

Anisakis survival in refrigerated fish products under CO₂ modified-atmosphere

S. Pascual *, J. Antonio, M.L. Cabo, C. Piñeiro

Instituto de Investigaciones Marinas, CSIC, Eduardo Cabello 6, 36208 Vigo, Spain

ARTICLE INFO

Article history:

Received 28 October 2009

Received in revised form 24 February 2010

Accepted 5 March 2010

Keywords:

Anisakis

CO₂ modified-atmosphere

ABSTRACT

With the aim to assess the survival of *Anisakis* infection in fish stored under CO₂ modified-atmosphere, third-stage larvae (L3) of *Anisakis simplex* were inoculated in samples of skinless fish musculature and then packaged in three different CO₂ modified-atmospheres commonly used to preserved fresh fish products. The microenvironment provided by CO₂ modified-atmosphere packaging (MAP) does not impair the well-being and somatic antigenic properties of *Anisakis* larvae in infected fresh fish. This result indicates that MAP does not protect against anisakiasis risk. Therefore proactive regular inspections in commercial fresh fish should be done by MAP operators in fishery industries to ensure that healthy products (i.e., parasite-free fish products) reach the consumer, thus accomplishing with the co-responsibility criterion established in the EU food hygiene legislation.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Modified atmosphere (MA) packaged foods have become increasingly more available to meet consumer demands for fresh and refrigerated foods with extended shelf-life. MA packaging of fishery products with an enhanced carbon dioxide level has been shown to inhibit the normal spoilage flora by retarding spoilage microbial growth without altering their organoleptic features, thus increasing shelf-life and quality of commercial fish (Cabo, Herrera, Sampedro, & Pastoriza, 2005; Pastoriza, Sampedro, Herrera, & Cabo, 1996a, 1996b; Sivertsvik, Jeksrud, & Rosnes, 2002). However, the possibility that parasites with anthroponozoonotic potential and associated gastroallergic clinical symptoms could survive in fish or fish-products packaged in low-oxygen atmospheres at refrigerated temperatures has not been checked till now. The aim of this work is to evaluate the effects of the CO₂ modified-atmosphere packaging on the survival and development of fish-borne Anisakids. The cosmopolitan parasite *Anisakis simplex* s.l. was chosen because of its highly prevalent infection in exploited fish stocks, and therefore in visceral mass, guts and hipoaxial musculature of commercial fishes (Abollo, Gestal, & Pascual, 2001).

2. Materials and methods

2.1. Experimental design and packaging conditions

For fish inoculation third larval stage (L3) type I (sensu Berland, 1961) of *Anisakis* were collected alive from the abdominal cavity

(mostly on the liver) of the blue whiting, *Micromesistius poutassou*, caught in Galician waters by local fisheries. Worms over 2.0 cm in length were washed twice in phosphate buffer saline (PBS) and stored in this solution at 4 °C. A total of six worms were inoculated by sterile handle after making an incision in each sample consisting on 32 muscular pieces of about 25 g (3 × 3 × 3 cm) of fresh European hake *Merluccius merluccius* packaged in 5% O₂ and ascending CO₂ concentrations (20%, MA1; 55%, MA2; 90%, MA3). Nitrogen was used as a filler gas. In order to simulate fish-processing industrial conditions, fish samples were packaged into multi-layer coextruded polyethylene barrier bags (Soplaril, SA, Barcelona). Once ready, the gas mixture was injected into the bags, which were immediately heat sealed with a modified-atmosphere packaging machine (Tecnotrip, SA, Barcelona). Samples were stored at 3 ± 1 °C and removed for analysis after 3 (sampling 1), 7 (sampling 2), 10 (sampling 3) and 15 (sampling 4) days of storage. Two bags of each treatment were then evaluated for parasites and microbiological analyses.

