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Assessing Portuguese health risks: *Anisakiks* parasite in Atlantic chub mackerel (*Scomber colias*) sold in Portuguese markets

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Abstract

Anisakiosis is a significant zoonotic disease caused by parasitic nematodes of the *Anisakis* genus. It can be contracted by humans through the consumption of raw or undercooked fish contaminated with the parasite, leading to gastrointestinal and allergic symptoms. While anisakiosis is not frequently documented in Portugal, the presence of allergic reactions to *Anisakis* in Spain suggests ongoing exposure in the Iberian Peninsula. To address this concern, the Interdisciplinary Centre of Marine and Environmental Research in Porto, Portugal, in collaboration with the Biology Centre of Czech Academy of Sciences in Ceske Budejovice, Czech Republic, has proposed a project entitled 'Assessing Portuguese Health Risks: *Anisakis* Parasite in Atlantic Chub Mackerel (*Scomber colias*) Sold in Portuguese Markets' under the European Food Risk Assessment Fellowship Programme. The primary objective of the project is to gather valuable epidemiological data on the host, Atlantic chub mackerel (*S. colias*) and the parasitic nematode (*Anisakis* spp.) with the focus on assessing contamination levels and evaluating potential health risks associated with anisakiosis in the Portuguese population. By conducting this research, the project aims to contribute to the understanding of anisakiosis and its impact on public health in Portugal. Investigation of the presence of the *Anisakis* parasite in Atlantic chub mackerel sold in Portuguese markets will provide crucial insights into the risks associated with consuming raw or undercooked fish. Ultimately, our findings will aid in the development of preventive measures and guidelines to ensure the well-being of the Portuguese population.

Keywords: *Anisakis*, fish parasite, risk assessment, disease, EFSA

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Summary

Risk assessment is an integral component in upholding the safety and quality of food within the European Union, adopting a methodical and scientific approach to evaluate potential hazards related to food products. This process facilitates informed decision-making and the implementation of effective risk management strategies. Acquiring a solid understanding of the principles and foundation of risk assessment is crucial for establishing a robust food safety system.

The evaluation of risks associated with parasites in fishery products is a fundamental process in protecting public health and ensuring the safety of seafood. By systematically assessing the risks linked to parasite contamination, appropriate measures can be employed to safeguard consumers and maintain elevated standards of food safety within the fishery industry.

By conducting meticulous risk assessments pertaining to parasites in fishery products, regulatory authorities and stakeholders can implement suitable control measures. These measures encompass the use of adequate processing techniques, application of freezing treatments and dissemination of consumer education. Their purpose is to mitigate risks and ensure the safety of seafood. Continuous monitoring and surveillance programmes further play a vital role in the detection and management of parasite presence, thus enhancing the safety and quality of fishery products.

This study aims to provide valuable insights into the potential risks associated with the consumption of untreated or undercooked mackerel. The findings of this study will contribute to the development of appropriate risk management strategies, thereby safeguarding public health. Ultimately, this project exemplifies the commitment of the fellow and collaborating institutions to address crucial food safety concerns and fulfil the objectives outlined within the EFSA programme.

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1. Introduction

1.1. European food risk assessment fellowship Programme

The European Food Risk Assessment Fellowship Programme (EU-FORA), established under the auspices of the European Food Safety Authority (EFSA), aims to bolster the scientific assessment capacity and knowledge community within the European Union, in line with EFSA's strategic goals for 2020. This initiative provides scientists employed in food safety organisations throughout Europe with a valuable opportunity to expand their expertise and practical skills in food risk assessment through immersive training experiences. In this case, the fellowship recipient was hosted by the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) in Porto, Portugal.

The fellow's specific work programme spanned 3 months of hands-on-practice and focused on 'Assessing Portuguese Health Risks: *Anisakis* Parasite in Atlantic Chub Mackerel (Scomber colias) Sold in Portuguese Markets.' This comprehensive EU-FORA programme entailed a 3-week induction training course, followed by four 1-week modules, each dedicated to distinct facets of risk assessment, identification, perception and communication. While the majority of modules were conducted online, Module 3 took place at EFSA's headquarters in Parma, Italy.

Upon the completion of the induction training, the fellow assumed responsibility and took over as the subsequent substitute for the EFSA project, overseeing its continuity and implementation.

