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Methods for inactivation of seafood *Anisakis* larvae and prevention of human anisakiasis: a mini-review

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Abstract. – Anisakiasis is an arising zoonosis induced by parasitic nematodes belonging to the family Anisakidae. Anisakiasis is often caused by the ingestion of larval nematodes in uncooked or minimally processed seafood dishes, which are regularly consumed by humans. Significant potential sources of infection are raw fish (e.g., sushi and sashimi) that can be found in traditional Japanese cuisine and can be part of the culinary tradition of consumption of raw or marinated fish that is particularly diffused in European countries. During the last five decades, the global prevalence of human anisakiasis has been rising, becoming an emergent major public health problem. Thus, there is an unmet need for well-defined and cost-effective methods aimed at killing *Anisakis* larvae, thus reducing the incidence of anisakiasis. In this mini-review, we discuss the clinical features of anisakiasis as well as the effectiveness and mechanisms of action of the main methods employed for increasing seafood safety and killing *Anisakis* larvae, including freezing, heating, use of high hydrostatic pressure, salting process, pepsin digestion method and use of garlic oil.

Key Words:
Seafood safety, *Anisakis*, Anisakiasis, Freezing, Heating, High hydrostatic pressure, Salting process, Artificial enzymatic digestion, Pepsin digestion, Garlic oil.

Introduction to Anisakiasis

In 2012, the World Health Organization (WHO)[1] estimated about 56 million cases of parasitic infections due to the consumption of fish products. Among the parasites implicated in these infections, there are *Anisakis* nematodes, which are largely distributed across all continents and can induce severe disease in humans[2].

Anisakiasis (also known as anisakidosis) is an emerging zoonosis induced by *Anisakis* spp., parasitic nematodes belonging to the family Anisakidae (genera *Anisakis*, *Pseudoterranova* and *Contracaecum*). This family has the potential to affect human health, even though not all the *Anisakis* species cause disease in humans. The term anisakiasis refers only to the disease caused by *Anisakis* species[3-5]. In particular, two species of the genus *Anisakis* are reported as the most frequent causative agents of human infections: *Anisakis simplex* (*A. simplex*) *sensu stricto* (s.s.) and *Anisakis pegreffii*[6,7].

Anisakiasis is due to the incidental ingestion of third-stage larvae (L3) in raw or minimally processed seafood dishes consumed by humans[8]. Humans represent incidental hosts since *Anisakis* third-stage larvae cannot reach the adult stage in the human intestinal tract and do not survive longer than a few days after ingestion. However, even the ingestion of a single L3 larva can cause anisakiasis.

In this mini-review, we aim to describe the clinical presentation of anisakiasis as well as the current state of the art regarding potential cost-effective strategies for the inactivation of *Anisakis* in fish, thus increasing the seafood safety and reducing the prevalence of anisakiasis, which would certainly have a positive impact on public health.

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Clinical Presentation of Anisakiasis

Within a few hours after ingestion of contaminated fish, the *Anisakis* larvae penetrate into the gastric or intestinal mucosa, leading to the manifestation of acute symptoms of gastrointestinal anisakiasis[9]. Since the gastrointestinal anisakiasis symptoms may be subtle and non-specific or may mimic other (more common) gastrointestinal disorders, the disease is often misdiagnosed as acute abdomen, appendicitis, stomach ulcer, peritonitis, or ileitis[9]. The majority of cases of anisakiasis are represented by gastric anisakiasis, which accounts for about 95% of all diagnosed cases, whereas intestinal anisakiasis represents the majority of the remaining cases[9,10]. The main gastrointestinal histopathologic findings of upper gastrointestinal anisakiasis are represented by edema, hyperemia, and bleeding in the surrounding mucosa around the area of penetration[11,13].

Incidental cases of colonic anisakiasis have increasingly been reported[14-16]. In rare instances, the larval nematodes can become invasive, migrating beyond the stomach and the intestine, and penetrating into different organs and tissues such as omentum, liver, pancreas, larynx, and even the lungs (ectopic or extra-gastrointestinal anisakiasis)[9,17,18].

