Absence of anisakis nematodes in smoked farmed Atlantic salmon (*Salmo salar)* products on sale in European countries

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Abstract

The increase of global demand of aquaculture products as compensation for the lowering of fishery sustainability has shown a parallel awareness by the consumers on the importance of the safety and quality of fish products. Among these, salmon industry has reached a leading position demonstrating the negligible risk of presence of zoonotic helminths such as *anisakis nematodes* in farmed salmon. Despite the massive production of data in literature on parasitological surveys carried out on fresh salmon, no data are published on processed farmed salmon such as smoked products. In 2016, 270 slices of smoked farmed Atlantic salmon (*Salmo salar*) and 13 smoked slices from wild sockeye salmon (*Oncorhynchus nerka*) have been analyzed by visual inspection and UV-press method searching for the presence of anisakid nematodes. No parasites were detected in samples from farmed Atlantic salmon, while 10 out of 13 from wild salmon were positive for *Anisakis simplex s.s.* larvae. This first survey on the possible presence of anisakid nematodes in processed smoked salmon confirms that this risk in farmed Atlantic salmon products has to be considered negligible.

Introduction

There is an extensive range of helminthic parasites of fish, but only a moderate number of species are capable of producing foodborne diseases in humans (Adams, Murrell, and Cross, 1997; Chai, Murrell, and Lymbery, 2005). These diseases are either caused by an infection following ingestion of viable larval stages of fish parasites, or as an allergic reaction against parasite antigens which occurs, so far, for nematodes of the family Anisakidae. *Anisakis simplex (s.l.)* is most frequently associated with human disease, denominated Anisakiasis, followed by *Pseudoterranova decipiens (s.l.)* (Buchmann and Mehrdana, 2016). In fact, *A. simplex* is the second most predominant biological hazard notified in seafood products, constituting 19.5% of the reported hazards (Kleter, Prandini, Filippi, and Marvin, 2009) and in recent years a blip is observed in the notifications for this nematode, with more than 700 alerts since 2010 according to the EU Rapid Alert System for Food and Feed - RASFF (https://ec.europa.eu/food/safety/rasff_en) and several fish species involved.

Among these, Atlantic salmon (*Salmo salar*) is susceptible of being infected by anisakid nematodes in natural environments (Noguera *et al.*, 2009; Wootten, Yoon, and Bron, 2010), although several studies carried out in Norway and Scotland did not show the presence of zoonotic anisakids in farmed Atlantic salmon (Angot, 1993; Deardorff and Kent, 1989; Lunestad, 2003; Wootten, Yoon, and Bron, 2009). This evidence drove the European Food Safety Authority (2010) to state that, for farmed Atlantic salmon, the risk of infection with anisakids is negligible and the European Commission to modify the Annex III of Regulation (EC) 853/2004 by issuing the Regulation (EU) 1276/2011, allowing to business operators the exemption of the freezing treatment for farmed Atlantic salmon products intended to be consumed raw or undercooked, if the indications reported in the Regulation are fully respected. Moreover a qualitative risk assessment analysis in Atlantic salmon has concluded the very low risk associated to commercialization of farmed salmon with viable anisakids (Crotta, Ferrari, and Guitian, 2016).

Despite the reassuring evidences emerging from these data and although, so far, no nematodes were found in farmed harvest quality salmon intended for human consumption, more recently the presence of anisakid parasites has been reported in runts of Atlantic salmon farmed in Norway (Levsen and Maage, 2016; Mo *et al*., 2014), questioning somehow the exemption for farmed Atlantic salmon from the freezing requirement included in the current EU legislation, with potential negative repercussion on the salmon products market.

Atlantic salmon (*Salmo salar*) is by quantity the largest species of salmonids produced worldwide (FAO, 2018). In 2017, the total supply of Atlantic salmon was 2.248 million tons GWE (gutted weight equivalent) and most commercially available is farmed. Atlantic salmon is a versatile product that can be used for a variety of primary and processed secondary products such as fillets, slices and smoked, respectively (MarineHarvest, 2018). Sliced fillets are the largest category of value added salmon products in terms of volume, with the 47%, followed by smoked salmon at 28% (Denstad, Lillevand, and Ulsund, 2015). The European market for smoked salmon is estimated to 185,000 tonnes in 2017 and the ten largest producers of smoked salmon in Europe are estimated to have a joint market share of more 60% (MarineHarvest, 2018). European smoked salmon production is part of a very complex food chain that can increase food safety risks after fillets manipulation from different origins. Food chain is longer and more complex than ever before and a failure of traceability from the farming through processing, transport, trade and catering can have significant food safety implications. However, despite of the commercial importance of this processed product, as far as we know, there are no surveys evaluating the possible presence of Anisakidae larvae in commercial smoked Atlantic salmon products.

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The present investigation aims to assess a food risk analysis of the presence of anisakid parasites in packaged slices of smoked salmon commercialized in Europe, carrying out a representative survey on this fish product and taking into consideration the main smoked salmon suppliers in supermarkets located in three of the main European markets: Italy, Spain and France.

