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Air-dried stockfish of Northeast Arctic cod do not carry viable anisakid nematodes

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ABSTRACT

A total of 80 stockfish fillets of Northeast Arctic cod (*Gadus morhua*), traditionally open-air-dried in northern Norway, was examined for the presence and viability of larval parasitic nematodes of the family Anisakidae. Anisakids (particularly those belonging to genera *Anisakis* and *Pseudoterranova*) are of public health and economic concern globally, since they are responsible for an underestimated fish-borne zoonotic disease called anisakidosis (anisakiasis when caused by members of the *Anisakis* genus). Stockfish fillets were inspected for anisakids by candling and artificial (pepsin) digestion methodologies. The recovered nematodes (n = 342) were morphologically identified to genus level and their viability assessed. Subsamples of anisakid larvae (n = 31) were identified by molecular/genetic markers inferred from sequences analyses and real time polymerase chain reaction (RT-PCR) of the mtDNA *cox2* gene, as *Anisakis simplex sensu stricto* (s.s.) (n = 29) and as *Pseudoterranova decipiens* (s.s.) (n = 2). This is the first time a RT-PCR primer/probe system was used to identify anisakids in a processed fishery product. *Anisakis simplex* (s.s.) larvae were found in 81% of the fillets, with average (range) 4 (0–35). In total, 338 *A. simplex* (s.s.) and 4 *P. decipiens* (s.s.) larvae, all dead, were recovered from the fillets. Anisakids were devitalised by the air-dried stockfish production process in 7.5 months (common stockfish production time from sea to plate). The results suggest that there is a negligible risk of acquiring anisakidosis from consumption of air-dried stockfish. Further research is recommended to evaluate if anisakids can be devitalised in five months (i.e. minimum stockfish production time). The health risk for sensitized consumers posed by the potential presence of anisakid allergens in stockfish needs to be assessed. This is the first report on the viability of anisakid larvae in an unsalted, naturally dried fishery product. Drying could represent an alternative and efficient treatment for the inactivation of anisakids in fishery products. Trimming of the belly flaps of highly parasitized cod may reduce the number of anisakids in stockfish by 74%.

1. Introduction

Stockfish (*tørrfisk* in Norwegian) is an unsalted, naturally dried fish product produced on open-air timber racks in Northern Norway. Stockfish is made from fresh, migrating Northeast (NE) Arctic cod (*Gadus morhua*) (*skrei* in Norwegian), caught during its annual southward migration from the Barents Sea to the spawning grounds off the Lofoten-Vesterålen archipelago, and other north-western coastal regions of Norway, between January and May (Bergstad, Jørgensen, & Dragesund, 1987; Sundby & Nakken, 2008). The NE Arctic cod population sustains the largest cod fishery in the world (Dahle, Johansen, Westgaard, Aglen, & Glover, 2018). Fishes are beheaded, gutted and hung outdoor for approximately 3 months at temperatures of around 0–2 °C before being moved to dry and ventilated warehouses for storage/maturation for 2–12 months prior to shipment. During the drying process, the product loses roughly 75% of its water content, but prior to final preparation for consumption, stockfish is usually rehydrated (and the water replaced) between 2 and 15 days in cold water (2–4 °C) (Luccia et al., 2005; Norwegian Seafood Council, 2018). Norway exported 5200 tonnes of stockfish to an estimated 77 million € in 2018, mainly to Italy (*stoccafisso* in Italian), where it has been traditionally consumed since the mid-15th century (Bertoja, Giaccone, Carraro, Mininni, & Cardazzo, 2009; Luccia et al., 2005). Other important export destinations are Nigeria, USA, Croatia, UK, Sweden, Canada and France

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Table 1
Fish biometric data and anisakid infection parameters in the fillets of fresh cod (n = 50) caught in Røst area (Lofoten, Norway) in March of 2017.

	Fresh cod data (n = 50)			Anisakid infection parameters in fillets*				
	TL (cm)	TW (kg)	WF (kg)	Left	Right	Ventral	Dorsal	Total
Average (mean abundance)	94	7.1	4.2	6.6	6.5	13.1	0.0	13.2
SD	8	2.3	1.3	6.7	7.0	12.6	0.2	12.6
Min.	75	3.5	2.3	0	1	3	0	3
Max.	116	15.8	9.1	30	34	61	1	62
Prevalence				94%	100%	100%	4%	100%
Density								3.1
Total number		355.7	211.8	331	327	656	2	658

TL, TW and WM, total length (cm), total weight (kg) and weight of the fillets (kg); mean abundance, prevalence (%) and density (total number of anisakid larvae in the fillets/kg of fillet) as described by Bush et al. (1997). * The anisakids were generically identified mainly as *Anisakis* spp. (> 90% of the total larvae) and the rest as *Pseudoterranova* spp.

