cm2)	3744	40.5	0.1285	47

Table 3

RT (MIN)	COMPOUNDS	GERANIUM EO		PEPPERMINT EO	
		Preformulation	Postformulation	Preformulation	Postformulation
7.16	α- pinene	-	-	1.92	-
8.36	β - pinene	-	-	1.85	-
9.87	Limonene	-	-	3.36	-
9.93	1-8 cineol	-	-	5.88	-
13.06	Linalool	12.67	9.95	-	-
13.55	Isomenthone	-	-	16.90	6.95
13.85	Menthone	11.14	1.38	-	-
14.10	Menthol	-	-	52.51	81.37
14.35	p-menten-3-ona	-	-	10.43	7.57
16.14	β-citronellol	26.14	38.12	-	-
16.48	Geraniol	23.19	47.89	-	-
16.98	Citronellyl Formate	10.37	1.71	-	-
17.70	Geranyl Formate	7.94	0.95	-	-
18.04	Menthol acetate	-	-	7.15	4.11
20.85	Geranyl Acetate	2.01	-	-	-
20.86	Caryophyllene	2.58	-	-	-
23.70	Neryl Acetate	2.98	-	-	-

4. Discussion

The *B. thuringiensis* serovar. *konkukian* and geranium EONP formulation gave promising results in our assays, and could be effective in reducing mite survival time. Different encapsulation strategies (for bacteria, Cry proteins and single spores) aimed to increase ingestion of *B. thuringiensis* Cry proteins, need to be tested. Moreover, host contact time and the effect of the temperature and UV radiation on the persistence of *B. thuringiensis* in the field must be addressed before performing *in vivo* assays (de Oliveira et al., 2021). The MXPA patent 02008705 (Ramírez, 2004) is a nanoencapsulation technique of a mixture of *B. thuringiensis* Cry proteins and spores with high residual activity. On the other hand, Ureña-Saborío et al. (2017) performed chitosan/TPP nanoparticles containing bacterial metabolic infiltrates of the strain *B. thuringiensis* SER-217, and achieved their efficient release in an aqueous medium, together with increasing protection and stability of such compounds.

B. thuringiensis strain GP 138, which has previously shown activity against the tick *Rhipicephalus microplus* (Fernández-Ruvalcaba et al., 2010), did not show any reduction in *Sarcoptes scabiei* survival in this study. *B. thuringiensis konkukian* strain 97-27, however, did show activity. This is a genome-sequenced strain of *B. thuringiensis* (Han et al., 2006) that, in contrast to other *B. thuringiensis* strains tested, is not recorded as producing known delta endotoxins or invertebrate-active toxins produced during the vegetative stage of growth. The genome does encode toxins with reported roles in mammalian food poisoning such as CytK, and the tripartite toxins Hbl and Nhe (in common with several strains of *B. thuringiensis*). The activity of these proteins against invertebrates has not been reported and it is possible that these or other, as yet uncharacterised, proteins or small molecule toxins are responsible for its activity

against *S. scabiei* in this study. This possibility warrants further investigation to identify the agent responsible for the activity observed.

This study used geranium and peppermint essential oils (EO) to formulate PEG-6000 nanoparticles, due to their bioactivity against various tick species (An and Tak, 2022; Awad et al., 2022; Klafke et al., 2021; Voronova et al., 2022). In the case of geranium oil, citronellol and geraniol are the important constituents that responsible for the acaricidal activity in *Rhipicephalus annulatus* (Ibrahium, et al., 2022). Similarly, the bioactivity of peppermint EO, attributed to menthol and isomenthone, has been observed against *Tetranychus cinnabarinus* and *Tetranychus urticae* (Abd-Allah et al., 2022). Enan (2001) suggested that the toxicity of constituents of essential oils against insect pests might be related to the octopaminergic nervous system of insects, while de Olivera et al. (1997) proposed that certain monoterpenes inhibit cytochrome P450-dependent monooxygenases. Moreover, Ryan and Byrne (1988) identified a connection between the toxicity of monoterpenes, their capacity to inhibit acetylcholinesterase (AChE), and their effectiveness against insects or ticks.