Materials and Methods

Smoked salmon sampling

The sampling was carried out temporarily (spring "P1" and autumn "P2") in local supermarkets in Basque region (Spain and France) and Italy during 2016 (Table 1). Since the expected prevalence of anisakid larvae should have been low, we established a sample size of 270 salmon slices between 100-200g, and the confidence level was set at 90% with an acceptable margin of error set at 5%. Sub samples size was split per suppliers, reflecting their salmon commercial production volume in 2015 in the countries under study.

In addition, 13 samples of smoked slices of wild sockeye salmon (*Oncorhynchus nerka*), 2 from supermarkets in Spain and 11 from Italy, were also collected to compare smoked farmed *vs* wild salmon products, since from literature wild salmon has very high prevalence values for *Anisakis* larvae (Deardorff and Kent, 1989; Karl, Baumann, Ostermeyer, Kuhn, and Klimpel, 2011).

The significance of differences between prevalence values of samples from farmed and wild salmon was calculated using Chi-squared test. Significant P-value was considered < 0.01.

Parasite inspection procedure

Immediately after sampling, all smoked salmon samples were placed in a cooler and transported to the laboratory where they were kept refrigerated and then processed. Each smoked portion included in the package was placed into a clear plastic bag ensuring there was enough space in the bag for compression. Using a UV-press method, samples were squeezed until they were approximately 1-2mm thick in a hydraulic pressing device at 7-8 bar. Then slices were frozen at -20 °C for at least 48 hours and examined under ultraviolet light (365 nm), where anisakid larvae, if present, would appear as a fluorescent body (Karl and Leinemann, 1993). All the samples were previously subjected to visual inspection by candling, as provided by the current regulatory framework (EC Regulation 2074/2005).

All anisakid larvae detected and isolated in the samples examined were studied morphologically according to Berland (1961), dissecting the anterior and posterior part of each larva and clarifying it with Amman's Lactophenol, while the middle part was subjected to molecular identification after DNA extraction from each parasite. In the Spanish control laboratory, parasite was mixed with 300 µL extraction buffer (1% (w/v) SDS, 150mM NaCl, 2mM EDTA, Tris-HCl pH 8.0) supplemented with 40 µL 5M guanidine thiocyanate, 50 µL proteinase K (600 UmL-1) and subsequently incubated at 56° C (±5) overnight. After centrifugation for 5 min at 16,000 g the supernatant was purified using Wizard Genomic DNA Purification Kit (Promega). DNA quality and quantity were determined with a NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc.). PCR amplification of mitochondrial cytochrome c oxidase II gene (COII) was carried out with the primers CYTCII-F and CYTCII-R following the protocol previously described (López and Pardo, 2010). The reaction was started by adding 1 unit/reaction Taq DNA polymerase (Life Technologies) and amplification reactions were developed in a Mastercycler Personal (Eppendorf) for 40 cycles with an annealing temperature of 58° C. The sequencing of amplified fragments was carried out directly on the purified fragments with a 3700 DNA Analyzer ABI PRISM, using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.0 (Applied

Table 1. Summary of the sampling carried out temporarily in local supermarkets during 2016.

Producer	Estimated GWE tonnes	Estimate annual production (%)	Supermarket	Number of samples			Country
				Total	P1	P2	
Marine Harvest - Morpol	80,000	30	ESSELUNGA	51	14	37	Italy
Marine Harvest - Harvest	80,000	30	ESSELUNGA	51	14	37	Italy
Marine Harvest - Freihofer (Laschinger Morpol)	80,000	30	Aldi	8	4	4	Spain
Labeyrie - Labeyrie	30,000	10	COOP	43	43	0	Italy
Labeyrie - Delpierre	30,000	10	Intermarché	13	7	6	France
Ubago	15,000	5	Mercadona	20	9	4	Spain
Norvelita	15,000	5	COOP	15	0	15	Italy
Suempol (Norfisk Berlin)	15,000	5	Lidl	13	7	6	Spain
Mer Alliance	15,000	5	Carrefour	14	7	7	France
Delpeyrat	15,000	5	Carrefour	14	7	7	France
Xantelmar	7,500	3	El Corte Inglés	9	5	4	Spain
Starlaks	7,500	3	ESSELUNGA	14	0	14	Italy
Salmon Sur	7,500	3	Eroski	8	4	4	Spain
La Balinesa	7,500	3	El Corte Inglés	8	4	4	Spain
Koral	7,500	3	Eroski	8	4	4	Spain
Intermarché (Odyssee)	7,500	3	Intermarché	8	4	4	France
Fjord	7,500	3	COOP	12	12	0	Italy
Ahumados Dominguez	7,500	3	Opencor	8	4	4	Spain
El Duende	<5,000	1	El Corte Inglés	4	2	2	Spain

Biosystems). In the Italian control laboratory the identification of the larvae has been carried out by PCR-RFLP of the ITS rDNA region (D'Amelio *et al.*, 1999; Kijewska, Rokicki, Sitko, and Wegrzyn, 2002; Zhu *et al.*, 2007) .