1.2. Overview of risk assessment for parasites in fishery products

1.2.1. Importance of risk assessment in ensuring seafood safety

The risk assessment process for parasites in fishery products plays a pivotal role in safeguarding public health and ensuring the safety and quality of seafood consumed within the population. Parasitic worms, such as *Anisakis* species, can be found in various fishes commonly used in fishery products. Ingestion of such parasites can lead to health risks, making it imperative to conduct comprehensive risk assessments to mitigate potential hazards and protect consumers. The risk assessment encompasses several key steps, including hazard identification, exposure assessment and risk characterisation.

1.2.2. Hazard identification: identifying parasites of concern

The first step in the risk assessment process is hazard identification, where the focus lies on identifying the parasites that pose a significant risk to human health. In the case of fishery products, this step involves evaluating the presence and prevalence of parasites across different fish species and potential impact on consumers.

1.2.3. Exposure assessment: evaluating consumer exposure to parasites

Once the parasites of concern are identified, the exposure assessment stage aims to quantify the level of exposure that consumers may face when consuming fishery products. This involves assessing factors such as consumption patterns, cooking methods and storage practices to estimate the likelihood and frequency of parasite ingestion. Understanding the potential routes and levels of exposure is crucial in determining the overall risk associated with consuming fishery products.

1.2.4. Risk characterisation: assessing the health risks and establishing safety measures

Risk characterisation is the final step in the risk assessment process, where the collected data on hazard identification and exposure assessment are combined to evaluate the actual health risks posed by parasites in fishery products. This step involves quantifying the risks and establishing safety measures such as tolerable levels of parasites, maximum acceptable limits and guidelines for processing techniques to ensure the safety and quality of seafood.

1.2.5. The role of risk assessment in regulation and risk management

Comprehensive risk assessments serve as a crucial foundation for the development of regulatory frameworks and risk management strategies in the fishery industry. The findings from risk assessments provide valuable information to regulatory authorities, enabling them to establish appropriate control

measures, such as adequate processing techniques and freezing treatments, that effectively reduce the risks associated with parasites in fishery products. Additionally, risk assessments support the development of consumer education programmes to raise awareness about proper handling and cooking practices, further enhancing seafood safety.

1.2.6. Ongoing monitoring and surveillance programmes

Risk assessment is not a one-time process but requires continuous monitoring and surveillance programmes to detect and manage the presence of parasites in fishery products (EFSA, 2010). Regular monitoring allows for timely identification of emerging risks and the implementation of appropriate control measures. These programmes contribute to the overall safety and quality of fishery products by ensuring ongoing compliance with established safety standards.

1.3. Overview of quantitative risk assessment in fishery products

Quantitative risk assessment (QRA) is a rigorous methodology utilised to estimate the probability and severity of hazards, providing a numerical expression of risk. In the context of fishery products, the presence of *Anisakis* spp. L3-stage larvae present a significant health concern for human consumption. Anisakiosis can manifest in various forms, ranging from mild to severe, affecting the gastric, intestinal, ectopic and allergic systems. Due to the nonspecific symptoms and infrequent outbreaks, anisakiosis is often misdiagnosed or underdiagnosed, and person-to-person transmission is considered improbable. A notable example of QRA application is evident in a study conducted by Lindqvist and Westöö (2000) concerning smoked fish in Sweden. The predicted annual number of illnesses attributed to *Listeria monocytogenes* ranged from 47 to 2,800 for consumers at the highest risk category. Bao et al. (2017) undertook the first QRA specifically focusing on fishery products contaminated by parasitic nematodes, utilising anchovies as the subject within the Spanish population. The study evaluated the risks associated with the consumption of raw, marinated and undercooked anchovy fillets. On average, the Spanish population was estimated to consume 0.66 *Anisakis* larvae per untreated (non-frozen) raw or marinated anchovy meal. The calculated probability of anisakiosis per meal was determined to be 9.56×10^{-5}, resulting in the prediction of annual anisakiosis cases requiring medical attention ranging between 7,700 and 8,320.