3. Parasites analyses

Each sample was visually inspected on a candling table under stereomicroscope. Displacement from the inoculation point (i.e., parasite motility) was noted. Then, all parasites were collected for further assays. One larval of *A. simplex* from each sample were homogenised in 1 ml PBS and the crude extract charged in a Bio-Merieux ApiZym25 biochemical test strip system in order to stand enzymatic activity. Another three *Anisakis* larvae from each sample were pelleted and homogenized at 4 °C in PBS using a glass potter. Parasite homogenates were centrifuged at 100,000g for 30 min also at 4 °C, and the supernatants removed and considered as crude

* Corresponding author. Tel.: +34 986 231930x181; fax: +34 986 292762.
E-mail address: spascual@iim.csic.es (S. Pascual).

extracts (CEs). Then, CEs were analysed in vertical SDS–PAGE discontinuous gels (10% acrylamide in the separating gel). Electrophoretic separations were carried out at 40 mA/slabT, 100 V and 150 W, using Tris–Tricine buffer (Schägger & von Jagow, 1987) in a Mini Protean® System (BioRad Laboratories, Hercules, USA). Broad range protein molecular weight markers (6–220 kDa) were employed as reference. The gels were stained with a Silver Staining kit (BioRad Laboratories, Hercules, USA) following the manufacturer's instructions.

3.1. Microbiological analyses

Microbial growth was monitored as a quality and freshness index (Wang & Brown, 1983) according to food legislation. Consequently, total viable aerobic bacteria count (TVC) was determined according with the Standard Microbiological Methods (Downes, 2001). Briefly, 25 g of fish were blended with 100 g of peptone water (Panreac, SA) in a Lab-blender-400 Stomacher, followed by serial dilutions and spreading in Plate Count Agar (Panreac, SA). Visible colonies were counted after 48 h of incubation at 31 °C by using an automatic colony counter based in image analysis (Flash and Go, IUL Instruments, S.L.).

4. Results

In all fish samples submitted to MAP *Anisakis* larvae were all found alive (motility), coiled in a flat spiral, actively moving or cleaved to the muscular tissue (Table 1). Otherwise, all nematodes showed a healthy aspect (shining and transparent cuticle) and they were found distant from the inoculation point, especially those maintained in 90% CO₂ were even found moving in the plastic bag away from the piece of fish muscle. Parasite behaviour was markedly different through time regarding the gas mixture. *Anisakis* maintained at 20% tend to a quiescence or diapause behaviour (mostly coiled, inside or outside the piece of fish musculature) at 15th day, whereas parasites at 55–90% CO₂ maintains active (stretched, mostly located at the outer part of the fish musculature).

The screening of enzymatic activities of *Anisakis* larvae under CO₂-atmosphere showed changes for all gas-mixture and the control sample (in air atmosphere) related to time after packaging (Table 2). From the 7th day, all *Anisakis* larvae (either those from the different gas-mixtures or from the control) become from a phosphoric and ester hydrolase pattern to a more reactive enzymatic pattern including peptide hydrolases and glycosidases. Regarding

Table 2

Screening of enzymatic activities for *Anisakis* larvae collected from different CO₂-atmosphere packaging trough time. Note that at each sampling the results are the same for *Anisakis* larvae maintained at the three-different atmosphere conditions as well as the control group.

Enzyme	Sampling			
	1	2	3	4
Phosphatase alkaline		+	+	+
Esterase	+	+	+	+
Esterase lipase	+	+	+	+
Lipase	–	–	–	–
Leucine arylamidase	–	+	+	+
Valine arylamidase	–	–	–	–
Cystine arylamidase	–	–	–	–
Trypsin	–	–	–	–
Chymotrypsin	–	–	–	–
Phosphatase acid	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+	+
α-Galactosidase	–	–	–	–
β-Galactosidase	–	–	–	–
β-Glucuronidase	–	–	+	+
α-Glucosidase	–	–	–	–
β-Glucosidase	–	–	–	–
N-acetyl-β-glucosaminidase	–	–	–	–
α-Mannosidase	–	–	–	–
α-Fucosidase (only in MA3)	–	+	+	+

the latter, α-fucosidase was only present in 90% CO₂ modified atmosphere.

The electrophoretic protein patterns of PBS crude extracts obtained from *Anisakis* L3 stored under different CO₂ gas mixtures and those from control samples did not show any differences (Fig. 1). It is likely that *Anisakis* antigens may not be affected by MAP treatments, although this statement requires an immunological extra-work.

As it was expected, air-stored fish showed TVC values higher than MAP-stored fish, especially in 90% CO₂ atmosphere (Fig. 2). Considering 10⁶ bacteria per gram is taken as a limit of acceptability for national consumption of fish (Boletín Oficial del Estado, BOE-O, 2/8/1991), MAP-stored sampled hake would have a shelf-life up to 10 days in the highest CO₂ atmosphere.

Table 1

Visual inspection of MAP *Anisakis* larvae. In/out refers to the inoculation point ("in") or "out" of this point. Data are referring to the number of larvae.

