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Inactivation by pulsed electric fields of *Anisakis* in naturally infected hake meat

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ABSTRACT

Anisakis is a zoonotic parasite found in the stomach of marine mammals. Its eggs are released into the sea and ingested by fish and cephalopods. Humans accidently become hosts when they consume raw or undercooked fish, or cephalopods, leading them to suffer from intestinal syndromes and allergic reactions. In Europe, the officially prescribed methods for inactivation of *Anisakis* are heat treatment or freezing, both of which can affect fish quality. Several studies have demonstrated the effectiveness of PEF for the inactivation of *Anisakis*; however, none of them have featured naturally infected samples. This study focuses on 1) the inactivation of *Anisakis* by PEF in naturally infected hake belly fillets (as hake is one of the most parasitized species in Europe) and 2) the evaluation of the quality of fish samples during their shelf life after PEF treatment. Results showed that it was necessary to apply higher PEF intensities of up to 5 kV/cm to inactivate *Anisakis* when it is naturally parasitized in comparison to artificial scenarios or when the parasite is present in water. The degree of inactivation increased over time when the samples were stored after PEF treatments in a modified atmosphere containing 50% CO_2. After PEF treatments, quality analyses during shelf-life indicated that fish microbiota evolved similarly to untreated samples; however, the modified atmosphere limited the growth of the microbiota. In PEF-treated samples, quality parameters (drip loss, moisture, water holding capacity, and cooking loss) were closer to those of fresh hake and superior, in terms of quality, to the values obtained in frozen/thawed samples during their entire shelf-life.

1. Introduction

Hake is one of the most common fish species in the North East Atlantic and the Mediterranean Sea (Pascual et al., 2018). In Spain, hake is one of the most highly consumed fish varieties; moreover, it tends to be consumed fresh rather than frozen (Mercasa, 2022). However, the percentage of *Anisakis* spp. in hakes is also high, thus making potential consumer rejection a critical economic issue (ELIKA, 2023; Llarena-Reino et al., 2015). In hake, the *Anisakis* spp. parasite is mainly found in the gonads; once the fish dies, it migrates to the muscle (Santos et al., 2022; Šimat et al., 2015). *Anisakis* are parasitic nematodes belonging to the phylum *Nemathelmintes*, class *Nematoda*, *Ascarida* order, suborder *Ascaridina*, superfamily *Ascaridoidea*, *Anisakidae* family and subfamily *Anisakinae* (Aibinu et al., 2019; Murata et al., 2011).

As *Anisakis* is a zoonotic parasite, it can raise notable food safety issues. Humans become accidental hosts by ingesting raw, undercooked, or improperly processed seafood products (Aibinu et al., 2019; Chai et al., 2005), thereby leading to nausea, vomiting, abdominal pain, or allergic manifestations ranging from urticaria to anaphylactic shock (EFSA, 2010; Hochberg & Hamer, 2010). According to European legislation (EU Commission Regulation, 2004), fishery products that have not undergone heat treatment guaranteeing the death of the parasite and which are intended for raw consumption (marinated, pickled, brined, smoked, or cold-smoked) must be kept frozen at a temperature of −20 °C or lower for a period of 24 h, or at a temperature of −35 °C or lower for a period of 15 h. Freezing has proven to be an effective technology for the inactivation of *Anisakis*. However, it has a considerable impact on the quality of the product: water holding capacity decreases, drip loss increases, the texture is softer, and significant changes in the flavor and color of the fish can be observed (Chai et al., 2005; Leygonie et al., 2012).

In recent studies, Abad, Alejandre, et al. (2023) and Onitsuka et al. (2022; 2024) demonstrated the efficacy of Pulsed Electric Fields (PEF) as a method for inactivation of *Anisakis* in water and in fish. Treatment via

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Pulsed Electric Fields consists in applying high-intensity electric fields (between 0.5 and 30 kV/cm) to a product immersed in an aqueous solution and placed between two electrodes by intermittently applying pulses of short duration (in the order of microseconds), causing only a minimal increase of the product's temperature (Raso et al., 2022; Raso & Heinz, 2006; Zhang et al., 1995). Studies by Onitsuka et al. (2024; 2020) and Abad, Alejandre, et al. (2023) have demonstrated that the percentage of immobilized parasites, associated with inactivation, increases with the number of pulses applied, field strength, and energy applied per pulse in artificial parasitization of horse mackerel (*Trachurus japonicus*), salmon (*Oncorhynchus keta*), or hake (*Merluccius merluccius*). The parasite's location within the fish also had a significant impact; moreover, the electrical conductivity of the treatment medium conditioned the survivability of *Anisakis* after applying PEF (Onitsuka et al., 2022, 2024). Quality analysis of PEF-treated fish samples immediately after application of PEF treatments, versus control and frozen/thawed samples, indicated that PEF had a lower impact on quality parameters than freezing and thawing. Despite the value of these studies, further data is required regarding the inactivation of *Anisakis* present in naturally infected fish as well as the impact of PEF on fish quality not only just after the PEF treatment but also during its shelf life. Our study's objective was to investigate the inactivation impact of PEF on *Anisakis*, evaluating several PEF parameters and using hake belly, which is one of the most heavily parasitized parts of hake, with the purpose of comparing this "worst-case scenario" with results previously obtained via artificial parasitization. Additionally, since hake fillets are usually packaged in modified atmosphere (MAP) to extend their shelf life, we evaluated the combined effect of PEF and MAP storage on *Anisakis* survivability as well as on fish quality during shelf life at cooling temperatures.

2. Material and methods

2.1. Anisakis spp. larvae and fish samples

All samples of hake (*Merluccius merluccius*) were supplied by Scanfisk Seafood S.L. (Zaragoza, Spain). Fillets were received with the belly, which was separated from the fillet; both portions were stored in separate trays covered with aluminum foil at 4 °C until use.

2.2. PEF treatment of Anisakis spp. larvae in the belly

Bellies containing the parasites were cut into pieces of 4 cm (length) x 2 cm (wide). The pieces were introduced into the PEF treatment chamber, which contained a salty solution of 0.7–1 mS/cm at 4±1 °C. The electrical conductivity and temperature of the treatment medium was measured with a conductivity probe (Almemo FYA641LF series, Alhborn, Germany).

The treatment chamber consisted of two parallel circular stainless-steel electrodes with a 5 cm radius. The pulse generation equipment used in this study was the EPULSUS-PM-10, 2 kW from Energy Pulse System (Lisbon, Portugal), which applies square wave pulses with a pulse width of 1–200 μs and a maximum frequency of 200 Hz. The system's maximum voltage is 10 kV, and the current is 180 A. This equipment has a touch screen for selecting voltage, pulse width, number of pulses, and frequency. Processing parameters were recorded using an oscilloscope (Tektronix, TDS 220, Wilsonville, OR, USA) to which a voltage probe (Tektronix, P6015A, Wilsonville, OR, USA) and an amperage probe (Stangenes Industries Inc. Palo Alto, CA, USA) were connected.

After each PEF treatment, five batches of 10 *Anisakis* larvae were extracted from each piece of belly, distinguishing between *Anisakis* larvae located in the outer belly layer and *Anisakis* situated in the deeper belly tissue. Each PEF treatment condition was applied at least in triplicate. Once the larvae had been extracted, they were stored in a 0.85% NaCl saline solution for 3 h at 4 °C. Previous studies indicated that the viability of *Anisakis* after PEF did not vary after 3 h (Abad, Alejandre, et al., 2023). We monitored the viability of *Anisakis* using the mechanical stimulation technique recommended by EFSA (2010), according to which L3 larvae are considered alive if they move when mechanically stimulated by forceps.

To evaluate the influence of PEF parameters (field strength, pulse width, and specific energy) on the viability of *Anisakis* in fish belly, a central composite experimental design was applied. The ranges of the evaluated parameters were as follows. Electric field strength: 3–5 kV/cm; specific energy: 10–30 kJ/kg; and pulse width: 10–30 μs. These conditions resulted in treatment times varying from 571 to 4800 μs, applying from 19 to 158 pulses of energies per pulse ranging from 0.06 (i.e. 3 kV/cm; 10 μs) to 0.53 kJ/kg (i.e. 5 kV/cm; 30 μs), depending on the pulse width and the applied specific energy. To calculate the specific energy, Equation (1) was used:

$$W = \frac{1}{m}\int_0^t \sigma E^2 dt \qquad \text{(Equation 1)}$$

where σ is the electrical conductivity of the treated medium or product (S/m), E is the electric field strength (V/m); dt is the total time (s) during the electric field strength is applied, and m the weight of the treated medium. Finally, immediately after PEF treatments, temperature of the center of the treated fish pieces was measured using a thermocouple K (NiCr-Ni-sensor FTA 15 P1, Almemo series, Alhborn, Germany). The temperature of the piece of fish never exceeded 10 °C after the most intense PEF treatment (5 kV/cm; 30 kJ/kg).

