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# Inactivation of *Anisakis simplex* Allergens in Fish Viscera by Acid Autolysis †

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**Abstract:** The evisceration of infested species on board commercial fleets and the throwing of viscera into the sea, as is mostly the case for hake, is common practice for reducing the occurrence of *Anisakis* in fishery products. Moreover, the high levels of infestation and the lack of technically and economically feasible solutions hinder the possibility of recovering viscera with other fish processing byproducts as raw material for feed without the risk of transmission of allergens to humans, with the subsequent risk for public health. The aim of this work was to study the fate of *Anisakis simplex* allergens during 11 days of silage of infested hake (*Merluccius merluccius*) viscera, as a potential method for eliminating this risk. While the viscera were almost completely liquefied, an increase in the allergenicity of the soluble fraction was observed, which decreased only slightly after day 9. As we are aware of the resistance of parasite allergens to gastrointestinal enzymes, silage was also analyzed after 12 and 15 months. While the lower molecular weight fragments of *Anisakis* proteins are fully digested, some larger fragments with potential allergenicity resisted autolysis after long silage periods, but they were present in a very low concentration. The study concludes that there is the potential of silage as a method for recovering fish viscera infested with *Anisakis*.

**Keywords:** fish byproducts; nematodes; parasites; IgE immunoreactivity; aquaculture feed; silage



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## 1. Introduction

Larvae of the genus *Anisakis* (Nematoda, Anisakidae) as intermediate and paratenic hosts can infest many predator fishes, crustaceans and cephalopods. Infested fishes accumulate *Anisakis simplex* nematodes in their tissues via ingestion of infested prey throughout their lifespan. Larvae can then be found in various parts of the body, mainly in the hypoaxial muscle and visceral cavity, and mostly in the viscera [1,2]. *Anisakis* larvae are distributed worldwide, and many of the most important pelagic and demersal commercial fish species caught by European fleets are potential hosts of these nematodes. In the extractive and marketing sector (and among consumers), there has been a growing awareness of increased infestation levels in high-value and high-demand fish species such as hake, anchovy, tuna and others. This accumulation of larvae in fish tissues is considered a significant factor affecting product quality in European fish markets [3–7]. When live larvae are accidentally ingested by humans, they can penetrate and encyst in the gastric or in the intestinal mucosa [8–11]. It has also been confirmed that *Anisakis simplex* nematodes can induce allergic and/or anaphylactic reactions in humans, even if the fish is well cooked [12–16].

Infestation with the *Anisakis simplex* nematode in fish species of high commercial interest (such as hake, monkfish, mackerel, anchovy, sardine and blue whiting) has become a problem for many fisheries in recent years because of the high infestation levels observed,

leading to economic losses in first-sale and retail services, as well as posing a risk to public health. Therefore, it is considered very important to implement measures aimed at reducing/minimizing the parasite load in fish, acting across the entire product chain, from the extraction and storage of fish, to marketing and sale to consumers.

The removal and discarding of viscera on board commercial fleets is a widely used method for preventing postmortem migration of nematode larvae from the viscera to the muscle when the fish are stored onboard; this approach helps reduce the presence of larvae in the product in fish auctions and markets [17]. According to some authors, discarded infested viscera can be ingested by other predator fishes and thus re-enter the life cycle [18–20]. However, a recent experiment with farmed seabasses free of *Anisakis* larvae showed that only a very small proportion of the nematodes ingested reinfected the host, and most of them were rapidly digested and defecated [21]. With respect to the removal of viscera, certain technological treatments aimed at inactivating *Anisakis* larvae before discarding them into the sea have been promoted [22], but the equipment necessary on board is often expensive and bulky. The space limitations on board and the lack of economic incentives for fishermen result in the viscera being thrown to the sea. Moreover, the presence of high infestation levels of *A. simplex* in the viscera casts doubt on this practice due to the unknown impact it may have in the trophic chain and the possibility of recovering the viscera with other fish processing byproducts as raw material for feed.

Total EU aquaculture production increased by 6% from 2011 to 2020 and is expected to increase from 13 to 32% between 2018 and 2030 [23]. Fishmeal and oil are the preferred sources of highly digestible essential nutrients for aquaculture species. A growing share of fishmeal and fish oil for aquaculture feeding, estimated at 25–35%, is produced from fish processing byproducts. However, previous studies have detected trace amounts of *Anisakis simplex* allergens in samples of sushi and slaughter-ready farmed salmon [24], suggesting a possible origin in the feed, and further studies have shown that *Anisakis* allergenic peptides can withstand aquaculture feed processing and be transferred to fish tissue, with the risk of causing symptoms in sensitized consumers [25].

The European hake (*Merluccius merluccius*) is a target species caught mainly by bottom trawlers in European multi-specific fisheries and by demersal and bottom gillnetters [26]. In the Basque Country, landings of European hake reached 4983 tons in 2021; medium-large individuals are normally gutted on board and marketed fresh. According to the total visceral weight relationship in hakes [27], the production of hake viscera in Basque ports was estimated at 438.5 tons in 2021. However, hake (*Merluccius merluccius*) captured on the Atlantic coast of Spain exhibits high infestation levels of *Anisakis* [28,29]. A simple and economically feasible processing method that can inactivate or destroy *Anisakis* allergens, allowing the use and exploitation of fish viscera, would be highly useful for the Basque fleet.

Although some studies have evaluated the resistance of *Anisakis simplex* allergens to human gastric enzymes [30,31], there are no previous studies on how acid autolysis, known as silage, can affect *Anisakis simplex* and its allergenic potential. Although a serine protease inhibitor similar to the trypsin inhibitor found in soya beans has been identified in *Anisakis* larvae [32] and may play a role in the defense against host digestion, it should not inhibit the acid aspartic protease (pepsin) present in the viscera, and the effect of long-term treatment has not yet been observed. Thus, the objective of this work was to study the silage of hake viscera as an alternative for the use or exploitation of discarded viscera to reintroduce them into the food value chain. Acid autolysis has been considered the most appropriate method for implementing both on board and ashore, due to its simplicity and relatively low investment cost [33]. Silage is a very simple process that is currently used to take advantage of the remains of fish or whole fish that are not for consumption. These materials are ground and mixed with an organic acid (usually formic acid) that preserves them and prevents them from rotting. The acid endoproteases that contain the fish viscera, which are highly stable at pH values of between 1.0 and 5.0 [34], digest the mixture in a process called autolysis and liquefy it in a few days or weeks depending on the ambient temperature. During the process, the fish oil floats and can be separated from the rest of

the mixture and used separately. The action of enzymes digests proteins, giving rise to peptides and amino acids, and improving the digestibility of the final product. Moreover, this approach makes it possible to obtain interesting byproducts such as peptides, amino acids and oils that can be valuable to specialized companies. The product, which is also called silage, has a nutritional composition similar to fishmeal, and its ideal final destination is animal feed. It can be used directly in the feed of farm animals or mixed with other raw materials such as grains and other dry products in wet feed. It can also be included in the formulation of granulated or pelletized dry feed.

