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Anisakis simplex larvae viability and potential pathogenicity in vitro is controlled by the essential oil from sea fennel (*Crithmum maritinum* L., Apiaceae)

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ABSTRACT

Essential oils, natural volatile products extracted from aromatic plants, exhibit a wide range of biological activities, including antimicrobial and antiparasitic properties. *Anisakis simplex* is a parasitic nematode commonly found in marine fish, which poses a significant health risk to humans through the consumption of raw or undercooked seafood, leading to anisakiasis, a zoonotic disease with both gastrointestinal and allergic manifestations. In this study, the impact of *Crithmum maritimum* L. (Apiaceae) essential oil on this nematode was evaluated in vitro. The chemical composition of *C. maritimum* essential oil was determined by gas chromatography–mass spectrometry (GC–MS). The analysis revealed γ-terpinene, limonene, and sabinene as the major components. The nematicidal activity, larval penetration ability, resistance to acidic pH, and inhibition of acetylcholinesterase were evaluated. The essential oil effects on interleukin production and cytotoxicity in HepG2 and Caco-2 cells were also assessed. *C. maritimun* exhibited larvicidal activity, with LD_{50} values of 619.11 μg mL^{-1} at 24 h and 353.95 μg mL^{-1} at 48 h. Additionally, it showed no significant cytotoxicity in HepG2 and Caco-2 cell lines at the concentrations tested. In Caco-2 cells stimulated with crude *Anisakis* extract, the essential oil modulated interleukin production. Furthermore, it demonstrated in vitro inhibitory activity against 5-lipoxygenase and phospholipase A_2, with IC_{50} values of 99.35 and 253.31 μg mL^{-1}, respectively. The traditional culinary use of *C. maritimum*, together with the observed anti-*Anisakis* activity in our study, suggest this essential oil may offer a promising complementary approach within broader integrated food safety strategies, especially in *Anisakis* control.

1. Introduction

Anisakiasis is a fish-borne zoonotic disease caused by the ingestion of third-stage larvae of nematodes of the genus *Anisakis* present in raw or undercooked fish and cephalopods. After ingestion, the larvae can invade the gastrointestinal mucosa and cause acute

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gastrointestinal symptoms, most commonly abdominal pain, nausea, and vomiting. The incidence of anisakiasis has increased in recent years, particularly in regions where the consumption of raw or minimally processed seafood is common (Suzuki et al., 2021).

These parasitic nematodes exhibit a complex heteroxenous life cycle in which odontocete cetaceans act as definitive hosts. Adult worms inhabit the gastrointestinal tract of these marine mammals, where sexual reproduction occurs and eggs are released into the marine environment via feces. Under suitable conditions, eggs embryonate and hatch, giving rise to early larval stages that are ingested by marine crustaceans, which act as the first intermediate hosts. Within these organisms, the larvae continue their development before reaching fish or cephalopods, where they persist as third-stage larvae and are subsequently transferred through trophic interactions among species acting as intermediate or paratenic hosts (Aibinu et al., 2019; Bao et al., 2025; Grabda, 1976).

Given the parasite's complex lifecycle and wide range of intermediate and paratenic hosts, complete control remains challenging. Prevention of anisakiasis primarily relies on avoiding the ingestion of viable *Anisakis* larvae, which are often present in raw or undercooked fish and cephalopods. Although traditional preparation methods such as marinating, smoking, brining, or pickling are widely used, they do not reliably inactivate all larvae, making thermal treatments, either freezing or cooking, the most effective strategy for ensuring food safety (Allende et al., 2024; Koutsoumanis et al., 2024). Immediate evisceration of fish after capture has been suggested as a method to reduce larval migration into muscle tissue; however, when done at sea, discarded viscera may be consumed by other fish, potentially increasing parasite prevalence in marine populations (Allende et al., 2024; Koutsoumanis et al., 2024).

The European Food Safety Authority (EFSA) considers *Anisakis* spp. a highly relevant zoonotic parasite in fishery products and uses it as a key indicator of parasitological risk in wild-caught marine fish, due to its widespread occurrence and ability to infect multiple fish species (Koutsoumanis et al., 2024). Furthermore, climate-related shifts in marine ecosystems, particularly rising sea surface temperatures may be contributing to changes in the distribution and prevalence of *Anisakis* spp. (Allende et al., 2024). The widespread habit of eating raw or lightly cooked fish in traditional dishes such as sushi, sashimi, ceviche, or marinated anchovies further increases exposure risk. While Japan reports the highest number of cases annually due to its culinary traditions, anisakiasis is also a growing public health concern in Mediterranean countries (Guardone et al., 2018; Herrador et al., 2019). In particular, Italy represents the country with more annual reports on the Rapid Alert System Feed and Food (RASFF) concerning the detection of *Anisakis* spp. in fishery products in recent years (Caldeira et al., 2021).

Despite this global distribution, the condition is frequently underdiagnosed or misdiagnosed as other gastrointestinal disorders such as appendicitis or diverticulitis, contributing to underreporting (Aibinu et al., 2019). The pathogenesis of anisakiasis involves larval invasion of the gastrointestinal wall, triggering acute inflammation. Clinically, it presents in gastric, intestinal, ectopic, or allergic forms. In ectopic cases, larvae can migrate beyond the gut and reach organs such as the liver or spleen (Nonković et al., 2025). Treatment of anisakiasis involves the endoscopic removal of larvae from the gastrointestinal tract, either via gastroscopy or colonoscopy, depending on the site of infection (Buchmann and Mehrdana, 2016; Shimamura et al., 2016). Although antiparasitic agents such as albendazole have shown activity against *Anisakis* larvae, their efficacy is not consistent and may be reduced under acidic conditions, suggesting limited effectiveness in some clinical scenarios (Arias-Diaz et al., 2006). Therefore, ongoing studies aim to develop new approaches to manage *Anisakis* infections (Kumas et al., 2025).