Results

No *anisakis* larvae were present in any of the smoked farmed Atlantic salmon samples commercialized and intended for human consumption. Conversely, 10 (76.9%) out of 13 smoked slices of wild sockeye salmons were positive for *Anisakis* larvae.

A significant difference ($p<0.001$) has been evidenced between farmed and wild salmon.

Twenty-eight parasites (including also fragments referable to a single parasite) were found under UV light examination (Figure 1); of these, two had not been detected by candling. Once those parasites were isolated from the samples the morphological study allowed to identify all the larvae as *Anisakis* sp. type 1 (*sensu* Berland, 1961). Molecular identification identified the nematodes as *A. simplex* (*s.s.*).

Discussion

It is generally assumed that farmed fish products have a very low or null prevalence of zoonotic parasites, due to the low likelihood to prey intermediate or paratenic hosts from the natural food chain, necessary for parasite transmission to the cultured fish stocks, fostered by the high level of domestication of farmed fish that are used to feed exclusively on extruded commercial pellets, in an optimized farm management (Crotta *et al*., 2016; EFSA, 2010). Since no parasites were found in the 270 smoked Atlantic salmon samples analyzed in our study, these results are consistent with those previously carried out and evidencing the absence of parasitic nematodes in Norwegian farmed harvest quality Atlantic salmon (Levsen and Maage, 2016) and in Scottish farmed Atlantic salmon (Wootten *et al.*, 2010). The survey is enough representative of the commercialization of this very appreciated seafood product in Europe, including the main European producers and countries where the consumption of smoked salmon is high. Moreover, the present research confirms that, processing, transport and trade of European commercialized smoked salmon are safe concerning to the presence of *anisakis* larvae, despite the current complexity of the food chain based on salmon products.

In contrast, the presence of *Anisakis simplex* larvae in wild smoked salmon confirms the cosmopolitan diffusion of this zoonotic species and the general assumption of a more negligible risk for the presence of zoonotic parasites in farmed fish products. Although all the parasites were found dead, their allergenic potential should not be overlooked or underestimated in a risk analysis (Sánchez-Alonso *et al.*, 2018). The possible derogation of freezing treatment for wild-salmon-derived products currently cannot be considered, based on data both from literature and data produced during this study.

The UV-press method used during the present survey, confirmed a sensitivity higher than visual inspection by candling provided by the current EU regulatory framework, since few larvae found in fillets had been missed by candling method. The standardization of this effective and feasible method could lead the industry to adopt it on large-scale for a self-assessed control system on *Anisakis* risk.

Conclusions

The present work represents the first comprehensive study on *Anisakis* risk in smoked products from farmed Atlantic salmon ever conducted in Europe. The results pointed out the absence of anisakid nematodes and therefore the negligible risk of anisakiasis linked to the consumption of smoked farmed Atlantic salmon products, while products from wild salmon still show a high risk for the presence of *Anisakis* larvae. As suggested by EFSA and EU Hygiene Package, for the other farmed fish species and relative fish products in EU, the pathway to assess the negligibility of zoonotic parasites risk should be similar to Atlantic salmon, contributing to improve the competitiveness of European aquaculture products in the global market.

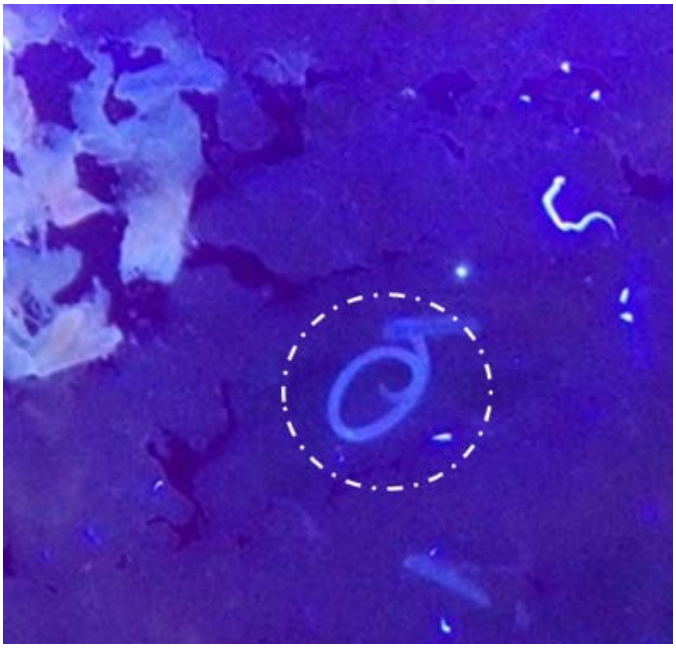

Figure 1. Single parasite detected under UV light examination in one of the smoked fillets of wild sockeye. *Anisakis* larvae is rounded with a white dotted circle.

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