Food and Waterborne Parasitology 23 (2021) e00121

Contents lists available at ScienceDirect

Food and Waterborne Parasitology

journal homepage: www.elsevier.com/locate/fawpar

The artificial digestion method underestimates the viability of *Anisakis simplex* (*s.l.*) L3 present in processed fish products

Isabel Sánchez-Alonso [a], Santiago Rodríguez [a], Margarita Tejada [a], Alfonso Navas [b], Miguel González-Muñoz [c], Mercedes Careche [a,*]

[a] Institute of Food Science, Technology and Nutrition (ICTAN), Spanish National Research Council (CSIC), c/José Antonio Novais 10, Madrid 28040, Spain
[b] Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales, CSIC, c/José Gutiérrez Abascal 2, Madrid 28006, Spain
[c] Department of Immunology, University Hospital La Paz, Paseo de la Castellana 261, Madrid 28046, Spain

ARTICLE INFO

Article history:
Received 27 October 2020
Received in revised form 11 March 2021
Accepted 14 March 2021

Keywords:
Anisakis larvae
Freeze-surviving larvae
Artificial digestion method
Viability
Fish muscle

ABSTRACT

This work studied the performance of the artificial digestion method in terms of recovery and viability of *Anisakis simplex* third-stage larvae (L3) when previous treatments given to the infected fish muscle may accidentally render viable larvae. For that: a) hake mince was spiked with 10 L3/75g mince, frozen at −10, −15, −20, and −30 °C and immediately thawed, or stored for 12 or 24 h, and subjected to pepsin digestion; b) the mince was spiked under the same conditions, frozen at the above temperatures and thawed immediately. After manual recovery, L3 were assessed for viability, used to spike again the minced fish and subjected to pepsin digestion; c) the mince was spiked with 10 L3 which were: i) living (i.e. chilled), ii) freeze-surviving (live L3 had been previously recovered after freezing at −10 °C), or iii) dead (frozen at −30 °C or − 80 °C), and then subjected to pepsin digestion. Results showed that the artificial digestion method kills a significant number of larvae that may have survived freezing and thus may underestimate the number of viable larvae in a given batch. The method may also underestimate the infection level of fish batches containing dead larvae. It is suggested to take these limitations into account when designing digestion protocols for specific applications, especially when there is a risk of insufficiently treated or cooked fish batches or ready-to-eat foods.

1. Introduction

The presence of *Anisakis* spp. third-stage larvae (L3) is widespread in many fish species of commercial value. These zoonotic parasites can infect humans following consumption of fish which has not been properly treated or sufficiently cooked to provoke the inactivation of all larvae (Audicana and Kennedy, 2008; Nieuwenhuizen and Lopata, 2014; Moneo et al., 2017). In humans this nematode cannot complete its life cycle, but it is capable of penetrating into the gastrointestinal mucosa causing anisakiasis, producing symptoms such as acute abdominal pain, nausea, vomiting, and diarrhea, as well as IgE-mediated allergic reactions (Moneo et al., 2017). According to EFSA BIOHAZ. Panel, (2010) *Anisakis* can mainly cause two allergic responses: gastroallergic anisakiasis, where gastric infection is accompanied by allergic symptoms, and *Anisakis* allergy as a result of allergen contamination of fish products, not requiring the presence of the live nematode. In addition, it poses an aesthetic problem, with consequences in the overall quality and marketability of the products, thus compromising quality and safety and constituting a matter of concern

* Corresponding author.
E-mail addresses: mcareche@ictan.csic.es, m.careche@csic.es. (M. Careche).

https://doi.org/10.1016/j.fawpar.2021.e00121

for consumers, official control authorities and fishing companies (D'Amico et al., 2014). According to Commission Regulation, 2004 (EC) No 853/2004 "Food business operators must ensure that fishery products have undergone a visual examination in order to detect visible parasites before being placed on the market. Fish products that are clearly contaminated with parasites shall not be placed on the market for human use".

Various methods are used for the detection of *Anisakis* larvae in fish muscle and viscera. Commission Regulation, 2005a (EC) No 2074/2005 of the EU Council establishes the conditions related to visual (Hartmann and Matern, 1988) and candling (Karl and Leinemann, 1993) inspections for parasite detection in food business operators. Other proposed methods include artificial digestion (Hauck, 1977; Leinemann and Karl, 1988; FAO CODEX STAN 244, 2004; EFSA BIOHAZ. Panel, 2010; Llarena-Reino et al., 2013; Fraulo et al., 2014; Karl et al., 2014; Cammilleri et al., 2016), those based on UV fluorescence imaging (366 nm) (Pippy, 1970; Karl and Leinemann, 1993; Karl, 2008; Levsen and Lunestad, 2010; Levsen and Karl, 2014; Gómez-Morales et al., 2018), image spectroscopy (Stormo et al., 2004; Heia et al., 2007; Sivertsen et al., 2012), magnetic resonance imaging (Bao et al., 2017), or electromagnetic detection (Choudhury et al., 2002). Parasite material detection methods include the measurement of specific DNA sequences or target proteins, including allergens, but they are not suited for routine inspection yet (Chalmers et al., 2020).

Although by visual counting up to 90 and 100% L3 can be detected in small fish such as anchovies and mackerel (Huang, 1990; Guardone et al., 2016), this efficiency may decrease to 60–70% depending on factors such as color, texture and thickness of the fillets, or the experience of the operator (Choudhury and Bublitz, 1994; Levsen et al., 2005). Moreover, these methods do not readily allow to distinguish between living and dead larvae (Huang, 1990; Bublitz and Choudhury, 1993).

Digestion with pepsin and HCl enables an easy separation of L3 from fish muscle or viscera, thus facilitating their counting. It may be employed as a confirmatory method (Chalmers et al., 2020) and it is also largely applied in specialized laboratories. In big pieces of fish, it has been reported that its use could detect 50% more parasites than using candling (Huang, 1990). The FAO CODEX STAN 244, 2004, is the official method for salted sprat and Atlantic herring, and the European Union Reference Laboratory for Parasites (EURLP) adopts this method (http://www.iss.it/binary/crlp/cont/SOP_Artificial_digestion_of_fish_fillet.pdf), although in proficiency testing, laboratories performing official controls use a variety of procedures (Rossi et al., 2015).

Artificial digestion protocols have been optimized in terms of pepsin type and activity, HCl and salt concentration, digestion solution/sample ratio, digestion time or stirring speed (Llarena-Reino et al., 2013; Karl et al., 2014). Modified methods based on the Commission Regulation, 2005b (EC) No 2075/2005 protocol for detection of *Trichinella* in meat have also been proposed (Fraulo et al., 2014; Guardone et al., 2016), including the sampling method and their adaptation into the mechanical digestion system called Trichineasy® (Cammilleri et al., 2016; Guardone et al., 2017).

When fish muscle or viscera infected with *Anisakis* L3 have undergone a previous treatment such as freezing, the larvae cuticle may be damaged becoming less resistant to the attack of digestive fluids (Rodríguez-Mahillo et al., 2008; Solas et al., 2009). Therefore, a lower percentage of intact L3 in previously treated (i.e. freezing) fish batches may be recovered as compared with untreated (i.e. chilled) fish. Moreover, the artificial digestion method itself may induce some mortality of L3, despite cuticle resistance to gastric fluids, and this mortality may be higher in the larvae subjected to treatments which were insufficient to kill them (Sánchez-Alonso et al., 2020). This poses the risk of underestimating the percentage of live larvae when using the artificial digestion method. These issues have not been systematically studied.