Once the parasite's viability had been measured after several different combinations, we performed a multiple regression in which non-significant values ($p < 0.05$) were eliminated. This step was carried out with the Design-Expert 6.0.6 software package (Stat-Ease Inc., Minneapolis, MN, USA). The obtained equation was validated with new experimental data of *Anisakis* inactivation in hake belly (up to 15 new conditions) measured in laboratory within the range of the equation. To determine the final equation's accuracy, we applied R^2, R^2-adjusted, and root mean square error (RMSE) (Baranyi et al., 1999).

2.3. Effect of MAP storage on survivability of Anisakis spp.

Three PEF treatments of 3, 4, and 5 kV/cm but with the same specific energy and pulse width (20 kJ/kg and 30 μs) were applied to pieces of parasitized bellies. After the treatments, samples were removed from the treatment chamber, dried with paper, placed in polystyrene trays (23 × 25 × 8 cm), and packaged in a thermosealing machine (ULMA, SMART-400, Gipuzkoa, Spain). Three trays were prepared for each treatment, containing three belly pieces each. The trays were packed using different atmospheres: without MAP (atmospheric air), 100% N_2, and 50% CO_2 – 50% N_2. After packaging, the samples were stored at 4 °C for 5 days. We evaluated the survivability of thirty *Anisakis* larvae on days 0, 2, and 5, following the same protocol described above. These tests were performed in duplicate.

2.4. Evaluation of fish microbiota after PEF treatments

Microbiological analysis of control and PEF-treated hake fillets free of parasites and bones provided by Scanfisk Seafood S.L. was performed. The PEF treatment was 4 kV/cm, 20 kJ/kg and 30 μs which was the maximum treatment intensity applicable with the available PEF equipment.

Hake fillets were cut into 4 × 2 cm pieces (10 g ± 2.5 g) under the most possible sterile conditions, and then introduced into the treatment chamber previously immersed in 70% isopropyl alcohol, and washed with sterile water. The fillets were covered with a sterile salty solution (0.7–1 mS/cm) at 4±1 °C, and the PEF treatment was applied. As previously indicated, under these conditions, the temperature of the piece

of fish never exceeded 10 °C after the applied PEF treatment.For control samples, the same protocol without applying the PEF treatments was applied; the samples were nevertheless also introduced in the PEF treatment chamber. Samples were removed from the treatment chamber with sterile clamps, placed in a tray, and packaged (ULMA, SMART-400, Gipuzkoa, Spain) without atmosphere and with 50% CO_2 – 50% N_2 atmosphere. The trays were stored at 4 °C for 7 days, and samples were taken on days 0, 2, 4, and 7. On each sampling day, pieces weighing 10 ± 0.3 g were taken, placed together with 90 mL of sterile buffered peptone water (APT, Oxoid, Basingstoke, UK) in sterile Stomacher plastic bags (VWR, Radnor, USA), and homogenized for 60 s at 230 rpm in a Stomacher® 400 Circulator (Sweard, Worthing, UK). Microbial groups, agar, temperatures, times and incubation conditions are described in Table 1, including the plating method, similarly to the procedure described in Antunes-Rohling et al. (2019c). Analyses were performed in triplicate; each replica contained two pieces of 10 g.

2.5. Evaluation of fish quality after PEF treatments

For fish quality analysis, we used the same raw material and applied the same protocol previously described for microbiota evolution, but using pieces weighing 20 g. In this case, sample points were days 0, 4, and 7 of storage at 4 °C under packaging in MAP (50% CO_2 – 50% N_2). As in the case of the microbiota assays, a PEF treatment of 4 kV/cm, 20 kJ/kg, and 30 μs was applied.

To determine impact on hake quality after PEF treatments, an indirect study of the treatments' effect by measuring a series of properties associated with fish meat quality was conducted: drip loss, moisture, water holding capacity (WHC), and cooking loss (CL). PEF samples were compared with untreated samples, and with samples frozen for 5 days at −20 °C then thawed. All samples were stored at 4 °C under MAP. The −20 °C/5 days freezing conditions we chose were based on recommendations provided by the Spanish Authority of Food Safety (AESAN, 2023) for clients using a domestic freezer. Seven samples of fish meat were used in each procedure. All analyses were performed in duplicate for each sample.

2.5.1. Drip loss

Pieces of hake were weighed before and after applying each technology (PEF, freezing/thawing, control) at day 0 as well as after the corresponding storage time, after previous removal of external moisture with paper on the day of analysis. Drip loss was calculated using Equation (2):

$$Drip\ loss\ \% = \frac{Pi - Pf}{Pi} x\ 100 \qquad \text{Equation 2}$$

where *Pi* and *Pf* are the initial and final weight in grams.

2.5.2. Moisture

Samples weighing 6 ± 0.5 g were placed in an oven at 105 °C for 24 h (AOAC, 2022). To calculate moisture content, Equation (3)was used:

$$Moisture\ \% = \frac{Pi - Pf}{Pi} x\ 100 \qquad \text{Equation 3}$$

where *Pi* is the initial weight in grams and *Pf* is the final weight in grams.

2.5.3. Water holding capacity (WHC)

Samples weighing 6 ± 0.5 g were wrapped in gauze and placed in a 50 mL Falcon tube with 5 glass beads. The tubes were centrifuged at 1300 rpm for 15 min (MEGAFUGE 1.0 R, Kendro, Germany). After centrifugation, samples were removed from the gauze, dried with paper, and weighed (Trout, 1988). WHC was determined according to Equation (4):

$$WHC\ \% = 100 - \frac{Pi - Pf}{Pi} x\ 100 \qquad \text{Equation 4}$$

where *Pi* and *Pf* are the initial and final weight of the sample (in grams), respectively.

2.5.4. Cooking loss (CL)

Samples weighing 6 ± 0.5 g were placed in a 50 mL tube and immersed in boiling water until a temperature of 75 °C was reached inside the sample (Honikel, 1998), according to measurement with a temperature sensor (ALMEMO® R2E4, Holzkirchen, Germany) at the product's thermal center. The sample was recovered, superficial water was removed with paper, and the sample was weighed. Equation (5) was used to determine CL:

$$CL\ \% = \frac{Pi - Pf}{Pi} x\ 100 \qquad \text{Equation 5}$$

where *Pi* and *Pf* are the initial and final weight of the sample (in grams), respectively.

2.6. Statistical analysis

GraphPad PRISM® program (GraphPad Software, San Diego, CA, USA) was used to determine whether there were statistically significant differences between the different parameters under study by performing one-way ANOVA with Tukey's post-test. Error bars in the figures correspond to the standard deviation of the mean.

3. Results and discussion

3.1. PEF survivability of Anisakis in hake belly

Two previous investigations, Abad, Alejandre, et al. (2023) and Onitsuka et al. (2022), studied the inactivation of *Anisakis* in saline solution and in artificially parasitized pieces of hake or horse mackerel. Recently, Onitsuka et al. (2024) evaluated the survivability to PEF of *Anisakis* that had naturally burrowed into the meat after the larvae had been artificially embedded in cut salmon. Since *Anisakis* in hake are

Table 1
Recovery conditions for the microbial groups under study.

Microbial group	Agar	Temperature	Time	Atmosphere	Plating
Aerobic Psychrotrophes	LH Agar[a]	7 °C	10 d	Aerobic	Spread
Anaerobic Psychrotrophes	LH Agar[a]	7 °C	10–12 d	Anaerobic	Spread
Pseudomonas	GSP Agar[b]	25 °C	24–48 h	Aerobic	Spread
Shewanella	Iron Agar[c]	25 °C	3–4 d	Aerobic	Spread
Lactic Acid Bacteria	Elliker Agar [d]	25 °C	24–48 h	Aerobic	Pour
Enterobacteriaceae	VRBG Agar [e]	37 °C	48 h	Aerobic	Spread (double layer)

[a] Long and Hammer Agar (Broekaert et al., 2011).
[b] Glutamate Starch Phenol Red Agar (Sigma-Aldrich, Steinheim, Germany) + Penicillin G (Sigma-Aldrich, Steinheim, Germany).
[c] Iron Agar (Lingby) (Conda, Madrid, Spain).
[d] Elliker Broth (Sigma-Aldrich, Steinheim, Germany) + Bacteriological Agar (Oxoid, Basingstoke Hants, UK).
[e] Violet Red Bile Glucose Agar (VRBG) (Oxoid, Basingstoke Hants, UK).

encysted and remain attached to internal tissues (Ángeles-Hernández et al., 2020), our study has explored the inactivation of the parasite in naturally parasitized hake. We used the belly area of the hake because it is the portion with the greatest concentration of *Anisakis* L3 (Roepstorff et al., 1993) after capture. Moreover, one of our study's aims was to determine whether the inactivation resistance of *Anisakis* is different when compared to artificial parasitization.