Regarding the bioactivity of nanoparticles, it was observed that geranium EONP exhibited greater efficacy compared to peppermint EOPN. This result could be attributed to the physicochemical characteristics of the nanoparticles. Peppermint EONP had size of 390 nm and were polydisperse these values are higher than geranium EONP. It is well-known that nanoparticle size plays a crucial role in the penetration of bioactive compounds through the cuticle. Hashem et al. (2018) demonstrated that EO nanoformulations enhance cuticle penetration, allowing products to penetrate insects more easily. Furthermore, the nanoscale size of EONP could extend the exposure time of bioactive compounds to insect pests, covering larger areas of the insect cuticle. Additionally, nanoparticles can alter the delivery pattern of EO active ingredients,

thereby enhancing their efficacy (Iavicoli et al., 2017). Moreover, the encapsulation efficiency (EE) of peppermint EONP was 72%, which is lower than that of geranium EONP. It will probably be necessary to use a higher amount of peppermint EONP for an effective pest management program.

In our study, temperature and relative humidity (RH) were maintained during the different assays. When off the host, *Sarcoptes* mites are unable to use water vapor actively from unsaturated ambient air (Arlian and Veselica, 1979) and, therefore, their survival time is strongly affected by ambient RH (Arlian et al., 1984). Mellanby et al. (1942) found that *S. scabiei* (obtained from human scrapings) did not move when temperature was below 15-16ºC, but did so rapidly above 20ºC; and heating at 50ºC for 10 minutes was enough to exterminate the mite. At cooler temperatures (e.g., 4 ºC) black bear-derived mites survived over a week (Niedringhaus et al., 2019). Moreover, at low temperatures, survival of *S. scabiei* increases with relative humidity (Davis & Moon, 1987; Arlian et al., 1989). Thus, environmental conditions (mainly temperature and relative humidity) will strongly affect mite survival when off the host and, therefore, its ability to be transmitted indirectly, to spread, to establish and to persist (Castro et al., 2016; Montecino-Latorre et al., 2019; Loredo et al., 2020; Browne et al., 2021).

5. Conclusion

In conclusion, the acaricidal activity of NP formulations of *B. thuringiensis konkukian* strain 97-27, of other *B. thuringiensis* strains and of other plant essential oils at different concentrations deserve to be studied in more detail, before considering *in vivo* assays. In a complementary way, the effect of both temperature and RH on the

312 survival of ibex-derived mites (when off the host) need to be analysed in depth by
313 maintaining mites *in vitro* at a wider range of such conditions.

314

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324 (Argentina) and the Jaén University (Spain) to conduct this research.

325 **CRediT authorship contribution statement**

326 All the coauthors conceived the work; MAG and CB prepared *Bacillus*
327 *thuringiensis* and *Lysinibacillus sphaericus* crystal proteins; EJ and JOW prepared
328 nanoparticles containing essential oils, JEG searched and captured donor scabietic ibex;
329 EJ, RC and JMP obtained live mites and performed the assays; EJ, JMP and AJLM
330 analysed the data obtained; all the authors contributed to writing this manuscript.

331 **Declaration of Competing Interest**

332 The authors declare that the research was conducted in the absence of any
333 commercial or financial relationships.

Data Availability

Original data are available upon reasonable request.

Ethics approval

Procedures carried out in this work were approved by the regional government (Junta de Andalucía): Project 15/12/2018/163, and also by the Ethics Committee of the Jaén University.

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571 **Tables**

572 **Table 1**. Dates of assays for analyse mite survival and the treatments tested.

573 **Table 2**. Average Size (AS), in nanometers, polydispersity (PDI), and Encapsulation
574 Efficiency (EE) of geranium and peppermint EOPN, after 7 Days Post-formulation.
575 Figures represent mean value ± standard error.

576 **Table 3.** Chemical analysis of pre / post-formulation of the oils from geranium and peppermint.