1.4. Additional information about the host species

The Atlantic chub mackerel (*S. colias*) is a pelagic species of moderate size (30–60 cm) mostly inhabiting warm waters. Population abundance and distribution in Portuguese waters significantly fluctuate over time, with maximum values observed in the middle of the last 3 decades. This rise is possibly correlated with the growing economical (canning industry, feeding of aquaculture species) and gastronomical interest on the species. The majority of chub mackerels caught in Iberian waters are juveniles, up to 2–3 years old, while older individuals are seldom encountered during surveys or by the commercial fleet. The Atlantic chub mackerel serves as a host for a diverse range of parasites. The symptoms exhibited by infected fish can vary depending on the parasite species and the severity of the infection. Common indications of parasitic infestation include lethargy, rapid breathing, reduced appetite, weight loss, fin clamping, laboured or accelerated respiration, the presence of yellow to rust-coloured dust on the fish's body, severe cases of skin peeling, sunken belly, the appearance of white stringy faeces, decreased appetite, respiratory distress, swollen and mucus-coated gills, irritation manifested through scratching behaviour (flashing) and diminished feeding activity.

2. Project description

2.1. Aims

A) Gaining basic knowledge on *Anisakis*.

   a) Morphological identification, life cycle and known hosts of *Anisakis*.
   b) Detection of parasites in the fish muscle using UV-Press method.
   c) Infectivity and vulnerability of the parasite *Anisakis* to humans.

B) Quantification of worms in fish individuals.

   a) Determination of infection levels (prevalence, mean intensity, mean abundance).

b) Identification of Anisakids species found in host by methods of molecular biology.
c) Assessment of food safety Microbiological Risk.

2.2. Methodology

2.2.1. Biometrics of the fish sample

One hundred and one fish samples were purchased from an auction in Aveiro (Portugal) in a time period from January to April 2023. Morphological identification of *S. colias* was conducted to separate *Scomber scombrus* from the samples. Fish biometric data was measured, including fork length, total length and total weight. The fish samples were placed in individual bags separately, with numerical identification and subsequently preserved in a freezer for 2 days before fish dissection and detection of infection by *Anisakis* spp.

2.2.2. Visual inspection of host

To visualise the viscera, fish samples were opened from the most ventral area of the opercula to the anal area. Sex and sexual maturity were evaluated on a scale of 1 (immature) to 5 (very mature, ready to spawn). Visual inspection of the abdominal organs was also evaluated by observation and estimation of overall level of *Anisakis* spp. infection on a scale of 0 (no parasites), 1 (slightly infected), 2 (infected) and 3 (very infected). Parasites found on the liver and in the viscera were counted. The visually detectable parasites were placed onto Petri dish with physiological solution. Encapsulated worms were cleaned, and morphological identification of Type I and Type II of *Anisakis* L3-stage larvae was carried out under a light microscope. The number of parasites per fish individual were counted and recorded. The fish viscera were measured for weight, extracted and placed in individual transparent bags for the UV-pressing method (described in Section 2.2.4).

2.2.3. Isolation of worms from the fish muscle

Each fish fillet, divided into two pieces (right and left side), was scaled, separated into four fragments (dorsal, ventral, anterior, posterior) from each side (eight pieces in total), skinned out and placed in individual transparent bags for the UV-pressing method (described in Section 2.2.4).

2.2.4. UV-pressing

After pressing, the viscera and muscle fragments changed their shape to a 'monolayer' with the thickness of 1–2 mm range. This structure allows investigation of the worm infection in muscles under UV light (Karl and Leinemann, 1993). Blue-fluorescing worms were counted and their precise localisation in the monolayer was marked. After thorough washing, the worms from liver and viscera or worms from fillet were used for morphological or molecular identification, respectively.

2.2.5. Morphological identification of *Anisakis* spp. from the fish liver and viscera

Isolated and cleaned worms were individually mounted on a microscopy slide, with a coverslip. The worm taxonomy (genus *Anisakis* or *Hysterothylacium*) and the identification of the type of *Anisakis* larvae were identified under the light microscope by observing anatomical characteristics, such as the type of mucron, ventricle and intestine.

2.2.6. Molecular identification of *Anisakis* spp. from the fish muscles

Each isolated *Anisakis* spp. sample found in different parts of the fish musculature was processed individually for DNA isolation and amplification of the Internal Transcribed Spacer rDNA (ITS) (Gasser et al., 1993; Gasser and Hoste, 1995) and the mitochondrial encoded Cytochrome C oxidase subunit 2 (COX-2) (Nadler and Hudspeth, 2000). HinfI endonuclease was used for the ITS region PCR product digestion and the product of restriction fragment length polymorphism (RFLP) was detected on agarose gel. COX-2 product was used for the Sanger sequencing. The sequences were trimmed in the Molecular Evolutionary Genetics Analysis (MEGA) software and compared with sequences available in the NCBI database.