Essential oils, mixtures of natural volatile compounds obtained from aromatic and medicinal plants through steam or hydrodistillation, have been proposed as potential agents for the management of *Anisakis* spp. in either food or health settings. In fact, some essential oil such as those from compact Oregano (*Origanum compactum* Benth.), syrian oregano (*Origanum syriacum* (Boiss.) Kuntze), and winter savory (*Satureja montana* L.) have previously shown effects against this parasite (Les et al., 2024; López et al., 2018, 2019; Valero et al., 2015). However, to our knowledge, the effects of *Crithmum maritimum* L. (Apiaceae) (*C. maritimum)* essential oil on this pathogen have not been described before. Commonly known as sea fennel, *C. maritimum* is a perennial facultative halophyte well adapted to saline environments. The plant's resilience to high salinity and its capacity to colonize harsh maritime habitats make it a characteristic species of temperate coastal ecosystems, including sandy beaches, rocky cliffs, breakwaters, and piers. In fact, it is native from the Mediterranean and Atlantic coasts of Southern and Western Europe, and it also extends to certain parts of North America, as well as Central and Western Asia (Kraouia et al., 2023; Renna, 2018). *Crithmum maritimum* is widely used in the cuisine of coastal areas, especially in Mediterranean countries, to accompany fish dishes, cured meats, cheeses and pizza and is also consumed as a side dish or incorporated into oils, sauces, and pestos (Renna and Gonnella, 2012). Given its historical use as a food *C. maritimum* has been included in the BELFRIT list of botanicals to be used in food supplements (Cousyn et al., 2013).

The aim of this study was to investigate the effects of *Crithmum maritimum* essential oil on *Anisakis* viability and on key processes involved in anisakiasis such as larval penetration capacity, resistance to acidic pH, and enzyme inhibition related to potential mechanisms of action. Additionally, the impact of the essential oil on mammalian cells was evaluated.

2. Material and methods

2.1. Plant material and essential oil isolation

The seeds of *C. maritimum* were kindly furnished by the company Rinci S.r.l. They came from a cultivation site located in the Municipality of Camerano (AN), Italy (N 43°32′08″; E 13°33′03″, 135 m a.s.l.) and were harvested in October 2021. For the preparation of the essential oil, the same methodology reported by Kavallieratos et al. (2024) with minor modification has been employed (Kavallieratos et al., 2024). A total of 500 g of schizocarps were inserted into a round bottom flask (Falc MA, Falc Instruments, Treviglio, Italy) with 5 L of deionized water and distilled for 6 h. The essential oil was collected from a Clevenger-type apparatus with a yield of 1.85%, estimated on a dry weight basis.

2.2. Gas chromatography-mass spectrometry (GC–MS) analysis of C. Maritimun essential oil

The GC–MS analysis was performed in an Agilent 8890 gas chromatograph combined with a 5977B mass spectrometer purchased from Agilent (Santa Clara, California, USA) and a PAL RTC 120 autosampler (CTC Analytics AG, Zwingen, Switzerland). The separation on the HP-5 MS capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness), identification and semi-quantification of compounds was achieved by following the same analytical conditions employed by Piatti et al. (2023). For peak assignment we also used co-elution with the following analytical standards purchased from Merck (Milan, Italy): α-pinene, β-pinene, myrcene, *p*-cymene, limonene, β-phellandrene, γ-terpinene, terpinolene, terpinen-4-ol, a-terpineol, thymol, dillapiole. *β*-Phellandrene and limonene were chromatographically separated using a DB-WAX capillary column (60 m, 250 μm id, 0.25 μm film thickness) as stationary phase and they were identified through the comparison between the retention indexes (RIs) and mass spectra reported in the NIST 17 library. Thus, the temperature of the oven was modified as follows: 5 min at 60 °C, then increased to 220 °C at a rate of 4 °C/min, followed by a ramp to 250 °C at 11 °C/min, and held at this temperature for 15 min.

2.3. Anisakis assays

2.3.1. Larvae collection

Third-stage larvae (L3) of *A. simplex* sensu lato (s.l.) were isolated from infected fish specimens, obtained from various fish markets in Zaragoza, Spain. The larvae were washed with a sterile 0.9% NaCl solution to remove residual matter. Only intact L3 of *A. simplex* s. l. with length over 2.0 cm were included, identification being performed by light microscopy following established morphological criteria previously described (López et al., 2019).

2.3.2. Nematicidal effect and LD_{50} determination

Nematicidal effect assessment was conducted in 6-well plates as previously described (Les et al., 2024). 10 larvae in a final volume of 2 mL sterile 0.9% NaCl with different concentrations of the essential oil solutions were added to each well. Negative control wells contained only sterile saline solution. After incubation at 37 °C, larval viability was assessed at 24 and 48 h by visual inspection, based on the presence or absence of spontaneous movement and movement after gentle mechanical stimulation. Larvae were considered dead when no movement was observed after stimulation. For each treatment concentration, larval mortality was calculated as the percentage of dead larvae and normalized to the negative control to account for background mortality. Assays were performed each with three technical replicates, meaning 30 larvae per condition. Three independent experiments were performed. Concentrations tested ranged from 31.25 to 1000 μg mL^{-1}. The mean mortality rate was calculated for each treatment and concentration.

To assess the nematicidal effect of the essential oil upon *Anisakis* larvae in acid media the same assay explained as described above was performed, changing the NaCl solution for the artificial gastric juice. Artificial gastric juice was prepared, consisting of 0.1% pepsin (Sigma-Aldrich, Barcelona, Spain), 0.1% porcine stomach mucin (Sigma-Aldrich, Barcelona, Spain), 0.12% NaCl, and 0.02% KCl, adjusted to pH 1.8. The plates were incubated at 37 °C for 24 h. Both a negative control in sterile saline solution and a negative control in artificial gastric juice were included, neither of which received any treatment with the essential oil. All assays were performed in triplicate.

2.3.3. Penetration assay

To assess the impact on the infective ability of *Anisakis simplex* s. l. L3 larvae, an agar penetration assay was conducted with slight modifications to previously described methods (López et al., 2019). Agar plates were prepared using RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% agar. The mixture was autoclaved and 2 mL per well were dispensed into 12-well plates and allowed to solidify at room temperature. The plates were stored at 4 °C until use. Larvae were incubated at 37 °C for 1 h with the essential oil at the determined LD_{50} concentration, after which only larvae exhibiting spontaneous movement were selected, washed twice with sterile 0.9% NaCl solution, and placed on the agar surface. Each well contained 5 larvae along with 100 μL of penetration medium (PBS + 1% commercial pepsin (Sigma-Aldrich, Barcelona, Spain), pH 4.0), while control wells contained untreated larvae. The plates were incubated at 37 °C. Three independent experiments each consisting of 20 larvae each (four wells) were performed. The penetration ability of *Anisakis* larvae was assessed at 2, 4, and 24 h. At each time point, the number of larvae that successfully penetrated the substrate was recorded. The percentage of penetration was calculated as the number of penetrating larvae relative to the initial number of larvae in each experimental group.