(Norwegian Seafood Council, 2018).

Parasitic nematodes of the family Anisakidae (Nematoda: Ascaridoidea) commonly occur in most commercially harvested, wild marine fish from NE Atlantic waters, such as Atlantic cod (*Gadus morhua*) (Gay et al., 2018; Levsen et al., 2018). Our understanding of their life cycle ecology is limited, for instance due to the high anisakid species diversity revealed by genetic studies the last decades. Generally, species of the genera *Anisakis* and *Pseudoterranova* reproduce in the digestive tract of marine mammals (i.e. cetaceans and seals, respectively) and the fertilised eggs are released to the marine environment with their faeces. Planktonic, semi-planktonic or benthic crustaceans serve as first intermediate hosts, while larger planktonic crustaceans, fish and squids act as transport hosts, transferring the larvae to their respective definitive hosts (for reviews of anisakid diversity, occurrence and life cycle in NE Atlantic waters, see reviews by Mattiucci, Cipriani, Paoletti, Levsen, & Nascetti, 2017; Mattiucci & Nascetti, 2008).

Anisakids (mainly *Anisakis* spp.) are of public health and socio-economic concern globally. The infective third stage larva (L3) of these nematodes, which may be present in the muscle of fish and squids, is the aetiological agent of an underestimated, emerging fish-borne zoonotic disease called anisakidosis (anisakiasis when caused only by *Anisakis* spp.) (reviewed by Bao et al., 2019; Buchmann & Mehrdana, 2016; Guardone, Armani, et al., 2018; Mattiucci, Cipriani, Levsen, Paoletti, & Nascetti, 2018). Humans may be infected and develop disease when eating raw or lightly cooked parasitized fishery products, such as traditionally marinated anchovies in Spain (*boquerones en vinagre*) and Italy (*alici marinate*), Japanese sushi and sashimi (currently eaten worldwide) and South American ceviche. The anisakids cannot mature in humans, but the L3 may try to penetrate the wall of the gastrointestinal tract, thereby causing gastric, intestinal or ectopic anisakidosis (reviewed by Audicana & Kennedy, 2008; EFSA-BIOHAZ, 2010; Mattiucci et al., 2018). In addition, *Anisakis simplex* (s.s.) and *Anisakis pegreffii* can cause clinical allergic responses, with symptoms that can vary from mild to severe (i.e. acute and chronic urticaria, angioedema, life-threatening anaphylaxis, etc.) (EFSA-BIOHAZ, 2010; Mattiucci et al., 2013).

According to EU-regulations, seafood businesses must ensure that fishery products have been subjected to visual examination in order to prevent any obviously parasitized product to be put on the market. In addition, if the product is intended to be consumed raw or only lightly processed, it must be frozen in all parts of the product to at least a) −20 °C for not less than 24 h; or b) −35 °C for not less than 15 h (EC, 2004a, 2004b, 2005; EU, 2011). Heating to ≥60 °C at the core of the product for at least 1 min also ensures destruction of the larvae (reviewed by EFSA-BIOHAZ, 2010; Franssen et al., 2019). Thus, freezing and heating are the most effective processes for inactivation of anisakid larvae. Alternative treatments for the killing of anisakid larvae such as marinating (Sánchez-Monsalvez et al., 2005; Šimat & Trumbić, 2019), cold-smoking (≤60 °C), salting (Anastasio et al., 2016; Guardone, Nucera, et al., 2018; Smaldone, Marrone, Palma, Sarnelli, & Anastasio, 2017), high hydrostatic pressure (Brutti et al., 2010), etc. have proven to be either ineffective if the treatment period is short or to lower the product quality and are, hence, not included in the regulations (reviewed by EFSA-BIOHAZ, 2010; Franssen et al., 2019). For instance, the traditional method of salting anchovies in southern Italy inactivates *A. pegreffii* larvae if the product is treated over a 15 days period (Anastasio et al., 2016). To date, there is still a lack of documentation with regards to drying as efficient treatment for the inactivation of anisakid larvae without altering the basic product quality (reviewed by EFSA-BIOHAZ, 2010; Franssen et al., 2019).

The main goal of the present study was to determine the infection levels and to assess the viability of anisakid nematodes that may be present in outdoor naturally air-dried stockfish of NE Arctic cod. In addition, the infection levels in the flesh of 50 fresh NE Arctic cod were studied to establish anisakid infection reference. A subsample of larvae from stockfish and fresh cod were identified to species level.