Fig. 1 shows the percentage of survivors to PEF treatments of different electric field strengths (from 1 to 5 kV/cm), pulse widths (from 10 to 200 μs), and specific energies (10 and 20 kJ/kg). As can be seen, lethal efficacy mainly depends on electric field strength. With a treatment of 1 kV/cm, 20 kJ/kg, and 10 μs (28 ms of total treatment time), hardly any inactivation occurred, but when the electric field was raised to 5 kV/cm for the same energy and pulse width (1.1 ms total treatment time), survivability decreased to 30%. Pulse width also affected the survival rate of *Anisakis*. Thus, at 2 kV/cm, the percentage of survivors varied from 85%, in the case of 10 μs pulse width, to 50% for pulsed widths of 200 μs. Although wider pulse widths can have certain advantages, they tend to cause long-term deterioration of the electrodes (Ho and Mittal, 2000; Pataro et al., 2014). In this study, we could not apply long pulses at higher field strengths due to limitations of the PEF system. Lastly, the specific energy applied to the parasites located in the meat also conditioned their viability. As shown in Fig. 1, the percentage of survivors after an electric field strength of 3 kV/cm with pulses of 30 μs was 80% with a specific energy of 10 kJ/kg (1.6 ms) and 65% with 20 kJ/kg (3.2 ms). Independently of all these results, Fig. 1 shows that the maximum inactivation of *Anisakis* obtained in hake belly reached around 90-80% by applying PEF treatments of 5 kV/cm, 20 kJ/kg, and 30 μs (1.1 ms of total treatment time).

As recently described by Onitsuka et al. (2024), the location of parasites in the fish, specifically in salmon, could be a determinant factor in their survivability to PEF treatments. Following a similar concept, Fig. 2 shows the survivability of *Anisakis* when hake bellies were treated with PEF, according to whether the parasite was naturally located on the surface of the muscle or in the meat. As observed, survivability was generally higher (10.9 % ± 1.4 higher) in larvae situated deeper inside

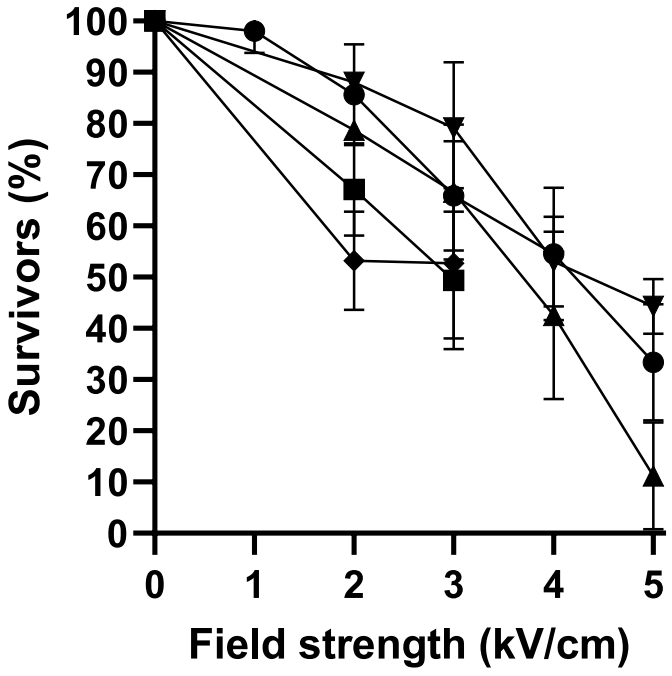

Fig. 1. Influence of electric field strength on the percentage of *Anisakis* survivors after the application of PEF treatments of different pulse widths and specific energies: 20 kJ/kg and 10 μs (●), 10 kJ/kg and 30 μs (▼), 20 kJ/kg and 30 μs (▲), 20 kJ/kg and 100 μs (■), 20 kJ/kg and 200 μs (◆).

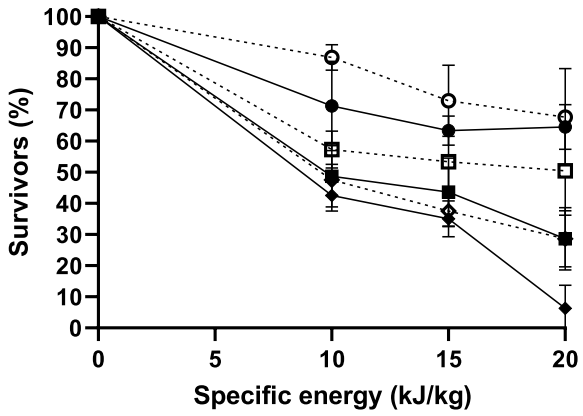

Fig. 2. Influence on the percentage of *Anisakis* survivors based on their position: on the surface (continuous lines) or inside the meat (dotted lines) of hake bellies after the application of PEF treatments of 30 μs and different specific energies and field strengths: 3 kV/cm (●), 4 kV/cm (■) and 5 kV/cm (◆) (n = 5).

the meat. This higher degree of survivability might be due to lower field strength inside the muscle, based on differences in electrical conductivity among the different compounds of the meat structure compared to those located on the surface (Abad, Grasa, et al., 2023). In the future, it would thus be advisable to conduct PEF inactivation studies of *Anisakis,* taking into account the PEF resistance of parasites located inside the meat.

Considering this point and in order to further explore the impact of PEF parameters (in which E = electric field strength; W = specific energy; P = pulse width) on the survivability of *Anisakis* when treated in hake belly, a central composite design was implemented. Table 2 shows the inactivation effect of different PEF combinations on *Anisakis*, evaluating the survivability of parasites located on the surface or in the internal part of the belly. As observed, inactivation under most of the evaluated conditions was higher for parasites located on the surface of the belly: they were 11.5 % ± 5.03% less resistant to PEF (similarly to the results displayed in Fig. 2). Independently of the parasites' location, inactivation generally increased in parallel with the three parameters; however, it was difficult to evaluate the importance and impact of each

Table 2
Central composite design evaluating the survivability of *Anisakis* L3 larvae when located inside the hake belly and on the surface after PEF treatments of different field strength, pulse width, and specific energy.

Field Strength (kV/cm)	Pulse Width (μs)	Specific Energy (kJ/kg)	Inside survivability (%)	Surface survivability (%)
3	30	10	86,9	71,3
3	10	20	76,7	65
3	20	20	78,1	72,9
3	30	20	67,7	64,5
3	20	30	81,5	40
4	30	10	57,3	48,7
4	10	20	61,7	46
4	20	20	42,5	36
4	20	20	42,5	36
4	20	20	40	38,8
4	20	20	45	34
4	30	20	50,5	28,6
4	20	30	51,9	22,5
5	30	10	47,5	42,5
5	10	20	33,3	33,6
5	20	20	28,3	30
5	10	30	21,9	6,3
5	20	30	14,8	0