2.3.4. Resistance in acidic medium

To evaluate the effectiveness of the essential oil under acidic conditions and at sublethal concentrations, an artificial gastric juice medium was prepared as described in 2.3.2. This assay aimed to determine whether exposure to the LD_{50} of the essential oil could reduce resistance to acidic conditions, potentially affecting their infectivity. Both a control without treatment in artificial gastric juice and a control in sterile phosphate-buffered saline (PBS, pH 7) were prepared. Prior to incubation, larvae were exposed to the essential oil or control solution (sterile PBS) for 1 h, then washed twice with sterile saline solution. The larvae were transferred to 6-well plates containing 10 larvae each well and 2 mL of either artificial gastric juice or PBS and incubated at 37 °C for 24 h. Larval motility was assessed at 2, 4, and 24 h. The assay was performed in triplicate to ensure reproducibility.

2.4. Enzymatic assays

2.4.1. Acetylcholinesterase

Acetylcholinesterase (AChE) mediates the degradation of acetylcholine, which functions as a neurotransmitter in *Anisakis* and other parasites. The AChE inhibition assay was conducted following a modified version of (López et al., 2019). A concentration range of 31.25–1000 μg mL^{-1} of *C. maritimun* essential oil was tested. Acetylthiocholine iodide (ATCI) (Panreac Barcelona, Spain) was used as the enzymatic substrate, while AChE enzyme (Panreac Barcelona, Spain) and Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid), DTNB) (Panreac Barcelona, Spain) were used for detection. Buffer solutions were prepared as follows: 50 mM Tris-HCl (pH 8) and 50 mM Tris-HCl (pH 8) supplemented with 0.1% bovine serum albumin (BSA) (Proquinorte Biscay, Spain). The test samples were dissolved in dimethyl sulfoxide (DMSO), and the enzymatic reaction was performed in 96 well plates. The assay was conducted by mixing the enzyme with the substrate and DTNB reagent, followed by the addition of the test compound. In order to determine AChE inhibition, absorbance was measured at 412 nm with a BioTek Synergy H1 microplate reader (BioTek® Instruments, Inc., Winooski, VT, USA). All reactions were performed in triplicate to ensure reproducibility.

2.4.2. 5-lipoxygenase (5-LOX) assay

The enzyme 5-LOX catalyzes the transformation of arachidonic acid into leukotrienes, key mediators in inflammatory processes. Its inhibitory activity was assessed by tracking the oxidation of linoleic acid to 13-hydroperoxyoctadecadienoic acid (13-HPODE), following the protocol described by Kachmar et al. (2019) (Kachmar et al., 2019). The essential oil was initially dissolved in ethanol at a final concentration of 0.5%, previously confirmed to have no effect on enzyme activity. Solvent-treated controls were also made. These stock solutions were then diluted in a sodium phosphate buffer ($Na_2HPO_4{\cdot}2H_2O$, pH 9). Concentrations ranging from 7.81 to 250 μg mL^{-1} were evaluated. Reactions were carried out in UV-transparent, flat-bottom 96-well microplates (Corning®), with each well containing buffer, the diluted sample, and 20 μL (100 units) of soybean-derived 5-LOX (*Glycine* max) obtained from Sigma-Aldrich (Barcelona, Spain). After a 5-min incubation at ambient temperature, 20 μL of a 4.18 mM linoleic acid solution (Sigma-Aldrich, Barcelona, Spain) were added to initiate the reaction. Absorbance was measured at 234 nm using a BioTek Synergy H1 microplate reader (BioTek® Instruments, Inc., Winooski, VT, USA). All assays were performed in triplicate to ensure reproducibility.

2.4.3. Phospholipase a_2 kit

The essential oil potential to inhibit the enzymatic activity of phospholipase A_2 (PLA_2) was determined using the sPLA₂ (Type V) Inhibitor Screening Assay Kit (PC Substrate) from Cayman Chemical. The assay was performed in 96-well clear flat-bottom plates using diheptanoyl thio-PC as the substrate, which upon hydrolysis by PLA_2 releases thiols that react with Ellman's reagent (DTNB), generating a detectable colorimetric signal. The reaction mixture consisted of assay buffer, substrate, DTNB, and recombinant human Type V sPLA₂ enzyme. Absorbance was measured at 405–420 nm using a BioTek Synergy H1 microplate reader (BioTek® Instruments, Inc., Winooski, VT, USA). A positive control inhibitor was included to validate the assay performance. The experiments were conducted in triplicate to ensure reproducibility. A concentration range of 15.62–500 μg mL^{-1} of *C. maritimun* essential oil was tested.

2.5. Cell assays

2.5.1. Cell culture

HepG2 (human hepatocellular carcinoma, HB-8065) and Caco-2 (human colorectal adenocarcinoma) were purchased from the American Type Culture Collection (Rockville, MD, USA), maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Barcelona, Spain) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, Barcelona, Spain) and 1% penicillin/streptomycin (Sigma-Aldrich, Barcelona, Spain). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.5.2. Cell viability assessment

To assess cell viability and evaluate the potential cytotoxicity of the essential oil, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay was performed, ensuring that any observed effects in subsequent experiments were not attributable to toxicity. A total of 20,000 cells per well were seeded into 96-well plates and allowed to adhere for 24 h. After this period, the culture medium was replaced with 100 μL of essential oil solutions prepared in DMEM at different concentrations. Cells were then incubated for an additional 24 h. Following incubation, the medium was removed and replaced with 100 μL of MTT solution in DMEM (0.4 mg/mL; L11939, Thermo Scientific, Waltham, MA, USA). The plates were incubated for 3 h to allow formazan crystal formation. Crystals were then dissolved in DMSO to facilitate quantification. Absorbance was recorded at 560 nm using a BioTek Synergy H1 microplate reader (BioTek® Instruments, Inc., Winooski, VT, USA). All experiments were conducted in triplicate across three independent assays.