With this as the objective, several silage trials were performed with hake viscera containing parasites. The effects of silage and pretreatment conditions (grinding) on *Anisakis* mortality were observed. Additionally, the allergenicity of silage products was monitored throughout the autolytic process, and the remaining allergens were characterized through electrophoresis and immunoblotting.

## 2. Materials and Methods

### 2.1. Hake Viscera

Fresh hake viscera were kindly provided by the commercial longline and trawl fleet of the Cofradía de Pescadores de Ondarroa (Fishermen's Association, Ondarroa, Spain). The viscera returned a high degree of infestation with *Anisakis* (Figure 1).

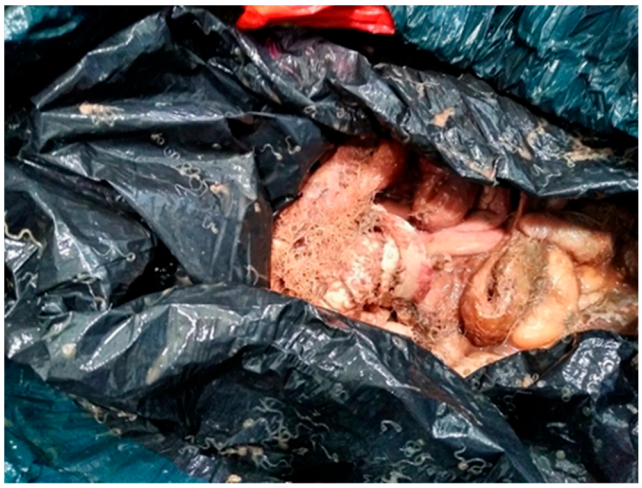

**Figure 1.** Hake viscera infested by *Anisakis*.

### 2.2. Silage Experiments

Approximately 1 kg of fresh viscera was gently ground, and the pH was adjusted with formic acid to 3.5 (initial viscera pH was 6.35) to perform silage for 11 days in closed 3 L glass vessels at an average temperature of 18 °C. The mixture was stirred every day, and the pH was monitored. The degree of liquefaction, calculated as the percentage of the total mass not retained through a 1 mm screen, was measured daily. Each day, an aliquot of 100 mL was separated to measure *Anisakis* mortality (see Section 2.3). Two aliquots were centrifuged in Falcon tubes at 3000× *g* for 10 min to separate the different phases and then preserved frozen at minus 80 °C until analysis (see Sections 2.5 and 2.6). Additionally, the protein content of the solid and liquid fractions obtained during the silage period was monitored daily for up to 264 h (Kjeldahl total nitrogen AOAC method 955.04).

### 2.3. Anisakis Mortality

The mortality of *Anisakis* was verified immediately after treatment and after 24 h, 48 h and 78 h in fresh samples and up to 96 h in silage. The motility of each individual was verified according to the criteria defined in Table 1.

**Table 1.** Description of qualitative (category) and quantitative (value) criteria for estimating the mortality rate of *Anisakis* after treatment.

| Category | Value | Description |
|---|---|---|
| Live | 1 | Vigorous motility without needle stimulation |
| Reduced motility | 0.5 | Motility requiring needle stimulation |
| Dead | 0 | Null motility when stimulated with the needle |

### 2.4. Patient Sera

To measure the allergenicity of all the silage fractions and its evolution during the ensiling period, an enzyme-linked immunosorbent assay (ELISA) was performed using a pool of sera from nine patients with a diagnosis of allergy to *Anisakis* (without previous anisakiasis) from the Allergy Department of the University Hospital of Araba (Gasteiz, Spain) and anti-*Anisakis*-specific IgE antibody concentrations above 30 kUA/L (measured with ImmunoCAP FEIA System, Thermo Fisher Scientific, Norristown, PA, USA). The use of the serum samples was approved by the ethics committee of the hospital (internal reference number PI2018118). All the donors signed the corresponding informed consent, and their anonymity was guaranteed throughout the study. The serum samples were mixed (with the same volume of each individual serum sample), and this mixture (pool) was used to evaluate potential allergenicity (IgE reactivity) via ELISA (Section 2.2).

### 2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

To detect the presence of traces of *Anisakis* allergens in silage, an ELISA was performed on the resulting products: an aqueous fraction with soluble protein, a solid pellet and floating solids.

As a control, a liquid extract of the raw material (viscera with *Anisakis*) was obtained on day 0 (untreated, 100% IgE binding) by homogenization (ultraturrax, 10,000 rpm for 1 min) of 1 g of sample in 10 mL of saline buffer (150 mM NaCl, 8 mM $K_2HPO_4$, 16 mM $KH_2PO_4$, pH 7.4, and PBS). After 2 to 9 days of processing, the samples were prepared in the same way (although divided into the different fractions obtained). For each extract (control and sample), the protein concentration was measured by a bicinchoninic acid (BCA) assay according to the manufacturer's instructions.

For the ELISA, 96-well plates were coated with 100 µL of an untreated (control) sample solution per well containing 10 µg/mL protein (including *Anisakis* proteins) in coating buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, pH 9.6). The plates were incubated overnight at 4 °C.

After coating, the wells were washed three times with PBST (0.05% Tween 20) and blocked with 300 µL of a 1% (*w*/*v*) solution of bovine serum albumin (BSA) in PBS for 1 h at room temperature (RT). Subsequently, after washing, 100 µL of a mixture (1:1) of the corresponding sample was diluted 1:1000 in PBS, and the pool of sera diluted 1:20 in PBS. The mixture was added to each well, after which it was incubated for 1.5 h at room temperature. After the corresponding washes, the plates were incubated with 100 µL of the corresponding secondary antibody (goat anti-human IgE-HRP; A9667; Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000 in PBS for 40 min at room temperature. Finally, the wells were washed again, and 100 µL of the peroxidase substrate (TMB) was added to each well. After color development, the reaction was stopped by adding 50 µL of 2 M $H_2SO_4$ to each well. The absorbance of each well was measured at 450 nm using a Thermo Scientific™ Varioskan Flash plate reader (Waltham, MA, USA).

