# Inactivation of *Anisakis simplex* in hake viscera by acid autolysis.

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## Introduction

Infestation with the nematode *Anisakis simplex* of fish species of high commercial interest (hake, monkfish, mackerel, anchovy, sardine and blue whiting) has become in recent years a problem for many fisheries due the high infestation levels observed with the economic loses it causes in the first sale and retail services and the risk for the public health. The evisceration of some species on board, as is mostly the case for hake, together with the maintenance of the cold chain, are key practices to extend the shelf life of fishery products. The space limitations on board and the lack of economic incentives for the fishermen finishes with the viscera being thrown to the sea. Meanwhile, the presence of high infestation levels with *Anisakis s.* in the viscera puts into question this practice due the unknown impact it may cause in the trophic chain but also the possibility of recovering the viscera with other fish processing by-products as raw material for feed. Previous studies have shown that *Anisakis* allergenic peptides can withstand the aquaculture feed processing and be transferred to the fish tissue, with the risk of causing symptoms in sensitized consumers (Fæste et al, 2015).

The objective of this work was to study the acid autolysis, known as silage, of hake viscera as an alternative for the use and/or exploitation of discarded viscera to obtain new products. Acid autolysis has been considered as the most appropriate method to implement both on board and ashore due to its simplicity and relatively low cost of investment (Toppe et al. 2018). At the same time, it allows to obtain interesting by-products such as peptides, amino acids and oils that can be valued by specialized companies. With this aim, several silage trials were done with hake viscera with parasites. The effect of silage and pre-treatment conditions (grinding) on *anisakis* mortality was observed. Also, the allergenicity of the silage products was monitored all along the autolytic process and the remaining allergens were characterized through electrophoresis and immunoblotting.

## Materials and methods

Each time, 1 kg viscera were gently grinded, and pH adjusted with formic acid up to 2.5. to 3.6 to perform silage for 11 days in closed glass vessels at an average temperature of 18 ºC. The degree of liquefaction as the percentage of the total mass not retained through a 1 mm screen was measured daily. Additionally, the protein content of the solid and liquid fraction obtained during the silage period was monitored daily up to 264 h (Kjeldahl total nitrogen AOAC method 955.04). The mortality of anisakis was verified visually immediately after treatment and at 24 h, 48 h, 78 h in fresh samples and up to 96 h in silage in 100 mL aliquots.

To measure the allergenicity in all silage fractions and its evolution along the silage period, Enzyme Linked Immunosorbent Assay technique (ELISA) was performed using a pool of sera from 9 patients with diagnosed allergy to anisakis (without previous anisakiasis) from the Allergy Department of the University Hospital of Araba (Gasteiz, Spain). SDS-Electrophoresis (SDS-PAGE) was used to investigate the presence of proteins with a molecular size similar to known *Anisakis* allergens and to monitor their presence all along the silage trial. Immunoblotting (IB) was used to detect immunoreactive proteins and /or their fragments within those identified in the EF.

## Results and discussion

In the initial sample in untreated viscera, 5% of dead anisakis were found, a value that increases to 100 % after 96 hours. However, the results of the silage samples showed that after 24 hours mortality was 100 % in all cases. Liquefaction achieved a maximum steady state after 200 h with a 90 %.

The evolution of protein recognition by IgE of patients' sera varies depending on the fraction analyzed (solid pellet, floating pellet and aqueous fraction). Results suggest that in the first days, IgE of the patients would recognize the somatic antigens of the larvae, while, in successive days, during the decomposition of the same due to hydrolysis, the secretory-excretory antigens (ESA) would become available, causing the increase in recognition by the IgE observed in all fractions (Fig. 1). The results of the IB of the SDS_PAGE were in accordance with this hypothesis (results not shown).

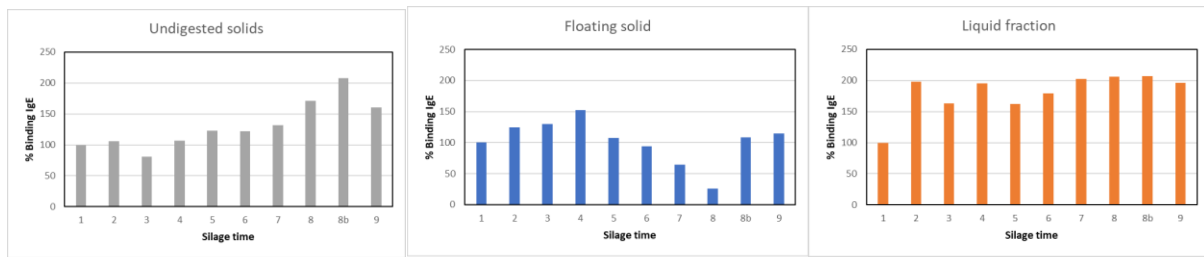


Figure 1. Evolution of the allergenicity of the fractions obtained along the silage period of the hake viscera.

As an additional test to observe the resistance of *Anisakis* allergenic proteins to the action of digestive enzymes present in the silage process, three of the experimental silage samples, namely F2, F5 and V3, were analyzed after 12 months of silage. After this time up to 82% of the total protein became part of the liquid fraction, 6% remained in the undigested pellet and 12-13 % in the floating fraction. In the SDS-PAGE profile it is observed that the solid fractions (P) and floating phase (M) are fully hydrolyzed and do not present protein bands (Fig. 2). In the liquid fraction (L) however there are still faint bands in all three samples. F5 and V3 showed residual IgE reactivity in a band around 15 kDa in the case of F5 and 20 kDa in the case of V3. No bands were observed below 15 kDa, due to the degree of hydrolysis achieved. The DNA analyses detected the presence of anisakis only in the solid pellet samples at any silage time, from 24 h to 264 h, while those of soluble protein did not present traces of the parasite.

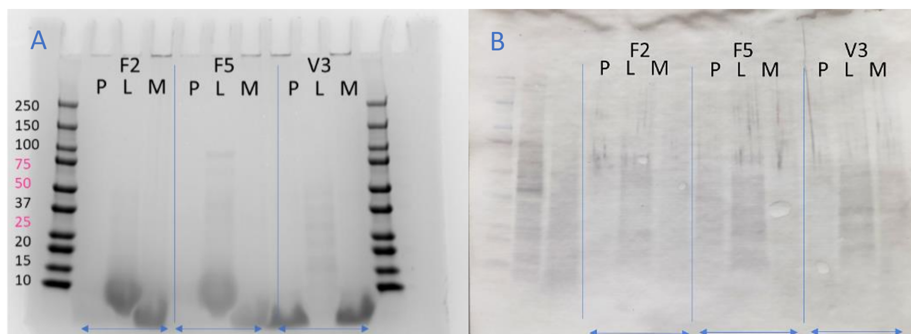


Figure 2. Image of electrophoresis (A) and immunoblotting (B) of the three fractions: P (pellet), L (liquid), M (floating fraction) of silages F2, F5 and V3 after 12 months of silage. In B the first three columns from left to right correspond to the molecular weight pattern, an anisakis extract and untreated viscera.

## Conclusions

In short time silage processes, 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. Since smaller proteins or fragments can be expected to be the most easily absorbed in the digestive tract, it would be necessary to check whether resistant and larger fragments with allergenic potential can pass through the intestinal tract without being absorbed and incorporated into the edible fraction of animals fed with a feed incorporating silage. This would ensure that feeding animals with this product does not pose a risk to allergy sufferers who consume them.

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