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# Inactivation of *Trichinella* spp. in naturally infected boar meat after Pulsed Electric Field (PEF) treatments

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ABSTRACT

Larvae of the nematode *Trichinella* are capable of causing parasitic infections in humans after the consumption of uncooked meat or meat products, leading to severe symptoms and even death. Meat inspection is costly and tends to progressively be derogated in holdings applying controlled housing conditions in certain regions. Our study focuses on the evaluation of the efficacy of Pulsed Electric Fields (PEF) in the inactivation of *Trichinella* spp., including excysted larvae isolated by artificial digestion and encapsulated larvae found in meat from naturally infected wild boars. Microscopic examination of unstained and propidium-iodide-stained larvae suspensions showed that most of the excysted larvae were inactivated 10 min after an intermediate PEF treatment (1 kV/cm, 0.41 kJ/kg). Treating excysted larvae with the mildest PEF treatment (0.5 kV/cm, 0.05 kJ/kg) combined with a 3% NaCl incubation resulted in synergistic inactivation. The application of 3 kV/cm (20 kJ/kg) to wild boar meat resulted in the inactivation of over 90% of encapsulated *Trichinella* larvae. The viability of *Trichinella* in meat was inversely correlated to the field strength applied (1–6 kV/cm) for equal energy input (20 kJ/kg). These findings demonstrate that PEF technology can potentially serve as a novel strategy for the inactivation of *Trichinella* larvae in meat.

## 1. Introduction

Trichinellosis is a serious disease in humans caused by nematodes belonging to the *Trichinella* genus. This widespread parasite comprises at least thirteen species distributed over all continents except Antarctica (Gherman et al., 2022; Pérez-Martín et al., 2000; Pozio, 2019). In humans, the ingestion of raw or undercooked meat harboring infective larvae leads to infestation (Zarlenga et al., 2020). Clinical symptoms include fever, abdominal pain, diarrhea, nausea, vomiting, myalgia, and even life-threatening complications, such as myocarditis and encephalitis (Pérez-Pérez et al., 2019). The worm completes its entire life cycle, from larva to adult, within the body of a single host, turning the muscles into a reservoir of larvae capable of long-term survival. There is no direct person-to-person transmission.

In the European Union (EU), most infections are caused by pork, especially wild boar meat and derived products consumed in rural areas. From 2017 to 2021, 525 cases of human trichinellosis were documented (EFSA-ECDC, 2022). While the number of cases in the EU has decreased over the past 20 years thanks to improved biosecurity in farms (Pozio, 2019), trichinellosis remains a significant zoonotic disease due to several factors: globalization of the food supply, gaps in surveillance and control, and general culinary habits that increasingly favor the consumption of raw food (Trevisan et al., 2019), in parallel with the increasing population of wild boars (Jori et al., 2021; Moral et al., 2022).

Pre-slaughter prevention involves swine management control in high-containment-level farms combined with biosafety and continuous surveillance programs. However, the growing trend toward raising animals outdoors to improve animal welfare and meat quality increases the risk of the reintroduction of *Trichinella* (Noeckler et al., 2019). Extensive rearing is common in Spain for the production of the well-known Iberian field bait ham from pigs with a diet based on natural pastures (Díaz-Caro et al., 2019). Regions with a strong tradition of backyard and free-ranging pigs are prevalent in the EU (Bandino et al., 2023). On the other hand, effective control of wild boars is difficult due to their

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wide-ranging habitat, frequent interactions with domestic pigs, and their status in Europe as a widely appreciated game animal (Vieira-Pinto et al., 2021). The post-slaughter strategy against Trichinellosis in the EU relies on official controls including systematical testing pork, wild boar and horse meat. However, inspection procedures are costly, time-consuming, and require skilled labor in slaughterhouses and game-handling establishments (Barlow et al., 2021).