2. Materials and methods

2.1. Sampling of fresh cod to establish anisakid infection reference

In March 2017, NE Arctic cod were caught off Røst island in the Lofoten archipelago, northern Norway, by Danish seine or longline fishing. As reference samples, to assess the general anisakid infection level in cod, 50 fish were taken from a freshly landed catch intended to be processed for stockfish production. All fish were measured and weighed (Table 1) prior to evisceration and filleting. Each fillet was individually labelled and placed in plastic bags in the freezer at −20 °C for > 24 h before inspection. Fillets were inspected for anisakids by applying the UV-press method (Karl & Leinemann, 1993), which basically involves pressing of thawed fillets to 1–3 mm thin layers, followed by visual inspection under a 366 nm UV-light source in a dark room. Dead anisakids show bluish fluorescence within the fish flesh and may be counted easily (Levsen & Lunestad, 2010; Pippy, 1970). The approximate larval infection site in the fillets (i.e. dorsal, ventral) was reported (Table 1).

2.2. Samplings of stockfish

2.2.1. Fish subsample

The remaining fishes of the above-mentioned catch were processed for stockfish production, hung outdoor for approximately 3 months at around 1 °C before being moved to a dry and ventilated warehouse for storage/maturation until the middle of November 2018 (approx. 20 months total production time). Sixty stockfish fillets from this batch were rehydrated for 10 days by the producer before being shipped to our laboratory for anisakid inspection.

In addition, 20 stockfish fillets were produced from NE Arctic cod caught in the Værøy area (Lofoten archipelago) in March 2018. The fishes were treated as above, i.e. 1) conventional outdoor drying until

Table 2
Rehydrated stockfish fillet measurements and infection parameters of *A. simplex* (s.s.) and *Pseudoterranova decipiens* (s.s.) recovered by candling and artificial pepsin digestion method (Note: just 300 g per fillet were digested).

	Rehydrated stockfish fillets (n = 80)		Infection parameters	
	L (cm)	WF (kg)	*A. simplex* (*s.s.*)	*P. decipiens* (*s.s.*)
Average	54	0.5	4.2	0.1
SD	4	0.1	4.8	0.3
Min.	46	0.3	0	0
Max.	64	0.9	35	2
Prevalence			81%	4%
Density*			14.1	0.2
Total number		42	338	4

L, length of the fillet (cm); WF, weight of the fillet after removal of fins, spines and skin if present (kg); average, prevalence or positivity rate (%) and density (total number of larvae in the stockfish products/kg of stockfish). * The density was calculated considering that only 300 g of flesh (always including belly flaps, if present) were sampled per fillet (n = 80), i.e. density = 338/(80 × 0.3) = 14.1 *A. simplex* (*s.s.*)/kg of fillet.

the middle of June; 2) indoor maturing until the end of October (approx. 7.5 months total production time); 3) rehydrated for 10 days before being shipped to our laboratory for inspection at the beginning of November 2018. All fillets (n = 80) were measured and weighed (Table 2).

Three inspection methods, i.e. candling, UV-press, and artificial pepsin digestion were applied for the assessment of nematode detection efficiency and larval viability.

2.2.2. Candling and UV-press

Candling, i.e. visual screening of fish fillets on a light table (Karl & Leinemann, 1993; Levsen, Lunestad, & Berland, 2005) was used to visualize superficially situated anisakids appearing as shadows in the flesh (EFSA-BIOHAZ, 2010). The UV-press method applied on stockfish followed the procedure as described in 2.1.

2.2.3. Artificial pepsin digestion and anisakid viability assessment

A modification of the artificial pepsin digestion methodology was used to recover all anisakids present, and to assess their viability (Codex Alimentarius, 2004; Gómez-Morales et al., 2018; Llarena-Reino et al., 2013).

Considering that 1) most of the anisakids present in cod fillets are likely to be located in the belly flaps (as found when studying the fresh cod), and also 2) to optimise the methodology in time, cost and efficiency; a total of approximately 300 g of flesh always containing at least the ventral part of the fillet (unless the belly flap was already removed by the producer) was digested per fillet. The fish bones and skin were removed from the fillets (if necessary). The flesh was smashed and reduced to smaller pieces by hand, in an attempt to facilitate the action of the pepsin solution, whilst not harming any possible anisakid present, and placed in a 5 l beaker containing a stirring rod and the solution (see details below) that was preheated at 30–37 °C in a magnetic stirrer with digital thermostat heating plate.

The digestion of the first 32 fillets was carried out following the protocol by Llarena-Reino et al. (2013). Digestions lasted on average 3.30 h, but the digestion process was not optimal for dried cod (i.e. undigested muscle residues were more than desirable for quick inspection of anisakids), and this slowed down further anisakid detection and identification under stereomicroscope (see section 2.3). Small modifications to the protocol were made in subsequent digestion attempts of the next 25 fillets (i.e. by adding 20 ml instead of 15 ml of liquid pepsin to a final 3 l solution), with slightly better results in terms of muscle residues.