Gastric anisakiasis can be suspected in the presence of a typical clinical presentation, which consists in acute and severe epigastric pain occurring a few hours (about 8-12 hours) after the ingestion of contaminated fish[9,19]. Other clinical manifestations of gastric anisakiasis include vomiting, nausea, ascites, and/or low-grade fever[9,20]. Sometimes, hematemesis can occur as a consequence of gastric ulceration[21-23]. Intussusception has also been reported as a rare consequence of intestinal anisakiasis[24,25]. Endoscopic examination with biopsy forceps has simplified both the diagnosis and treatment of gastric anisakiasis, allowing for the identification and removal of all the *Anisakis* larvae by experienced endoscopists[9,11].

Intestinal anisakiasis usually presents with colicky or diffuse abdominal pain, vomiting and/or nausea. As compared to gastric anisakiasis, symptoms of intestinal anisakiasis generally develop more slowly and usually occur within 5 days after the ingestion of contaminated fish[19]. The definitive diagnosis of intestinal anisakiasis is frequently challenging since direct identification of the nematode from the small intestine is often not feasible and patients may not remember if or when they ingested contaminated food[9]. Therefore, particularly patients with intestinal anisakiasis are often misdiagnosed with other (more common) gastrointestinal diseases such as acute appendicitis, diverticulitis, cholecystitis, ileitis, inflammatory bowel disease, peptic ulcer, or small bowel obstruction[9]. Although surgical treatment can be the first-line treatment for intestinal anisakiasis, there are increasing reports[26,27] on the efficacy of conservative therapy.

It is worth reminding that clinical manifestations of anisakiasis are not confined to gastrointestinal symptoms but can also consist of allergic symptoms. Allergic sensitization represents one of the most concerning presentations of anisakiasis, which potentially results in a wide range of clinical consequences such as angioedema, urticaria, and even anaphylaxis[28,29]. *Anisakis* predominantly promotes the production of T-helper 2 (Th2) cytokines and subsequently causes mastocytosis, eosinophilia, and IgE-mediated reactions, which are typical immune responses to tissue parasitic helminths[30,31].

Seafood Consumption and Anisakiasis

Anisakis spp. usually parasitize adult marine mammals, with paratenic hosts of the larvae represented by fish and cephalopods[28]. The parasite transmission to humans is clearly related to the consumption of raw or inadequately treated or cooked fish/shellfish meals. Thus, humans are the only incidental hosts for anisakid nematodes. Anisakiasis has therefore been directly associated with eating habits[2]. Significant potential sources of infection are contaminated dishes of raw fish (e.g., sushi and sashimi), which are typically part of both Japanese cuisine and culinary tradition of European countries such as Italy[32,33]. Japan has the highest annual anisakiasis reported cases, namely 2,000-3,000 cases per year[32]. However, the true incidence of anisakiasis may be potentially higher than that reported in the literature since several cases can remain undiagnosed[9]. With regard to Europe, regions of Southern Italy display a higher prevalence of clinical cases of anisakiasis due to the higher consumption of traditional undercooked/raw fish dishes in these geographic areas. Current data on reported anisakiasis cases are thought to be markedly underestimated[6].

Over the last years, the globalization of Japanese cuisine undoubtedly raised a greater awareness of anisakiasis, which led to an increased in-

cidence of disease diagnosis in most continents[32]. Globally, over 20,000 anisakiasis cases/year have been diagnosed[34]. The most important step of anisakiasis prevention is to educate the public about the potential risk of contracting the disease when eating raw or inadequately treated/cooked fish. Thus, seafood products should always be inspected visually to examine the presence of visible parasites.