An alternative strategy to prevent trichinellosis and improve food safety without affecting food quality can be found in technologies that reduce the infectivity of *Trichinella* spp. larvae in meat. One such technology is Pulsed Electric Fields (PEF). PEF treatments consist of subjecting a product placed between two electrodes, usually immersed in an aqueous solution, to high-intensity electric fields (between 0.5 and 30 kV/cm) by applying intermittent pulses of short duration (microseconds to milliseconds) without increasing the product's temperature. While PEF is commonly used for the nonthermal inactivation of microorganisms in foods (Raso et al., 2022), there is limited scientific literature on the inactivation of zoonotic parasites, apart from some very recent studies that report the inactivation of *Anisakis* in fish (Abad et al., 2023; Onitsuka et al., 2022, 2024). A generic patent for the paralysis of parasites by PEF was recently filed (EP4039096A1), but data on the effect of PEF on *Trichinella* have not yet been reported.

The aim of the study was to evaluate the potential of PEF technology to inactivate *Trichinella* spp., both in excysted larvae isolated by artificial digestion and in encapsulated larvae in meat from naturally infected wild boars.

## 2. Material and methods

### 2.1. Excysted Trichinella larvae

*Trichinella spiralis* larvae were recovered from the muscle of infected mice using a standard pepsin-hydrochloric acid digestion protocol as described by the European Union Reference Laboratory for Parasites (https://www.iss.it/en/eurlp-chi-siamo). Briefly, muscles were minced with a small amount of digestion solution consisting of 250 mL of water at 46–48 °C, 2 mL 25% HCl, and 1.25 g of Pepsin. For every gram of minced meat, 10 mL of the solution was poured into a flask (beaker) equipped with a magnetic stirrer and incubated in shaking at 44–46 °C for 30 min. The mixture was then run through a sieve with a pore size of approximately 180–200 μm. The larvae were allowed to settle for approximately 30 min, after which the supernatant was removed and the larvae were collected from the bottom of the beaker. The larvae were re-suspended in Phosphate Buffered Saline (PBS, Sigma, Burlington, Massachusetts, USA). Larva motility and morphology were microscopically assessed (see section 2.4), and the larvae count was determined. Stock suspensions containing 200 larvae/mL were prepared and stored at 4 °C in the period between the digestion procedure and the PEF treatments.

For all experiments, larvae were carefully handled to avoid any damage. Stock tubes were gently mixed, and 100 μL aliquots, containing approximately 20 larvae each, were poured into individual Eppendorf tubes. These tubes were then filled to 0.5 mL with PBS with adjusted electrical conductivity (0.1 mS/cm). Two control tubes containing untreated larvae as well as two tubes for each of the different PEF treatments were prepared. In each round of experiments, the control tube (untreated) underwent the same protocol except for the PEF treatment, in order to compare the results.

### 2.2. Encapsulated Trichinella larvae

Meat from wild boars (*Sus scrofa*) hunted in the South-West of Spain during the 2022–2023 season was delivered to our laboratory. Natural infection in these wild boars was confirmed following the ISO 18743/2015 standard, based on European Regulation 2015/1375. Parasitic intensities in the samples ranged from 26 to 693 larvae per gram corresponding to *T. spiralis*. Muscular tissue samples from eight different hunted animals were used for PEF experiments. After subjecting meat to PEF, untreated and treated samples were digested to extract the larvae with the standard technique described in 2.1.

### 2.3. PEF treatment

The PEF setup used in this investigation featured commercial equipment (Vitave, Prague, Czech Republic) previously described by Berzosa et al. (2023). The actual voltage of each treatment was measured using a high-voltage probe (Tektronik, P6015A, Wilsonville, Oregon, USA) connected to an oscilloscope (Tektronik, TDS 220). For the excysted larvae treatments, a suspension of larvae (0.44 mL) was introduced into a static parallel treatment chamber with specific dimensions (gap: 0.25 cm; diameter: 1.6 cm) using a 1 mL sterile syringe (TERUMO, Leuven, Belgium) with a 20Gx 1" (0.9 × 25 mm) needle (TERUMO). Tubes for the investigation of the effect of PEF on excysted larvae were subjected to 30 monopolar square waveform pulses of 3 μs of different field strengths (0.5, 1, and 2 kV/cm). These treatments corresponded to specific energies ranging from 0.05 to 0.79 kJ/kg. Experiments involving control larvae and treated larvae were performed in duplicate. Tubes treated with PEF were immediately processed for microscopic observation in order to minimize the time lapse between treatment and evaluation.