Further modifications of the pepsin digestion protocol were made to optimise the conditions in terms of speed of digestion and minimization of muscle residues. The optimized protocol was tested on the last 23 fillets as follows: the pepsin solution was prepared by adding 41.6 ml of 18% HCl (0.063 M) to 3 l temperate tap water in a 5 l beaker; 25 ml of liquid pepsin (proteolytic activity 660 u Ph. Eur./ml) was added to a 0.6% final concentration; and finally temperate tap water was added up to 4 l of final volume (stockfish weight (300 g)/volume (4 l) ratio for digestion solution was 1:13). The flesh was digested for approximately 2–4 h (in average 3.07 h) until the fish muscles got reduced to the minimum possible.

The digestion fluid was poured through a sieve, and the flesh residues and anisakids were carefully transferred by forceps and spoon on to Petri dishes for further counting, identification and examination for viability under stereomicroscope. A given anisakid larva is considered viable when it is physically intact and motile, as demonstrated by spontaneous movements upon repeated mechanical stimulation with forceps or needles (Codex Alimentarius, 2004; EFSA-BIOHAZ, 2010). The viability of anisakid larvae was therefore tested by visual assessment of motility and integrity of cuticle and whether internal organs were intact under a stereomicroscope.

2.3. Nematode identification

Anisakid larvae recovered from stockfish fillets were counted and assigned to genus level by microscopy, using morphological characters such as the shape and length of the oesophageal ventricle, position of the excretory pore relative to the boring tooth, presence/absence of a caudal mucron, and presence of any clear cuticle ornamentation (Berland, 1961, 1989).

A subsample of n = 60 *Anisakis* spp. larvae from the flesh of fresh cod, and n = 31 (29 *Anisakis* spp. and 2 *Pseudoterranova* spp.) from rehydrated stockfish fillets were identified by direct sequence analysis of the mtDNA *cox*2 gene (629 bp) following the procedure of Mattiucci et al. (2014). PCR products were shipped to Bio-Fab Research (Rome, Italy) for sequencing. The sequences obtained at the mtDNA *cox2* of the larval nematodes were compared with corresponding sequences for *Anisakis* spp. and *Pseudoterranova* spp. deposited in GenBank (Mattiucci et al., 2014; Timi et al., 2014).

In addition, 31 larvae were also identified using RT-PCR, employing assays for the mtDNA cox2 sequences of both *A. simplex* (s.s.) and *P. decipiens* (s.s.) following the procedure of Paoletti et al. (2018). A cross-reactivity verification tests with the host tissue was conducted by testing the fish genomic DNA (Paoletti et al., 2018).

2.4. Statistical analyses

Quantitative descriptors of anisakid infection, such as prevalence, mean abundance, mean intensity and density were calculated for fresh cod as defined in Bush, Lafferty, Lotz, and Shostak (1997) (Table 1). The following descriptors were calculated for the rehydrated stockfish products according to Guardone et al. (2019): prevalence or positivity rate (number of stockfish with at least 1 anisakid larva/number of total stockfish products sampled), average (mean number of larvae in the stockfish sample) and density (total number of larvae in the stockfish products/kg of stockfish). Spearman rank tests were run to analyse the relationship between larval abundance in fillets (of fresh cod) and fish length and weight, using Statistica 13.4.0.14.

3. Results

3.1. Nematode identification

The anisakids present in the flesh of fresh cod were generically identified as *Anisakis* spp. (> 90% of the total larvae) or *Pseudoterranova* spp. (< 10%).