2.5.3. Anisakis crude extract (CE) preparation

Anisakis CE was prepared according to the following procedure. The larvae were obtained as described in 2.3.1. The larvae were washed and transferred to a falcon and incubated on ice for 30 min in phosphate buffer (0.1 M sodium phosphate buffer (pH 7.4)) containing 0.15% *n*-octyl-glycoside to permeabilize the cuticle and release intracellular contents. The larvae were subjected to repeated freeze–thaw cycles. Protease inhibitors (0.08 mL L^{-1}) were added to prevent protein degradation in the resulting homogenate. The homogenates were kept at −80 °C until use and thawed on ice prior to analysis. Bicinchoninic Acid Assay (BCA) was performed to determine the amount of protein in the CE (Supplementary data).

2.5.4. Exposition to CE

Caco-2 cells seeded at a cell density of 20,000 cells per well and were allowed to adhere for 24 h. After the incubation, cells exposed to the CE filtered with a 0.22 μm filter and different concentrations of *C. maritimun* in DMEM (62.5, 125 and 250 μg mL^{-1}). Both a control not exposed to essential oil and CE and a control only exposed to CE were made. After 24 h of treatment, cell supernatants were collected and stored at −80 °C before used. For each condition, supernatants were obtained in triplicate.

2.5.5. IL-6 and IL-8 kits

Bicinchoninic Acid Assay (BCA) was performed to standardize total protein loading before performing the IL-6 and IL-8 kits (Supplementary data). The concentrations of interleukin-6 (IL-6) and interleukin-8 (IL-8) in cell culture supernatants were quantified using ELISA kits (Human IL-6 ELISA Kit, Cat. No. A101912; Human IL-8 ELISA Kit, Cat. No. A2982; Antibodies.com, Cambridge, UK), following the manufacturer's instructions. Absorbance was recorded at 450 nm using a microplate reader, and cytokine concentrations were calculated by interpolating the values from standard curves generated using the respective standards supplied with each kit. Three independent assays were performed in triplicate.

2.6. Statistical analysis

The statistical analysis was performed using GraphPad Prism 10. Significant outliers were identified through Grubbs' test. The different concentrations tested for each sample were compared with the control using one-way analysis of variance (ANOVA) followed by Dunnett's and Bonferroni's post hoc tests, except for the impact of the essential oil on anisakis pH resistance and penetration ability, where significance was determined by *t*-test comparing controls and treatments at the same incubation times. The lethal dose 50 (LD_{50}) values were estimated by nonlinear regression analysis of dose–response curves using GraphPad Prism version 10. Mortality data were fitted using a four-parameter logistic model with variable slope. LD_{50} values were interpolated from the fitted four-parameter logistic curve. Half-maximal inhibitory concentration (IC_{50}) values were estimated by nonlinear regression analysis of enzyme activity data using a four-parameter logistic model. The IC_{50} was also interpolated from the fitted inhibition curve.

Table 1
Chemical composition of *Crithmum maritimum* essential oil obtained by GC–MS analysis.

No	Component[a]	RI[b]		RI Lit[c]		% ± SD[d]	ID[e]
		HP5-MS	DB-WAX	Apolar c.	Polar c.		
1	α-thujene	926	1026	924	1025	0.3 ± 0.0	RI,MS
2	α-pinene	932	1023	932	1021	6.5 ± 0.2	Std, RI,MS
5	sabinene	972	1124	969	1124	9.2 ± 0.8	RI,MS
6	β-pinene	975	1110	974	1112	0.3 ± 0.0	Std, RI,MS
7	myrcene	991	1161	988	1167	0.5 ± 0.1	Std, RI,MS
8	α-phellandrene	1004	1165	1002	1161	0.1 ± 0.0	RI,MS
10	α-terpinene	1016	1180	1014	1179	0.3 ± 0.1	RI,MS
11	*p*-cymene	1024	1272	1020	1270	6.8 ± 0.9	Std, RI,MS
13	limonene	1028	1200	1024	1198	20.9 ± 1.2	Std,RI,MS
14	β-phellandrene	1028	1209	1024	1208	2.8 ± 0.2	Std, RI,MS
15	(*Z*)-β-ocimene	1039	1232	1032	1235	0.3 ± 0.0	RI,MS
16	γ-terpinene	1058	1246	1054	1241	35.5 ± 1.1	Std, RI,MS
17	(*Z*)-sabinene hydrate	1066		1065		0.1 ± 0.0	RI,MS
18	terpinolene	1088	1282	1086	1282	tr[f]	Std, RI,MS
19	terpinen-4-ol	1176	1606	1174	1603	0.7 ± 0.1	Std, RI,MS
20	α-terpineol	1190	1699	1186	1705	tr	Std, RI,MS
21	thymol, methyl ether	1235	1590	1232	1590	7.9 ± 1.9	RI,MS
22	thymol	1292	2172	1289	2172	0.2 ± 0.0	Std, RI,MS
23	dillapiole	1626	2357	1620	2351	7.5 ± 1.7	Std, RI,MS
	Total identified					99.8 ± 0.0	
	Monoterpene hydrocarbons					83.6	
	Oxygenated monoterpenes					8.7	
	Phenylpropanoids					7.5	

The separation was carried out on an HP-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). β-Phellandrene and limonene were chromatographically separated on a DB-WAX capillary column (60 m × 250 μm i.d., 0.25 μm film thickness).[a]Compounds are listed in order of their elution from a HP-5MS column. [b]Linear retention index on HP-5- MS and DB-WAX capillary columns, experimentally determined using homologous series of C_7–C_{30} alkanes. [c]Linear retention index deriving from Adams (2007) and NIST08. [d]Relative area percentage values are means of two independent determinations ± Standard Deviation (SD). [e]Identification methods: Std, based on comparison with pure compounds; MS, based on comparison with WILEY, ADAMS and NIST 08 MS databases; RI, based on comparison of RI with those reported in ADAMS, and NIST 08. [f]tr, % < 0.05%. Abbreviations: RI, linear retention index; RI Lit, linear retention index reported in Adams (2007) and NIST 08; SD, standard deviation; ID, identification methods; Std, identification based on comparison with pure compounds; MS, identification based on comparison with WILEY, ADAMS and NIST 08 mass spectral databases.