In experiments designed to assess the effect of PEF on larvae encapsulated in *Trichinella*-infected meat, the meat was cut into 3 cm cubes and PEF-treated in batches using a parallel-electrode semicircular chamber (10 cm diameter) half-filled with tap water (0.7 mS/cm). Three different electric field strengths were tested (1, 3, and 6 kV/cm), each with a varying number of pulses of 20 μs width (1400, 160, and 20, respectively), corresponding to a specific energy of 20 kJ/kg for all PEF treatments.

The potential effect of PEF on the inactivation of both excysted and encapsulated *Trichinella* larvae was evaluated through microscopic examination, observing morphological parameters in unstained larvae as well as the use of propidium iodide fluorescent staining.

### 2.4. Microscopic observation

Immediately after PEF treatments on excysted *Trichinella* larvae or after digestion of PEF-treated meat with encysted larvae, 15 μL aliquots of larvae suspensions from both control and treated tubes were observed under a microscope (Eclipse E400, Nikon, Tokyo, Japan) connected to a camera (AxioCam MRC, Zeiss, Oberkochen, Germany). Brightfield and phase contrast observation techniques were both applied. Observation of the larvae was renewed 10 min after PEF treatment. The criteria used to classify the larvae as either alive or dead were as follows: living larvae exhibited a rolled-up configuration, were motile and displayed their typical morphology, whereas dead larvae appeared unrolled, non-motile, and showed structural loss or abnormal morphology, as described by Johne et al. (2021) (Fig. 1).

The assessment of electroporation in *Trichinella* larvae was also conducted by evaluating the inclusion/exclusion of the fluorescent dye propidium iodide (PI; Sigma-Aldrich, Barcelona, Spain) in both excysted and encapsulated larvae after digestion. PI is a small (660 Da) hydrophilic molecule that is unable to cross through intact cytoplasmic membranes. Prior experiments were carried out to adapt published protocols to this specific species (Ferreira et al., 2015; Jasmer et al., 2020). Optimization of PI concentration, incubation times, and temperatures was performed. In the optimized protocol, 15 μL of staining solution (0.1 mg/mL PI in buffer) were added to 15 μL larvae suspensions. Samples were then incubated at 37 °C for 20 min. The presence of PI trapped inside the cells was assessed using an epi-fluorescence microscope (Nikon, Mod. L-Kc).

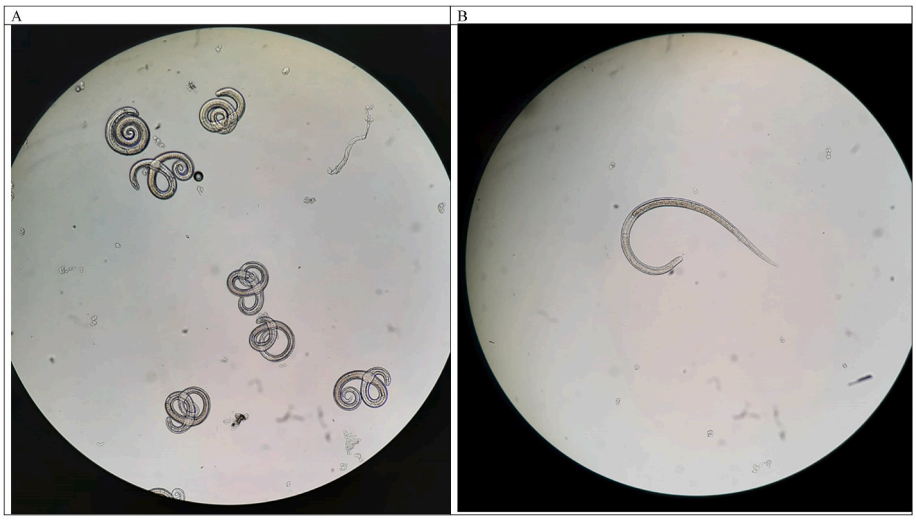


**Fig. 1.** Optical microscopy observation (100x) of live (untreated) (A) and dead (PEF 2 kV/cm, 90 μs, 0.8 kJ/kg) (B) excysted Trichinella spp. larvae.