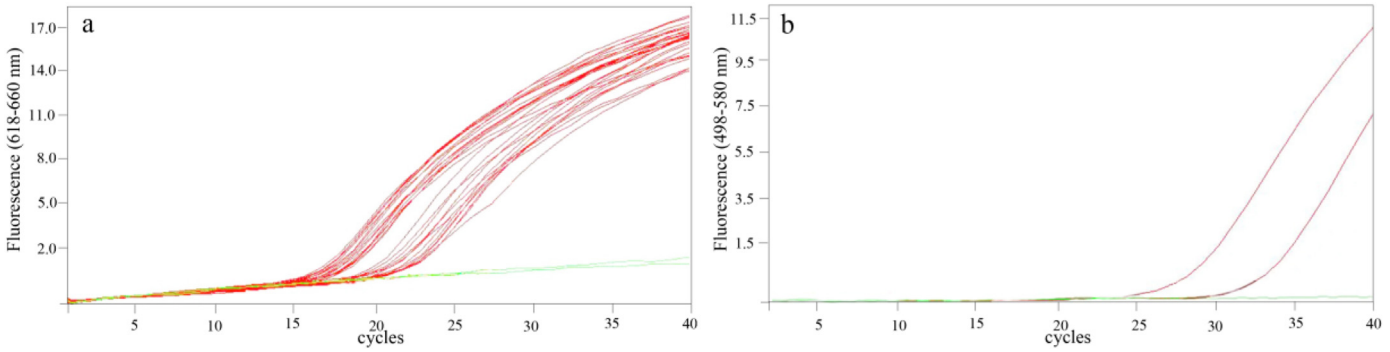

Fig. 1. Real Time-PCR probe system procedure showing a consistent fluorescent signal at 618–660 nm for 29 specimens of *A. simplex* (s.s.) (a) and at 498–580 nm for the two specimens identified as *P. decipiens* (s.s.) (b). The green line represents the negative control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The majority of the anisakid larvae found in the stockfish fillets was assigned to genus *Anisakis* (N = 338), while only four larvae belonged to genus *Pseudoterranova*.

According to the anisakid mtDNA cox2 obtained, all 89 *Anisakis* sp. larvae analysed (60 from fresh cod and 29 from dried fish) were identified as *A. simplex* (s.s.). The mtDNA *cox2* sequences obtained (563 bp; GenBank accession numbers MT347693 and MT347694) matched 99%–100% with the mtDNA *cox2* sequences of *A. simplex* (s.s.) obtained in previous works and deposited in GenBank (Mattiucci et al., 2014).

According to the *cox2* gene sequences obtained, 2 *Pseudoterranova* sp. larvae from dried fillets samples were molecularly identified as *Pseudoterranova decipiens* (s.s.). The mtDNA *cox2* sequences obtained (519 bp; GenBank accession numbers MT347695 and MT347696) matched 99% with the mtDNA *cox2* sequences of *P. decipiens* (s.s.). obtained in previous works and deposited in GenBank (Mattiucci et al., 2014).

The RT-PCR primer/probe system procedure showed a consistent fluorescent signal at 660 nm for the specimens tested (n = 29), corresponding to *A. simplex* (s. s.) (Fig. 1a), in accordance with the mtDNA *cox2* sequencing results. Similarly, a consistent fluorescent signal at 580 nm allowed to identify two specimens as *P. decipiens* (s.s.) (Fig. 1b); the results were in accordance with the direct DNA sequencing at the same gene locus.

3.2. Fresh cod

3.2.1. Infection level and location in fillets

A total of 658 anisakids were found in the flesh of 50 cod, almost equally distributed between left and right fillets (Table 1). The prevalence of larvae in flesh was 100%, with mean abundance ± standard deviation (SD) of 13.2 ± 12.6, and density of 3.1 larvae/kg of fillet (mean abundance equals mean intensity at 100% prevalence). Virtually all the larvae were located in the ventral part of the fillets (656 out of 658 larvae (99.7%)), especially in the belly flaps (484 out of 658 larvae (73.6%)).

Spearman's rank correlation coefficients between larval abundance and fish host biometrics (i.e. fish total length and total weight, and weight of fillets) were positive but non-significant (r = 0.14, p = 0.32; r = 0.12, p = 0.38; r = 0.12, p = 0.39, respectively).

3.3. Stockfish

3.3.1. Candling and UV-press

A total of 67 *A. simplex* (s.s.) and 1 *P. decipiens* (s.s.) were detected and recovered from 20 rehydrated stockfish fillets by candling, and all of them were dead. Some larvae were clearly visible in the belly flaps by candling (Fig. 2). At subsequent artificial digestion, 62 *A. simplex* (s.s.) and 3 *P. decipiens* (s.s.) were recovered from these fillets, corresponding to 51% anisakid detection efficiency of candling. Thus, candling was not able to detect all *Anisakis* spp. larvae that were present in the fillets.

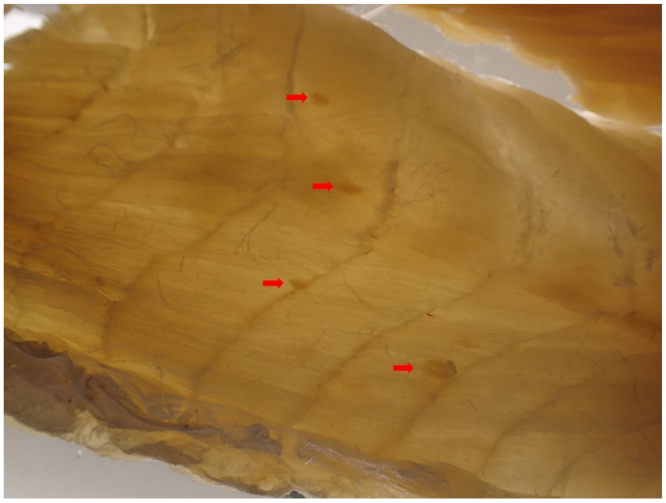

Fig. 2. Four *Anisakis simplex* (s.s.) larvae in the belly flap of a rehydrated stockfish fillet revealed by candling.