Anisakis Life Cycle

Anisakis life cycle is complex, as it requires several stages and involves a variable number of hosts. However, a profound comprehension of the propagation mechanisms of this parasite is critical for preventing such a widespread food safety risk and contributing to ensuring stronger food safety systems[34]. *A. simplex* and *Pseudoterranova decipiens* represent the most prevalent nematode worm species involved in human infection[35,36]. *Anisakis* biological cycle includes four larval stages (L1-L4): the first stages occur within the eggs (L1-L3), while the preadult (L4) and adult stages occur in the so-called definitive hosts, namely cetaceans and other marine mammals such as whales, sea lions, seals, dolphins, porpoises and walruses. Adult anisakid nematodes can be found free-living in the stomach of the aforementioned animals. The female is fertilized by the male, laying eggs that are excreted with the definitive host feces[37]. The nematode unembryonated eggs excreted in the host feces become embryonated in the aquatic environment (seawater). Then, L3 larvae form in the eggs[34]. L3 larvae hatch from eggs (as ensheathed, free-swimming forms) and are then ingested by intermediate hosts such as krill and crustaceans. The ingested larvae grow within the crustacean hemocoel, and become infective to paratenic hosts, such as fish and cephalopods (e.g., squids) that eat infected crustaceans[34]. Afterward, the digested L3 larvae migrate from the paratenic host intestine into the abdominal cavity, and ultimately to the tissues of the mesenteries and skeletal muscle. More specifically, the parasites encyst on the visceral cavity of these hosts or in the squid mantle, or in the fish flesh, growing up to 8.8 mm in size[38]. Paratenic hosts maintain third-stage larvae (L3) in tissues that are infective to definitive hosts (marine mammals), but also to humans. Indeed, third-stage larvae (L3) do not undergo a further period of growth until marine mammals (which serve as definitive hosts) ingest an infected paratenic host. In the definitive host marine mammals, the parasite develops to L4 larval stage and subsequently to the adult stage. The *Anisakis* life cycle is completed when new eggs are excreted in the feces of the definitive hosts[31].

The *Anisakis* life cycle may continue and not progress to the definitive host as long as an undefined number of paratenic hosts could take part in it (L3 larvae can be transferred to other paratenic hosts *via* predation). Humans become incidental hosts in the parasite life cycle when they ingest contaminated raw, undercooked, pickled, or smoked seafood. Particularly, muscle tissues represent the most dangerous parts of seafood products, unlike the visceral cavities (that are usually removed from seafood and not consumed)[31]. Figure 1 illustrates the *Anisakis* spp. life cycle, as well as the main methods employed to kill *Anisakis* larvae and prevent human anisakiasis (discussed later in the text).

Methods for Inactivation of Anisakis spp. Larvae

Freezing

Thermal treatment of raw fish prior to consumption has been identified as one of the most effective control measures for preventing anisakiasis[39]. Notably, cold treatment can affect nematode viability and kill *Anisakis* larvae (*A. simplex* and *Pseudoterranova* spp.). Indeed, the latter method has been adopted since 1953[40]. However, many factors might affect the freezing process and, as a consequence, the survival of the larvae, including the type of seafood, the quantity and volume of the product, as well as the freezer unit features[41]. In addition, studies[42] reported that anisakid nematodes can survive under stress conditions, including cold temperatures. This mechanism is attributed to trehalose, a sugar produced in response to environmental stress, which has been reported to promote thermotolerance at both low and high temperatures.

In 2010, the European Food Safety Authority (EFSA) provided instructions to assert the hazard to human health of ingesting viable parasites in raw/undercooked fishery products. Thus, many countries developed sanitary regulations: according to EU Regulation No. 1276/2011, freezing has become a mandatory treatment of fishery products intended for culinary purposes in EU countries. The process requires lowering the temperature in all parts of the product to either