### 2.5. Sublethal injury evaluation

To assess the potential for sublethal injury following mild PEF treatment (0.5 kV/cm, 90 μs), excysted larvae were suspended and then incubated in buffer solutions containing different salt concentrations (1, 2 or 3 % NaCl). This evaluation aimed to determine if the treatment, in combination with salt, caused any damage to the larvae's membranes or altered their behavior or morphology, indicating sublethal damage associated to the PEF treatment.

## 3. Results

### 3.1. Effect of PEF on excysted Trichinella spp. larvae

Control samples (untreated tubes) showed 100 % viability of *Trichinella* larvae after sample manipulation and the digestion protocol. The results of the impact of PEF on excysted *Trichinella* larvae are shown in Table 1. Immediately after the mildest PEF treatment (0.5 kV/cm and 0.05 kJ/kg; 90 μs), the percentage of live larvae remained high (92.6% and 95.0%) in each of the replicates, respectively, and these figures remained unchanged even after 1 h of PEF treatment. Immediately after the intermediate PEF treatment (1 kV/cm; 0.4 kJ/kg, 90 μs), the percentage of live larvae decreased to 64.0 and 71.4 % in each replicate, respectively. Nevertheless, most of the larvae became inactivated when microscopic examination was repeated 10 min after the PEF treatment. Photographs captured over time within the same microscopic field revealed morphological changes (Fig. 2). Slides were prepared with extra volume to prevent sample drying. It was observed that intracellular content leaked into to the extracellular medium over time. Finally, after the most intense PEF treatment (2 kV/cm, 0.79 kJ/kg; 90 μs), all nematodes appeared dead immediately after the treatment. Within 10 min, even the morphology of the nematodes began to disaggregate. Untreated larvae did not take PI using the protocol described in section 2.4, even after longer incubation times, nor were they affected in terms of motility, shape, or morphology. However, some PEF-treated larvae were stained with PI, indicating permeabilization (>1 kV/cm). Significantly, a leakage of fluorescent PI-labeled genetic material could also be observed in some apparently live (rolled-up) larvae (Fig. 3).

**Table 1**
Inactivation of excysted *Trichinella* larvae by Pulsed Electric Field treatments of different electric field strengths (30 pulses of 3 μs).

| Electric Field Strength (kV/cm) | Energy input (kJ/kg) | Immediately after PEF | After 10 min incubation |
|---|---|---|---|
| 0.5 | 0.05 | R1: 25 alive, 2 dead<br>R2: 19 alive, 1 dead | Unchanged even after 1 h |
| 1 | 0.41 | R1:16 alive, 9 dead<br>R2: 15 alive, 6 dead | R1: 20 dead<br>R2: 18 dead |
| 2 | 0.79 | R1: 25 dead | Dead and disintegrating |

### 3.2. Sublethal injury evaluation

Excysted *Trichinella* larvae were subjected to PEF treatment under the mildest conditions (0.5 kV/cm, 90 μs, 0.05 kJ/kg) and subsequently incubated at varying salt concentrations (1%, 2%, and 3 % NaCl) with the aim of assessing the potential synergistic effect of PEF and non-physiological media (Table 2). After 10 min of incubation in a 1% salt solution, all PEF-treated nematodes remained coiled up and exhibited motility, indicating that they were viable. Extending the incubation to 30 min had no impact on their viability. PI staining yielded negative results, suggesting that the selective permeability of their membranes remained intact. Similarly, following 10 and 30 min of incubation in a 2% salt solution, all larvae appeared viable and continued to exhibit motility. However, PI uptake indicated some membrane damage, with 14.3% and 15.4 % of larvae testing PI-positive, depending on the replicate. In contrast, while a 10-min incubation in a 3% salt solution did not affect the morphology or motility of the larvae, extending the incubation to 30 min resulted in the cessation of motility in 50 % of larvae, accompanied by a change to a right-angle morphology. At this point, PI uptake was positive in 46 % of the larvae. Untreated nematodes did not experience any changes in larvae morphology or motility, even after several hours of incubation in a 3% salt solution.