577 **Table 4**. Parameters estimated by treatment via Kaplan-Meier analysis. From left to right:
578 type of treatment, number of mites subjected to this treatment, mean survival time,
579 standard error of the mean and median survival time. Mean and median are measured in
580 hours; se: standard error.

581

582 **Figure Captions**

583 **Figure 1. A:** Skin of the scabietic donor ibex. **B:** Several ibex skin pieces were placed
584 into a painted glass Petri dish; note that the center of the plate remains transparent. **C:**
585 the light applied to the bottom of the plate generated a temperature gradient into the
586 plate. This gradient favoured mite migration from the skin to the centre of the plate. **D:**
587 protective wear was needed for skin and plates manipulation.

588 **Figure 2.** Kaplan-Meier survival curves. Left graph (A) shows the overall survival
589 curve without considering any treatments. Right graph (B) shows the survival curves by
590 treatment, the p-value of the log-rank test and the pairwise multiple comparisons with
591 Bonferroni correction. Significant differences were indicated by different lowercase
592 letters ($p<0.05$).

593 **Figure 3.** Kaplan-Meier survival curves separated for better visualization.

In vitro acaricidal activity of several natural products against ibex-derived *Sarcoptes scabiei*.

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ABSTRACT

In this study we analysed the effect of the temperature, diverse strains of *Bacillus thuringiensis*, *Lysinibacillus sphaericus* and nanoformulations with essential plant oils (EONP) on the survival of *Sarcoptes scabiei* mites derived from naturally-infested Iberian ibex (*Capra pyrenaica*). In general, mites maintained at 12ºC survived more than those maintained at 35ºC (40.7 hr and 31.2 hr, respectively). Mites with no treatment survived 27.6 h on average. Mites treated with *B. thuringiensis* serovar. *konkukian* and geranium EONP showed significant reduction in their survival. Despite the fact that these agents seem to be promising candidates for controlling sarcoptic mange in the field, further research is still needed to get stable, efficient and eco-friendly acaricides.

Keywords: *Bacillus thuringiensis*, essential plant oils, nanoparticles, *Sarcoptes scabiei*, survival

1. Introduction

Sarcoptes scabiei is an astigmatid mite causing a dermal disease, namely sarcoptic mange, in domestic and wild mammalian hosts, including man, worldwide, reaching high morbidity and mortality rates (Bornstein et al., 2001; Pence &

Ueckermann, 2002; Arlian & Morgan, 2017). Transmission of this mite between susceptible hosts may be direct, indirect, or a combination of both (Browne et al., 2022).

Control of this disease in wild populations is a challenging task. Although multiple doses of subcutaneous ivermectin (200-400 µg/kg) is the treatment most commonly used (Rowe et al., 2019), its implementation with free-ranging animals is very difficult from a logistic viewpoint. Moreover, this approach may have undesirable impact on non-target organisms and favours the development of resistance by the mite (Walton et al., 2000), among other "secondary" effects (Moroni et al., 2020).

The development of resistance of *S. scabiei* against acaricidal compounds is increasing (Currie et al., 2004; Mounsey et al., 2010; Andriantsoanirina et al., 2014). Therefore, it is crucial to develop new drugs for treating scabies (Walton et al., 2004). *Bacillus thuringiensis* is a Gram-positive bacterium which produces one or several crystalline proteins referred to as δ-endotoxins (Hill & Pinnock, 1998). After being ingested by susceptible arthropods, the *B thuringiensis* δ-endotoxin crystals are dissolved in the midgut with a consequent production of activated toxic polypeptides commonly known as δ-endotoxin crystal proteins (Cry proteins) (Höfte & Whiteley, 1989), which may belong to a number of distinct structural families (Crickmore et al., 2021). These toxins seem to disrupt the selective permeability of the cell membrane, which ultimately causes the arthropod death from starvation and/or septicemia (Knowles & Dow, 1993). Like *B. thuringiensis*, *Lysinibacillus sphaericus* is another gram positive bacterium able to produce a range of insecticidal proteins and which exerts its effects in a similar manner (Berry, 2012).