The UV-press method was shown to be inefficient for larval detection in stockfish. Rehydrated stockfish fillets could not be pressed to an evenly thin layer, most fillets became fragmented and thus, were not suitable for subsequent screening under UV-light. Hence, no larvae were detected by applying UV-press.

3.3.2. Artificial pepsin digestion

In the 80 rehydrated stockfish fillets, 338 *A. simplex* (s.s.) (271 by digestion and 67 by candling) and 4 *P. decipiens* (s.s.) (3 by digestion and 1 by candling) larvae were found (Table 2). The prevalence of *A. simplex* (s.s.) in the fillets was 81%, with average ± SD (range) 4 ± 5 (0–35) and density 14.1 larvae per kg of fillet (Table 2). In addition, 95% and 77% *A. simplex* (s.s.) prevalence with average ± SD (range) of 6 ± 8 (0–35) and 3 ± 3 (0–11) were found in the stockfish samples from Værøy and Røst, respectively.

3.3.3. Viability assessment

No larvae showed motility after stimulation with forceps and needles. The cuticle of recovered larvae was damaged, showing severe effects of dehydration (even considering that the stockfish fillets were rehydrated), and the larval body contents appeared partially digested (Fig. 3). Thus, all larvae (n = 342) were considered dead.

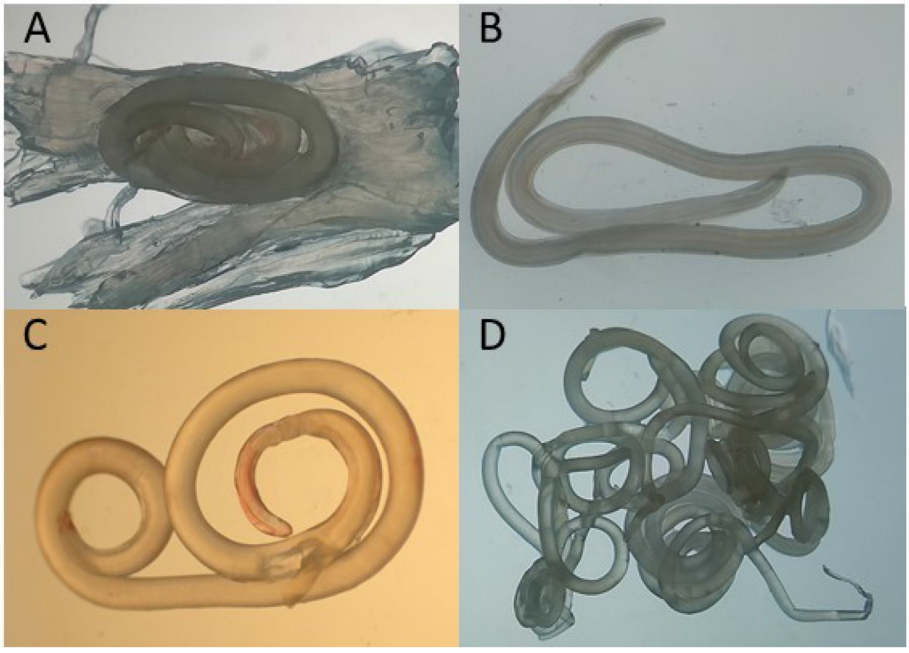

Fig. 3. Dead *Anisakis simplex* (s.s.) larvae examined under stereomicroscope. A: dead larva still coiled in the muscle. B: dead larva showing cuticle damage. C: dead larva with broken cuticle. The reddish colour of the anterior body part of the larva was observed in many other larvae. Presumably, this phenomenon might be originated by substances released by the stressed larva when dying due to extreme dehydration. D: Several dead larvae showing cuticle damage. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

The infection levels of anisakids (most of them being *A. simplex* (s.s.)) in the flesh of freshly caught migrating NE Arctic cod from the spawning grounds off the Lofoten archipelago are presented here. The prevalence was 100% with mean abundance ± SD 13.2 ± 12.6. These infection values were higher than those from stockfish where the prevalence of *A. simplex* (s.s.) was 77% with average of 3 larvae in the Røst stockfish sample. The lower infection level found in stockfish may be due to the removal of the ventral part of some fillets (i.e. belly flaps) during stockfish production. Consequently, those larvae that were located in these parts will not be present in the final product. In addition, some larvae that were more damaged during the drying process may have been dissolved during the artificial pepsin digestion and were no longer detectable.