### 2.6. Allergen Characterization

SDS-Electrophoresis in polyacrylamide gel (SDS-PAGE) was used to investigate the presence of proteins with a molecular size compatible with known *Anisakis* allergens and to monitor their presence throughout the silage trial. Immunoblotting (IB) was used to detect immunoreactive proteins or their fragments within those identified via electrophoresis (EF).

Electrophoresis provides information on the molecular weight of the proteins in suspension and makes it possible to identify most allergens found in the samples compared with the known allergens of *Anisakis.* It also determines whether they are somatic antigens (SAs) or secretory antigens (SEs).

The electrophoresis of the proteins present in each fraction throughout the digestion period of the viscera (days 1–9) was performed on gels with a gradient of 4 to 20% polyacrylamide in a Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, CA, USA) system according to the conditions set for these samples. The different samples were diluted in 10 mM Tris-HCl buffer (pH 8.0) with 2.5% SDS and 50 mM DTT as the reducing agent and heated in a water bath for 6 min at 95 °C. The proteins were separated at 125 V for 1 h. In each gel, a molecular weight standard (10–250 kDa, Precision Plus Protein™ Dual Color Standards, Bio-Rad) was used, and a sample of a complete *Anisakis* protein extract was also included as a reference. After separation, the proteins were stained by immersing the gels in a solution of Coomassie blue for 1 h. The excess dye was removed by immersion in distilled water for 2 h until the background was colorless. The gels were photographed and analyzed in an EZ Gel Doc™ system with Image Lab™ software version 6.0.1 build 34, © 2017 (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Immunoblotting makes it possible to identify the specific proteins or the proteins recognized by antibodies, determining whether there is a modification throughout the digestion process of the viscera and also determining the degree of hydrolysis (measured by molecular weight size) in which the specific IgE antibodies cannot recognize the hydrolyzed proteins.

First of all, the proteins were separated via electrophoresis under the same conditions as those described above. The separated proteins of each fraction were transferred to a nitrocellulose membrane under the current conditions of Am, V for 7 min. Immunoblotting was performed with a commercial Amplified Opti-4CN Substrate Kit (Bio-Rad) following the manufacturer's instructions and optimized for the conditions of this assay. The nitrocellulose membranes were allowed to dry completely, and the free sites were blocked with the provided blocker diluted 3% in PBST for 1 h. After blocking, each membrane was washed twice with PBST and incubated with the pooled sera diluted 1/100 in a 1% solution of bovine serum albumin (BSA) in PBST. Subsequently, the membranes were washed again and incubated with the secondary antibody labeled with peroxidase (the same as for the ELISA) diluted 1/4000 in PBST. The following steps were performed according to the instructions of the aforementioned kit for signal amplification and colorimetric detection with 4-chlor-1-naphtol substrate.

### *2.7. Presence of Anisakis in the Products*

To detect the presence of traces of *Anisakis* DNA in the silage products and as a complement to the allergenicity tests, real-time PCR was performed on the water samples with the soluble protein and on the solid pellet on a MicroAmp Optical 96 reaction plate. The amplification reaction was carried out with TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA) with the primers QCYTCII-F (5′-AGTAAGAAGATTGAATATCAGTTTGGTGA-3′) and QCYTCII-R (5′-AAGTAAACTCAAAGAAGGCACCATC-3′) and the specific probe CYTCII (5′-FAM-TTCCTACTTTAATTTTTTGGTTGCTC-MGB-3′). For the amplification reactions, a LightCycler 480 sequencer (Roche Diagnostics, Indianapolis, IN, USA) was used under the following conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

### *2.8. Amino Acid Profile*

The amino acid profiles of the hydrolysates obtained after 12 months of storage were determined via high-performance liquid chromatography with fluorescence detection (HPLC/FD) as described by Gutierrez et al. [35]. Briefly, aliquots of the samples were neutralized with HCl (6 N) for 24 h at 100 °C and derivatized using an AccQ Fluor Reagent Kit (Waters, Milford, MA, USA). Chromatographic analysis was performed in a gradient

system comprising 60% acetonitrile and 40% AccQ-Tag buffer (10%, $v/v$) for 5 min and then in 100% Waters AccQ-Tag buffer (10%, $v/v$) (Waters, Milford, MA, USA) for another 9 min. The column used was an Agilent Polaris C18- 4 µm (83.9 × 150 mm) (Agilent Technologies, Madrid, Spain). The excitation and emission wavelengths used for detection were 250 and 396 nm, respectively. For the quantification of the amino acids, AccQ-Tag, Pico-Tag and Accu-Tag Ultra (Waters, Milford, MA, USA) were used as amino acid standards.

### 2.9. Molecular Size Profiling

Size exclusion high-performance liquid chromatography (SE-HPLC) was performed to assess the peptide molecular weight distribution in the silages obtained. Samples were analyzed using an AdvanceBio SEC LC column (130 Å, 7.8 × 150 mm, 2.7 µm) (Agilent Technologies, Spain) connected to a diode array detector (DAD) (Agilent Technologies, Spain). A total of 1.5 µL of sample was injected into the column, which was kept at room temperature. The mobile phase consisted of pH 7 sodium phosphate buffer (150 mM). The samples were eluted at a flow rate of 0.5 mL/min, and the UV signal was measured at 214 nm. Each sample was filtered through a 0.45 µm PVDF filter. The AdvanceBio SEC 130 Å Protein Standard (Agilent Technologies, Spain) and SIGMA-HPLC peptide standard mixture Product No. H 2016 (SIGMA ALDRICH®, Merck KGaA, Darmstadt, Germany) were used to determine the elution time depending on the weight of the molecules. Every sample was diluted to a protein concentration of 3 g/L.

## 3. Results and Discussion

### 3.1. Silage Evolution

When centrifuging the silage samples, four fractions could be observed at all times: oil, an interphase or "floating fraction" (apparently an emulsion of water-oil-protein), the liquid hydrolysate and the insoluble pellet (Figure 2). At the end of the ensiling period, the yield in each fraction was as follows: 6% oil, 77% liquid hydrolysate, 12% interphase and 5% pellet. The liquid contained 79% of the total protein in the sample, which is higher than the 63% protein recovery reported by Bhaskar et al. [36] after enzymatic hydrolysis of fish viscera. The pellet contained 7%, and the interphase contained 14% total protein (this protein can be later recovered in the liquid fraction by breaking the emulsion).