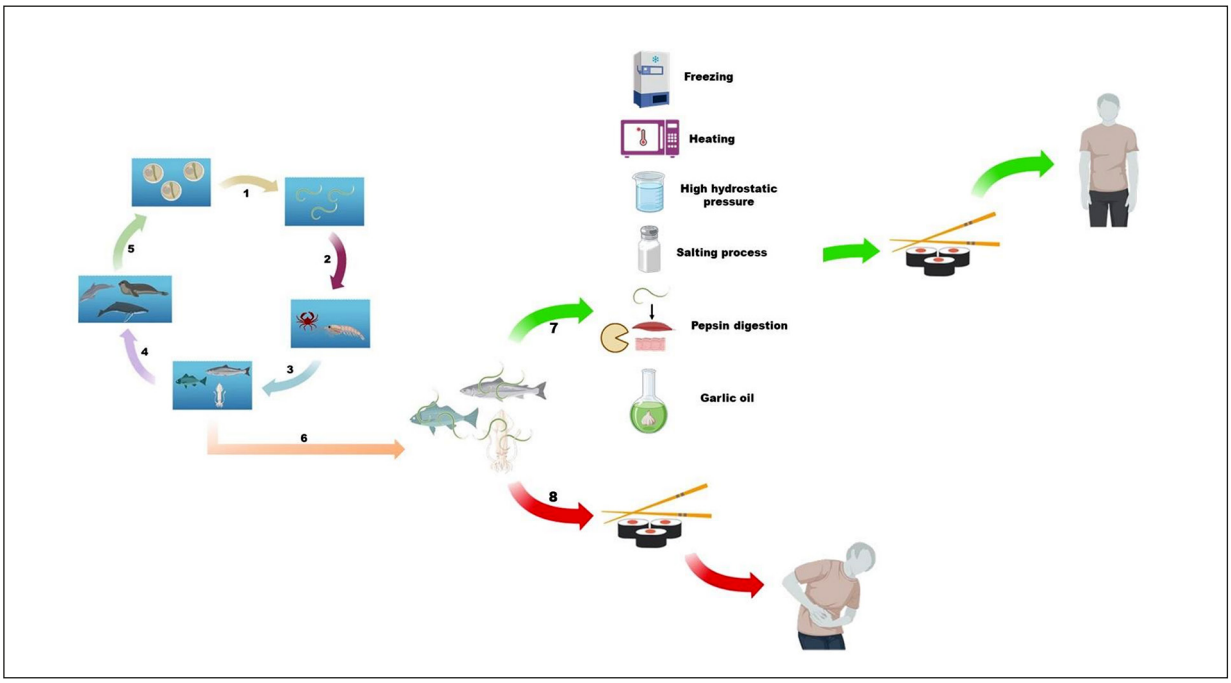

Figure 1. Illustration of *Anisakis* spp. life cycle, human infection *via* infected seafood consumption, and main methods to kill *Anisakis* larvae and prevent human anisakiasis. **1**, Eggs produced by adult anisakid nematodes are excreted in the feces of definitive host marine mammals and released in the seawater (where eggs embryonate). **2**, The unembryonated eggs excreted in the definitive host feces become embryonated in the aquatic environment (seawater). Then, L3 larvae form in the eggs and hatch from eggs as ensheathed, free-swimming forms. **3**, Intermediate hosts such as krill and crustaceans ingest the L3 larvae, which grow within the crustacean hemocoel. **4**, Paratenic hosts such as fish and cephalopods (e.g., squids) eat infected crustaceans. **5**, Definitive hosts (cetaceans and other marine mammals such as whales, sea lions, seals, dolphins, porpoises and walruses) become infected when they eat a contaminated paratenic host. In the definitive host marine mammals, the parasite develops to L4 larval stage and subsequently to the adult stage. **6**, Seafood products may be inadequately processed and prepared for culinary purposes, thus posing a significant human health risk. **7**, The main methods employed to kill *Anisakis* larvae and prevent anisakiasis include freezing, heating, high hydrostatic pressure, salting process, pepsin digestion, and use of garlic oil. **8**, Humans become incidental hosts when they consume contaminated seafood products. Then, anisakiasis ensues, usually resulting in abdominal pain and other gastrointestinal symptoms (such as nausea and vomiting). Moderate-to-severe allergic reactions (such as angioedema, urticaria, and even anaphylaxis) can also occur as a consequence of consumption of *Anisakis*-contaminated seafood. The figure was created by using BioRender (available at: www.biorender.com/). The sushi illustration source was Macrovector-Canva (available at: www.canva.cn/p/macrovector).

-20°C for not less than 24 hours, or to -35°C for not less than 15 hours[39]. Similarly, the U.S. Food and Drug Administration (FDA) established that fish products must undergo a freezing treatment (-20°C or below for 7 days, or -35°C or below for 15 hours)[43]. However, this seems to be not sufficient to completely ensure consumer safety. Hence, there have been numerous studies aimed at testing the most suitable and effective freezing procedures to kill *Anisakis* larvae. Herein, we report four studies[41,44-46] that analyzed the effectiveness of the freezing technique (alone or in combination with other procedures) to kill *Anisakis* larvae.