Anisakis simplex (s.l.) occurrence in cod from Norwegian waters is relatively well known (Aspholm, 1995; Gay et al., 2018; Hemmingsen, Halvorsen, & MacKenzie, 2000; Heuch et al., 2011; Platt, 1975; Smith & Hemmingsen, 2003; Strømnes & Andersen, 1998). However, data with regard to muscle infection are still insufficient, especially considering the vast importance of cod as food resource.

Most studies on cod from the Barents Sea that reported *A. simplex* (s.l.) infection levels in the flesh were based on plain visual inspection, candling and/or slicing methodologies. However, these methods are considered non-efficient for the detection and accurate quantification of larvae in fish muscle (EFSA-BIOHAZ, 2010; Karl & Leinemann, 1993; Levsen et al., 2005). Nevertheless, the prevalence of infection in cod fillets has apparently been high for decades. Platt (1975) and Aspholm (1995) reported prevalence of 96% and Smith and Hemmingsen (2003) found 88.5% prevalence of anisakids (95% being grossly identified as *Anisakis* sp.) using candling methods.

Recently, Gay et al. (2018) reported 90% prevalence and mean abundance (mA) of 5 *A. simplex* (s.l.) in cod fillets screened for anisakids by UV-press. In the present study, 100% prevalence was found for migratory NE Arctic cod caught in the Lofoten area. Fish size (and consequently fish age) is reported as one of the main drivers of overall infection burden in several fish species, along with geographical area of catch and season (Levsen et al., 2018). The almost threefold higher anisakid mA found in the present study (i.e. 13 mA, Table 1) compared to the results of Gay et al. (2018), could thus be explained considering the different fishing ground and the body size of the present cod, considerably bigger (see, Table 1 here and in Gay et al. (2018)).

Candling resulted not an appropriate methodology to detect all *Anisakis* spp. larvae that may be present in stockfish, as is the case when detecting the larvae present in the fillets of fresh pelagic fish (Levsen et al., 2005). Larvae embedded in the fish muscle tissue may appear as a darkish shadowlike spot, thus not easily detectable. Only larvae present in smaller/thinner fillets may be spotted. However, any larvae located more deeply in the flesh, e.g. in the dorsal and thicker part of the fillets, can easily be overlooked. Thus, candling could be useful to detect and subsequently remove manually only the superficially located larvae in products of stockfish.

Artificial pepsin digestion and UV-press are considered practically equally reliable methodologies for detection and quantification of *A. simplex* (s.l.) larvae in fish flesh (Gómez-Morales et al., 2018). The UV-press method, known to be very efficient to detect larvae in the flesh of fresh fish (Gómez-Morales et al., 2018), was shown here to be most inefficient (i.e. not useful) for larval detection in rehydrated stockfish fillets. Furthermore, in order to assess larval viability of anisakids, pepsin digestion is considered the most appropriate method. The digestion procedure mimics the conditions that prevail in the stomach and foregut of marine mammals, which, due to their role as definitive hosts, facilitate anisakid survival. All anisakid larvae (i.e. *A. simplex* (s.s.) and *P. decipiens* (s.s.)) obtained by pepsin digestion from rehydrated stockfish fillets resulted dead.

In the present study, a RT-PCR primers/probe system procedure was tested for the first time to identify anisakid larvae in a processed fishery product. The *Anisakis* larvae from dried cod fillets and analysed by the probe system showed a consistent fluorescent output signal, which permitted to define the species as *A. simplex* (s.s.). Similar results were obtained for *Pseudoterranova* larvae from dried cod fillets, that were identified as *P. decipiens* (s.s.). These identifications were in accordance with the DNA sequencing results obtained from the same specimens. When applied on fish tissue only, without presence of any parasite DNA, no amplification took place. In recent years, a RT-PCR procedure has been validated for several anisakid species (Cavallero et al., 2017). The present species-specific RT-PCR probe systems was recently developed and validated on several specimens of ascaridoid nematodes belonging to the genera *Anisakis* and *Pseudoterranova* (e.g. *A. simplex* (s.s.) and *P.decipiens* (s.s.)) (Paoletti et al., 2018). The main advantage of using

RT-PCR probes is rapid identification of several ascaridoid individuals simultaneously. It may be used whenever a rapid and highly sensitive (detection limit lower than 0.0006 ng/μl of DNA) tool for detection of zoonotic species (e.g. *A. simplex* (s.s.) and *P. decipiens* (s.s.) in fishery products is needed, e.g. by the fish processing industry.