Essential oils (EO) are mixtures of diverse volatile compounds synthesized by plants to protect themselves and are considered as new ecofriendly insecticides, since they may show good biological activity against a number of insect pests, low toxicity to

humans and rapid degradation in the environment (Jesser et al., 2020a). However, EO show some disadvantages such as instability, volatility, and low solubility in water, which may limit their applications (Jesser et al., 2020b).

In recent years, the integration of nanotechnology in the field of biopesticides has garnered significant attention. An innovative method, the nanoformulation of EO, has emerged as a solution to shield active compounds from environmental conditions and prevent the gradual loss of EO (Kumar et al., 2020). Several materials, including proteins, synthetic emulsifiers, polysaccharides (such as starch and chitosan), and polyethers (like polyethylene glycol and poly-ε-caprolactone), have been explored for their effectiveness in nanoformulating EO or its constituents (Athanassiou et al., 2018; De Luca, et al., 2021). Polyethylene glycol 6000 (PEG 6000) has been extensively investigated over the past few decades for medical, food industry, and pest control applications. This material showed a broad range of solubility, lacks antigenicity and immunotoxicity, and is easily excreted from living organisms without toxicity concerns. PEG 6000 polymeric nanoparticles loaded with EO (EONP) are considered as one of the most important emerging trends in insect pest control (Campolo et al. 2018; Werdin et al. 2014; 2017).

Nanoformulations of *B. thuringiensis* Cry proteins and EO contribute to increase the activity period of such active compounds in the environment and allow reductions in the amount to be used (De Oliveira et al., 2014; De Oliveira et al., 2021).

The aim of this study was to test the potential acaricide effect of several treatments with *B. thuringiensis* and *Lysinibacillus sphaericus* Cry proteins and essential oils nanoparticles formulations.

2. Materials and methods

Preparation of Bacillus thuringiensis *and* Lysinibacillus sphaericus

Bacillus thuringiensis and *Lysinibacillus sphaericus* spore/crystal preparations were produced using a method previously described (Jones et al., 2008), by growing the strains in Embrapa medium (Monnerat et al., 2007) until approximately 95% sporulation (judged by phase contrast microscopy) after which spores and crystals were harvested by centrifugation and washed in distilled water before lyophilisation and storage at 4˚C. Bacterial strains used were obtained as follows: *Lysinibacillus sphaericus* strain IAB59, *Bacillus thuringiensis* serovar. *higo* strain T44001, *Bacillus thuringiensis* serovar. *israelensis* strain 4Q7, *Bacillus thuringiensis* serovar. *kurstaki* strain HD1 from the *Bacillus* Genetic Stock Center: *Bacillus thuringiensis* GP 138, a kind gift from Prof Alejandra Bravo, UNAM, Mexico; and *Bacillus thuringiensis* serovar. *konkukian* strain 97-27 and *Lysinibacillus sphaericus* strain 2362 from the collection of the Pasteur institute, France.

Preparation of nanoparticles

The EONP was synthesized using the melt-dispersion method, a procedure previously outlined by Werdin González et al. (2014). Initially, 20g of PEG 6000 were heated to 65°C on a hotplate stirrer. Subsequently, 2g of geranium (*Geranium maculatum*) or peppermint (*Mentha piperita*) EO were added to the molten PEG 6000. Geranium and peppermint oils were purchased from Swiss-Just (Switzerland). PEG 6000 was acquired from Merck, Germany. Concurrently, the mixture of PEG 6000 and EO was stirred with a D-500 Handheld Homogenizer (D-lab instrument limited) for 15 minutes at 15,000 rpm. The EONP spontaneously formed when the mixture was cooled to -4°C. After 45 minutes at that temperature, the resulting mixture was thoroughly

ground in a refrigerated mortar box at 0°C, and the product was sifted through a stainless-steel sieve with a mesh size of 230. The EONP were stored in airtight polyethylene pouches at 27 ± 2°C within desiccators containing calcium chloride for seven days before further experimentation.