The protein extracted in the liquid fraction appeared to reach a maximum on day 9 of silage (Figure 3), and the degree of liquefaction reached its maximum and stabilized after approximately 240 h (Figure 4).

### 3.2. Anisakis Survival

For the immediate mortality sample, all dead and living nematodes (12 + 249) were counted. For the following periods of 24, 48, 72 and 96 h, four subsamples of 10 individuals were taken, and mortality was analyzed according to the criteria described above. In the initial sample of the untreated viscera, only 5% of the *Anisakis* died, a value that increased to 100% after 96 h (Table 2). However, the results of the four silage samples showed that after 24 h, the mortality rate was 10% in all cases. It appears that the cause of *Anisakis* mortality was not slight grinding but likely the addition of formic acid.

**Table 2.** Mortality (% *Anisakis* and number of individuals) in samples of untreated viscera and silage viscera.

| Mortality (%) | Untreated Viscera | | Silage Viscera | |
|---|---|---|---|---|
| Initial | 5% | 249 L + 12 D | 50% | 10 RM |
| 24 h | 0% | 10 L | 100% | 10 D |
| 48 h | 85% | 7 D + 3 RM | 100% | 10 D |
| 72 h | 95% | 9 D + 1 RM | 100% | 10 D |
| 96 h | 100% | 10 D | 100% | 10 D |

L: live; D: dead; RM: reduced motility.

**Figure 2.** Fractions obtained after centrifugation (3000× *g*, 10 min) of the silage of the hake viscera (from top to bottom: oil, floating interphase, liquid and pellet).

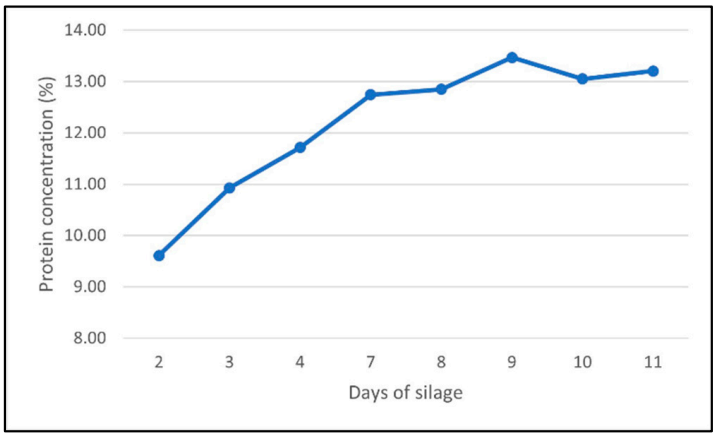


**Figure 3.** Changes in the protein concentration in the liquid fraction of the silage over the 11 days of the experiment.

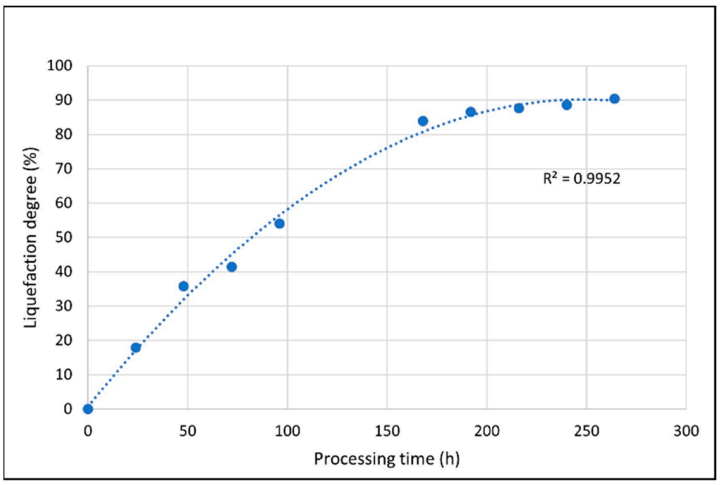


**Figure 4.** Degree of liquefaction obtained during the 11 days of silage of hake viscera.

### *3.3. Presence of Anisakis allergens*

As shown in Figure 5, the extent of protein recognition by IgE in patient serum varies depending on the fraction analyzed (solid pellet, floating interphase and liquid fraction). However, although the evolution of the three fractions follows a different pattern, the results obtained are related to each other, considering the type of process to which the samples are subjected.

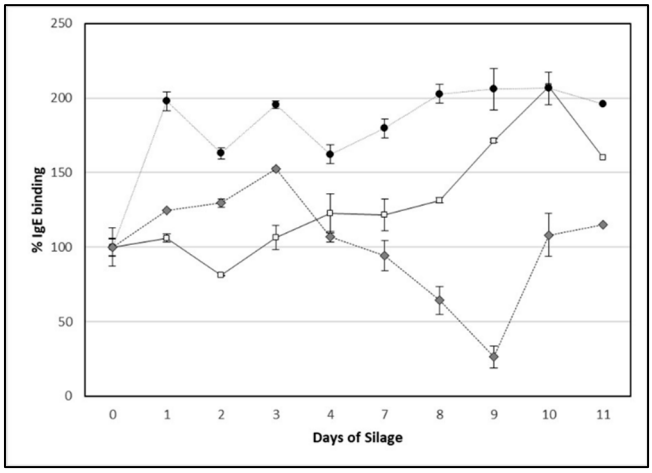


**Figure 5.** Evolution of the allergenicity in the different fractions obtained during the ensiling period of the hake viscera: solid fraction (□), floating interphase (♦) and liquid fraction (●). The vertical bars correspond to the SDs ($n$ = 6).

Thus, in the solid pellet, no changes were observed during the first days, while in the final days, there was an increase in the binding of IgE to the proteins present in that fraction. This may be because, during the hydrolysis process, the allergenic proteins initially present inside the body of *Anisakis* become solubilized; consequently, the epitopes (fractions of the protein that recognize the antibodies) change from a state where they are "hidden" (days 1–3) to a soluble or superficial state that is more easily recognized by antibodies, gradually increasing IgE binding (days 4–10). These results are also consistent with the degree of liquefaction and the protein content in the liquid fraction (Figures 3 and 4). presented above. On the last day, a decrease in the binding of IgE to *Anisakis* proteins was observed again due to further hydrolysis of the solubilized proteins. However, IgE binding continued to be stronger than that in the control group. It is necessary to verify that this

downward trend was maintained in the following days and that the IgE epitopes of the *Anisakis* allergens present were completely hydrolyzed.