Adams et al[44] assessed the survival of *A. simplex* larvae in fresh arrowtooth flounder (*Atheresthes stomia*) after storage at four freezing temperatures for selected periods. Authors documented that all larvae were killed by exposure to 96, 60, 12, and 9 hours at temperatures of -15, -20, -30, and -40°C, respectively.

Podolska et al[41] tested numerous time-temperature conditions to kill *A. simplex* and *Pseudoterranova* spp. on two species of fish, aiming to optimize the freezing process. Particularly, authors froze fish samples at -15, -18 or -20°C for 24 hours, or at -20°C for 48 hours in a single-compressor freezer, and at -20, -25, or -35°C for 24 hours in a double-compressor freezer. Then, authors assessed *Anisakis* larvae viability based on their mobility. Only freezing at -20°C for 48 hours in a single-compressor freezer, and at -35°C for 24 hours turned out to be effective procedures. These observations suggested that

fish species and freezing device capability are essential aspects to be taken into account for the *Anisakis* larvae inactivation process[41].

Furthermore, in an *in vitro* experiment, Guan et al[45] investigated the effect of temperature, carbon dioxide (CO_2) and oxygen (O_2) on larval motility and mobility. After collecting and processing, larvae were transferred to a semi-solid phosphate buffered saline (PBS) agar and were exposed to different temperatures and various combinations of O_2, CO_2 and nitrogen (N_2). N_2 was added to reproduce the modified atmosphere packaging system, in order to extend the shelf life of fresh fish products. This study demonstrated that temperature has a remarkable impact on larval movement (highest motility registered at 22°C for *A. simplex*, and at 37°C for *Pseudoterranova* spp.), while CO_2 and O_2 did not exert a significant role in the short term[45].

Oh et al[46] investigated the *Anisakis* larvae inactivation by combining freezing (-20°C and -40°C for 6 hours, 12 hours and 1-21 days) and salting [5, 10, 15, 20% sodium chloride (NaCl) for 3, 6, 12 hours, and 1-7 days] with chlorine (500, 1,000, 1,500, and 2,000 ppm) and ultrasound (37 kHz frequency and 1,200 W for 5, 10, 15, 20, 30 minutes). Authors aimed to test the effectiveness of the combination of all these procedures. The salting process is recognized to be a powerful method to kill larvae[47]. Chlorine has been long employed as a food disinfectant, mainly during the washing process[48]. Chlorine water solution has also been combined with ultrasound since the latter technique can kill pathogenic bacteria in the frequency range between 20 and 100 kHz[49]. In the aforementioned study, freezing at -20°C for 48 hours and at -40°C for 24 hours was found to inactivate the larvae. Ultrasound turned out to be effective in reducing larval viability from 43% to 13%. Among the tested combinations, 24 h-freezing and salting in 15% NaCl for 7 days proved to be a valid choice to inactivate *Anisakis* larvae in seafood[46].

Yet, even when L3-infected fishery products are treated with freezing procedures, human health hazards can still occur following their consumption. As reported by EFSA[50], allergens released by the parasites can cause allergic reactions in sensitized patients[51]. This phenomenon could be attributed to the modifications of the larval cuticle that occur upon freezing. *Anisakis* larvae have a multi-layered cuticle that is mostly composed of proteins, glycoproteins, and collagen[52]. Low temperatures can alter these structures, leading to lower resistance to the human gastric juice. A correlation between the number of broken larvae and the antigen release has been noted. Particularly, it seems that the fast-freezing process leads to a greater loss of larval integrity, which may cause an increased release of allergens and, as a consequence, a higher risk of allergic reactions[53].