parameter separately. Using multiple regression analysis, polynomial equations for the two locations were developed (Equations (6) and (7)). Table 3 shows the obtained polynomial equations, including the coefficients for each parameter, their statistical significance (p value), and the 95% confidence limits of each coefficient. In addition, the equation's goodness of fit in terms of R^2, R^2-adjusted, and RMSE are indicated. Similar equations were obtained for both data sets and for all three parameters affecting the parasites' resistance to PEF. Field strength and mainly the specific energy were the main parameters affecting survival rates. Although we were able to obtain an equation for each location, we focused on the equation related to parasite survivability inside the fish meat (Equation (6)), as parasites located inside the meat showed a higher degree of resistance to PEF. Based on that equation, the influence of pulse width (Fig. 3A), field strength (Fig. 3B), specific energy (Fig. 3C) and treatment time (Fig. 3D) of PEF treatments on the survivability of *Anisakis* L3 larvae located inside hake belly was predicted. Fig. 3D has been plotted based on the predictions done by Equations (6) and (7) and transforming the resulted specific energy by the number of pulses applied depending on the applied pulse width and the specific energy per pulse. In all figures, for comparison purposes, we include an estimation of the case in which parasites would be located on the surface of the belly (thicker line). In addition, the standard deviation of the predictions are included as grey (for inside inactivation) and blue (superficial inactivation) shadowed area at both sides of the prediction lines. As previously indicated, the predictions display that the most influential parameters are electric field and specific energy. Maximum inactivation in hake bellies (parasites internally located) would be achieved with 5 kV/cm, 30 μs, and 30 kJ/kg requiring processing times of 1.7 ms. These treatments would be stronger than the ones measured when treating *Anisakis* in salty solution or in artificially parasitized fish (Abad, Alejandre, et al., 2023), Abad, Grasa, et al., 2023nd as observed when parasites are located on the surface of the belly (the blue lines in Fig. 3ABCD). The observed inactivation in hake belly resulted slightly lower to that described by Onitsuka et al. (2024). For a complete inactivation, those authors required 3.75 kV/cm and energies of around 42.6 kJ/kg in a cut salmon where *Anisakis* had naturally burrowed into the meat after larvae had been artificially embedded. The slightly higher inactivation could be both to different fish species were treated which could be affecting the lethality of PEF and high energy levels were used by the other authors. In our study, the maximum specific energy was 30 kJ/kg, which resulted in an increment of fish meat temperature of less than 7 °C. Higher specific energies might negatively affect the product's temperature and, consequentially, its quality (Abad, Alejandre, et al., 2023).

Table 3
Polynomial equations describing the percentage of survivability (S) of *Anisakis* when located inside (upper table) or on the surface (lower table) of hake belly after PEF treatments of different electric field strength (E), specific energy (W), and pulse width (P). Statistical significance (p value) and the 95% confidence limits of each parameter are included.

%S internal = $b_0 + b_1 \times E + b_2 \times W + b_3 \times P + b_4 \times E{*}W + b_5 \times W{*}W$ **(Equation (6))**
$R^2 = 0.942$; R^2 adjusted = 0.918; RMSE = 5.944

	Coefficient	p value	CL (-95%)	CL (+95%)
b_0	167.08	0.000105	102.79	231.37
b_1	−10.87	0.00959	−23.98	2.241
b_2	−2.484	0.00164	−6.131	1.164
b_3	−0.577	0.04637	−1.143	−0.01090
b_4	−0.706	0.02521	−1.309	−0.104
b_5	0.107	0.00062	0.03658	0.177

%S surface = $b_0 + b_1 \times E + b_2 \times W + b_3 \times P + b_4 \times E{*}E$ **(Equation (7))** $R^2 = 0.930$; R^2 adjusted = 0.909; RMSE = 6.027

	Coefficient	p value	CL (-95%)	CL (+95%)
b_0	284.16	3.461×10^{-5}	184.42	383.91
b_1	−74.99	0.00619	−124.66	−25.31
b_2	−2.054	4.0543×10^{-6}	−2.640	−1.468
b_3	−0.778	0.00868	−1.271	−0.284
b_4	6.857	0.03245	0.669	13.04

In order to more specifically elicit the effect of parasite location in fish on a parasite's resistance to PEF, and based on Equation (6) (Table 3) corresponding to internal survivability, we plotted Fig. 4. It shows the relationship between the estimated inactivation based on eEquation 6 and the observed inactivation of *Anisakis* when located inside the meat (triangles). Fig. 4 shows that the developed equation adequately describes the new set of experimental data obtained to plot the figure. However, Fig. 4 also includes the relationship between the survivability obtained with Equation (6) (the one for the internal survivability) and the experimental inactivation of *Anisakis* when located on the belly surface (open squares) or immersed in saline water solution (circles). Data corresponding to the inactivation in saline solution are the values obtained by Abad, Alejandre, et al. (2023). In these cases, the developed Equation (6) for internal survivability to PEF underestimated the inactivation data for *Anisakis* when the parasite was located closer to the belly surface; this resistance at the surface was more similar to the values obtained when parasites had been treated in saline solution. These results confirm the higher resistance of parasites when they are located internally in the meat; then it can be concluded that the obtained equation could be used to predict *Anisakis* inactivation by PEF in a worst-case scenario. Thus, Equation (6) obtained to predict the internal survivability of parasites can be regarded as a reliable equation for purposes of defining PEF treatment conditions to achieve a desired level of *Anisakis* inactivation in the studied range of conditions. Therefore, and based on that equation, PEF treatments of 5 kV/cm and 30 kJ/kg applying pulses of 20–30 μs would reduce the survivability of *Anisakis* located inside the belly to 10% and almost completely inactivate the parasite when it is situated on the surface.

3.2. Effect of MAP storage on the PEF inactivation of Anisakis in hake belly

Previous studies have shown that the survivability of *Anisakis* after PEF treatments decreases with time (Onitsuka et al., 2022, 2024). This would indicate possible damage to the parasite due to PEF, as occurs with bacteria (Pillet et al., 2016). In bacteria, such damage results in higher inactivation levels when combined with other preservation strategies: such hurdle combinations, in turn, make it possible to reduce the intensity of the PEF treatment. However, such a combined effect has not yet been investigated in the case of parasites subjected to PEF treatments. As hake fillets packaged in a modified atmosphere (MAP) composed of 50% CO_2 and 50% N_2 are one of the main retail varieties of hake available for consumer consumption in Spain (Antunes-Rohling et al., 2019a, 2019b, 2019c), PEF combined with MAP could represent a thoroughly effective synergistic combination for the elimination of *Anisakis*. Fig. 5 shows the percentage of *Anisakis* survivors after the application of PEF (3-4-5 kV/cm, 20 kJ/kg, 30 μs) to pieces of hake belly packed with three different kinds of modified atmospheres (without MAP, 100% N_2, and 50% CO_2 – 50% N_2) and stored at 4 °C during 5 days. As observed, when the MAP contained 50% CO_2, the survivability of *Anisakis* decreased with storage time and was more pronounced at 3 kV/cm. Thus, inactivation increased from 30% at day 0, to 40% and 60% after 2 and 5 days of storage, respectively, after having applied PEF at 3 kV/cm. An additional degree of inactivation of the order of 20% and 10% was observed when the treatment was of 4 and 5 kV/cm, respectively. In cases of control and MAP with 100% of N_2, there was no effect on *Anisakis* inactivation. Based on these results, after a PEF treatment of 5 kV/cm, 20 kJ/kg, and 30 μs where the survivability was around 15%, storage in MAP (50% CO_2 – 50% N_2) at 4 °C decreased the number of survivors to under 10%.

Since the observed effect was only occurring in the presence of CO_2, this gas can be regarded as an essential factor behind the observed effect. However, CO_2 by itself has not been shown to affect *Anisakis* survivability. The impact of an atmosphere with a high percentage of CO_2 on the inactivation of *Anisakis* was not described in the studies carried out