3. Results

3.1. Essential oil composition

The chemical characterization of the essential oil was obtained by GC–MS analysis (Table 1). The essential oil was dominated by γ-terpinene which represented the 35.5% of the essential oil composition. Limonene constituted the 20.9% of the EO while other compounds such as sabinene (9.2%), thymol methyl ether (7.9%), and dill apiole (7.5%) accounted for less than 10% of the EO.

3.2. Impact of essential oil on Anisakis mortality

Fig. 1 shows the impact of *C. maritimun* essential oil on L3 *Anisakis* larvae as the percentage of mortality of the larvae after 24 and 48 h of exposure to the essential oil in NaCl. For the 24 h exposure (Fig. 1A), the calculated LD_{50} was 619.11 μg mL $^{-1}$. The activity was dose-dependent and reached statistical significance at the two highest concentrations tested, 500 μg mL $^{-1}$ (32.8% mortality; $p < 0.05$) and 1000 μg mL^{-1} (87.2% mortality; $p < 0.0001$). The essential oil displayed an LD_{50} of 353.95 μg mL $^{-1}$ when the larvae were exposed for 48 h (Fig. 1B). Mortality was significant when compared to the untreated control at concentrations of 125 μg mL^{-1} (30% mortality; $p < 0.05$), 250 μg mL^{-1} (40% mortality; $p < 0.01$), 500 μg mL^{-1} (80% mortality; $p < 0.0001$), and 1000 μg mL^{-1} (100% mortality; $p < 0.0001$).

The impact of *C. maritimun* essential oil on L3 *Anisakis* larvae in artificial gastric juice is shown in Fig. 1C as the percentage of mortality of the larvae after 24 h exposure. Similarly to what was observed after 24 h exposure in NaCl, the mortality was dose-dependent reaching significance at the two highest concentrations tested, namely 500 μg mL^{-1} (33.3 % mortality; $p < 0.01$) and 1000 μg mL^{-1} (100% mortality; $p < 0.0001$). The calculated LD_{50} in this case was 546.94 μg mL^{-1}, slightly lower than the calculated in Fig. 1A. Fig. 1D shows the impact of the essential oil in the resistance to acid pH of the pathogen. After a previous 2 h exposure to the LD_{50} (619.11 μg mL^{-1}) of the essential oil, the viable larvae were washed and incubated for 2, 4, and 24 h in artificial gastric juice.

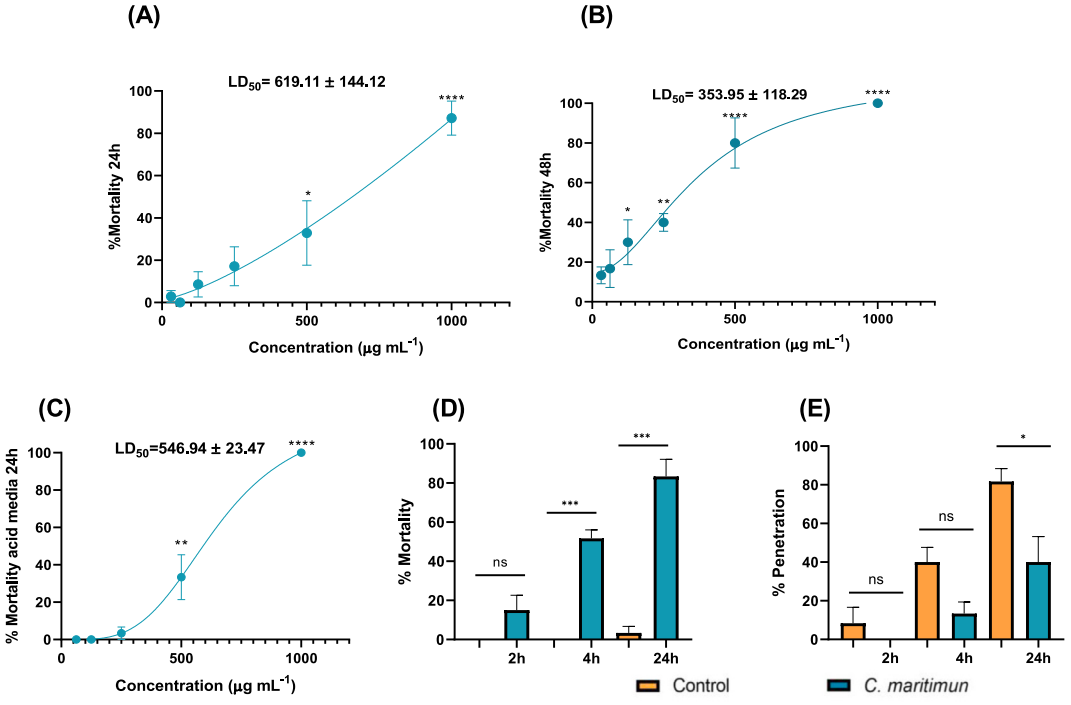

Fig. 1. Anthelmintic Activity of *C. maritimum* essential oil against Anisakis Larvae under Different Experimental Conditions. (A) Percentage of *Anisakis* mortality after 24 h of exposure to the essential oil (B) Percentage of *Anisakis* mortality after 48 h of exposure. (C) Percentage mortality of *Anisakis* larvae in artificial gastric juice after 24 h of exposure. (D) Impact of the essential oil on anisakis pH resistance determined as percentage mortality of *Anisakis* larvae after a 2 h pretreatment with essential oil at the LD_{50} concentration followed by exposure to artificial gastric juice. Dead larvae were quantified after 2, 4, and 24 h of essential oil exposure. (E) Impact of essential oil at the LD_{50} concentration on the penetration ability of *Anisakis* larvae, expressed as the percentage of penetration (calculated at 2, 4, and 24 h relative to the initial number of larvae). For both (C) and (D) a non-represented control in sterile 0.9% NaCl was performed and resulted in a 0% mortality. Data are presented as mean ± SEM from three different experiments performed in triplicate. Non-linear regression was performed to determine the lethal dose 50 (LD_{50}) values. Statistical significance was determined by one-way ANOVA, except for the impact of the essential oil on anisakis pH resistance and penetration ability, where significance was determined by *t*-test comparing controls and treatments at the same incubation times (ns, no significant differences; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$).