The objective of this work was to study the performance of the artificial digestion method in terms of larvae recovery and viability when previous treatments given to the fish flesh such as freezing may accidentally render viable larvae. These results were compared with those from untreated fish (i.e. containing live L3) or fully treated fish (i.e. containing dead L3).

2. Materials and methods

2.1. Raw material and Anisakis L3

Seven batches of hake (*Merluccius merluccius*) were caught in the Atlantic Northeast fishing area (FAO area 27). The gutted fish (n = 12, ~1.5–2 kg per individual) was purchased from a local fishmonger. Once filleted, the fish fillets were transported refrigerated to the laboratory. On arrival, the fish fillets were visually inspected and any larvae detected were removed. The fish were stored at 4 °C or at −80 °C until needed.

Nine batches of *Anisakis* L3 from heavily infected ovaries and viscera of hake caught in the same fishing grounds were obtained from the central fish market in Madrid (Mercamadrid). After receiving the larvae, they were removed from the tissue with forceps, cleaned by successive washings with water or NaCl 0.85%, placed in groups of 50 in tubes containing 10 mL NaCl 0.85%, and stored at 4 °C until use. Before using the larvae, they were checked for viability (see Section 2.4).

The percentage of representation of species or their heterozygote genotypes (Mattiucci et al., 2016) was performed in the batches of *Anisakis* spp. For that, at least 40 clean, frozen stored (−20 °C) L3 per batch were subjected to taxonomic identification according to Sánchez-Alonso et al. (2020).

2.2. Preparation of spiked mince

Fish were visually inspected for possible natural parasitization, belly flaps were cut and any visible *Anisakis* L3 were removed. Fillets were minced in a meat mincer machine with 3 mm diameter holes. Petri dishes (diameter 88 mm and thickness 11 mm) were half-filled with minced fish, spiked with 10 L3, and filled with additional mince (final weight 74.7 ± 2.5 g). Depending on

the experiment, minced fish samples were spiked with live-non treated, freeze-surviving, or dead L3. Temperature was recorded in all the process and was typically below 12 °C.

2.3. Freezing

Freezers set at temperatures ranging from −10 to −80 °C were used. The freezing finished when each Petri dish reached the target temperature and they were immediately thawed, although in some experiments, samples were frozen stored for some hours or days.

Freezing was also applied to isolated larvae placed in ice cubes with water (10 L3/10 mL) as described by Sánchez-Alonso et al. (2018). Some of the L3 were taken out when samples reached −10 °C, and immediately thawed. The rest were stored frozen at −30 °C or − 80 °C for more than 24 h.

Temperatures were measured both in the thermal centre of the samples and within the freezer using T-type thermocouples and recorded with a DaqPRO model 5300 data logger (Fourier Systems Ltd.). After thawing, L3 were checked for viability (see 2.4).

2.4. Recovery of larvae and assessment of viability

Larvae were recovered manually from the samples under visible light and in some cases with the aid of UV light (366 nm). L3 were placed in NaCl 0.85% for further analyses. Viability, measured as movement of the larvae, spontaneously or in response to stimulation with tweezers (EFSA BIOHAZ. Panel, 2010), was observed according to Sánchez-Alonso et al. (2018).

2.5. Artificial digestion method

This was performed according to Osanz-Mur (2001) and Rodríguez-Mahillo et al. (2008). The mince was placed in 250 mL Erlenmeyer flasks (75 g muscle/flask) with an acid and pepsin solution to a final concentration of 0.3 M HCl, 10 mg mL^{-1} pepsin (proteolytic activity 1:10,000 NF [U.S. National Formulary], equivalent to 2000 International Pharmaceutical Federation [FIP]-U/g; Panreac, Castellar del Vallés, Spain) with pH ~ 1 yielding a proportion of 2:1 (solution: muscle). The capped flasks were placed in a water bath (37 ± 0.5 °C) for 4 or 24 h with continuous shaking (100 rpm). After treatment, the L3 were separated from the incubation liquid with a sieve.

2.6. Electron microscopy

Environmental scanning electron microscopy (ESEM) and scanning electron microscopy (SEM) of L3 was performed according to Tejada et al. (2006).

2.7. Optical microscopy

Anisakis L3 were placed in Petri dishes with NaCl 0.85% and observed under a stereomicroscope (LEICA MZ16F; Leica Microsystems, Meyer Instruments, Houston, USA) from the Service of Confocal and Multidimensional Microscopy from the Centro de Investigaciones Biológicas Margarita Salas (CIB-CSIC).

2.8. Experimental design

In a first block of experiments (Supplementary Fig. S1) 32 Petri dishes containing fish mince spiked with 10 L3 each were frozen at different final freezing temperatures (−10, −15, −20, and − 30 °C) and stored for 0, 12, and 24 h, thawed and subjected to pepsin digestion. L3 recovery and viability were analysed. Additional experiments were used for microscopy observations (−20 °C and − 30 °C).

A second experiment was set up by freezing infected muscle contained in 12 Petri dishes (10 L3/75 g muscle) at −10, −15, −20, and − 30 °C and immediately thawed. After visual recovery of L3 from the mince and viability assessment, L3 were carefully placed again into the minced fish and subjected to pepsin digestion. L3 recovery and viability were analysed after thawing and also after pepsin digestion.

In a third experiment, 52 Petri dishes with minced fish were spiked with 10 L3 which were either: a) living (i.e. untreated), b) freeze-surviving (live L3 had been previously recovered after freezing at −10 °C), or c) dead (frozen at −30 °C or − 80 °C), and then subjected to pepsin digestion. Five to six replicated experiments per group (A-F) were performed.

The freeze-surviving and dead L3 were obtained by previously freezing them in water. For that sufficient L3 were subjected to water-freezing, recovered, assessed for viability and used in the spiking studies.

Thus, ~1460 L3 were studied in these experiments, considering all specimens used: a) for taxonomic analyses (i.e. 320 L3), and b) to obtain freeze-surviving and dead L3 (i.e. L3 frozen in water) plus those used in the spiking experiments themselves (i.e. 1140 L3).

2.9. Statistical analysis

The statistical analysis was performed with IBM SPSS Statistics software V26 for Windows (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was done and the Levene test was used to check homocedasticity of the data. The difference between means was analysed by the F test where variances were equal, and Welch and Brown-Forsythe's robust test for equality of means was used otherwise. Significant differences among means were determined by a Bonferroni multiple range test in those cases in which the prerequisite of homogeneity of variances was fulfilled. Otherwise, Tamhane T2 post hoc test was employed. Also, *t*-tests of independent or related samples were used using the Levene test to analyse the equality of variances.

2.10. UNE-EN ISO 9001 certification

The Institute of Food Science, Technology and Nutrition (ICTAN-CSIC) has been certified under UNE-EN ISO 9001 for "Management and execution of research projects and contracts in the area of Food Science and Technology and Nutrition" (certificate number ER-0366/2015).