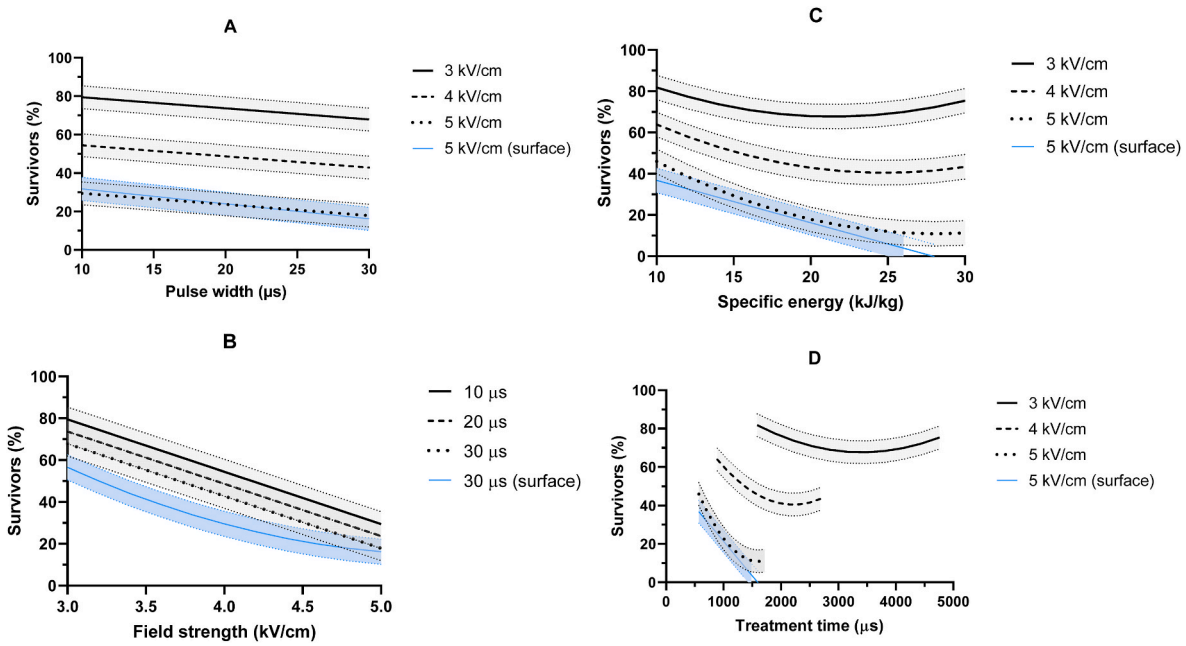

Fig. 3. Influence of pulse width (3A), field strength (3B), specific energy (3C), and treatment time (3D) on the survivability of *Anisakis* inside hake belly based on the equation shown in Table 3. In Fig. 3A and 3B, all treatments were applied at 20 kJ/kg; in Fig. 3C and 3D, 30 μs pulses were used. The thicker and blue lines indicate the parasite's survivability on the surface of the belly. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

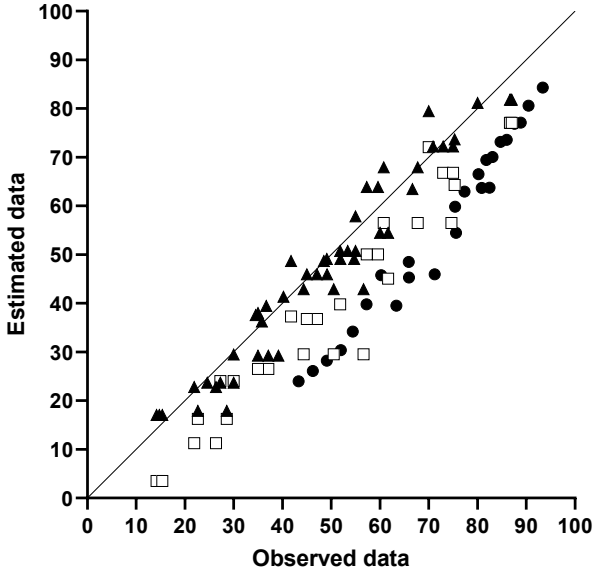

Fig. 4. Relationship between the estimated inactivation based on the obtained equation shown in Table 3 corresponding to the internal survivability and the experimentally observed survivability of *Anisakis* when located inside the meat (▲), on the surface of the belly (□), or immersed in saline water solution (●, data from Abad, Alejandre, et al., 2023).

by Guan et al. (2021) and Pascual et al. (2010). The only effect they observed when using a high CO_2 concentration was a noticeable migration of the parasites from the inner parts of the fish to the surface (Pascual et al., 2010). Based on these results, one could infer that the storage of *Anisakis* in a CO_2-rich atmosphere after PEF treatments had a synergistic lethal effect on *Anisakis*, resulting in a higher degree of inactivation. This is the first time such an effect has been described in the literature; practical implications for the application of PEF technology are of considerable interest. More research is necessary to evaluate the mechanism of action exerted by this combined effect.

3.3. *Evaluation of fish microbiota after PEF treatments and storage in MAP*

Fig. 6 shows the evolution of microbial groups under study, including Aerobic (Fig. 6A) and Anaerobic Psychrotrophic microorganisms (Fig. 6B), *Pseudomonas* (Fig. 6C), *Shewanella* (Fig. 6D), lactic acid bacteria (Fig. 6E), and *Enterobacteriaceae* (Fig. 6F), in untreated hake pieces and in pieces treated with PEF (4 kV/cm; 20 kJ/kg; 30 μs), followed by storage at 4 °C with and without MAP (50% CO_2 – 50% N_2). The initial counts of each microorganism were quite similar to those of the samples analyzed by Antunes-Rohling et al. (2019a, 2019b, 2019c). Aerobic and Anaerobic Psychotropic bacteria always had the highest initial counts; their subsequent growth had high similarities. In contrast, the lowest initial counts were of *Enterobacteriaceae*, as observed by other authors (Antunes-Rohling et al., 2019a, 2019b, 2019c).

In PEF-treated samples (continuous lines in Fig. 6), the evolution of microbiota was similar, thus indicating that PEF did not affect the initial counts of microbiota or their growth. On the other hand, MAP slowed down growth during storage of the microbiota under study, regardless of whether the samples were PEF-treated or untreated. Such a growth rate slowdown in MAP was also observed by Ordóñez et al. (2020), using an atmosphere featuring a CO_2 concentration similar to the one used in this investigation. In the specific case of *Pseudomonas*, which is generally regarded as one of the primary agents responsible for the spoilage of fish species stored under aerobic conditions (as *Pseudomonas* is a strictly aerobic microorganism), storage under modified atmosphere without oxygen limited the bacterium's growth (Fig. 6C).

Based on these results, PEF treatments applied to reduce the survivability of *Anisakis* in hake did not affect the evolution of microbiota during storage at cooling temperatures and packaged with atmospheric air or in MAP with 50% CO_2 – 50% N_2. In the latter case, only the MAP was responsible for growth limitation of the microbiota present in hake.

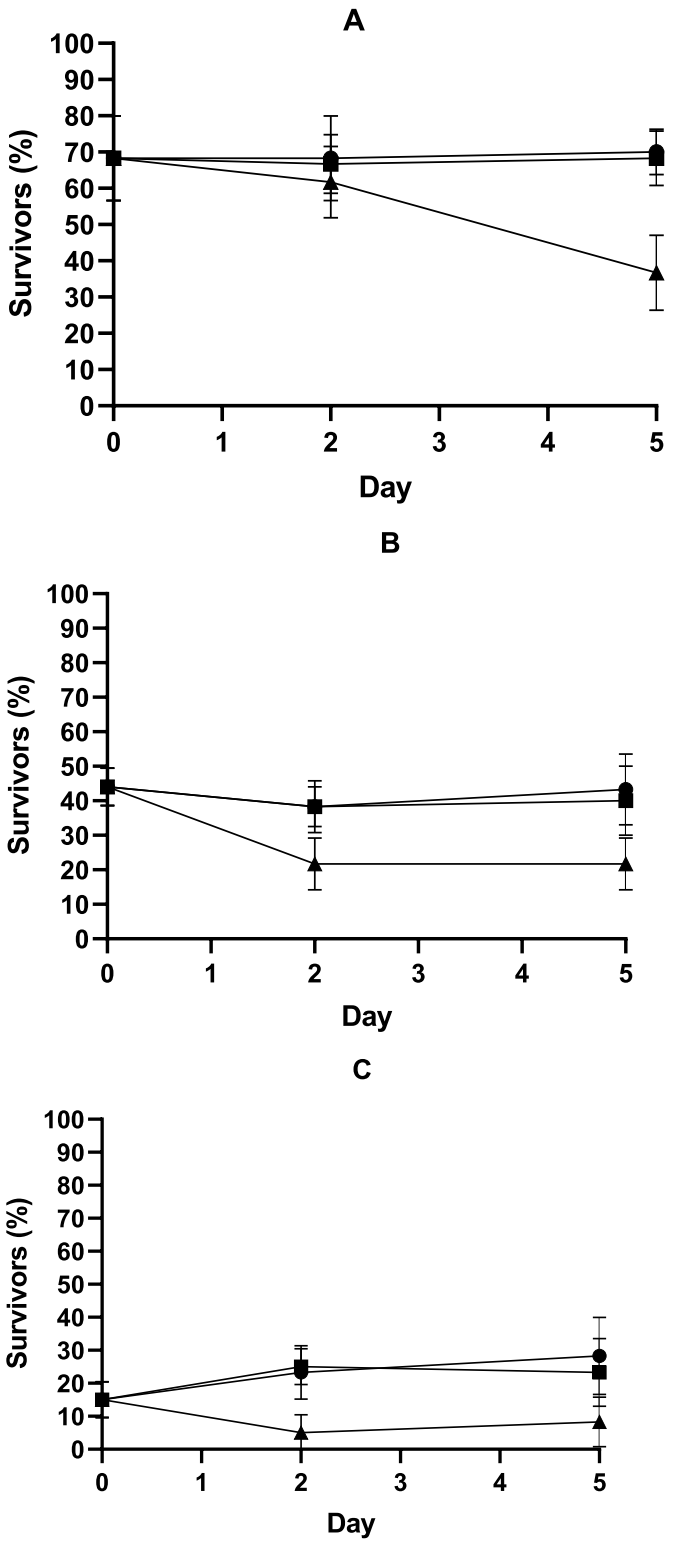

Fig. 5. Survivability of *Anisakis* L3 larvae in hake belly after applying PEF treatments of 3 kV/cm (5A), 4 kV/cm (5B), and 5 kV/cm (5C), and then packaged: without MAP (●), in MAP with 100% N_2 (■), and in MAP with 50% CO_2 – 50% N_2 (▲), and storage at 4 °C. All PEF treatments were applied at 20 kJ/kg, and with pulses of 30 μs.