After 2 h of incubation no significant differences were observed between the pretreated larvae and the control ones. However, after 4 h (51.7% of mortality) and 24 h (83.3% of mortality) of exposure to the acid media, significant differences in mortality were found between the treated *Anisakis* and the control group ($p < 0.001$). In Fig. 1E the impact of the essential oil on the penetration ability of the pathogen is displayed. Despite all controls at different times showing higher percentage of penetration than those conditions treated with essential oil, only after 24 h, the differences between the treatment and the control were statistically significant ($p < 0.05$), with the control displaying a percentage of penetration of 81.7% and the treatment 40.0%.

3.3. Enzymatic assays

Fig. 2 shows the impact of the essential oil as an inhibitor of different enzymes involved in parasite survival and host tissue responses. Fig. 2A shows the essential oil inhibition of the AChE enzyme, responsible for the degradation of acetylcholine, a neurotransmitter in *Anisakis* and other parasites. *C. maritimun* showed concentration dependant activity, being active the concentrations from 500 μg mL^{-1} ($p < 0.01$) to 1000 μg mL^{-1} ($p < 0.0001$). However, none of the concentrations tested reached 50% inhibition, therefore, the IC_{50} value could not be calculated. In Fig. 2B the inhibition of the 5-LOX enzyme is depicted. The essential oil showed significant inhibition at all concentrations tested ($p < 0.01$ at 15.12 μg mL^{-1}; $p < 0.001$ at 31.25 μg mL^{-1}; $p < 0.0001$ from 62. 5 to 250 μg mL^{-1}), displaying an IC_{50} of 99.35 μg mL^{-1}. Fig. 2C displays the activity of the essential oil upon PLA_2. The essential oil managed to significantly inhibit the enzyme at 250 (45.2% inhibition; $p < 0.01$) and 500 μg mL^{-1}(79.8% inhibition; $p < 0.001$). The IC_{50} value obtained for this enzyme was 253.31 μg mL^{-1}.

3.4. Cell assays

3.4.1. Impact upon cell viability

Fig. 3 shows the impact of *C. maritimum* essential oil upon cell viability in different cell lines at different times of exposure. Cell viability was expressed as a percentage relative to the viability of untreated control cells. No significant differences were observed at any tested concentration, regardless of cell line or exposure duration.

The Selectivity Index (SI), based on the comparison between LD_{50} values from the *Anisakis* assays and CC_{50} values from the cell assays, could not be determined., since none of the tested concentrations reduced cell viability by 50%, CC_{50} values could not be determined for any of the cell lines or exposure times. Nonetheless, given that the CC_{50} value exceeded 1000 μg mL^{-1}, and LD_{50} values were obtained for both 24 and 48 h of exposure, the calculated SI would be greater than >1.62 and > 2.83, respectively, for those exposure times, indicating greater selectivity towards the nematode.

3.4.2. Impact upon IL production in Caco-2 cells

In Fig. 4, the impact of *C. maritimum* essential oil upon IL-6 and IL-8 production in Caco-2 cells stressed with *Anisakis* CE is depicted. The exposure to the CE increased the production of both IL compared to the unexposed control (IL-8, $p < 0.0001$; IL-6, $p < 0.001$). For the IL-8 production, the essential oil managed to reduce the amount of IL showing significant differences between the stressed control and the different concentrations tested (62.5 μg mL^{-1}, $p < 0.0001$; 125 μg mL^{-1}, $p < 0.001$; 250 μg mL^{-1}, $p < 0.05$). However, for the IL-6 production, the treatment with the essential oil showed no differences between the stressed control and the concentrations tested.

4. Discussion

Several secondary metabolites, in particular monoterpene hydrocarbons, oxygenated monoterpenes, and phenylpropanoids are usually described in *C. maritimun* essential oil. Among monoterpene hydrocarbons, sabinene and γ-terpinene are the most prevalent, with their concentrations varying widely depending on plant part and growth stage. In the oxygenated monoterpene group, key constituents include terpinen-4-ol, carvacrol methyl ether, and thymol methyl ether, with the latter sometimes reaching dominant levels (up to 89%). The main phenylpropanoid identified is dillapiole, often reported as a characteristic marker in sea fennel essential

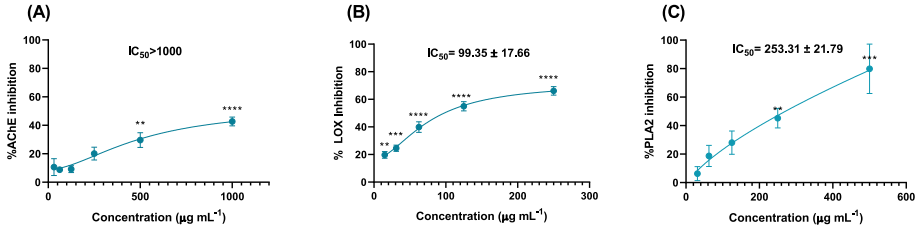

Fig. 2. Activity of *C. maritimum* essential oil upon different enzymes. (A) Essential oil inhibition of AChE tested from 1.25 to 1000 μg mL^{-1}. (B) Essential oil inhibition of LOX tested from 15.12 μg mL^{-1} to 250 μg mL^{-1}. (C) Essential oil inhibition of PLA2 tested from 31.25 μg mL^{-1} to 500 μg mL^{-1}. All data were normalized relative to the untreated control group (non-represented). Data are presented as mean ± SEM from three independent experiments, each performed in triplicate. Non-linear regression displayed in the figure was performed to determine the half-maximal inhibitory concentration (IC_{50}) values. Statistical significance was determined by one-way ANOVA (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