In the floating interphase, this phenomenon of increased IgE binding due to the initial solubilization of proteins was also observed but, after day 5, there was a rapid decrease in the recognition of the allergens present due to the hydrolysis process that gradually destroyed the epitopes (reduction in the size of the peptides and proteins present). In this case, the reduction in allergenicity can be faster than in the solid pellet since, in this fraction, there are no nematode carcasses (bodies) at the beginning, and the potential allergens present are more accessible to hydrolysis. However, in the last two days, an increase was again observed, possibly related to the increase in allergenicity in the solid and aqueous fractions because the allergens present inside the bodies of *Anisakis* (solid fraction), initially hidden, are released and consequently migrate to the other fractions.

Finally, a significant increase in the IgE binding of the *Anisakis* proteins present in the aqueous fraction was observed after the first day. This could be due to the advance of hydrolysis, which would cause the proteins to pass to a soluble state while maintaining hydrolyzed protein fragments and peptides still recognizable by the IgEs. Consequently, a high "allergenic potential" was observed until the end of the process. These results are also in accordance with previous results, where it has been observed that the passage of proteins from the bodies of *Anisakis* to a more available and recognizable state is continuous during the period under study.

There are currently 14 recognized allergenic proteins of A. simplex [37], although this list may be extended with the identification of new potential allergens. These allergens are classified according to their function and location within the body of *Anisakis* as somatic antigens (AS) and excretory-secretory antigens (ES), which are produced by the internal glands of the parasite. Some *Anisakis* allergens have been described as very resistant to heat and pepsin digestion [30,37,38]. Most of these allergens are classified in the ES fraction. Additionally, previous research has established that most *Anisakis* allergenic patients (<80%) have specific IgEs to pepsin-resistant allergens [30]. These characteristics of *Anisakis* allergens explain the results obtained in this work. First, as mentioned above, the IgE antibodies of patients recognize the somatic antigens of the larvae, but on successive days, during the decomposition of the body due to hydrolysis, the epitopes from the ES antigens become available, causing an increase in recognition by the IgE antibodies in all fractions.

Later, in some fractions, the degradation of somatic allergens continues, reducing the potential allergenicity, for example in the floating fraction; while, on the other hand, the liberation of ES antigens becomes continuous, increasing allergenicity. As these allergens are more resistant to digestion, the desirable reduction in recognition by IgE did not occur until very intense hydrolysis was achieved—more than 11 days for the liquid fraction and solid pellet—while further hydrolysis may be necessary.

It can be concluded that in the silage process, hydrolysis triggers the passage of the excretory/secretory and somatic allergens of *Anisakis* to a more soluble or available state, which causes IgE antibodies to gradually recognize them throughout the hydrolysis process. However, the evolution of this potential allergenicity varies depending on the fraction of the sample we are considering. In this sense, although initially discarded in terms of productivity and efficiency/simplicity/speed of the process on board, it could be interesting to evaluate whether the treatment of the viscera with *Anisakis* prior to the hydrolysis process (e.g., crushing) would accelerate the liberation of ES allergens and their subsequent solubilization and hydrolysis, allowing a faster reduction in allergenicity.

### *3.4. Allergen Characterization and Amino Acid Profile*

Electrophoresis and immunoblotting of the different fractions revealed changes in the protein fraction during hydrolysis. As shown in Figure 6, variations in IgE binding in the different fractions were not associated with any specific protein since, in general, no clear bands were observed in the immunoblotting images; this is probably due to the

low sensitivity of this method. Since the *Anisakis* extract lane (Ani) showed clear reactive bands, the *Anisakis* protein concentration present in other fractions (where *Anisakis* proteins make up only a part of the total proteins) was insufficient for detection via this method. However, the electrophoresis images and faint bands in the immunoblot showed differences among the fractions and the different days of evolution, which are in accordance with the ELISA results.