Heating

Different studies[54,55] have been conducted to test the use of conventional ovens and microwaves for killing microorganisms in various foods, but there are only a few reports on the impact of microwaves on parasites. Fish *Anisakis* infestation is a significant issue for the fishing industry and food safety authorities[50]. Furthermore, fish *Anisakis* infestation poses a significant human health risk because some of the secretion products and somatic and cuticle proteins released from the parasite can cause an allergic response[35]. In general, thermal treatments involve applying high (heating) or low (freezing) temperatures to seafood before its consumption. *Anisakis* larvae have been reported to moderately tolerate heat treatments[56]. EU Regulation No. 1276/2011 indicates that reaching a temperature of 60°C for 1 minute in the central part of the fish products minimizes the risk of anisakiasis[39]. Yet, the most advantageous pattern of time-temperature conditions needed to kill *Anisakis* larvae still remains unclear and some studies[57] have been conducted to address this question.

Sánchez-Alonso et al[56] analyzed three different heating-timing combinations to treat *Anisakis* L3-infected fish fillets, in order to define the exact point at which the fishery product loses its infective power. Their experiments were conducted in a water bath or in a conventional oven. According to the results, a 1-minute heating treatment is not sufficient to kill the parasites, but at least 8 minutes of high temperature (>60°C) are required for this purpose. However, some *A. simplex* antigens are highly resistant to thermal treatments[58]. Besides the clinically adverse events resulting in anisakiasis, a potential risk to consumers is represented by *Anisakis* allergens. Some allergens have been found in fish samples even after heating treatments that killed the larvae[59]. Indeed, results reported in the literature consider unsafe to heat the seafood product at 60°C even for 10 minutes[57]. Vidaček and colleagues determined the lowest time-tem-

perature set to kill the *Anisakis* larvae (≥70°C for ≥1 minute) and to observe no larval movement. Nonetheless, antigenic proteins were still detected in larvae who were exposed to a temperature of 94±1°C for 3 minutes[57]. As we previously mentioned, the inactivation of parasites can also be obtained through microwave (MW) treatment. MW treatment effectiveness may depend on different variables regarding both microwave ovens and food peculiarities. MW cooking has some limitations since microwaves do not reach all areas of the food samples, which can differ in thickness. Comparison between conventional and microwave heating, upon controlled conditions, showed that MW ovens killed the larvae in a lower amount of time[59]. Although MW cooking can cause larval death and prevent anisakiasis, *Anisakis* allergens may remain within the fish muscle tissue. Thus, this treatment may be unsafe for consumers who are already sensitized to *Anisakis* allergenic proteins[59].

High Hydrostatic Pressure

High hydrostatic pressure has been proven to be a useful method for treating food to reduce the pathogenic microorganisms and to extend the product shelf life[60]. High-pressure (HP) methodology is now the topic of numerous studies about its application to different features of food processing. High-hydrostatic pressure technology has recently made its entrance into the food industry and represents an alternative to traditional preservation methods (like heat processing)[61]. HP processing is a novel technique used to produce minimally processed food with better retention of the naturally characteristic aroma and fresh-like taste (without food additives)[62]. A study by Molina-García and Sanz[63] investigated the viability of *Anisakis* larvae isolated from fish tissue and their survival in distilled water and physiological isotonic solution. Treatments at a pressure of 200 MPa for 10 minutes (at a temperature between 0°C and 15°C) killed all *Anisakis* larvae. Authors found that lower pressures can be successfully used down to 140 MPa, although with lower pressures the treatment time must be increased by up to 1 hour to kill all larvae. In this study, lack of motility and autofluorescence methods were used to determine larval death. The authors also documented that those cycles of compression and decompression enhanced the larval death compared to a single-pressure treatment employed for a similar treatment time. In particular, the authors reported that a long treatment time at low pressure could lead to the equivalent effect exerted by a short treatment time at high pressure. All different conditions in fish experiments (200 MPa for 10 minutes, 190 MPa for 15 minutes, and 170 MPa for 25 minutes; at a temperature of 10 to 15°C in all experimental conditions) yielded 100% of dead larvae and non-motile larvae (immediately after the experiment and 2, 6, and 12 hours later). It is also important to consider that all the non-treated *Anisakis* larvae (control group) were alive[63].