EO and EOPN composition

According to Yeguerman et al. (2022), the chemical composition of both pre- and post-formulation essential oils (EOs) was analysed using gas chromatography-mass spectrometry (GC-MS) with an Agilent 7890B gas chromatograph coupled to an Agilent 5977A mass spectrometer. A HP-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) was utilized, with helium serving as the carrier gas at a flow rate of 1.0 mL min−1. The oven temperature was initially set at 50 °C for 2 minutes, then ramped at 5 °C min−1 to 200 °C and held for 15 minutes. Injection block temperature was maintained at 280 °C, and 1 μL aliquots of samples were injected. Ionization energy of 70 eV was used for mass spectrometry, scanning from 35 to 550 m/z. Retention indices (RI) of components were determined using a series of n-alkanes (C8-C20). Further identification was accomplished using NIST 2.0 database. Relative percentages of individual components were calculated by averaging gas chromatography with flame ionization detection (GC-FID) peak areas obtained on a DB-5 column under similar conditions. Key components like α-pinene, limonene, menthol, pulegone, and geraniol were confirmed by comparing with their standard samples (Sigma-Aldrich) via co-injection. Essential oils were extracted from polymeric nanoparticles (EOPN) by dissolving 0.5 g of each sample in 5 mL of distilled water, heating at 65 °C for 30 minutes with magnetic stirring. Upon melting of PEG 6000, 4 mL of petroleum ether was added, and the mixture was stirred for 2 hours. Afterward, the ether phase containing the extracted EOs was collected, diluted to a concentration of

143 0.001 mg mL−1 (0.1% v/v), and subjected to GC-MS and GC analysis for component
144 identification.

145 *EONP Size Measurement*

146 A Malvern Nano ZS90 instrument was used to determine the size of the EONP.
147 The Polydispersity Index (PDI) was calculated as the square of the standard deviation
148 divided by the square of the mean size, serving as an indicator of the homogeneity or
149 heterogeneity in the size distribution of the particles, following the method described by
150 Pascoli et al. (2018). Each sample, consisting of 0.2 g of EONP, was suspended in 10
151 mL of distilled water for 30 minutes. Subsequently, the dispersion was filtered using
152 Whatman N° 1 filter paper and allowed to equilibrate for 2 hours. Data were statistically
153 compared using one-way analysis of variance (ANOVA), followed by the LSD test (N
154 = 4).

155 *EONP Encapsulation Efficiency*

156 As outlined by Werdin González et al. (2014) the encapsulation efficiency was
157 assessed using spectrophotometric methods. For this, 0.1 g of EONP were individually
158 dissolved in 2 mL of an absolute ethanol-water solution (75:25). The resulting mixture
159 was then centrifuged at 9000 rpm for 10 minutes. The supernatant was carefully
160 collected and subjected to analysis via UV–vis spectrophotometry, employing a
161 Shimadzu UV-1203 photometer with the Kinetics-2-Program Pack (P/N 206-62029-10;
162 Shimadzu Corp., Kyoto, Japan) at a wavelength of 290 nm. This process was repeated
163 for four samples, and the quantity of EO was determined by referring to an appropriate
164 calibration curve for free EO in ethanol.

165 Encapsulation efficiency (EE) was determined from:

166 $$\text{EE (\%)} = \frac{\text{weight of loaded EO}}{\text{weight of initial EO}} \times 100$$

167 One way analysis of variance (ANOVA) and LSD were used in order to compare
168 the data (N = 4). The mean physical and chemical characteristics of the EONP
169 formulations are included in tables 2 and 3, respectively.

170 *Mite collection*

171 Ibex (*Capra pyrenaica*) with severe mange in the consolidation and chronic
172 stages (Espinosa et al., 2017), with lesions affecting ≥ 50% of the host skin surface were
173 selected as mite donors (Fig. 1a). The ibex were chemically immobilized with a mixture
174 of ketamine (3 mg/kg) and xylazine (3 mg/kg) (Casas-Díaz et al., 2011), and then
175 euthanized with T-61 Intervet® (combination of embutramide and mebezonium iodide)
176 at a dose of 1ml/1.5 kg.