### 3.3. Impact of PEF on encapsulated Trichinella spp. larvae

Wild boar meat containing encapsulated *Trichinella* spp. larvae was subjected to PEF treatment and subsequently digested to assess the treatment's impact on the larvae. Fig. 4 shows the appearance of digested products from untreated (control) and PEF-treated encapsulated *Trichinella* larvae. The effect of PEF, particularly above 3 kV/cm, was readily apparent. PEF-treated larvae displayed an open arrangement instead of the typical rolled-up configuration of viable larvae. Additionally, the interior of PEF-treated larvae appeared empty compared to untreated ones. Fig. 5 shows the percentages of motile

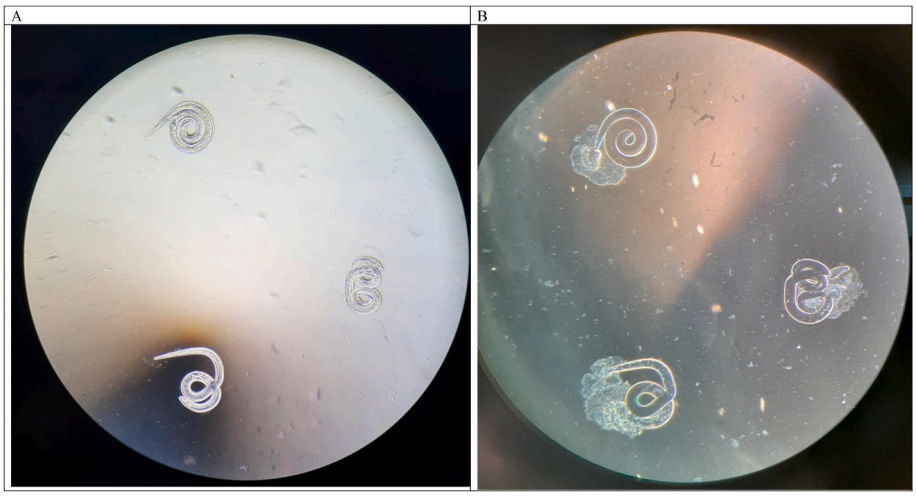


**Fig. 2.** Optical microscopy observation (200x) of PEF-treated (1 kV/cm; 90 μs; 0.41 kJ/kg) excysted *Trichinella* spp. larvae immediately after the PEF-treatment (A) and after 10 min incubation (B).

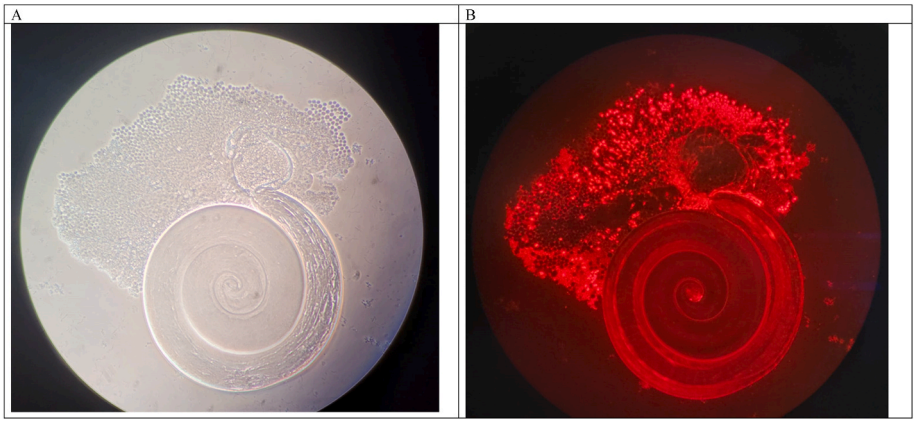


**Fig. 3.** Optical microscopy observation (400x) (A) and epi-fluorescence (B) of PEF-treated (1 kV/cm; 90 μs; 0.41 kJ/kg) excysted *Trichinella* spp. larvae after 10 min incubation.