3. Results

Taxonomic identification of the *Anisakis* L3 batches showed that the most abundant species were *A. simplex s.s.*, which coexisted with *A. pegreffii* as well as with heterozygous genotypes (Supplementary Table S1).

To study the effect of the artificial digestion method on the recovery of dead and live *Anisakis* L3, a first group of experiments was conducted (Supplementary Fig. S1). No viable L3 was found and some fragments were observed (Table 1). One-way ANOVA did not show significant differences on the number of damaged L3 as a function of temperature. Although some visible larvae present in the fish muscle were removed, some escaped the visual examination and hence, in some of the samples the percentage of recovery was higher than 100%. Changes in appearance of the *Anisakis* L3 after freezing and thawing were observed by microscopy in separate studies. These changes were evident by electron microscopy (−20 °C, two days) so that the typical cylindrical shape of the body of the L3 was damaged acquiring a dehydrated appearance (Fig. 1a) and altered areas in the cuticle that cause the emission of their internal content into the medium (Fig. 1b) were also seen. In addition, larvae frozen stored at −30 °C for up to 18 days were observed after pepsin digestion by optical microscopy. Fig. 2 shows transparent areas in the body as well as ruptures in the larval cuticle which would allow the action of pepsin in their body cavity and the digestion of their internal contents.

In the second experiment, where spiked mince was immediately thawed after freezing at −10, −15, −20, and − 30 °C (Supplementary Fig. S1), the recovery percentage from the muscle was of 99.2%, and the percentage of viable L3 extracted from the muscle ranged from 0 to 20%, depending on the freezing temperature (Table 2). When larvae were again placed into the minced fish, digested with pepsin and then recovered, a) none of those larvae that had survived a freeze/thaw cycle survived the digestion (Table 2), and b) recovery of intact L3 decreased. A *t*-test of related samples showed differences in mortality before and after artificial digestion, and although in the limit of significance ($P = 0.087$) owing to the small number of samples, results suggested that the artificial digestion method could underestimate both the number of live and of total larvae present when fish had been subjected to freezing.

In a third experiment, minced fish (supplementary Fig. S1) were spiked with either: a) living (untreated), b) freeze-surviving, or c) dead, L3 and then subjected to pepsin digestion. As expected, the percentages of freeze-surviving L3 obtained by previously freezing them in water, and used in the different replicated trials of the third experiment (Supplementary Fig. S1), ranged from 59% to 64%, and none of the L3 present in spiked mince frozen at −30 or −80 °C survived freezing.

Under these conditions, the percentage of total recovery after artificial digestion was the highest in untreated L3 and the lowest in dead L3, with intermediate values in larvae that had survived a previous freezing and thawing treatment (Fig. 3). These intermediate recovery values were obtained a) from mince where 100% larvae were recovered dead, thus they did not survive the subsequent digestion, and b) from those samples where some live L3 were recovered. In order to make a clearer distinction on the effect of dead and live larvae in terms of recovery, samples (i.e. Petri dishes) were grouped into "all dead after pepsin" and "living", with at least one surviving L3 (i.e. untreated plus freeze-surviving) after digestion. The percentage of recovery was 77 and 92% respectively (Fig. 4) and the t-test of independent samples showed that there were significant differences at $P < 0.01$ level.

As regards viability, the digestion of minced fish with freeze-surviving L3, also rendered a significantly lower ($P < 0.001$) percentage of live larvae (i.e. 40%) as compared with untreated L3 (82%) (Fig. 5). No significant differences in this parameter were observed as a function of the digestion time (i.e. 24 h for experiments A–C, F and 4 h for experiments D,E).

4. Discussion

Food business operators must ensure that fishery products have been subjected to visual examination to detect visible parasites, so that no obviously contaminated fish product is put on the market (Commission Regulation, 2004 (EC) N° 853/2004). No fishing area can be considered free from *Anisakis* (EFSA BIOHAZ. Panel, 2010) and the subjectivity and ambiguity of "obviously contaminated" leaves the limit between zero risk and tolerable risk open (Llarena-Reino et al., 2015). Therefore, the need to define threshold values to discriminate between marketable and non-marketable products has been pointed out by

Table 1
Recovered *Anisakis* L3 in spiked minced fish muscle (10 L3/75 g muscle) frozen at different final freezing temperatures (−10, −15, −20, and −30 °C) stored for 0, 12, or 24 h, thawed and subjected to artificial digestion.

Temperature (°C)	Frozen storage time (hours)	Introduced larvae into minced fish[a]	Recovered larvae after artificial digestion	Larvae fragments[b]	Recovered larvae after artificial digestion (%)
-10	0	30	32	2	107
	12	30	29	–	97
	24	20	19	–	95
−15	0	30	26	6	87
	12	30	28	–	93
	24	20	19	–	95
−20	0	30	32	2	107
	12	30	25	–	83
	24	20	20	–	100
−30	0	30	30	1	100
	12	30	31	–	103
	24	20	20	–	100

[a] Sum of total larvae per trial (corresponding to Petri dishes spiked with 10 L3)
[b] -, not measured.

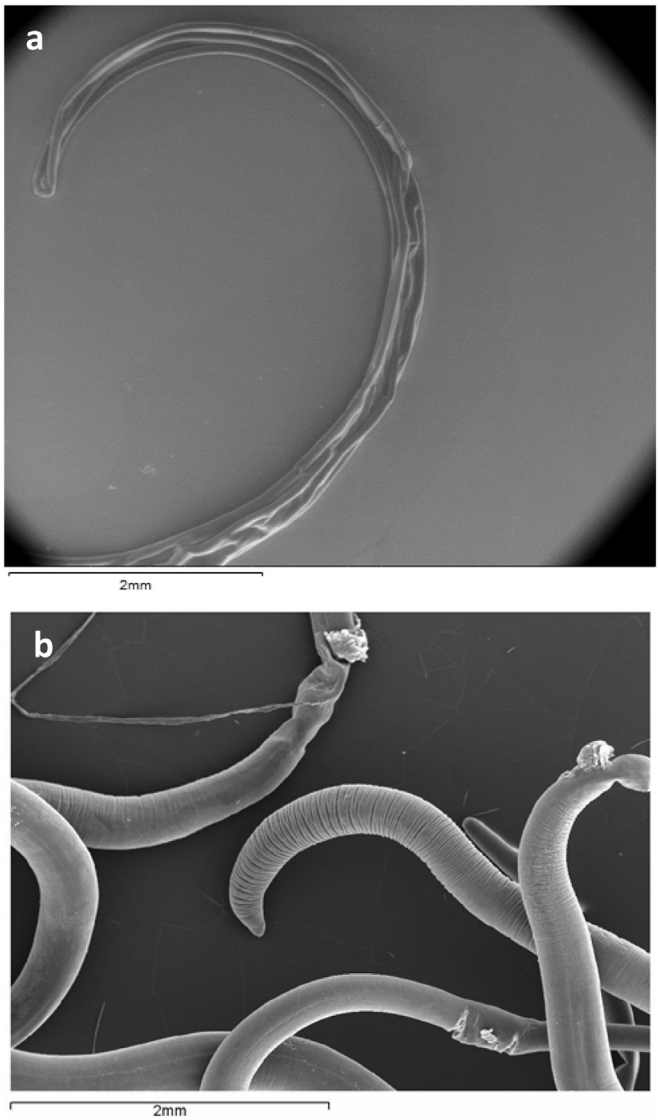

Fig. 1. ESEM (a) and SEM (b) of *Anisakis* L3 recovered from spiked fish muscle frozen at −20 °C for two days.