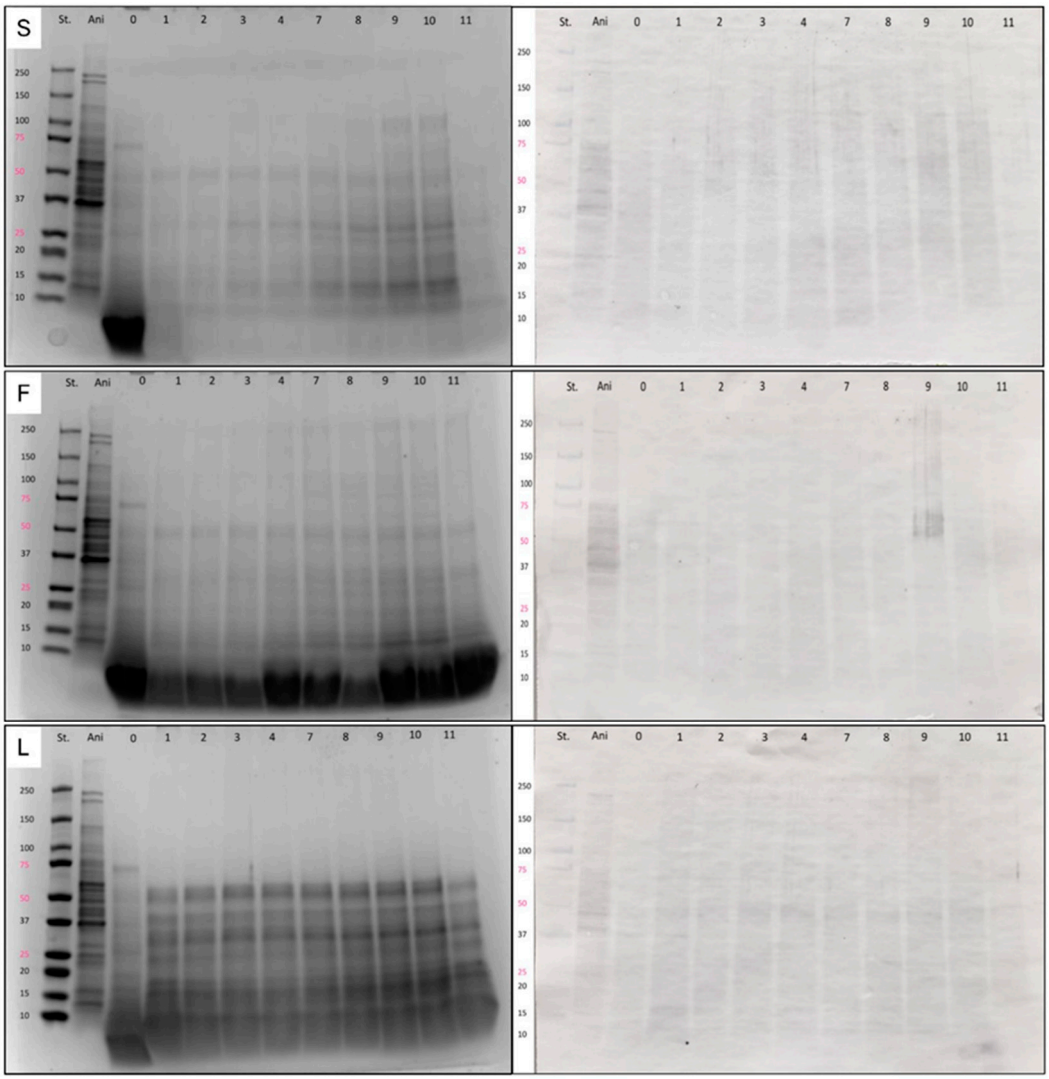


**Figure 6.** Images of SDS-PAGE (**left**) and immunoblotting (**right**) of the solid pellet (S), floating interphase (F) and liquid (L) fractions. The first three columns from left to right in each picture correspond to the pattern of molecular weights (St.), an *Anisakis* extract (Ani) and untreated viscera (Day 0-Control). The other lanes, from left to right, correspond to days 1 to 11 of the silage experiment for the corresponding fraction of the hydrolysate under study.

The solid pellet (S) fraction analysis revealed a gradual increase in soluble protein concentration (as indicated by more intense bands), and immunoblotting also revealed an increase in IgE binding, albeit somewhat diffuse, until day 10 (Figure 6), in accordance with the results observed in the ELISA analysis (Figure 5). This confirms that proteins are gradually digested and become more soluble and accessible to IgE during silage hydrolysis.

In the floating (F) fraction, on the other hand, an increase in protein concentration was observed, except on the last day (Figure 6); however, the allergenicity gradually decreased until day 9 (Figure 5). This is in accordance with the decrease in allergenicity observed in the ELISA. In contrast, in the last two days, although the protein bands detected via

electrophoresis were equal to or less intense than those detected on day 9, an increase in allergenicity was observed. In this sense, in immunoblotting analysis (Figure 6), a slight increase in the reactivity toward proteins with a molecular weight of approximately 50 kDa was observed during the last 2 days; this could explain the increase in IgE reactivity. This reactivity could be related to allergens Ani s 3 (41 kDa), a somatic antigen; or Ani s 11 (55 kDa), but also to fractions of the ES antigen Ani s 7 (139 kDa) [39]. This again confirms that due to hydrolysis, proteins would pass from the body of *Anisakis* and the soluble (liquid) fraction to other fractions, increasing immunoreactivity. In other studies, the most relevant IgE-binding heat- and pepsin-resistant *Anisakis* proteins were identified to be Ani s 4 (9 kDa) and Ani s 5 (15 kDa) [30], which seems to coincide with our electrophoresis results. Although these proteins were not reactive to IgE, according to the immunoblotting results, the higher concentrations of these proteins (or protein fragments), especially on the last day of study, could explain the higher IgE binding detected in the ELISA (Figure 5), the most sensitive method.

Finally, in the case of the liquid fraction, the increase in reactivity observed from the first day compared to untreated viscera (not yet "solubilized") is also related to the fact that the proteins are already in a soluble state from the beginning of the process. In this case, a reactive band of approximately 50 kDa (possibly Ani s 3, a somatic antigen) was also observed in immunoblotting during the experiment, although other proteins could also be involved in IgE immunoreactivity.

On the other hand, DNA analyses detected the presence of *Anisakis* only in solid pellet samples at any silage time (from 24 h to 264 h), while other fractions (liquid and interphase) did not present detectable traces of parasite DNA. This finding confirmed that parasite bodies remained mainly in the solid pellet, while during the hydrolysis process, only proteins and their fragments were solubilized.

Additionally, long-term resistance of *Anisakis* allergenic proteins to the action of digestive enzymes present in the silage process was observed in three of the experimental silage samples that were kept for up to 12 months (V3) or 15 months (F2, F5). The differences between the samples were also in the grinding step (F2 and F5 were intensively ground, while V3 was slightly crushed) and in the pH (3.6 in V3 and 2.2 and 2.5 in F2 and F5, respectively).

After this period, up to 82% of the total protein became part of the liquid fraction, 6% remained in the undigested pellet, and 12–13% remained in the floating interphase fraction. According to the SDS-PAGE profile, the solid fraction (S) and floating interphase (F) were fully hydrolyzed, and protein bands above 10 kDa were not observed (Figure 7). However, in the liquid fraction (L), there were still faint protein bands (>10 kDa) in all three samples, which also showed slight immunoreactivity in immunoblotting, especially in silages F5 and V3, confirming the presence of highly resistant allergens.

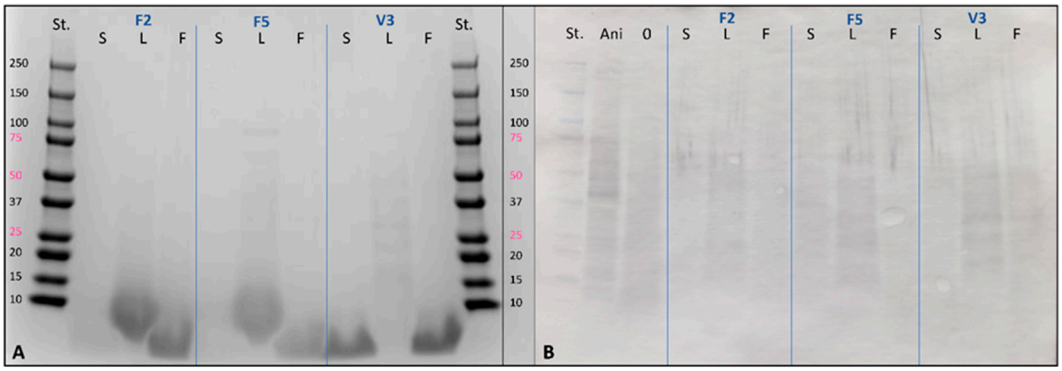


**Figure 7.** Images of electrophoresis (**A**) and immunoblotting (**B**) of the three fractions: S (solid pellet), L (liquid fraction) and F (floating interphase) of silages F2, F5 and V3. In B (immunoblotting), the first three columns from left to right correspond to the molecular weight pattern (St.), an *Anisakis* extract (Ani) and untreated viscera (0).