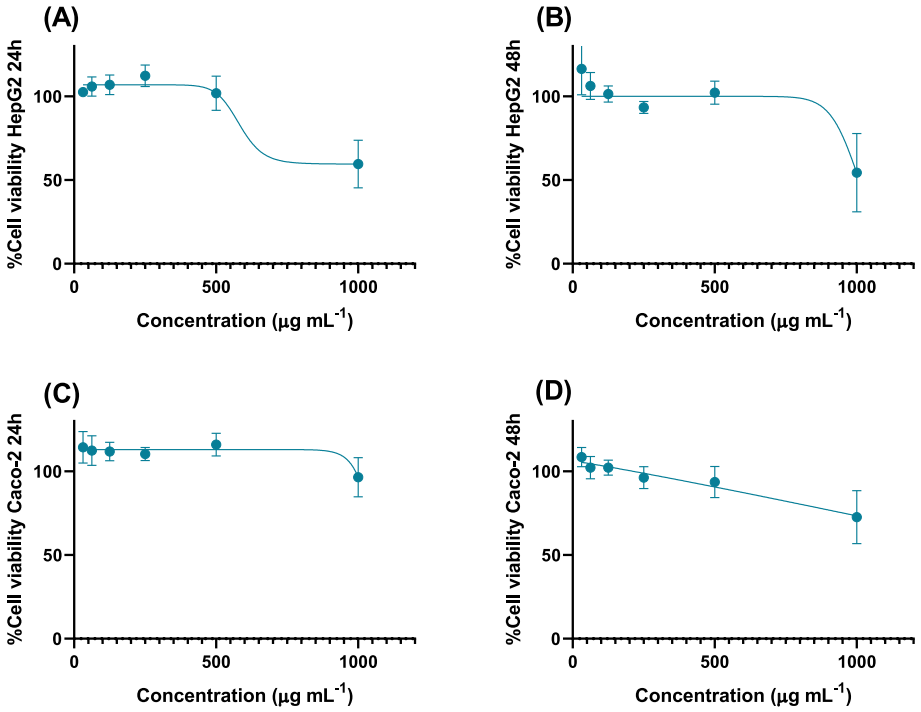

Fig. 3. Impact of *C. maritimum* essential oil upon cell viability in different cell lines at different times of exposure. (A) Impact upon HepG2 cells after 24 h of exposure to different concentrations of the essential oil. (B) Impact upon HepG2 cells after 48 h of exposure to different concentrations of the essential oil. (C) Impact upon Caco-2 cells after 24 h of exposure to different concentrations of the essential oil. (D) Impact upon Caco-2 cells after 48 h of exposure to different concentrations of the essential oil. Concentrations from 15.63 to 1000 µg mL^{-1} were tested. All data were normalized relative to the untreated control group. Data are presented as mean ± SEM from three independent experiments, each performed in triplicate. Statistical significance was determined by one-way ANOVA, although none of the tested concentrations showed significant differences when compared to the untreated control.

oil (Giordano et al., 2021; Kraouia et al., 2023). The composition of the essential oil herein studied aligns with previous reports, since γ-terpinene (35.5%) and sabinene (9.2%) were two of the major compounds found in the essential oil. Limonene, which is also a major compound in the essential oil herein studied, was classified in a previous work as infrequent despite being present in other few studies (Kraouia et al., 2023). The yield of this compound has been described to increase with techniques such as microwave assisted hydrodistillation (Politeo et al., 2023). Comparison of the essential oil chemical profile with those obtained from seeds collected in other regions revealed some differences. For instance, the essential oil composition from seeds collected in France was dominated by phenylpropanoids (53.1%), with lower contribution of monoterpene hydrocarbons (33.4%) and oxygenated monoterpenes (12.2%). Dillapiole (39.9%) and myristicin (12.8%), *g*-terpinene (21.2%), and thymol methyl ether (11.1%), were the most representative compounds, respectively (Pavela et al., 2017). On the other hand, an overlapping profile was observed with essential oil samples obtained from the same collection area and collected in different years (Kavallieratos et al., 2024; Piatti et al., 2023). Overall, variability in essential oil composition among studies may be explained by factors such as geographic origin, plant developmental stage, and year of collection. (Renna, 2018).

Previous reports have described the antimicrobial potential of *C. martitimun* essential oil, as well as the nematicidal effect of this essential oil against pinewood nematode (*Bursaphelenchus xylophilus)* from the Aphelenchoididae family (Barbosa et al., 2010; Campana et al., 2022; Jallali et al., 2014; Pedreiro et al., 2023). To our knowledge, the activity of *C. maritimun* essential oil against other nematodes has not been explored, particularly its potential upon *Anisakis* spp. The essential oil showed significant activity upon the parasite in both 24 and 48 h exposition, with higher activity observed at the latter exposition time. The results also show that the impact on *Anisakis* mortality was similar when the assay was performed in artificial gastric juice. This is particularly noteworthy since it has been described that some antiparasitic drugs, such as albendazole, reduce its efficacy in pH acidic medium (Arias-Diaz et al., 2006). Out of the three main components identified in the essential oil, only limonene has been previously reported to show activity against *Anisakis* larvae. This monoterpene was effective during the marination of anchovy fillets and their subsequent storage in sunflower oil (Nalbone et al., 2022).

Gastric and intestinal anisakiasis result from the active penetration of larvae into the gastrointestinal mucosa, causing tissue damage and inflammatory responses (Aibinu et al., 2019; Rama and Silva, 2022). Therefore, the ability of larvae to penetrate host tissues plays a key role in the development of the infection. Given this critical role, in this work, the ability of the essential oil to avoid penetration in an in vitro assay was assessed and a significant reduction in larval penetration ability was observed following exposure to the essential oil. The penetration assay in agar herein studied represents a simplified model that does not fully capture tissue