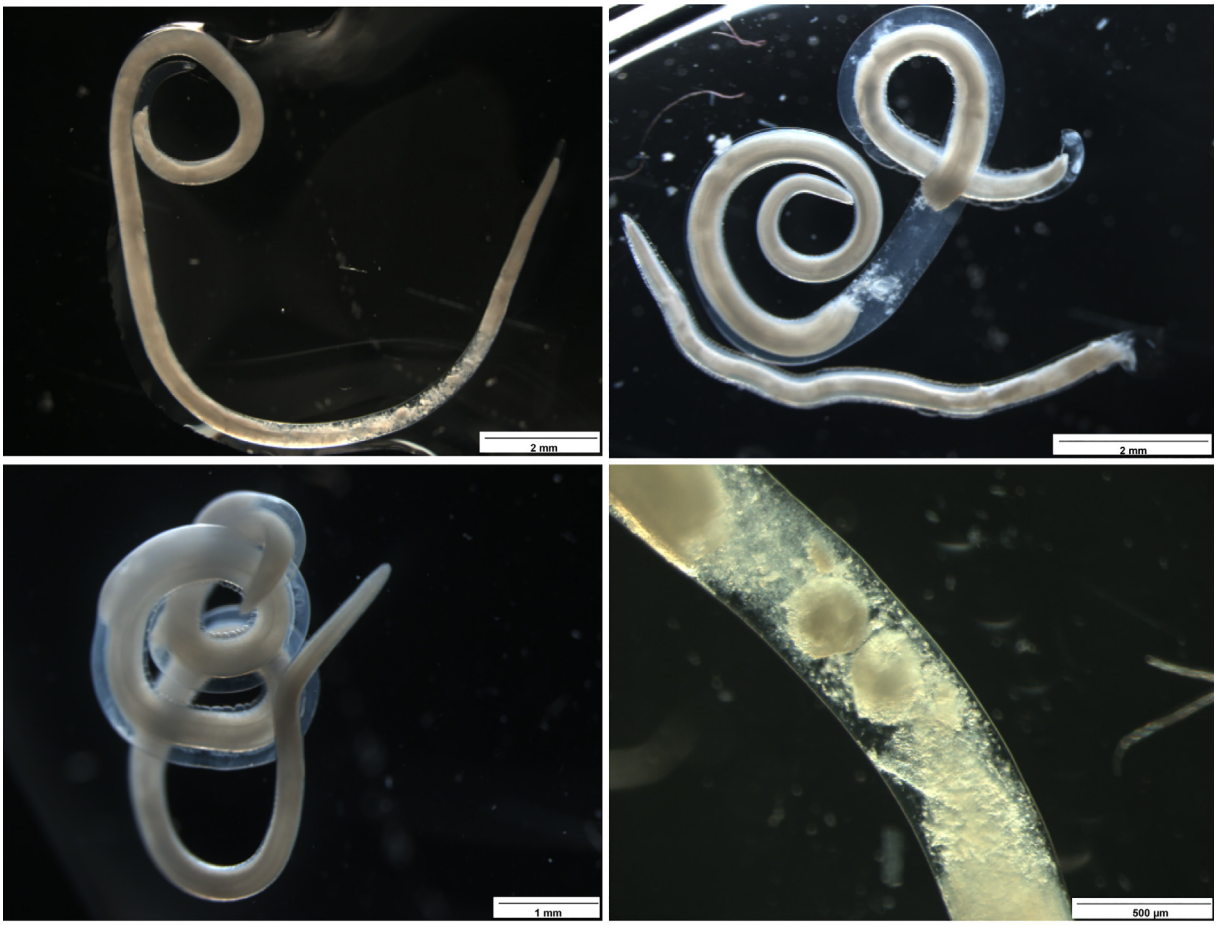

Fig. 2. Photographs of *Anisakis* L3 recovered after artificial digestion from hake mince spiked with L3 and stored frozen at −30 °C for 18 days.

Table 2
Recovered *Anisakis* L3 in spiked minced fish muscle (10 L3/75 g muscle) frozen at different final freezing temperatures (−10, −15, −20, and − 30 °C), thawed, analysed for mobility, reintroduced in minced muscle and subjected to artificial digestion.

Temperature (°C)	Number of larvae				Larvae fragments
	Introduced into minced fish[a]	Mobile after freezing/thawing	Mobile after artificial digestion	Recovered after artificial digestion	
−10	30	6	0	14	9
−15	30	2	0	18	15
−20	30	0	0	19	13
−30	30	0	0	18	17

[a] Same as in Table 1.

several authors, e.g. by means of scoring systems such as the fish parasite rating method (Rodríguez et al., 2018; Smaldone et al., 2020) or by setting pre-defined thresholds for particular products such as anchovies (e.g. Guardone et al., 2016, 2017).

Given this level of tolerance, there are a number of scenarios where it would be important to know, not only the actual number of larvae, but also if larvae are dead or still alive, especially when food business operators must ensure that the products put on the market have undergone a physiochemical or culinary treatment which is sufficiently strong to kill all parasites present in the fish (Commission Regulation, 2011 (EU) No 1276/2011of 8 December 2011 as regards the treatment to kill viable parasites in fishery products for human consumption). This could be the case for, e.g., ready-to-eat products.

The results presented in this piece of work show that artificial digestion may underestimate the number of total and viable larvae under certain conditions. Despite the fact that no viable L3 was observed after pepsin digestion at any of the temperatures and times used in the first experiment (Table 1), direct recovery from the muscle rendered up to 50% live L3 after freezing at −10 °C and immediate thawing under conditions similar to the ones of this experiment (i.e. spiked mince in Petri dishes) (Sánchez-Alonso et al., 2020). This fact suggests that the 100% mortality in some of the conditions assayed in Table 1 could be