The results of the SE-HPLC were in accordance with the high degree of hydrolysis observed in V3, F2 and F5 (Figure 8). The average molecular size was smaller in V3 (Table 3). However, in V3, no peptides were observed in the range that could correspond to the bands of higher molecular size and the 20 and 15 kDa bands captured in the EF. This could be due to differences in the pretreatment of the sample and/or to the very low concentration of these fragments in the hydrolysate. As discussed later, in V3, most of the protein is hydrolyzed into small peptides and free amino acids.

**Table 3.** Distribution of the molecular size profiles as a percentage of the defined range for each hydrolysate obtained in the long-term silage experiments.

| | F2 | F5 | V3 |
|---|---|---|---|
| >10 kDa | 0.27 | 0.74 | 0.00 |
| 10 > 6 kDa | 2.10 | 2.13 | 0.00 |
| 6 > 3 kDa | 13.44 | 14.18 | 0.00 |
| 3 > 1 kDa | 52.19 | 52.88 | 35.76 |
| 1 > 0.3 kDa | 25.34 | 23.45 | 38.07 |
| <0.3 kDa | 6.66 | 6.72 | 26.17 |
| Average mw (kDa) | 1.98 | 2.02 | 0.96 |

Regarding the nutritional quality of silage for further use, prolonged silage could lead to partial deamination and loss of protein and some essential amino acids (EAA); however, other authors argue that maintaining silage at lower pH can minimize these losses even over very long storage periods [40]. Previous studies reported protein losses of 17.91% after 91 days of storage at pH 4–4.5 and a reduction in EAA mainly during the first month of silage [41]. In this study, the average protein content (total N2 × 6.25) in the liquid fraction of the hydrolysates on day 9 of silage was 45.73 ± 2.48% (dry matter basis, n = 4), 73.38 ± 0.38% after 12 months and 54.83 ± 1.23% after 15 months of storage. The amino acid profile was not determined at the beginning of the experiment, but the three silage samples after 12 and 15 months contained a similar pattern of amino acids (Table 3). However, there was a big difference in total amino acid (AA) and free amino acid (FAA) contents between the two groups. Similarly, the ratios of essential (EAA) to nonessential (NEAA) amino acids were 0.9 and 1.0; this is similar to what was observed in the aforementioned studies by Bhaskar et al. [36] and Opheim et al. [42], respectively, for the autolysis of salmon viscera at 52 °C and 120 min, which may indicate that no significant losses of amino acids were produced despite the long duration of silage.

The FAA/AA (total amino acid) ratio in the V3 hydrolysate was quite high (0.85) (Table 4). This ratio may indicate the degree of hydrolysis achieved in the V3 sample. The ratios in F2 and F5 were only 0.25 and 0.28, respectively. In previous studies, other authors reported a ratio of 0.49 for the autolysis of salmon viscera [42] and 52–62% after 91 days of silage, as measured by the OPA method [41]. The latter gives slightly higher results, as it measures the total number of peptide bonds cleaved (FAA plus peptides). The difference between the V3 and F batches cannot be explained by the difference in pH conditions, as acid proteases in fish viscera are reported to work optimally at pH 2.0–4.0 [34]. However, when formic acid is used, it is not necessary to work at such an acidic pH to preserve silage from microbial growth as when mineral acids are used. As no differences in the amino acid profiles between the V3 and F samples were observed, the difference in the degree of hydrolysis could be due to differences in the composition of the initial sample or differences in the feeding activity of the fishes, which could also result in differences in enzymatic activity.