177 For mite extraction we painted glass Petri dishes black (14 cm diameter), except
178 in a central circle at the bottom (5.5. cm diameter). Then, a 25 W lamp was placed 7-8
179 cm below the central circle and several skin pieces from the donor ibex were placed
180 around this circle (Figure 1b). In this way, a temperature gradient was created allowing
181 the mites to concentrate in the central area of the dish (Andrews, 1981) after overnight
182 exposure to the lamp (Fig. 1c). Once the mites left the host skin, the skin pieces were
183 removed (Fig. 1d). The aim of this method was to obtain live mites without
184 manipulating them, therefore, avoiding mechanical damage to mites which could affect
185 their survival.

186 The first assay (including mite extraction and following counts) was carried out
187 at 12ºC and 70% relative humidity (RH). During the remaining assays the plates were
188 maintained in an incubator at 35ºC and 45% RH. The number of control plates and
189 treatments of each assay are included in Table 1. After an initial count (including both

live and dead mites), live mites (those showing some kind of movement) were counted twice a day until the death of all the mites.

The acaricidal activity of geranium EONP and peppermint EONP was evaluated at 35 µg cm^{-2} and 70 µg cm^{-2}, respectively. The decision to use the concentration of peppermint for this nanoparticle was based on research by Jesser et al. (2020a), which indicated that the bioactivity of geranium EONP was higher than that of peppermint EONP against *Plodia interpunctella* (Lepidoptera: Pyralidae). The nanoparticles were dispersed in the central circle at the bottom base of glass Petri dish.

Statistical analysis

Survival of mites subjected to the different treatments was analysed using the non-parametric Kaplan-Meier estimate via survival curves (Kaplan and Meier, 1958). The Log-rank test (Kleinbaum and Klein, 2012) with Bonferroni correction allowed for multiple pairwise comparisons between the survival curves of each treatment in order to determine significant differences between them.

All statistical analyses were performed using R version 4.3.1 (R Core Team, 2023). We used the survfit() function to conduct the Kaplan-Meier estimations with the survival package (Therneau, 2023). The survival curves were drawn using ggsurvplot() function of the survminer package (Kassambara et al., 2020). The log-rank test was carried out using survdiff() function of the survival package. Multiple pairwise comparisons were conducted with the pairwise_survdiff() function of the survminer package. The statistical significance level set in all statistical analyses was 0.05.

3. Results and discussion

First, the chemical analysis of EOPN revealed that β-citronellol and geraniol were the predominant compounds in geranium EOPN (Table 3). Moreover, components such as linalool, menthone, citronellyl formate, and geranyl formate, which, in the pre-formulation sample, were between 8 and 11%, had a significant reduction after formulation (<1.7%). Additionally, minor components present in the original sample (<3%) were undetectable after formulation. In contrast, menthol emerged as the primary compound in peppermint oil and its nanoparticles, as indicated in Table 3. After formulation, a slight decrease was noted in the concentrations of isomenthone, p-menthen-3-one, and menthol acetate. Furthermore, minor components present in the initial sample (<6%) were not detected after formulation.

Mites in control plates survived, on average, 27.6 h. Mean and median survival times are shown in Table 3. We must take into account that this time measurement started with mite extraction, but, on average, ibex death to laboratory mite extraction took around 10.9 ± 6.1 h. As expected, average survival of mites maintained at low temperature (12 ºC) reached the highest values: 40.7 h, compared with that at 35ºC: 31.2 hr.

Kaplan-Meier survival analysis is depicted in figures 2 and 3. The overall survival function can be seen in Fig. 2a. Log-rank test (Fig. 2b) showed statistically significant differences in the mean survival time for most of the treatments (χ^2 = 38309, $p < 0.0001$). In particular, *B. thuringiensis* serovar. *konkukian* 97-27 and geranium EONP reduced mite survival significantly. Conversely, mites treated with *B. thuringiensis* GP 138, *B. thuringiensis higo* T44001, Ls IAB59 and peppermint EONP survived more than those maintained in the control plates (Table 4; Fig. 3). The remaining treatments did not show significant differences in mite survival compared with controls.