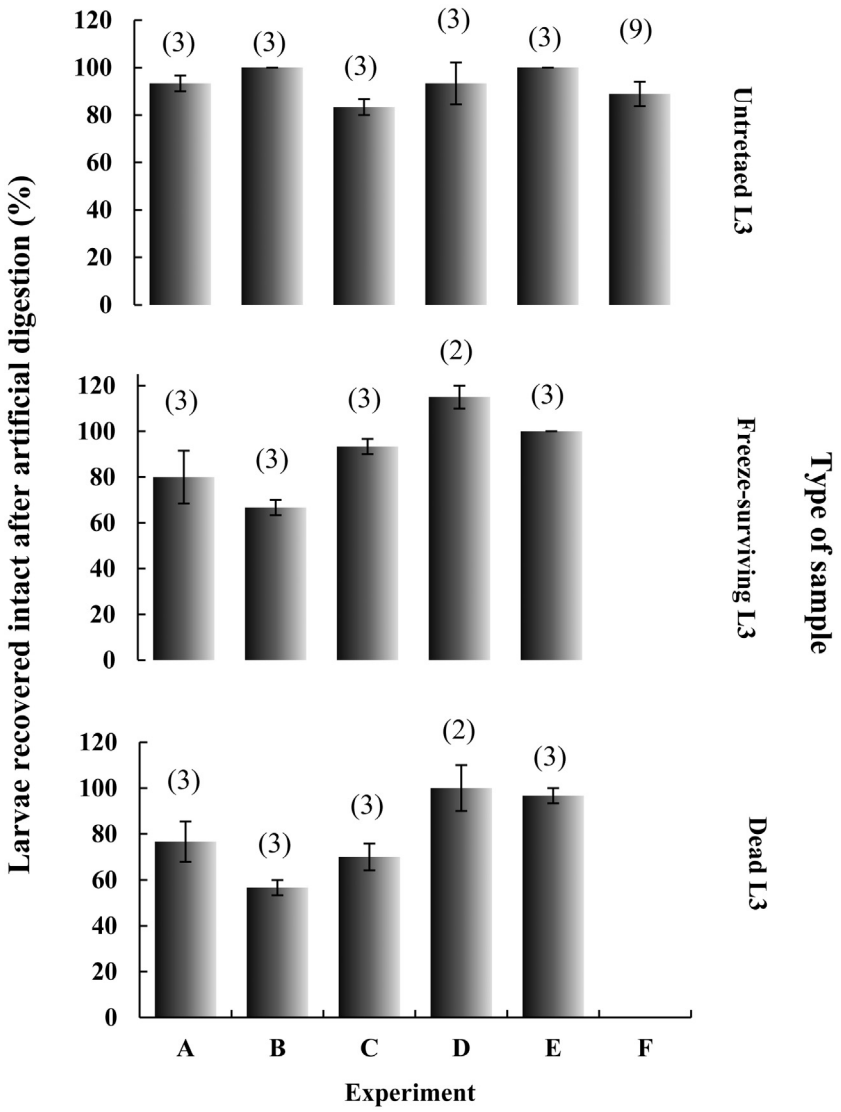

Fig. 3. Percentage of L3 recovered intact after artificial digestion as a function of the previous treatment given to the larvae. Hake mince was spiked with *Anisakis* L3 (10 L3/75g) which were: a) live, chilled L3, upper figure, b) living after freezing-thawing, middle figure, c) dead L3 after frozen storage at −30 °C. Bars show the standard deviation of the mean. In brackets the number of experimental units (i.e. Petri dishes) per experiment. Digestion time was 24 h for experiments A-C, and F and 4 h for experiments D-E.

due to the combined action of freezing and the artificial digestion method. Evidences of body-shape changes and ruptures due to freezing and observed by electron microscopy (Fig. 1a, b), in accordance to previous results (Tejada et al., 2006; Solas et al., 2009), are compatible with some lability of treated L3 upon further treatments (Fig. 2). On the other hand, *Anisakis* larvae posses strategies such as the production of protease inhibitors to inhibit host gastric enzymes, which play an important role in the penetration ability and survival of the parasite (Dzik, 2006; Knox, 2007). However, they are vulnerable to the attack of gastric enzymes (Morris and Sakanari, 1994), and it has been reported that freeze-surviving L3 have lower resistance to simulated gastric juices as compared to untreated L3 (Sánchez-Alonso et al., 2018, 2020, 2021).

In the second experiment, where the effect of freezing was separated from the effect of digestion (Supplementary Fig. S1), viable larvae were found at the highest freezing temperature, as previously reported (Sánchez-Alonso et al., 2020). Differences observed before and after pepsin digestion (Table 2) supported the hypothesis that both the number of total and viable *Anisakis* may be underestimated after digestion, albeit the small number of surviving L3 did not allow rendering statistically significant results.

This was overcome in the third set of experiments, which was designed to have, prior to pepsin digestion, the same number of untreated, freeze-surviving, and dead L3 per Petri dish. Under these conditions, the total recovery obtained after artificial digestion depended on the status of the larvae, and the highest differences were found between untreated and dead L3 (Figs. 3, 4). In untreated L3 the recovery percentage was similar to that found by other authors (Llarena-Reino et al., 2013; Fraulo et al., 2014; Gómez-Morales et al., 2018) whereas for dead L3 it was in the range found in a collaborative work (Karl et al., 2014), although

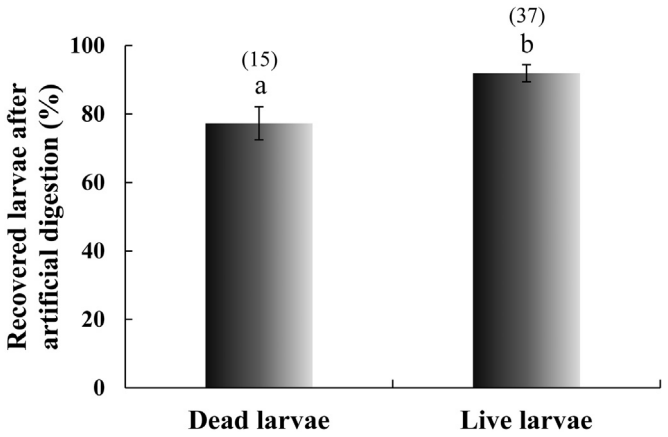

Fig. 4. Percentage of recovered L3 as a function of the status of the larvae in the experimental unit (i.e. Petri dish containing fish muscle spiked with 10 L3/75g) after artificial digestion: a) all L3 dead, b) at least one live L3. Bars show the standard deviation of the mean. In brackets the number of experimental units (i.e. Petri dishes) per group. Different letters (a, b) indicate significant differences ($P < 0.001$).

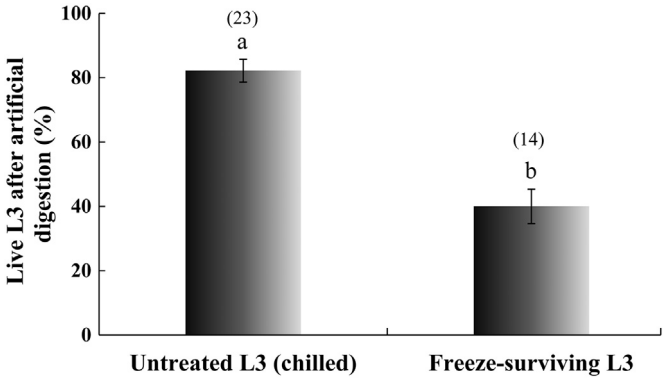

Fig. 5. Percentage of live L3 recovered after artificial digestion. Hake mince was spiked with *Anisakis* L3 (10 L3/75g) which were: a) living, chilled L3 or b) living after freezing-thawing. Bars show the standard deviation of the mean. In brackets the number of experimental units (i.e. Petri dishes) per group. Different letters (a, b) indicate significant differences ($P < 0.001$).

lower than the mean values obtained in that study. Similarly, the results of this experiment confirmed that the viability after digestion depended on the status of the larvae, so that for freeze-surviving L3 it was about half of that of the untreated L3. It is more difficult to compare the viability values obtained in this work with the literature, since values differ greatly among research works. For example, the percentage of untreated, live larvae after artificial digestion (Fig. 5) was much higher than that found by other authors using the adaptation of Commission Regulation, 2005b (EC) No 2075/2005 method (Cammilleri et al., 2016) (i.e. 29%), but lower than by using both the Codex standard and a modified method based on liquid pepsin (Llarena-Reino et al., 2013).