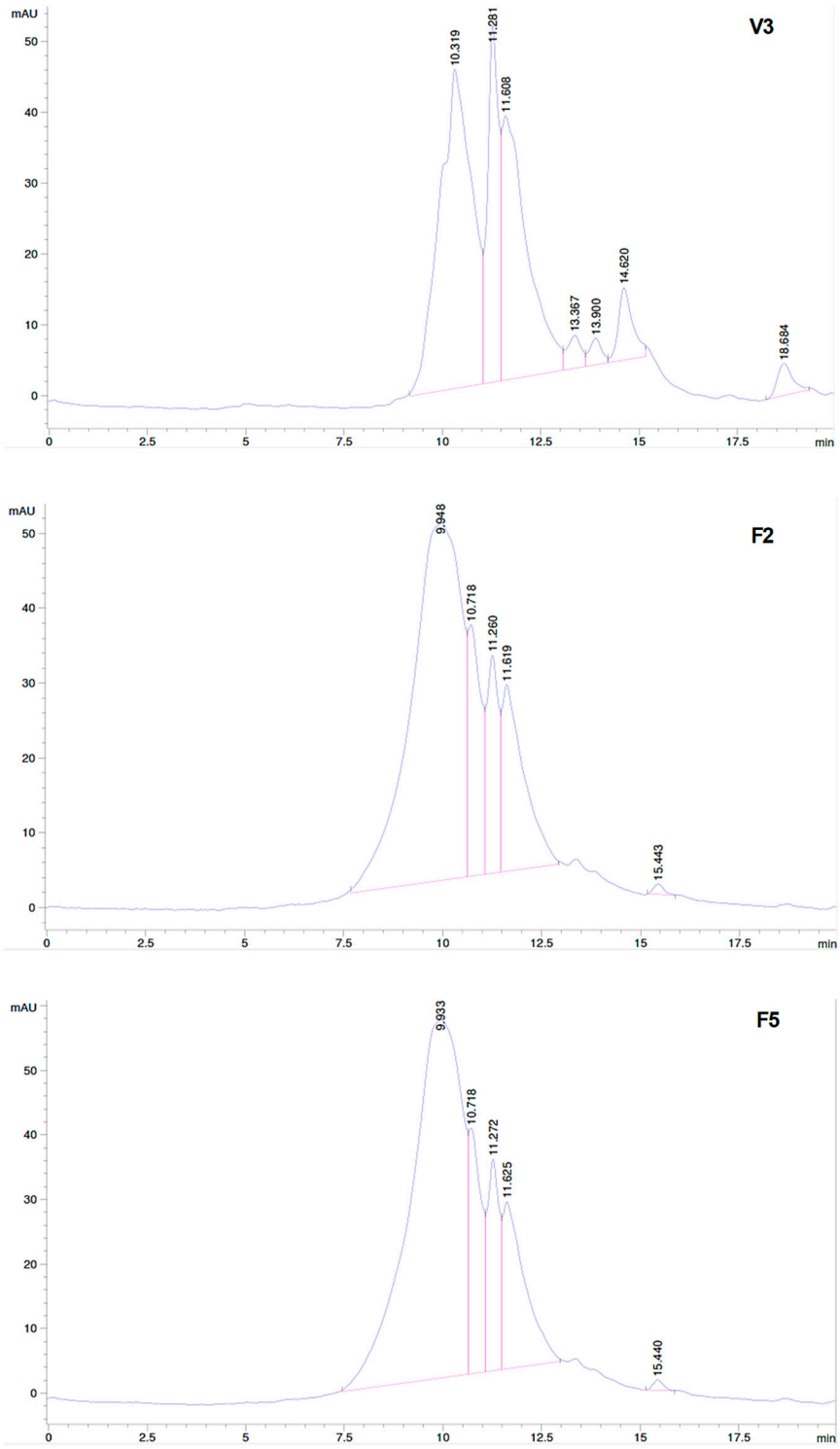


**Figure 8.** SE-HPLC profile of the liquid fraction of silage V3 (12 months of storage), F2 and F5 (15 months of storage). The standard molecular sizes corresponding to the retention times were as follows: 45 kDa, 6.297 min; 17 kDa, 7.103 min; 6.7 kDa, 8.223 min; 1.0 kDa, 10.860 min; 0.379 kDa, 12.880 min. The points used to define the selected intervals were 10 kDa, 7.968 min; 6 kDa, 8.683 min; 3 kDa, 9.652 min; 1 kDa, 11.189 min; and 300 Da, 12.873 min.

**Table 4.** Essential amino acids (EAAs), nonessential amino acids (NEAAs), total standard amino acids (AAs) and free amino acids (FAA) in the liquid fractions of the three silages obtained after 12 months of storage (V3) and 15 months of storage (F2 and F5). Single amino acids are expressed in g/100 g AA.

| | V3 | F2 | F5 | Reference * |
|---|---|---|---|---|
| *Essential amino acids* | | | | |
| Histidine | 3.3 | 3.3 | 3.2 | 2.1 |
| Threonine | 5.2 | 5.2 | 5.1 | 3.9 |
| Arginine | 6.4 | 6.8 | 6.9 | 1.3 |
| Valine | 6.1 | 5.5 | 5.2 | 3.6 |
| Methionine | 3.2 | 2.9 | 2.9 | 3.1 |
| Phenylalanine | 4.2 | 4.0 | 3.8 | 6.5 |
| Isoleucine | 5.3 | 4.4 | 4.3 | 2.5 |
| Leucine | 8.2 | 7.5 | 7.0 | 3.3 |
| Lysine | 9.2 | 8.3 | 8.0 | 5.7 |
| Tryptophan | 0.25 | <0.010 | <0.010 | 0.8 |
| *Non-essential amino acids* | | | | |
| Aspartic | 9.7 | 9.7 | 9.7 | |
| Glutamic | 14.2 | 13.7 | 13.8 | |
| Serine | 5.0 | 5.4 | 5.3 | |
| Glycine | 6.3 | 5.4 | 7.2 | |
| Alanine | 6.6 | 6.5 | 6.6 | |
| Tyrosine | 1.1 | 3.8 | 3.9 | |
| Hydroxyproline | 0.9 | 1.1 | 1.5 | |
| Proline | 4.9 | 5.3 | 5.4 | |
| EAA (g/100 g silage DM) | 29.35 | 20.83 | 20.44 | |
| NEAA (g/100 g silage DM) | 27.96 | 22.77 | 23.36 | |
| TAA (g/100 g silage DM) | 57.31 | 43.60 | 43.80 | |
| FAA (g/100 g silage DM) | 48.88 | 10.75 | 12.29 | |

* Reference amino acid requirements of juvenile common carp (NRC,1993 [43]).

Compared with the amino acid requirements of juvenile common carp listed by the NRC [43], phenylalanine and tryptophan were present in less than the required proportions, while the other amino acids were present and even exceeded the necessary relative contents. Tryptophan is usually known as the first limiting amino acid in fish silage because it is acid sensitive [44]. Similar results were observed by Bhaskar et al. [36] in an experiment in which fish viscera were hydrolyzed with Alcalase®, except that they reported methionine as well as a limiting amino acid.

There is only one published feeding trial investigating whether the protein fragments of Anisakis ingested by zebra fish can be found in their tissues. After a two-week feeding experiment, Anisakidae peptides were detected in zebrafish tissue [25]. However, this approach was based on the percentage of intact Anisakis larvae included, which was much greater than what could be expected from fishmeal from naturally infested fish. In a survey of farmed salmon and several processed seafood products in the Norwegian market, only trace amounts (<10 mg/kg) were detected, and the authors considered that this contamination does not represent a health risk [24]. Whether or not the resulting resistant fragments detected from silage of infested viscera may be a risk for consumers through their inclusion in fish feed must be investigated by means of an adequate exposure assessment [45].

## 4. Conclusions

In short, hydrolysis triggers the passage of *Anisakis* excretory/secretory and somatic allergens to a more soluble or available state, initially increasing their allergenicity. After long silage periods, the lower molecular weight fragments of *Anisakis* proteins become fully digested. However, some higher-molecular size fragments with potential allergenicity still appeared, but at very low concentrations. The silage duration should be adjusted to the

minimal time necessary to minimize or eliminate allergens from *Anisakis* while avoiding potential loss of sensitive amino acids. Although this product is not intended for human consumption, but rather for animal feed, smaller proteins or fragments can be expected to be the most easily absorbed in the digestive tract. Therefore, it is necessary to determine whether resistant and larger fragments with allergenic potential can pass through the intestinal tract of animals without being absorbed or incorporated into the edible fraction of animals. Thus, further work and additional sensitive proteomic techniques must be performed to identify the risks of potentially remaining IgE reactive proteins in foods obtained from animals fed with silages. This approach ensures that feeding this product to animals does not pose a potential risk to *Anisakis* allergy sufferers.

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**Institutional Review Board Statement:** This study was conducted according to the guidelines of the Declaration of Helsinki. The collection of sera was approved by the Ethics Committee for Clinical Research (CEIC) of the Araba Integrated Health Organization (Vitoria, Spain), with internal Ref. PI2018118 (date of approval: 10 August 2018).

**Informed Consent Statement:** All participants signed the corresponding informed consent. The anonymity of the participants was guaranteed throughout the study.

**Data Availability Statement:** The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

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