Another pilot study conducted by Dong et al[60] analyzed the impact of high hydrostatic pressure on the viability of *A. simplex* larvae by evaluating the effects of this treatment on the color and texture of the fish fillets. 100 grams of fish containing *A. simplex* larvae were exposed to pressures of up to 552 MPa for up to 180 seconds. The conditions (time and pressures) required to kill all the larvae were: 414 MPa for 30 to 60 s, 276 MPa for 90 to 180 s, and 207 MPa for 180 s. Although high hydrostatic pressure was efficient in killing all the larvae in raw fish fillets, the impact of such a procedure on the color and appearance of the fillet may limit its application in fish processing for the raw fish industry[60]. However, how pressure treatments damage these microorganisms must be considered. High-pressure acts by modifying macromolecular assembly, whereas it has a mild effect on small molecules. High external pressure might damage the nematodes in different ways, such by altering the mechanisms to preserve hydro-skeletal turgidity or by driving solutes and water through the cuticle. The high-hydrostatic pressure-induced larval death can be associated with internal alterations, such as protein coagulation, that may suggest disruption of the internal compartmentalization and a mixture of inner fluids. All these conditions could represent a potential cause of larval death. However, in order to avoid the diffusion of allergens in the fish tissues and to reduce the potentially detrimental effects of these allergens on sensitized consumers, it is critical to study novel strategies aimed at preserving cuticle integrity during high-pressure-mediated larval inactivation. The main advantage of HP processing for modern food

manufacturing is that this technique does not alter the organoleptic and nutritional properties of treated food. Thus, the high-hydrostatic pressure processing method can be considered an alternative technique to enhance seafood safety when the destruction of the larvae of *A. simplex* or other types of parasites is desired[63].

Salting Process

Salting process (especially brining) has long been employed as a method to inactivate nematodes of the genus *Anisakis*[47]. The salting process determines osmotic damages to the larval cuticle and digestive tract, thus modifying the permeability of the cell membranes with subsequent leakage of cell content (e.g., ions). Smaldone et al[64] assessed the efficacy of the salting process for the inactivation of *Anisakis* spp. in naturally infected cod fillets. Fillets were treated with a double salting process: fresh cod fillets were pickle salted in a NaCl brine solution (13%) at 5°C for 24 hours in a 1:1 fish-to-brine ratio. Then, fillets were dry salted and matured for 3 months at 5°C by stacking salt and fish (weight ratio 1:1) in alternating layers. Notably, authors demonstrated that the salting process of cod with a salt (NaCl) concentration of 18.6% for at least 15 days effectively devitalized all Anisakidae larvae present in flash[64]. Similarly, Anastasio et al[65] showed that dry salting process of naturally contaminated European anchovies (*Engraulis encrasicolus*) with a salt concentration of 21% in all the edible parts was able to devitalize *Anisakis pegreffii* larvae (present in ripened anchovies) in a 15-day period, with the final product showing a good panel acceptance along with an acceptable quality grade. Indeed, a 15-day period is deemed as the minimum time required to obtain commercial salted cod[66]. However, Oh et al[46] showed that *Anisakis* larvae in salt-fermented squid and pollock tripe were inactivated after only 7 days in 15% NaCl and after 6 days in 20% NaCl.

In 2007, the Spanish Agency for Food Safety and Nutrition (Agencia Española de Seguridad Alimentaria y Nutrición) defined the technical salting parameters and curing period (except for product freezing) able to inactivate Anisakidae larvae[67]. When salt concentration in fish results higher than 9% for at least 6 weeks, between 10% and 20% for at least 4 weeks, or more than 20% for at least 3 weeks, fish product freezing can be avoided[67].

Pepsin Digestion Method

Artificial enzymatic digestion procedure by CODEX (STAN 244-2004: standard for salted Atlantic herring and salted sprat) currently represents the recommended protocol for research on anisakid nematodes. The performance of the artificial pepsin digestion technique in terms of recovery and viability of L3 *A. simplex* larvae has also been investigated, since such technique combined with the use of hydrogen chloride (HCl) facilitates easy separation of third-stage larvae (L3) from fish muscle or viscera, thereby simplifying their counting.