2.3. The risk assessment analysis of *Anisakis* spp. in the fish *Scomber colias*

2.3.1. Quantification of infection parameters

Quantitative Parasitology (QPweb) online tool was applied to calculate prevalence, mean intensity and mean abundance of the isolated worms in the analysed fish samples. The fish samples were grouped by time points of the fish collection (January, February and March) and the fish size ('small fish' or 'big fish'). The 'small fish' is of the length from 20.7 to 24.2 cm and the weight from 85.39 to 95.26 g, while the 'big fish' length is from 24.3 to 31.0 cm and the weight is from 100.14 to 268.01 g. The infection parameters were calculated for each tissue, including liver, fillet and viscera.

2.3.2. Quantitative risk assessment

Anisakis spp. L3-stage parasitic larvae can pose a health hazard to humans when ingested in raw fish meal. To evaluate the likelihood of contracting anisakiosis from a regular portion of mackerel fish meal served in local restaurants, we initially employed the @RISK software (trial version) as a QRA tool. However, due to licensing limitations, we subsequently conducted all essential calculations using Microsoft Excel. In addition to the collected experimental data, two types of input data were utilised in the QRA. The first type of data was the annual consumption of mackerel in Portugal, which was extracted from webpages of the European Market Observatory for Fisheries and Aquaculture Products (EUMOFA). The second type of data was from a survey published in Golden et al. (2022), which focused on the total number of meals containing untreated raw mackerels consumed by Portuguese respondents. The current number of Portugal population as of 25 July 2023 was taken from online resources (https://www.worldometers.info/world-population/portugal-population).

3. Conclusion

Despite the initial turnover of fellows during the project, the current fellow, in collaboration with the sending and hosting site supervisors, has established all the necessary materials and requirements for conducting successful experiments at the hosting institution.

The process of isolating parasites from the host provided a novel experience for the fellow. Within the limited time spent at the hosting institution, the fellow acquired valuable skills in fish dissection, parasite isolation and differentiation of morphological characteristics among closely related host species (*S. colias* and *S. scombrus*), as well as the worm parasite species (*Anisakis* type I and II). The implementation of the 'UV-pressing' method proved instrumental in detecting parasites within the host fillet. Additionally, the fellow utilised molecular techniques to identify parasite species. By collecting biometric data on the host and determining parasite distribution in different body regions, the fellow was able to independently analyse the acquired data.

Under the guidance of the sending institution supervisor, the fellow categorised the studied fish samples based on size and collection time points, evaluating the infection parameters in each specific tissue (liver, fillet and viscera) both individually and collectively. This categorisation is crucial for understanding the influence of both seasonal variations (collection time) and host traits (size) on the levels of infection in *S. colias*. The sending institution supervisor has signed up for the European Association of Fish Pathologists (EAFP) conference in 2023 to present the project's results through a poster. This poster presentation at the EAFP conference serves as a platform for sharing the fellow's work with a broader scientific community, providing an opportunity to emphasise the significance of considering host variables and seasonal variations in comprehending the infection dynamics of the fish species *S. colias*. These novel findings contribute to the expanding knowledge in fish pathology and showcase the fellow's unwavering dedication to research in this field.

The QRA has yielded significant information regarding the potential health risk for the Portuguese population associated with consuming untreated (marinated, cold smoked) or undercooked mackerel. Since the fellow did not have the opportunity to attend the induction training, she familiarised herself with the @RISK software with assistance from the @RISK customer service and independently learned the analytical approaches employed in similar risk assessment studies. The principle of the risk model was discussed with Miguel Boa (Institute of Marine Research in Norway). Despite encountering licensing limitations, we proceeded with conducting all essential calculations using Microsoft Excel. The objective of this study is to establish a foundational knowledge base for food safety in Portugal under the auspices of the EFSA programme.

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Abbreviations

CIIMAR	Interdisciplinary Centre of Marine and Environmental Research
COX-2	Cytochrome C oxidase subunit 2
EAFP	European Association of Fish Pathologists
EU-FORA	European Food Risk Assessment Fellowship Programme
EUMOFA	European Market Observatory for Fisheries and Aquaculture Products
ITS	Internal Transcribed Spacer rDNA
L3	Third-stage larvae
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
QPweb	Quantitative Parasitology online tool
QRA	Quantitative risk assessment
RFLP	Restriction Fragment Length Polymorphism