**Table 2**
Evaluation of viability and PI uptake in PEF-treated (0.5 kV/cm; 90 μs, 0.05 kJ/kg) excysted *Trichinella* larvae incubated in PBS buffer with different NaCl concentrations.

| Salt concentration (NaCl) | Viability after 10 min | Viability after 30 min | PI uptake after 30 min |
|---|---|---|---|
| 1 | 100 % rolled-up and motile | 100 % rolled-up and motile | 100 % PI negative |
| 2 | 100 % rolled-up and motile | 100 % rolled-up and motile | R1: 2 positive, 11 negative<br>R2: 1 positive, 7 negative |
| 3 | 100 % rolled-up and motile | 50 % rolled-up and motile | R1: 6 positive, 7 negative |

*Trichinella* larvae following treatments with equivalent energy inputs (20 kJ/kg) at various electric field strengths (0, 1, 3, or 6 kV/cm). The percentages of motile larvae were respectively 99, 45, 5 and 0 % and exhibited an inverse correlation with the electric field strength applied. The application of 1 kV/cm resulted in the inactivation of approximately half of the larvae observed after digestion. However, after 3 kV/cm treatment, the inactivation rate exceeded 90 %, and with 6 kV/cm, none of the larvae survived in any of the replicates.

## 4. Discussion

Microscopic examination of unstained and propidium-iodide-stained larvae suspensions showed that most of the excysted larvae were inactivated (unrolled, non-motile, and exhibiting structural loss or abnormal morphology) within 10 min after the intermediate PEF treatment (1 kV/cm, 0.41 kJ/kg).

To date, research regarding the efficacy of PEF treatment on parasites and larger organisms is still ongoing. Evidence on the effectiveness of PEF on parasites in solid foods has been limited to the study of *Anisakis*. Abad et al. (2023) investigated the impact of PEF treatment on *Anisakis* larvae, which were either isolated in a saline solution or artificially embedded in hake fillets. Their results showed that treatments of 3 kV/cm (40–50 kJ/kg) effectively inactivated 90–100% of the larvae, regardless of whether they were isolated in a saline solution or artificially implanted in the fish fillets.

To the best of our knowledge, the present study is the first report of a PEF treatment on solid food naturally infected with nematodes. Most of the excysted larvae were inactivated following a treatment of 1 kV/cm (0,41 kJ/kg); moreover, the application of 3 kV/cm (20 kJ/kg) to 3 cm cubes of wild boar meat resulted in the inactivation of 95 % of encapsulated *Trichinella* larvae. This finding demonstrates that PEF treatments have an impact not only on unicellular microorganisms but also on more complex multicellular organisms, even when they are encysted in meat. *Trichinella* larvae encysted in muscle are approximately 1 mm long. This