3.4. Evaluation of fish quality after PEF treatments and storage in MAP

To evaluate the impact of PEF treatments on fish quality during storage at 4 °C in MAP (50% CO_2 – 50% N_2), drip loss, moisture, water holding capacity (WHC), and cooking loss (CL) of pieces of hake after applying PEF treatments of 4 kV/cm, 20 kJ/kg, and 30 μs was measured. Those quality parameters of PEF-treated samples were compared with those of control samples (fresh hake non-PEF treated), and with samples that had been stored for 5 days at −20 °C and subsequently thawed. Results are shown in Fig. 7, where Fig. 7A represents drip loss of the samples; Fig. 7B shows the moisture content; Fig. 7C, the water holding capacity; and Fig. 7D, cooking loss of pieces of hake that were either PEF-treated, frozen/thawed, or fresh.

Fig. 7A shows that weight losses due to drip loss were more pronounced in frozen/thawed samples on all tested days. PEF-treated samples also showed higher losses compared to control, but the differences were not statistically significant. For all tested days, the losses in PEF-treated samples were lower compared to frozen samples.

Similarly to drip loss, moisture measurement is an important parameter for the assessment of water content in muscle before and after a treatment. In general, sample moisture decreased during the storage period for all treatments, with the exception of day 7 for the control and PEF samples, but not to a statistically significant extent (Fig. 7B). As shown in Fig. 7B, PEF treatments did not affect water content, but water content was significantly reduced in the case of the frozen/thawed samples. These results, together with those shown for drip loss (Fig. 7A), infer that the eventual electroporation of muscle cells would not be sufficiently significant to produce a loss of water as great as the loss observed in frozen pieces.

WHC indicates the loss of the capacity of fish muscle to retain water after centrifugation, which gives an idea of the juiciness of the flesh (Offer et al., 1989). Fig. 7C shows that the samples' WHC generally tended to decrease during the storage period for all treatments. The PEF samples showed WHC values similar to control samples throughout storage except for day 4, where a statistically significant difference could be observed. In other words, the electroporation that PEF could instill in the muscle cells would not affect water retention capacity. On the other hand, the frozen samples showed the greatest differences compared to control, therefore indicating that freezing would tend to affect WHC more than PEF. This implies that the juiciness of fish treated with PEF would be more pronounced than that of frozen/thawed fish and similar to that of fresh samples.

Finally, cooking loss indirectly measures the level of damage suffered by proteins after treatments and subsequent cooking (Toldrá, 2010). When samples are subjected to high temperatures (75 °C), the affected proteins become denatured and can no longer retain the water they previously contained (Skipnes et al., 2007). Fig. 7D shows that PEF and control samples behaved similarly, with higher values of CL for PEF-treated samples at day 0. During storage, the CL values of frozen samples were slightly higher than control and PEF samples on days 4 and 7.

Results obtained on day 0 of storage in our study are in agreement with those of Abad, Alejandre, et al. (2023) in hake pieces. In horse mackerel, Onitsuka et al. (2022) did not observe any impact of PEF treatments on fish quality. Our study describes quality parameters of hake during shelf-life in MAP. In general, we conclude that PEF-treated samples would tend to be of superior quality than frozen ones and similar in quality to untreated samples.

4. Conclusions

Inactivation of *Anisakis* in naturally parasitized hake bellies was highly dependent on PEF parameters, among which the two most important were field strength and specific energy. In addition, *Anisakis* survivability depended on the parasite's location, as it was more resistant to PEF when located inside the fish meat. Based on these observations, a mathematical equation was developed that enabled to estimate the survivability of *Anisakis* under worst-case circumstances. Complete inactivation of the parasite in hake belly was achieved with a treatment of 5 kV/cm and 30 kJ/kg, applying pulses of 30 μs. Since the belly is the

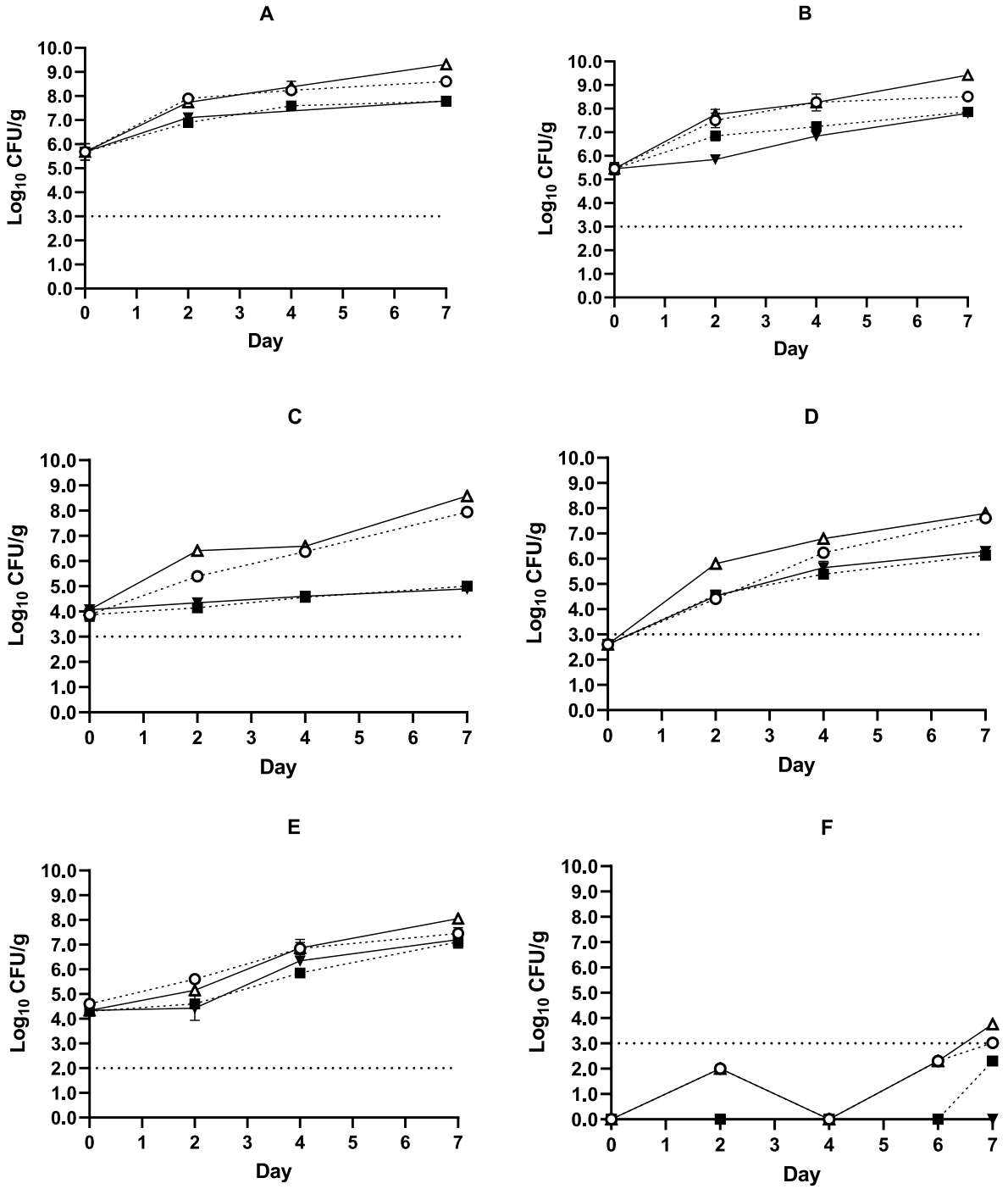

Fig. 6. Growth curves of Aerobic Psychrotrophes (6A), Anaerobic Psychrotrophes (6B), *Pseudomonas* (6C), *Shewanella* (6D), Lactic Acid Bacteria (6E), *Enterobacteriaceae* (6F) in control (dotted lines) and PEF samples (continuous lines), stored under (■,▼) MAP (50% CO_2 – 50% N_2) and without MAP (◯,△) at 4 °C. Error bars represent the standard deviation.

hake portion containing the highest amount of parasites, lower PEF intensities would be required for a complete *Anisakis* inactivation in hake fillets which are much less parasited. On the other hand, PEF technology combined with the storage of samples in a MAP containing CO_2, appears to have a synergistic lethal effect, thereby further increasing the potential degree of inactivation of *Anisakis*.