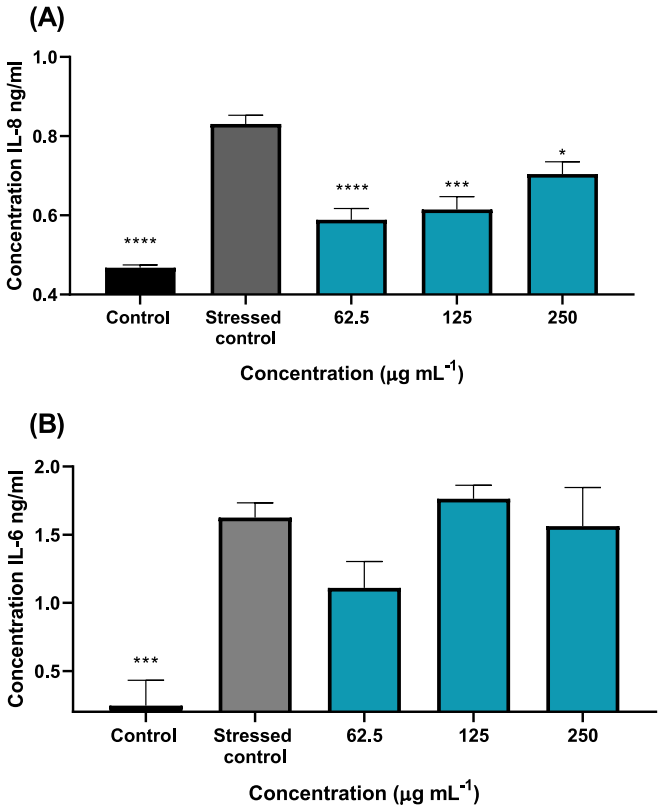

Fig. 4. Impact of *C. maritimum* essential oil upon IL-8 and IL-6 production in Caco-2 cells stressed with *Anisakis* CE. Impact upon interleukin-8 (A) production and interleukin-6 (B) production in Caco-2 cells. Concentrations ranging from 62.5 to 250 µg mL^{-1} of essential oil were tested. Both non-stressed (depicted as control) and stressed with CE (depicted as stressed control) controls without essential oil treatment were made. Data are presented as mean ± SEM from at least three independent experiments, each performed in duplicate. Statistical significance was determined by comparing the different conditions with the stressed non-treated control. Statistical significance was determined by one-way ANOVA (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

complexity. Despite its limitations, the impact of the essential oil on larval penetration seems promising. Additionally, a pre-exposition to the essential oil showed modifications in the parasite ability to survive in an acidic environment that mimics the conditions in the stomach. Therefore, treatment with the essential oil could diminish the pathogen resistance to acidity suggesting efficacy under gastric conditions without impairing nematicidal activity. Taken together, these findings suggest that treatment with *C. martini* may reduce the infective potential of the pathogen by affecting both its penetration ability and its tolerance to gastric pH, although additional studies are necessary to confirm this potential.

The inhibition of the AChE enzyme was evaluated to identify a possible mechanism of action. Inhibition of AChE activity may result in increased levels of acetylcholine, leading to excessive stimulation of nicotinic acetylcholine receptors in some parasitic nematodes, which produces spastic paralysis (Wolstenholme, 2011). *C. maritimun* essential oil showed inhibition of AChE at the highest concentrations tested however, this effect was not very pronounced, suggesting that it may not be the sole mechanism of action involved. The AChE inhibition potential of *C. maritimum* had been previously assessed in another work displaying lower IC_{50} values, however, the composition of the essential oil was different form the one studied in this work, since it had a higher content of thymol methyl ether (Ismail et al., 2024).

Larval penetration is associated with tissue damage and a marked inflammatory response, including eosinophilic infiltration, mast cell activation, and cytokine release. In this work, the interleukin production in Caco-2 cells exposed to *Anisakis* CE was studied. The levels of both IL-6 and IL-8 were quantified, revealing notable differences in cytokine production between the unstimulated control and the stressed control. These results suggest that exposure to *Anisakis* leads to an upregulation in the production of these pro-inflammatory interleukins. A previous work studied the impact of CE on Caco-2 cells showing a significant secretion of IL-6 after 24 h of exposition, however, for the IL8 no significant differences were found when compared with the control (Bellini et al., 2022). The differences observed may be related to different experimental conditions and the use of different crude extract preparation methods, such as mechanical homogenization in buffered solution or detergent-assisted permeabilization combined with freeze–thaw cycles. The essential oil herein tested decreased the IL-8 production in Caco-2 cells but had no effect on IL-6. IL-6 and IL-8 are key cytokines involved in nematode-induced intestinal inflammation, playing complementary roles. While IL-6 shows pleiotropic activity by promoting early innate immune responses, it also contributes to anti-inflammatory and tissue-repair processes that help limit

epithelial damage and support mucosal healing. (Smith and Maizels, 2013; Vilotić et al., 2022). In contrast, IL-8 functions primarily as a proinflammatory chemokine that drives the recruitment of neutrophils to sites of infection (Vilotić et al., 2022). While essential for early parasite containment, sustained IL-8-driven neutrophil infiltration may lead to epithelial injury due to the release of reactive oxygen species and proteolytic enzymes by activated neutrophils (Cambier et al., 2023; Fournier and Parkos, 2012). In this work, the essential oil managed to reduce the IL-8 production, showing a noteworthy potential regarding the adverse effects of excessive immune response and inflammation in intestinal cells previously discussed.

Additionally, the in vitro assays performed proved that the essential oil is also capable of inhibiting LOX and PLA2 enzymes, involved in the eicosanoid cascade (Fig. 2B and C). Moreover, the main compounds identified in the essential oil evaluated in the present study (limonene, sabinene, and γ-terpinene) have been shown to inhibit leukotriene biosynthesis via 5-LOX inhibition in RBL-1 cells (Lee et al., 2012), while γ-terpinene, in particular, has also been described to reduce edema, pro-inflammatory cytokine production, and leukocyte recruitment in animal models, further supporting its anti-inflammatory profile (Ramalho et al., 2015). In addition, a previous work reported the potential of *C. maritimum* to reduce nitric oxide production in LPS-stimulated macrophages without inducing cytotoxic effects (Alves-Silva et al., 2020).

5. Conclusions

The essential oil of *C. maritimun*, characterized by the presence of γ-terpinene, limonene, and sabinene, exhibited promising activity in vitro against *Anisakis simplex* s. l. Notably, the essential oil demonstrated significant nematicidal activity at 24 and 48 h of exposition to the pathogen in both saline solution and artificial gastric juice media, impairing viability, acid resistance of the larvae, and penetration capacity. Additionally, the essential oil showed no significant cytotoxicity in HepG2 and Caco-2 cell lines. Furthermore, it reduced the production of IL-8 in Caco-2 cells stimulated with *Anisakis* CE and displayed in vitro inhibitory activity against key enzymes in the eicosanoid inflammatory cascade. These findings suggest that *C. maritimum* essential oil could serve as an effective natural agent for the control of *Anisakis* infections and the regulation of host response mechanisms.

CRediT authorship contribution statement

Pilar Cebollada: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Eleonora Spinozzi:** Writing – review & editing, Investigation. **Marta Ferrati:** Writing – review & editing. **Cristina Moliner:** Methodology. **Riccardo Petrelli:** Writing – review & editing. **Filippo Maggi:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Carlota Gomez-Rincón:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Víctor López:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2026.e00331.

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