4. Discussion

The *B. thuringiensis* serovar. *konkukian* and geranium EONP formulation gave promising results in our assays, and could be effective in reducing mite survival time. Different encapsulation strategies (for bacteria, Cry proteins and single spores) aimed to increase ingestion of *B. thuringiensis* Cry proteins, need to be tested. Moreover, host contact time and the effect of the temperature and UV radiation on the persistence of *B. thuringiensis* in the field must be addressed before performing *in vivo* assays (de Oliveira et al., 2021). The MXPA patent 02008705 (Ramírez, 2004) is a nanoencapsulation technique of a mixture of *B. thuringiensis* Cry proteins and spores with high residual activity. On the other hand, Ureña-Saborío et al. (2017) performed chitosan/TPP nanoparticles containing bacterial metabolic infiltrates of the strain *B. thuringiensis* SER-217, and achieved their efficient release in an aqueous medium, together with increasing protection and stability of such compounds.

B. thuringiensis strain GP 138, which has previously shown activity against the tick *Rhipicephalus microplus* (Fernández-Ruvalcaba et al., 2010), did not show any reduction in *Sarcoptes scabiei* survival in this study. *B. thuringiensis konkukian* strain 97-27, however, did show activity. This is a genome-sequenced strain of *B. thuringiensis* (Han et al., 2006) that, in contrast to other *B. thuringiensis* strains tested, is not recorded as producing known delta endotoxins or invertebrate-active toxins produced during the vegetative stage of growth. The genome does encode toxins with reported roles in mammalian food poisoning such as CytK, and the tripartite toxins Hbl and Nhe (in common with several strains of *B. thuringiensis*). The activity of these proteins against invertebrates has not been reported and it is possible that these or other, as yet uncharacterised, proteins or small molecule toxins are responsible for its activity

against *S. scabiei* in this study. This possibility warrants further investigation to identify the agent responsible for the activity observed.

This study used geranium and peppermint essential oils (EO) to formulate PEG-6000 nanoparticles, due to their bioactivity against various tick species (An and Tak, 2022; Awad et al., 2022; Klafke et al., 2021; Voronova et al., 2022). In the case of geranium oil, citronellol and geraniol are the important constituents that responsible for the acaricidal activity in *Rhipicephalus annulatus* (Ibrahium, et al., 2022). Similarly, the bioactivity of peppermint EO, attributed to menthol and isomenthone, has been observed against *Tetranychus cinnabarinus* and *Tetranychus urticae* (Abd-Allah et al., 2022). Enan (2001) suggested that the toxicity of constituents of essential oils against insect pests might be related to the octopaminergic nervous system of insects, while de Olivera et al. (1997) proposed that certain monoterpenes inhibit cytochrome P450-dependent monooxygenases. Moreover, Ryan and Byrne (1988) identified a connection between the toxicity of monoterpenes, their capacity to inhibit acetylcholinesterase (AChE), and their effectiveness against insects or ticks.

Regarding the bioactivity of nanoparticles, it was observed that geranium EONP exhibited greater efficacy compared to peppermint EOPN. This result could be attributed to the physicochemical characteristics of the nanoparticles. Peppermint EONP had size of 390 nm and were polydisperse these values are higher than geranium EONP. It is well-known that nanoparticle size plays a crucial role in the penetration of bioactive compounds through the cuticle. Hashem et al. (2018) demonstrated that EO nanoformulations enhance cuticle penetration, allowing products to penetrate insects more easily. Furthermore, the nanoscale size of EONP could extend the exposure time of bioactive compounds to insect pests, covering larger areas of the insect cuticle. Additionally, nanoparticles can alter the delivery pattern of EO active ingredients,

thereby enhancing their efficacy (Iavicoli et al., 2017). Moreover, the encapsulation efficiency (EE) of peppermint EONP was 72%, which is lower than that of geranium EONP. It will probably be necessary to use a higher amount of peppermint EONP for an effective pest management program.