PEF treatments did not affect the microbiota of hake fillets; however, the presence of CO_2 limited the growth of microbiota during storage at 4 °C. In addition, quality parameters of PEF-treated hake were thoroughly similar to those of fresh samples and superior to those of frozen/thawed ones, even after a storage time of 7 days in MAP (50% CO_2 – 50% N_2). These results suggest that PEF could represent a promising alternative to freezing as a strategy for the elimination of *Anisakis* in fish, specifically in hake. In any case, further studies on other fish species would be required to support this conclusion.

CRediT authorship contribution statement

V. Abad: Validation, Investigation, Writing – original draft, Visualization. **J.M. Martínez:** Writing – review & editing. **M.P. Mañas:** Data Curation. **J. Raso:** Methodology, Formal analysis. **G. Cebrián:** Software, Resources, Funding acquisition. **I. Álvarez-Lanzarote:**

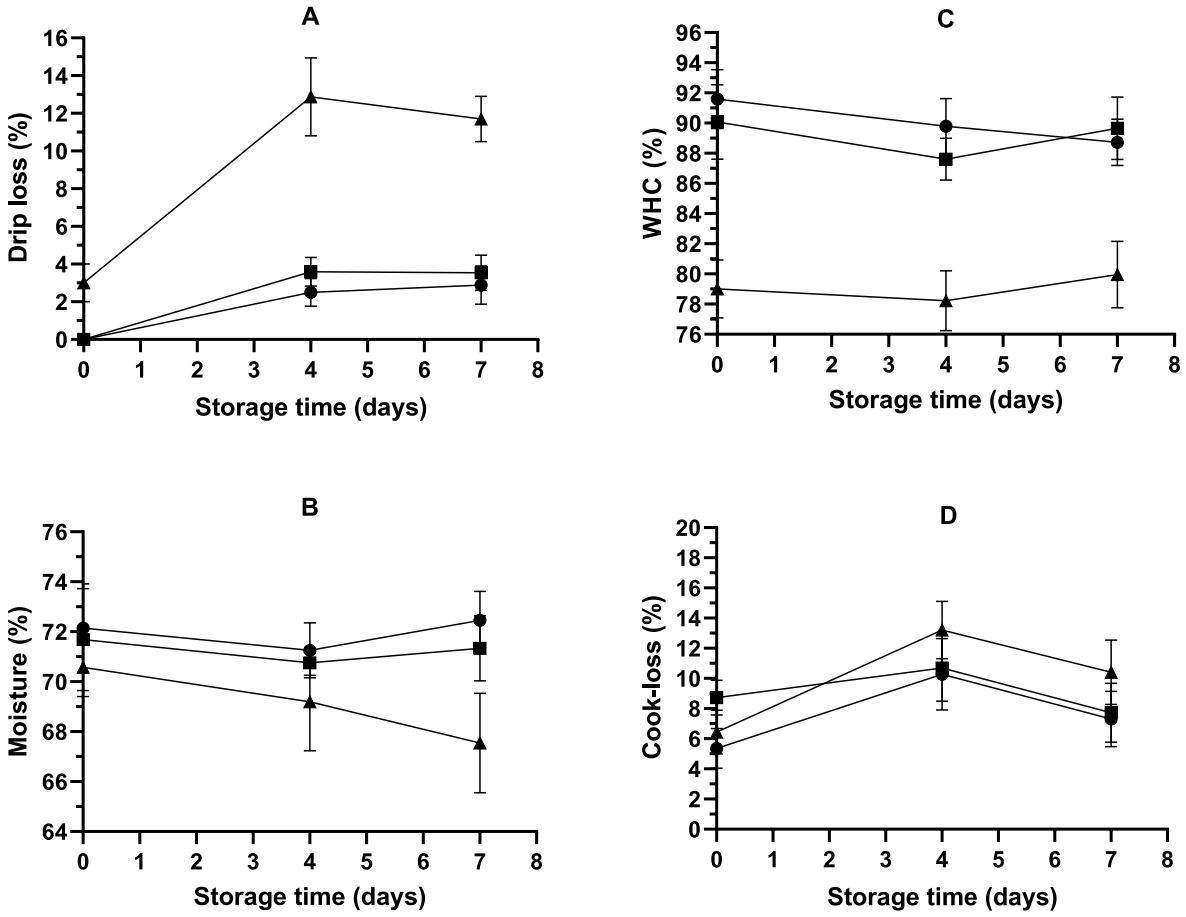

Fig. 7. Drip loss (7A), moisture content (7B), WHC (7C), and CL (7E) of control (●), PEF (■), and frozen/thawed (▲) hake fillet samples during storage at 4 °C and packaged in MAP (50% CO_2 – 50% N_2).

Conceptualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors of the manuscript entitled "Inactivation of *Anisakis* in naturally infected hake meat by Pulsed Electric Fields", Abad, V.; Martínez, J.M.; Mañas, M.P.; Raso, J., Cebrián, G.; Álvarez-Lanzarote, I., declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Álvarez-Lanzarote, I., as the corresponding author complete this document on behalf of all the authors of a submission.

Data availability

Data will be made available on request.

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References

Abad, V., Alejandre, M., Hernández-Fernández, E., Raso, J., Cebrián, G., & Álvarez-Lanzarote, I. (2023a). Evaluation of pulsed electric fields (PEF) parameters in the inactivation of anisakis larvae in saline solution and hake meat. *Foods, 12*(2), 264. https://doi.org/10.3390/FOODS12020264

Abad, V., Grasa, J., Calvo, B., Ospina, S., Cebrián, G., & Álvarez, I. (2023b). Inactivation of Anisakis in a real scenario and numerical simulation when applying Pulsed Electric Fields (PEF). *Poster session presentation at European Federation of Food Science and Technology (EFFoST)*. Valencia, Spain.

AESAN. (2023). Anisakis. Retrieved from https://www.aesan.gob.es/AECOSAN/web/para_el_consumidor/ampliacion/anisakis.htm. (Accessed 12 November 2023).

Aibinu, I. E., Smooker, P. M., & Lopata, A. L. (2019). Anisakis nematodes in fish and shellfish- from infection to allergies. *International Journal for Parasitology, 9*, 384–393. https://doi.org/10.1016/J.IJPPAW.2019.04.007. Parasites and Wildlife.

Ángeles-Hernández, J. C., Gómez-De Anda, F. R., Reyes-Rodríguez, N. E., Vega-Sánchez, V., García-Reyna, P. B., Campos-Montiel, R. G., Calderón-Apodaca, N. L., Salgado-Miranda, C., & Zepeda-Velázquez, A. P. (2020). Genera and species of the Anisakidae family and their geographical distribution. *Animals, 10*(12), 1–23. https://doi.org/10.3390/ANI10122374

Antunes-Rohling, A., Artaiz, Á., Calero, S., Halaihel, N., Guillén, S., Raso, J., Álvarez, I., & Cebrián, G. (2019a). Dataset on the use of the Ratkowsky model for describing the influence of storage temperature on microbial growth in hake fillets (Merluccius merluccius) stored under MAP. *Data in Brief, 27*. https://doi.org/10.1016/j.dib.2019.104743

Antunes-Rohling, A., Artaiz, Á., Calero, S., Halaihel, N., Guillén, S., Raso, J., Álvarez, I., & Cebrián, G. (2019b). Modelling microbial growth in modified-atmosphere-packed hake (Merluccius merluccius) fillets stored at different temperatures. *Food Research International, 122*, 506–516. https://doi.org/10.1016/J.FOODRES.2019.05.018

Antunes-Rohling, A., Calero, S., Halaihel, N., Marquina, P., Raso, J., Calanche, J., Beltrán, J. A., Álvarez, I., & Cebrián, G. (2019c). Characterization of the spoilage microbiota of hake fillets packaged under a modified atmosphere (MAP) rich in CO2 (50% CO2/50% N2) and stored at different temperatures. *Foods, 8*(10), 489. https://doi.org/10.3390/FOODS8100489, 473.