The artificial digestion method has been considered as the gold standard (Guardone et al., 2017) for the detection of *Anisakis*, although some authors have found better results for chilled samples with UV press. Gómez-Morales et al. (2018) performed a ring trial comparing both methods with chilled fish muscle spiked with *Anisakis* L3. They found that UV test was superior in terms of a better accuracy and sensitivity although no significant differences were found. However, it is known that the fluorescence may be lost upon prolonged frozen storage or it may be weak when L3 are subjected to other physiochemical or culinary treatments (Tejada et al., 2006), and hence both methods can be used in different scenarios and can be considered as complementary. Actually, ISO standards for detection of Anisakidae larvae in fish and fishery products are currently being development based on both methods (i.e. the UV-press, ISO/DIS 23036-1, 2020; and artificial digestion, ISO/DIS 23036-2, 2020).

Evidences in the literature show that incorrect application of treatments aiming at killing fish parasites to avoid human infection (Commission Regulation, 2011 (EU) No 1276/2011) may lead to the occurrence of surviving parasites (Wharton and Aalders, 2002; Lanfranchi and Sardella, 2010; Oh et al., 2014; Sánchez-Alonso et al., 2018, 2020; Podolska et al., 2019). The infective potential of freeze-surviving larvae has been recently addressed (Sánchez-Alonso et al., 2018, 2019, 2020, 2021). These larvae showed impaired agar-penetration capacity and survival ability in simulated gastric fluids. However, their basal respiration rate was not different from that of the controls although their maximum respiration ability was significantly lower. The percentage of *Anidakis* species used in this work was within the range reported for L3 from the same fishing area (Sánchez-Alonso et al., 2018, 2020), and it is expected

to have similar potential infectivity as the ones in the former studies. These results support the precautionary principle expressed by EFSA BIOHAZ. Panel, 2010 so that any larva with mobility after a given physiochemical treatment should be treated as capable of infecting a human.

Although no method for general application has been prescribed for the detection of viable parasites (Fraulo et al., 2014), there are some standards for particular products. Thus, the Codex Standard for Salted Atlantic Herring and Salted Sprat, CXS 244-2004 indicates that "Fish flesh shall not contain living larvae of nematodes. Viability of nematodes shall be examined according to Annex I (pepsin digestion)". Fraulo et al. (2014) proposed modifications to the Codex method as well as their incorporation into specific monitoring programs for the control of ready-to-eat foods containing raw or practically raw fish products where some larvae may remain viable as a consequence of insufficient physiochemical or culinary treatments.

If fish products (e.g. ready-to-eat) have been subjected to improper freezing, the possible living larvae may be killed by the pepsin digestion and thus, false negatives could occur. We may speculate that not only freezing but there may be other treatments insufficient to kill the larvae that may leave the cuticle less resistant to the attack of the pepsin digestion conditions. In this context we consider important to know the limitations of the method since it would allow putting the results obtained in perspective and also help in the design of improved procedures or conditions to avoid this problem.

5. Conclusion

Artificial digestion kills a significant number of larvae that have survived a freezing procedure and thus may underestimate the number of viable larvae in a given batch. The method may also underestimate the infection level of fish batches containing dead larvae. It is suggested to take these limitations into account when designing protocols for specific applications, especially when there is a risk of insufficiently treated or cooked fish batches or with ready-to-eat foods.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work has been financed by the European Union's Seventh Framework Programme for Research, Technological Development and Demonstration under Grant Agreement 312068, EU PARASITE and Spanish ANIRISK (AGL2015-68248-C2-1-R) MINECO/FEDER. Thanks are due to Dr. Maria Teresa Solas from the Cellular Biology Department, Faculty of Biology of the University Complutense of Madrid (UCM) and to ICTS Centro Nacional de Microscopía Electrónica for the SEM analyses and to Dr. Angel Mendizábal for provision of *Anisakis* larvae.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2021.e00121.

References

Audicana, M.T., Kennedy, M.W., 2008. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. Clin. Microbiol. Rev. 21, 360–379. https://doi.org/10.1128/CMR.00012-07.

Bao, M., Strachan, N.J.C., Hastie, L.C., MacKenzie, K., Seton, H.C., Pierce, G.J., 2017. Employing visual inspection and magnetic resonance imaging to investigate *Anisakis simplex s.l.* infection in herring viscera. Food Control 75, 40–47. https://doi.org/10.1016/j.foodcont.2016.12.030.

Bublitz, C.G., Choudhury, G.S., 1993. Effect of light intensity and color on worker productivity and parasite detection efficiency during candling of cod fillets. J. Aquat. Food Prod. Technol. 1, 75–89. https://doi.org/10.1300/j030v01n02_08.

Cammilleri, G., Chetta, M., Costa, A., Graci, S., Collura, R., Buscemi, M.D., Cusimano, M., Alongi, A., Principato, D., Giangrosso, G., Vella, A., Ferrantelli, V., 2016. Validation of the TrichinEasy® digestion system for the detection of Anisakidae larvae in fish products. Acta Parasitol. 61, 369–375. https://doi.org/10.1515/ap-2016-0048.

Chalmers, R.M., Robertson, L.J., Dorny, P., Jordan, S., Kärssin, A., Katzer, F., La Carbona, S., Lalle, M., Lassen, B., Mladineo, I., Rozycki, M., Bilska-Zajac, E., Schares, G., Mayer-Scholl, A., Trevisan, C., Tysnes, K., Vasilev, S., Klotz, C., 2020. Parasite detection in food: current status and future needs for validation. Trends Food Sci. Technol. 99, 337–350. https://doi.org/10.1016/j.tifs.2020.03.011.

Choudhury, G.S., Bublitz, C.G., 1994. Electromagnetic method for detection of parasites in fish. J. Aquat. Food Prod. Technol. 3, 1–4. https://doi.org/10.1300/J030v03n01_06.

Choudhury, G.S., Jenks, W.G., Wikswo, J.P., Bublitz, C.G., 2002. Effects of parasite attributes and injected current parameters on electromagnetic detection of parasites in fish muscle. J. Food Sci. 67, 3381–3387. https://doi.org/10.1111/j.1365-2621.2002.tb09594.x.

Commission Regulation, 2004. (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. Off. J. Eur. Union 139, 55–205. http://data.europa.eu/eli/reg/2004/853/oj (accessed July 2020).

Commission Regulation, 2005a. (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under regulation (EC) No 853/2004 of the European Parliament and of the council and for the organization of official controls under regulation (EC) No 854/2004 of the European Parliament and of the council and regulation (EC) No 882/2004 of the European Parliament and of the council, derogating from regulation (EC) No 852/2004 of the European Parliament and of the council and amending regulations (EC) No 853/2004 and (EC) No 854/2004. Off. J. Eur. Union 383, 27–59. http://data.europa.eu/eli/reg/2005/2074/oj (accessed July 2020).