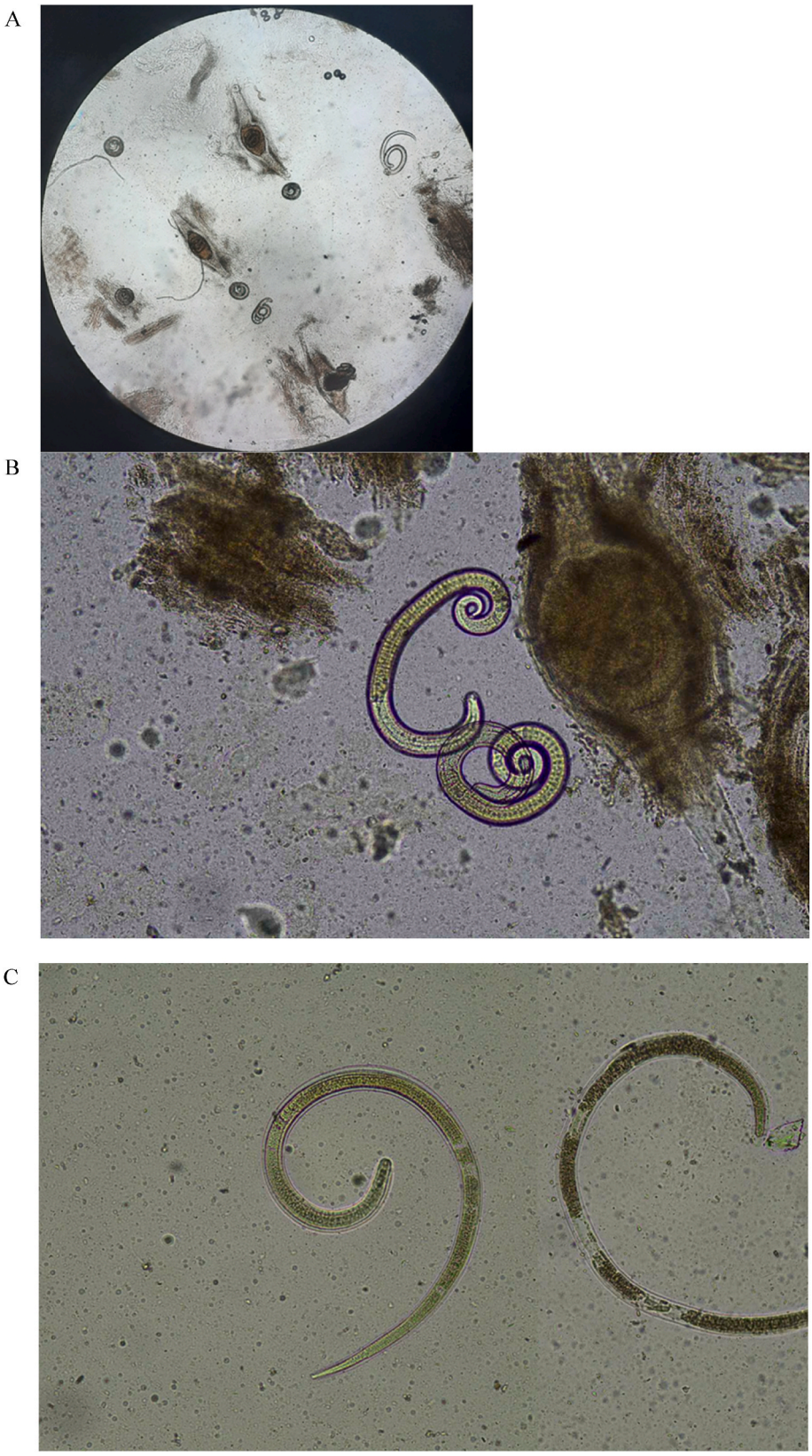


**Fig. 4.** Optical microscopy observation (100x) of control (naturally infected) parasitized meat after digestion (A), 400x detail of untreated encysted *Trichinella* spp larvae after digestion (B) and image at the same magnification of PEF-treated (3 kV/cm; 20 kJ/kg, 3200 μs) encysted *Trichinella* spp. larvae after digestion (C).

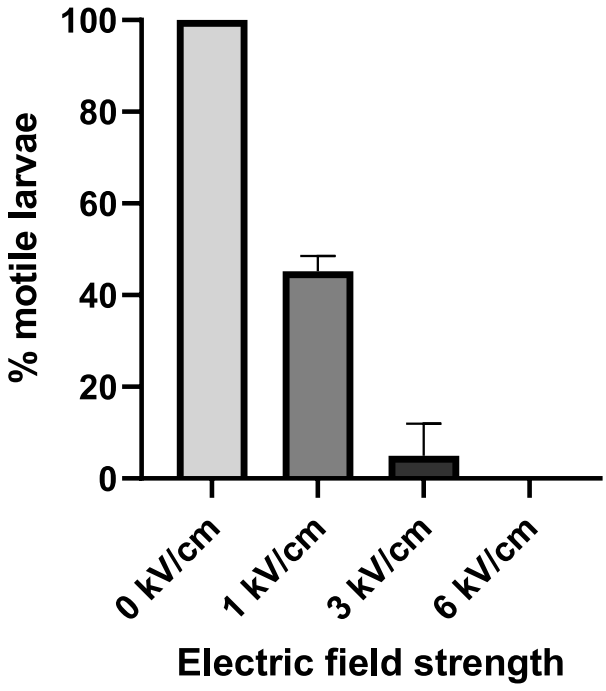


**Fig. 5.** Influence of electric field strength on motility after digestion of previously encapsulated *Trichinella* spp larvae from naturally parasitized wild boar meat. Energy input was 20 kJ/kg for all treatments.

parasite stage exhibits a worm-like, coiled, spiral appearance, significantly different from the spherical morphology and simplicity of unicellular organisms. The mechanisms underlying the electroporation of the cytoplasmic membrane of the cells that constitute the larvae are yet to be fully elucidated. The arrangement of a parasite encysted in muscle tissue differs significantly from the typical cell structure, consisting of an extracellular medium-lipid bilayer-intracellular medium unicellular structure, which behaves as a capacitor and often conforms to electroporation theory (Martínez et al., 2023). Therefore, further research is required in this field to optimize PEF treatments. This could involve enhancing the electrical conductivity of the media in which food is embedded, refining the direction of the electric field, and taking further parameters into account that may potentially influence the outcome.