AOAC. (2022). *Official methods of analysis* (17th ed.).

Baranyi, J., Pin, C., & Ross, T. (1999). Validating and comparing predictive models. *International Journal of Food Microbiology, 48*(3), 159–166. https://doi.org/10.1016/S0168-1605(99)00035-5

Broekaert, K., Heyndrickx, M., Herman, L., Vlaemynck, G., & Devlieghere, F. (2011). Seafood quality analysis: Molecular identification of dominant microbiota after ice storage on several general growth media. *Food Microbiology, 28*(6), 1162–1169. https://doi.org/10.1016/j.fm.2011.03.009

Chai, J. Y., Murrell, K. D., & Lymbery, A. J. (2005). Fish-borne parasitic zoonoses: Status and issues. *International Journal for Parasitology, 35*(11–12), 1233–1254. https://doi.org/10.1016/J.IJPARA.2005.07.013

EFSA. (2010). Panel on Biological Hazards. Scientific Opinion on risk assessment of parasites in fishery products. *EFSA Journal, 8*(4). https://doi.org/10.2903/J.EFSA.2010.1543

ELIKA. (2023). Anisakis. Retrieved from https://seguridadalimentaria.elika.eus/fichas-de-peligros/anisakis/. (Accessed 8 November 2023).

EU Commission Regulation. (2004). Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Official Journal of the European Union*, 139–155. Retrieved from https://eur-lex.europa.eu/eli/reg/2004/853/oj. (Accessed 17 October 2023).

Guan, A., Van Damme, I., Devlieghere, F., & Gabriël, S. (2021). Effect of temperature, CO2 and O2 on motility and mobility of Anisakidae larvae. *Scientific Reports, 11*(1), 1–11. https://doi.org/10.1038/s41598-021-83505-5

Ho, S., & Mittal, G. S. (2000). High voltage pulsed electrical field for liquid food pasteurization. *Food Reviews International, 16*(4), 395–434. https://doi.org/10.1081/FRI-100102317

Hochberg, N. S., & Hamer, D. H. (2010). Anisakidosis: Perils of the deep. *Clinical Infectious Diseases, 51*(7), 806–812. https://doi.org/10.1086/656238

Honikel, K. O. (1998). Reference methods for the assessment of physical characteristics of meat. *Meat Science, 49*(4), 447–457. https://doi.org/10.1016/S0309-1740(98)00034-5

Leygonie, C., Britz, T. J., & Hoffman, L. C. (2012). Impact of freezing and thawing on the quality of meat: Review. *Meat Science, 91*(2), 93–98. https://doi.org/10.1016/J.MEATSCI.2012.01.013

Llarena-Reino, M., Abollo, E., Regueira, M., Rodríguez, H., & Pascual, S. (2015). Horizon scanning for management of emerging parasitic infections in fishery products. *Food Control, 49*, 49–58. https://doi.org/10.1016/J.FOODCONT.2013.09.005

Mercasa. (2022). Alimentación en España 2022. Retrieved from https://www.mercasa.es/publicaciones/alimentacion-en-espana/. (Accessed 12 October 2023).

Murata, R., Suzuki, J., Sadamasu, K., & Kai, A. (2011). Morphological and molecular characterization of Anisakis larvae (Nematoda: Anisakidae) in Beryx splendens from Japanese waters. *Parasitology International, 60*(2), 193–198. https://doi.org/10.1016/J.PARINT.2011.02.008

Offer, G., Knight, P., Jeacocke, R., Almond, R., Cousins, T., Offer, G., Knight, P., Jeacocke, R., Almond, R., Cousins, T., Elsey, J., Parsons, N., Sharp, A., Starr, R., & Purslow, P. (1989). The structural basis of the water-holding, appearance and toughness of meat and meat products. *Food Structure, 8*(1), 151–170.

Onitsuka, C., Nakamura, K., Wang, D., Matsuda, M., Tanaka, R., Inoue, Y., Kuroda, R., Noda, T., Negoro, K., Negoro, T., & Namihira, T. (2022). Inactivation of anisakis larva using pulsed power technology and quality evaluation of horse mackerel meat treated with pulsed power. *Fisheries Science, 88*(2), 337–344. https://doi.org/10.1007/S12562-022-01593-2/FIGURES/7

Onitsuka, C., Nakamura, K., Wang, D., Matsuda, M., Tanaka, R., Inoue, Y., & Namihira, T. (2024). Dependence of anisakid larva inactivation by pulsed power on various parameters. *Journal of Food Engineering, 360*. https://doi.org/10.1016/j.jfoodeng.2023.111715

Ordóñez, J. A., López-Gálvez, D. E., Fernández, M., Hierro, E., & De La Hoz, L. (2020). Microbial and physicochemical modifications of hake (Merluccius merluccius) steaks stored under carbon dioxide enriched atmospheres. *Journal of the Science of Food and Agriculture, 80*(13), 1831–1840. https://doi.org/10.1002/1097-0010(200010)80:13<1831::AID-JSFA707>3.0.CO;2-Z

Pascual, S., Antonio, J., Cabo, M. L., & Piñeiro, C. (2010). Anisakis survival in refrigerated fish products under CO2 modified-atmosphere. *Food Control, 21*(9), 1254–1256. https://doi.org/10.1016/J.FOODCONT.2010.03.002

Pascual, S., Rodríguez, H., Pierce, G. J., González, A. F., & Hastie, L. C. (2018). The NE atlantic European hake: A neglected high exposure risk for zoonotic parasites in European fish markets. *Fisheries Research, 202*, 69–78. https://doi.org/10.1016/j.fishres.2017.12.008

Pataro, G., Falcone, M., Donsì, G., & Ferrari, G. (2014). Metal release from stainless steel electrodes of a PEF treatment chamber: Effects of electrical parameters and food composition. *Innovative Food Science & Emerging Technologies, 21*, 58–65. https://doi.org/10.1016/j.ifset.2013.10.005

Pillet, F., Formosa-Dague, C., Baaziz, H., Dague, E., & Rols, M. P. (2016). Cell wall as a target for bacteria inactivation by pulsed electric fields. *Scientific Reports, 6*. https://doi.org/10.1038/srep19778

Raso, J., & Heinz, V. (2006). *Pulsed electric fields technology for the food industry*. Springer (Chapter 4)).

Raso, J., Heinz, V., Alvarez, I., & Toepfl, S. (2022). *Pulsed electric fields technology for the food industry: Fundamental and applications* (2nd ed.). Springer (Chapter 2).

Roepstorff, A., Karl, H., Bloemsma, B., & Huss, H. H. (1993). Catch handling and the possible migration of anisakis larvae in herring, Clupea harengus. *Journal of Food Protection, 56*(9), 783–787. https://doi.org/10.4315/0362-028X-56.9.783

Santos, M. J., Matos, M., Golden, O., Caldeira, A. J. R., Vieira-Pinto, M., Guardone, L., & Armani, A. (2022). Preliminary data on the occurrence of anisakis spp. in European hake (Merluccius merluccius) caught off the Portuguese coast and on reports of human anisakiosis in Portugal. *Microorganisms, 10*(2), 331–345. https://doi.org/10.3390/microorganisms10020331

Šimat, V., Miletić, J., Bogdanović, T., Poljak, V., & Mladineo, I. (2015). Role of biogenic amines in the post-mortem migration of Anisakis pegreffii (Nematoda: Anisakidae Dujardin, 1845) larvae into fish fillets. *International Journal of Food Microbiology, 214*, 179–186. https://doi.org/10.1016/J.IJFOODMICRO.2015.08.008

Skipnes, D., Østby, M. L., & Hendrickx, M. E. (2007). A method for characterising cook loss and water holding capacity in heat treated cod (Gadus morhua) muscle. *Journal of Food Engineering, 80*(4), 1078–1085. https://doi.org/10.1016/J.JFOODENG.2006.08.015

Toldrá, F. (2010). *Handbook of meat processing*. Wiley-Blackwell (Chapter 5)).

Trout, G. R. (1988). Techniques for measuring water-binding capacity in muscle foods—a review of methodology. *Meat Science, 23*(4), 235–252. https://doi.org/10.1016/0309-1740(88)90009-5

Zhang, Q., Barbosa-Cánovas, G. V., & Swanson, B. G. (1995). Engineering aspects of pulsed electric field pasteurization. *Journal of Food Engineering, 25*(2), 261–281. https://doi.org/10.1016/0260-8774(94)00030-D