Commission Regulation, 2005b. (EC) no 2075/2005 of 5 December 2005 laying down specific rules on official controls for *Trichinella* in meat. Off. J. Eur. Union 338, 60–82. http://data.europa.eu/eli/reg/2005/2075/oj (accessed July 2020).

Commission Regulation, 2011. (EU) no 1276/2011 of 8 December 2011 amending annex III to regulation (EC) no 853/2004 of the European Parliament and of the council as regards the treatment to kill viable parasites in fishery products for human consumption. Off. J. Eur. Union 327, 39–41. http://data.europa.eu/eli/reg/2011/1276/oj. (Accessed July 2020).
D'Amico, P., Malandra, R., Costanzo, F., Castigliego, L., Guidi, A., Gianfaldoni, D., Armani, A., 2014. Evolution of the Anisakis risk management in the European and Italian context. Food Res. Int. 64, 348–362. https://doi.org/10.1016/j.foodres.2014.06.038.
Dzik, J.M., 2006. Molecules released by helminth parasites involved in host colonization. Acta Biochim. Pol. 53, 33–64. https://doi.org/10.18388/abp.2006_3361.
EFSA BIOHAZ. Panel, 2010. Scientific opinion on risk assessment of parasites in fishery products. EFSA J. 8 (4), 1543 [91 pp]. https://www.efsa.europa.eu/de/efsajournal/pub/1543 (Accessed Jul 2020).
FAO CODEX STAN 244, 2004. Standard for Salted Atlantic Herring and Salted Sprat. http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B244-2004%252FCXS_244e.pdf (Accessed July 2020).
Fraulo, P., Morena, C., Costa, A., 2014. Recovery of Anisakid larvae by means of chloro-peptic digestion and proposal of the method for the official control. Acta Parasitol. 59, 629–634. https://doi.org/10.2478/s11686-014-0294-3.
Gómez-Morales, M.A., Castro, C.M., Lalle, M., Fernández, R., Pezzotti, P., Abollo, E., Pozio, E., 2018. UV-press method versus artificial digestion method to detect Anisakidae L3 in fish fillets: comparative study and suitability for the industry. Fish. Res. 202, 22–28. https://doi.org/10.1016/j.fishres.2016.12.011.
Guardone, L., Malandra, R., Costanzo, F., Castigliego, L., Tinacci, L., Gianfaldoni, D., Guidi, A., Armani, A., 2016. Assessment of a sampling plan based on visual inspection for the detection of Anisakid larvae in fresh anchovies (Engraulis encrasicolus). A first step towards official validation? Food anal. Methods 9, 1418–1427. https://doi.org/10.1007/s12161-015-0316-2.
Guardone, L., Nucera, D., Pergola, V., Costanzo, F., Costa, E., Tinacci, L., Guidi, A., Armani, A., 2017. Visceral larvae as a predictive index of the overall level of fish batch infection in European anchovies (Engraulis encrasicolus): a rapid procedure for food business operators to assess marketability. Int. J. Food Microbiol. 250, 12–18. https://doi.org/10.1016/j.ijfoodmicro.2017.03.011.
Hartmann, F., Matern, K., 1988. Apparatus for Handling Fish Fillets for the Purpose of Quality Inspection US Patent. Patent Number: 4,744,131.
Hauck, A.K., 1977. Occurrence and survival of the larval nematode *Anisakis sp.* in the flesh of fresh, frozen, brined, and smoked pacific herring, *Clupea harengus pallasi*. J. Parasitol. 63, 515–519.
Heia, K., Sivertsen, A., Stormo, S., Elvevoll, E., Wold, J., Nilsen, H., 2007. Detection of nematodes in cod (*Gadus morhua*) fillets by imaging spectroscopy. J. Food Sci. 72, E011–E015. https://doi.org/10.1111/j.1750-3841.2006.00212.x.
Huang, W., 1990. Méthodes de recherche des larves d'anisakidés dans les poissons marins. Possibilités d'application à l'inspection des poissons commercialisés en région parisienne. Réc. Méd. Vet. 166, 895–900.
ISO/DIS 23036-1, 2020. Microbiology of the Food Chain — Methods for the Detection of Anisakidae L3 Larvae in Fish and Fishery Products — Part 1: UV-Press Method. Under Development. https://www.iso.org/standard/74372.html (Accessed July 2020).
ISO/DIS 23036-2, 2020. Microbiology of the Food Chain — Methods for the Detection of Anisakidae L3 Larvae in Fish and Fishery Products — Part 2: Artificial Digestion Method. Under Development. https://www.iso.org/standard/74373.html (Accessed July 2020).
Karl, H., 2008. Nematode larvae in fish on the German market 20 years of consumer related research. Arch. Leb. 59, 107–116. https://doi.org/10.2376/0003-925X-59-107.
Karl, H., Leinemann, M., 1993. A fast and quantitative detection method for nematodes in fish fillets and fishery products. Arch. Leb. 44, 124–125.
Karl, H., Ostermeyer, U., Bauer, H., Miller, A., Mohn, K., Müller-Hohe, E., Neuhaus, H., Pölzelbauer, C., Stumme, B., Walter, M., Wernusch, J., Werth, B.M., Wittmann, C., 2014. Collaborative study for quantification of *Anisakis* larvae in spiked salmon fillets (*Salmo salar*) by a modified Codex digestion method. J. fur Verbraucherschutz und Leb. 9, 359–365. https://doi.org/10.1007/s00003-014-0907-7.
Knox, D.P., 2007. Proteinase inhibitors and helminth parasite infection. Parasite Immunol. 29, 57–71. https://doi.org/10.1111/j.1365-3024.2006.00913.x.
Lanfranchi, A., Sardella, N., 2010. Anisakids survival after microwaving, freezing and salting fish from Argentina. Food Sci. Technol. Res. 16, 499–504. https://doi.org/10.3136/fstr.16.499.
Leinemann, M., Karl, H., 1988. Untersuchungen zur Differenzierung lebender und toter Nematodenlarven (*Anisakis* sp.) in Heringen und Heringserzeugnissen. Arch. Leb. 39, 133–156.
Levsen, A., Karl, H., 2014. *Anisakis simplex* (s.l.) in Grey gurnard (*Eutrigla gurnardus*) from the North Sea: food safety considerations in relation to fishing ground and distribution in the flesh. Food Control 36, 15–19. https://doi.org/10.1016/j.foodcont.2013.07.006.
Levsen, A., Lunestad, B.T., 2010. *Anisakis simplex* third stage larvae in Norwegian spring spawning herring (*Clupea harengus* L.), with emphasis on larval distribution in the flesh. Vet. Parasitol. 171, 247–253. https://doi.org/10.1016/j.vetpar.2010.03.039.
Levsen, A., Lunestad, B.T., Berland, B., 2005. Low detection efficiency of candling as a commonly recommended inspection method for nematode larvae in the flesh of pelagic fish. J. Food Prot. 68, 828–832. https://doi.org/10.4315/0362-028X-68.4.828.
Llarena-Reino, M., Piñeiro, C., Antonio, J., Outeriño, L., Vello, C., González, Á.F., Pascual, S., 2013. Optimization of the pepsin digestion method for anisakids inspection in the fishing industry. Vet. Parasitol. 191, 276–283. https://doi.org/10.1016/j.vetpar.2012.09.015.
Llarena-Reino, M., Abollo, E., Regueira, M., Rodriguez, H., 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.
Mattiucci, S., Acerra, V., Paoletti, M., Cipriani, P., Levsen, A., Webb, S.C., Canestrelli, D., Nascetti, G., 2016. No more time to stay "single" in the detection of *Anisakis pegreffii, A-simplex* (*s.s.*) and hybridization events between them: a multi-marker nuclear genotyping approach. Parasitology 143, 998–1011. https://doi.org/10.1017/S0031182016000330.
Moneo, I., Carballeda-Sangiao, N., González-Muñoz, M., 2017. New perspectives on the diagnosis of allergy to *Anisakis* spp. Curr Allergy Asthma Rep 17, 27. https://doi.org/10.1007/s11882-017-0698-x.
Morris, S.R., Sakanari, J.A., 1994. Characterization of the serine protease and serine protease inhibitor from the tissue-penetrating nematode *Anisakis simplex*. J. Biol. Chem. 269, 27650–27656.
Nieuwenhuizen, N., Lopata, A., 2014. Allergic reactions to *Anisakis* found in fish. Curr Allergy Asthma Rep 14, 455. https://doi.org/10.1007/s11882-014-0455-3.
Oh, S.-R., Zhang, C.-Y., Kim, T.-I., Hong, S.-J., Ju, I.-S., Lee, S.-H., Kim, S.-H., Cho, J.-I., Ha, S.-D., 2014. Inactivation of *Anisakis* larvae in salt-fermented squid and Pollock tripe by freezing, salting, and combined treatment with chlorine and ultrasound. Food Control 40, 46–49. https://doi.org/10.1016/j.foodcont.2013.11.023.
Osanz-Mur, A.C., 2001. Presencia de larvas de anasákidos (Nematoda: *Ascaroidea*) en pescado de consumo capturado en la zona pesquera de Tarragona. Tesis Doctoral. Universidad Autónoma de Barcelona, Facultad de Veterinaria.
Pippy, J., 1970. Use of ultraviolet light to find parasitic nematodes *in situ*. J. Fish. Res. Board Can. 27, 963–965. https://doi.org/10.1139/f70-107.
Podolska, M., Pawlikowski, B., Nadolna-Ałtyn, K., Pawlak, J., Komar-Szymczak, K., Szostakowska, B., 2019. How effective is freezing at killing *Anisakis simplex*, *Pseudoterranova krabbei*, and *P. decipiens* larvae? An experimental evaluation of time-temperature conditions. Parasitol. Res. 118, 2139–2147. https://doi.org/10.1007/s00436-019-06339-1.
Rodríguez, H., Abollo, E., González, A., Pascual, S., 2018. Scoring the parasite risk in highly valuable fish species from southern ICES areas. Fish. Res. 202, 134–139. https://doi.org/10.1016/j.fishres.2017.06.019.
Rodríguez-Mahillo, A., González-Muñoz, M., Moneo, I., Solas, M., Mendizábal, A., Heras, C., Tejada, M., 2008. Allergenic properties and cuticle microstructure of *Anisakis simplex* L3 after freezing and pepsin digestion. J. Food Prot. 71, 2578–2581. https://doi.org/10.4315/0362-028X-71.12.2578.
Rossi, P., Marucci, G., Lalle, M., Casulli, A., Possenti, A., Pozio, E., 2015. Proficiency testing carried out by the European Union reference Laboratory for Parasites. Accred. Qual. Assur. 20, 311–317. https://doi.org/10.1007/s00769-015-1142-3.
Sánchez-Alonso, I., Carballeda-Sangiao, N., González-Muñoz, M., Navas, A., Arcos, S.C., Mendizábal, A., Tejada, M., Careche, M., 2018. Pathogenic potential of *Anisakis* L3 after freezing in domestic freezers. Food Control 84, 61–69. https://doi.org/10.1016/j.foodcont.2017.07.010.
Sánchez-Alonso, I., Navas, A., Arcos, S.C., González-Muñoz, M., Carballeda-Sangiao, N., Careche, M., 2019. Respiratory analysis as a tool to detect physiological changes in *Anisakis* larvae subjected to stress. Parasitol. Res. 118, 1127–1135. https://doi.org/10.1007/s00436-019-06260-7.
Sánchez-Alonso, I., Carballeda-Sangiao, N., González-Muñoz, M., Navas, A., Arcos, S.C., Mendizábal, A., Cuesta, F., Careche, M., 2020. Freezing kinetic parameters influence allergenic and infective potential of *Anisakis simplex* L3 present in fish muscle. Food Control 118, 107373. https://doi.org/10.1016/j.foodcont.2020.107373.