To the best of our knowledge, this is the first study to assess the viability of anisakids (*A. simplex* (s.s.), and *P. decipiens* (s.s.)) in a dried, unsalted fishery product. All anisakids found in naturally air-dried stockfish of NE Arctic cod were dead. Many treatments for inactivation of *A. simplex* (s.l.) in fishery products have been assessed for their effectivity, such as salting (Anastasio et al., 2016; Guardone, Nucera, et al., 2018; Smaldone et al., 2017), marinating (Sánchez-Monsalvez et al., 2005; Šimat & Trumbić, 2019), cold-smoking, vegetable compounds (Gómez-Mateos Pérez, Navarro Moll, Merino Espinosa, & Valero López, 2017; López, Cascella, Benelli, Maggi, & Gómez-Rincón, 2018), high hydrostatic pressure (Brutti et al., 2010) and irradiation (see reviews by EFSA-BIOHAZ (2010); Franssen et al. (2019)). However, heating at $\geq$ 60 °C at the core of the product for at least 1 min, and freezing to ≤ -20 °C in all parts of the product to at least 24 h, -35 °C not less than 15 h or at -15 °C to at least 96 h for those fishery products intended to be consumed as raw, marinated, or generally if the treatment is insufficient to destroy the nematodes, remain the reference processes guaranteeing the killing of parasitic nematodes (EC, 2004a; EFSA-BIOHAZ, 2010; EU, 2011).

Smaldone et al. (2017) performed a preliminary study to evaluate the inactivation of anisakids by salting in *baccalà* (salted cod fillets prepared traditionally in Italy). Results suggest that anisakid larvae are devitalised by the salting process in a 15-day period.

In the present study, results show that *A. simplex* (s.s.) is devitalised by the drying process of stockfish in 7.5 months. All 274 larvae obtained from fillets after digestion and examined for vitality according to standard procedures (Codex Alimentarius, 2004; EFSA-BIOHAZ, 2010) resulted dead. No larval movements were observed after stimulation with forceps and needles. The larvae appeared clearly dehydrated, with the cuticle broken and the body contents frequently partially digested. It is important to highlight that all larvae recovered from stockfish in the present study were non-motile and, particularly, that their bodies were clearly damaged and, hence, considered non-viable, since mobile larvae may not be infective to humans and, most importantly, immobile larvae may be erroneously considered as non-viable (Sánchez-Alonso et al., 2018, 2019). It seems likely that larvae would also be killed in much less time, as water is fundamental for all living organisms. It appears therefore not surprising that anisakid nematodes cannot survive the drastic reduction in water content achieved during the production of stockfish (from 65%-95% to 10–15%) (Luccia et al., 2005). Further research is recommended to determine if *A. simplex* (s.l.) would also be killed at the minimum period in which stockfish may be ready for consumption (approx. 5 months), and/or the minimum drying time needed for the devitalization of all larvae that may be present in the product. In addition, future research is needed to assess the viability of anisakids in artificially dried stockfish recently produced in Lofoten.

Thus, results suggest that consumption of stockfish would pose no risk for acquiring anisakidosis, i.e. live infection with *A. simplex* (s.s.) and *P. decipiens* (s.s.). However, the existence of heat- and/or pepsin-resistant *A. simplex* (s.l.) allergens might still pose a health risk to sensitized consumers (reviewed by Bao et al. (2019)), and this risk should be assessed.

In addition, results suggest that candling may be used to detect and subsequently remove manually any visible anisakid from the fillets, as required by EU regulations (EC, 2004a; 2004b, 2005). In relation to this, trimming the belly flaps would reduce the anisakid burden in at least 74% in the final product, as virtually all the larvae (99.7%) were present in the ventral part of the fillets in fresh cod.

5. Conclusions

The current results suggest that all *A. simplex* (s.s.) and *P. decipiens* (s.s.) that may be present in naturally air-dried stockfish are dead, and therefore, there is a negligible risk of acquiring anisakidosis from consumption of traditionally produced stockfish. The potential presence of anisakid allergens in the product may still pose a health risk for sensitized consumers and, thus, warrants further investigation. The outdoor drying process devitalize anisakids in 7.5 months (normal stockfish production time from sea to plate). Further research is needed to assess if anisakid larvae would also be devitalised at the minimum period in which stockfish may be ready for consumption (approx. 5 months). Results suggest that drying is an efficient alternative treatment for the inactivation of anisakids present in fishery products. Trimming the belly flaps of highly parasitized cod may reduce the number of anisakid larvae in stockfish by 74%. Candling may be used by producers to remove those anisakids that may be visible in the stockfish fillet.

CRediT authorship contribution statement

Miguel Bao: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Paolo Cipriani:** Conceptualization, Investigation, Resources, Writing - review & editing, Visualization. **Lucilla Giulietti:** Methodology, Resources, Writing - review & editing. **Irja Sunde Roiha:** Conceptualization, Writing - review & editing, Funding acquisition. **Michela Paoletti:** Methodology, Investigation, Writing - review & editing. **Marialetizia Palomba:** Methodology, Investigation, Writing - review & editing. **Arne Levsen:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

None.

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