Furthermore, it is essential to investigate other potential side effects on meat quality, as specific negative impacts on food quality may vary depending on the PEF treatment and the particular product. No previous studies have been published regarding the effects of PEF on wild boar meat. Wild boar meat is characterized by low intramuscular fat content and a fatty acid composition within the range of values reported for pork, yet it is darker and tougher in comparison. The application of PEF to muscle tissue could create pores and facilitate water movement, which may be disadvantageous depending on the meat's intended use, as the procedure could reduce water-holding capacity (Astráin-Redín et al., 2019; Faridnia et al., 2015). On the other hand, there is a potential for positive effects, such as improved meat tenderization through increased proteolysis, or enhanced meat drying in curing processes, which could take place alongside parasite inactivation (Martínez et al., 2023). Another non-thermal technology applied directly to solid food for the inactivation of nematode parasites is high hydrostatic pressure (HHP) (Dong et al., 2003). However, while HHP has been effective in killing *A. simplex* larvae in raw fish fillets, its significant impact on the color, texture, and overall appearance of the fillet, due to the extreme pressures and time combinations required, poses limits to its practical application. PEF offers a significant advantage in this regard, as the applied energy input is reasonable, and the adverse effects are thus presumably minimal.

EU legislation establishes specific rules for the control of trichinellosis, including the requirement for systematic testing for *Trichinella* in all slaughtered pigs, wild boars, and horses, with the exception of pigs from holdings or compartments officially recognized as applying controlled housing conditions, in which a percentage of carcasses shall be exempt for *Trichinella* examination (Comission implementing regulation (EU) 2015/1375). In the EU, approximately two hundred million domestic pig units (animals and/or slaughter batches) are tested each year (EFSA-ECDC, 2022). The estimated cost of artificial digestion varies between 0.87 and 1.62 € per unit, depending on the facility where samples are processed, whether it be the slaughter facility or an external commercial laboratory (Barlow et al., 2021). Therefore, excluding tests for wild boar and horses, the EU spends over 200 M euros annually on *Trichinella* inspections for swine alone. Therefore, any technology which could be useful to eliminate these tests would be of great interest. PEF could be offering a possibility for this. However, much more research is necessary and new results are necessary to consider that possibility.

## 5. Conclusion

This study has demonstrated the potential of Pulsed Electric Field (PEF) treatments for the inactivation of both excysted and encapsulated *Trichinella* spp. larvae. The variable of electric field strength played a crucial role in determining the impact of PEF on larvae motility, morphology, and propidium iodide (PI) uptake. Our results suggest that PEF technology could serve as a novel strategy for inactivating *Trichinella* larvae in meat and controlling this zoonosis. However, further research is necessary to assess a broader range of PEF treatment conditions and to evaluate meat quality parameters after treatment for various culinary purposes, such as braising, curing, or pâté production.

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## CRediT authorship contribution statement

**J.M. Martínez:** Writing – original draft, Methodology, Investigation, Data curation. **V. Abad:** Validation, Data curation. **J. Quílez:** Writing – review & editing, Supervision, Investigation. **D. Reina:** Resources. **J.E. Pérez-Martin:** Writing – review & editing, Supervision. **J. Raso:** Formal analysis, Data curation. **G. Cebrián:** Visualization, Supervision, Project administration. **I. Álvarez-Lanzarote:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Funding acquisition, Conceptualization.

## Declaration of competing interest

All authors disclose there are no interests to declare. There are neither financial nor personal relationships with other people or organizations that could inappropriately influence (bias) our work.

## Data availability

Data will be made available on request.

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