Sánchez-Alonso, I., Carballeda-Sangiao, N., Rodríguez, S., Tejada, M., Navas, A., Arcos, S.C., González-Muñoz, M., Careche, M., 2021. *Anisakis simplex* (s.l.) resistance to the action of gastric enzymes depends upon previous treatments applied to infected fish mince and affects antigen release. J. Sci. Food Agric. https://doi.org/10.1002/jsfa.11031 In press.

Sivertsen, A.H., Heia, K., Hindberg, K., Godtliebsen, F., 2012. Automatic nematode detection in cod fillets (*Gadus morhua* L.) by hyperspectral imaging. J. Food Eng. 111, 675–681. https://doi.org/10.1016/j.jfoodeng.2012.02.036.

Smaldone, G., Abollo, E., Marrone, R., Bernardi, C.M.E., Chirollo, C., Aniello Anastasio, A., del Hierro, S.P., 2020. Risk-based scoring and genetic identification for anisakids in frozen fish products from Atlantic FAO areas. BMC Vet. Res. 16, 65. https://doi.org/10.1186/s12917-020-02286-7.

Solas, M.T., García, M.L., De Las Heras, C., Rodríguez-Mahillo, A.I., Gonzalez-Muñoz, M., Moneo, I., Mendizábal, A., Tejada, M., 2009. *Anisakis simplex* antigens in fresh and frozen-thawed muscle of anchovies in vinegar. Food Sci. Technol. Int. 15, 139–148. https://doi.org/10.1177/1082013208105171.

Stormo, S., Ernstsen, A., Nilsen, H., Heia, K., Sivertsen, A., Elvevoll, E., 2004. Compounds of parasitic roundworm absorbing in the visible region: target molecules for detection of roundworm in Atlantic cod. J. Food Prot. 67, 1522–1525. https://doi.org/10.4315/0362-028X-67.7.1522.

Tejada, M., Solas, M.T., Navas, A., Mendizábal, A., 2006. Scanning electron microscopy of *Anisakis* larvae following different treatments. J. Food Prot. 69, 1379–1387. https://doi.org/10.4315/0362-028x-69.6.1379.

Wharton, D.A., Aalders, O., 2002. The response of *Anisakis* larvae to freezing. J. Helminthol. 76, 363–368. https://doi.org/10.1079/JOH2002149.