Sánchez-Alonso et al[68] showed that the artificial digestion method is able to kill a remarkable number of *A. simplex* larvae that may have survived freezing. In addition, the authors concluded that this method could underestimate the infection level of fish batches containing dead L3 larvae[68]. Llarena-Reino et al[69] conducted a study aimed at optimizing the actual artificial digestion protocol by CODEX in an attempt to offer a rapid, cheaper, safer, and more convenient technique for unskilled personnel, such as factory and fish market workers. Authors found that a novel digestion method based on liquid pepsin format represented an accurate, reproducible, and user-friendly off-site tool, which was also effective for the implementation of screening programs aimed at detecting anisakid nematodes and subsequently preventing human anisakiasis. Interestingly, the novel liquid enzyme formula was also able to avoid allergic reactions that may instead arise from pepsin in powder[69].

Garlic Oil

It has been reported[70] that garlic and its bioactive compounds exert immunomodulatory, anti-inflammatory, antioxidant, and antimicrobial properties, among others. Morsy et al[71] recently evaluated the protective effect of garlic oil (GO) in Wistar albino rats after oral inoculation of fresh and thermally treated L3 *Anisakis* spp. larvae that were isolated from the Red Sea fish (*Dicentrarchus labrax*). Rats inoculated with fresh and thermally treated L3 larvae exhibited abnormal liver and kidney histologic changes, such as: renal tubules with cloudy swelling, some renal tubules with hyaline casts in their lumen, perivascular inflammation thickening the walls

of blood vessels, lobulated glomerular tufts with narrowed Bowman's spaces, marked vacuolar degeneration of the epithelial cells lining tubules, interstitial fibrosis with perivascular edema, some tubules affected by coagulative necrosis, hepatocytes with vacuolated cytoplasm, focal inflammatory cell aggregates with perivascular edema and inflammation, dilated sinusoids with inflammatory cells, ballooning and degenerated hepatocytes, and focal necrotic areas in the liver. Surprisingly, GO produced a protective effect in rat groups inoculated with L3 extracts plus GO, as it was supported by the evidence of normal liver and kidney histologic features[71]. Based on these promising findings and on the known antiparasitic properties exerted by garlic, authors suggested that fish meals, in general, should be eaten with the addition of garlic (before and after seafood consumption), which may represent a potentially effective prophylactic method to avoid the detrimental health consequences of anisakiasis[71]. Figure 1 illustrates the abovementioned methods that are employed to kill *Anisakis* larvae and prevent human anisakiasis.

Conclusions

The consumption of inadequately processed fish is a public health risk due to the possible propagation of *Anisakis* spp. larvae causing anisakiasis in humans that ingest larval nematodes present in raw or minimally treated seafood dishes. During the last five decades, the global prevalence of human anisakiasis has been growing, thus becoming an emergent major public health issue. Hence, several studies evaluated the effectiveness of different methods and techniques to kill viable anisakid nematodes in fishery products and simultaneously ensure food safety and product quality. The main techniques investigated and employed for such purposes include freezing, heating, exposure to high hydrostatic pressure, salting process, pepsin digestion, and use of garlic oil. All these techniques have demonstrated a certain degree of efficacy across different studies and may therefore represent valid and cost-effective strategies (to be employed alone or in combination) aimed at enhancing seafood safety and reducing the incidence of human anisakiasis.

Indeed, over recent years, the consumption of raw and undercooked fishery products has substantially increased, particularly due to the globalization of Japanese cuisine. Current recommendations for increasing seafood safety may appear limited due to the marked seafood diversity and the high specificity required for the inactivation processes (depending on the type of microorganism involved and on the specific seafood product). Therefore, further studies are needed to better establish which of the aforementioned methods carries the highest efficacy in killing *Anisakis* larvae in seafood products (especially raw fishery products such as sushi and sashimi) and/or whether a combination approach involving the use of two or more of these techniques may be valuable for enhancing the seafood safety. These studies would certainly inform public health policies to define novel and more specific guidelines for manufacturers and consumers concerning the different techniques to kill *Anisakis* larvae in a wide range of seafood products.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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