In our study, temperature and relative humidity (RH) were maintained during the different assays. When off the host, *Sarcoptes* mites are unable to use water vapor actively from unsaturated ambient air (Arlian and Veselica, 1979) and, therefore, their survival time is strongly affected by ambient RH (Arlian et al., 1984). Mellanby et al. (1942) found that *S. scabiei* (obtained from human scrapings) did not move when temperature was below 15-16ºC, but did so rapidly above 20ºC; and heating at 50ºC for 10 minutes was enough to exterminate the mite. At cooler temperatures (e.g., 4 ºC) black bear-derived mites survived over a week (Niedringhaus et al., 2019). Moreover, at low temperatures, survival of *S. scabiei* increases with relative humidity (Davis & Moon, 1987; Arlian et al., 1989). Thus, environmental conditions (mainly temperature and relative humidity) will strongly affect mite survival when off the host and, therefore, its ability to be transmitted indirectly, to spread, to establish and to persist (Castro et al., 2016; Montecino-Latorre et al., 2019; Loredo et al., 2020; Browne et al., 2021).

5. Conclusion

In conclusion, the acaricidal activity of NP formulations of *B. thuringiensis konkukian* strain 97-27, of other *B. thuringiensis* strains and of other plant essential oils at different concentrations deserve to be studied in more detail, before considering *in vivo* assays. In a complementary way, the effect of both temperature and RH on the

312 survival of ibex-derived mites (when off the host) need to be analysed in depth by
313 maintaining mites *in vitro* at a wider range of such conditions.

314

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325 **CRediT authorship contribution statement**

326 All the coauthors conceived the work; MAG and CB prepared *Bacillus*
327 *thuringiensis* and *Lysinibacillus sphaericus* crystal proteins; EJ and JOW prepared
328 nanoparticles containing essential oils, JEG searched and captured donor scabietic ibex;
329 EJ, RC and JMP obtained live mites and performed the assays; EJ, JMP and AJLM
330 analysed the data obtained; all the authors contributed to writing this manuscript.

331 **Declaration of Competing Interest**

332 The authors declare that the research was conducted in the absence of any
333 commercial or financial relationships.

Data Availability

Original data are available upon reasonable request.

Ethics approval

Procedures carried out in this work were approved by the regional government (Junta de Andalucía): Project 15/12/2018/163, and also by the Ethics Committee of the Jaén University.

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571 **Tables**

572 **Table 1**. Dates of assays for analyse mite survival and the treatments tested.

573 **Table 2**. Average Size (AS), in nanometers, polydispersity (PDI), and Encapsulation
574 Efficiency (EE) of geranium and peppermint EOPN, after 7 Days Post-formulation.
575 Figures represent mean value ± standard error.

576 **Table 3.** Chemical analysis of pre / post-formulation of the oils from geranium and peppermint.

577 **Table 4**. Parameters estimated by treatment via Kaplan-Meier analysis. From left to right:
578 type of treatment, number of mites subjected to this treatment, mean survival time,
579 standard error of the mean and median survival time. Mean and median are measured in
580 hours; se: standard error.

581

582 **Figure Captions**

583 **Figure 1. A:** Skin of the scabietic donor ibex. **B:** Several ibex skin pieces were placed
584 into a painted glass Petri dish; note that the center of the plate remains transparent. **C:**
585 the light applied to the bottom of the plate generated a temperature gradient into the
586 plate. This gradient favoured mite migration from the skin to the centre of the plate. **D:**
587 protective wear was needed for skin and plates manipulation.

588 **Figure 2.** Kaplan-Meier survival curves. Left graph (A) shows the overall survival
589 curve without considering any treatments. Right graph (B) shows the survival curves by
590 treatment, the p-value of the log-rank test and the pairwise multiple comparisons with
591 Bonferroni correction. Significant differences were indicated by different lowercase
592 letters ($p<0.05$).

593 **Figure 3.** Kaplan-Meier survival curves separated for better visualization.

Graphical Abstract