	MA1	MA2	MA3	Control
3rd day				
Site infection (in/out)	3/3	4/2	6/0	5/1
Coiled/stretched	3/3	1/5	2/4	3/3
Motility	6/6	6/6	6/6	6/6
7th day				
Site infection (in/out)	5/1	3/3	3/3	3/3
Coiled/stretched	3/3	2/4	2/4	4/2
Motility	6/6	6/6	6/6	6/6
10th day				
Site infection (in/out)	4/2	3/3	2/4	4/2
Coiled/stretched	3/3	2/4	1/5	4/2
Motility	6/6	6/6	6/6	6/6
15th day				
Site infection (in/out)	6/0	3/3	2/4	5/1
Coiled/stretched	5/1	2/4	3/3	5/1
Motility	6/6	6/6	6/6	6/6

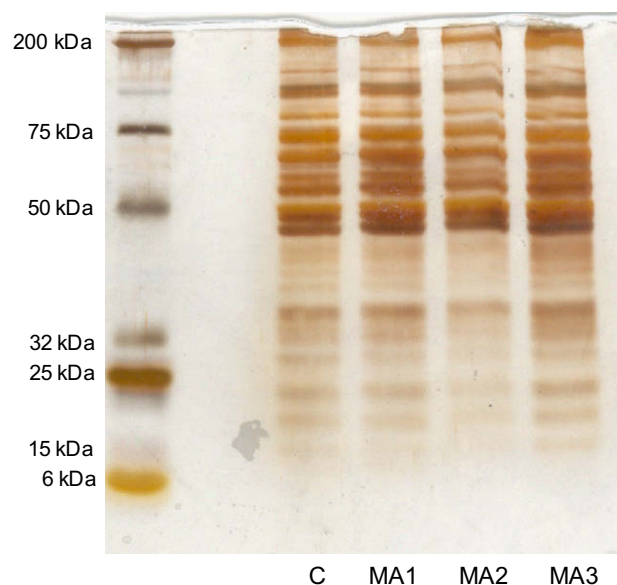


Fig. 1. SDS–PAGE of crude extracts of *Anisakis* larvae collected from control (C), modified-atmosphere 1 (MA1), modified-atmosphere 2 (MA2) and modified-atmosphere 3 (MA3) samples.

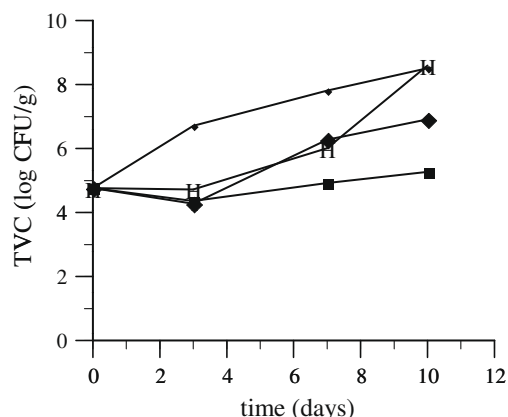


Fig. 2. Total Viable Count (TVC) in *Merluccius merluccius* packaged under different atmospheres (♦ control, □ H MA1, ◆ MA2, ■ MA3) during storage in the refrigerated stage.

5. Discussion

The major finding in the present study is that *Anisakis* larvae can survive after MAP. Especially in gas mixtures (50–90% CO₂) where the MAP has been showed as an effective preservation technique to maintain quality of fresh fish live spontaneously moving larvae were found. After being packaged in this suitable stimuli (CO₂), motile cultured larvae from MA2 and MA3 showed a higher reactive enzymatic pattern, suggesting the change from a “hypometabolic dormancy” (see Petronijevic & Rogers, 1987) of L3 to an active 3rd moulting stage. Furthermore, the above “trade-off” of MAP larvae is congruent with the SDS–PAGE profiles of ECs as they did not change when comparing with those of control larvae. This suggests that crude-extract antigens of *Anisakis* remain stable after MAP.

From the above results it is clear that viable *Anisakis* larvae in fresh fish preserved in MAP reach the consumer who in fact is the only responsible to manage the zoonotic risk and gastroallergic-associated symptoms. Prophylactic measures have been introduced in EC legislation and informative campaigns were carried out at fish markets in order to minimize the risk of zoonotic and allergy symptoms by frozen or heating infected fish. Nevertheless, some highly thermostables *Anisakis* antigens (Caballero & Moneo, 2004) and the persistence and emergent culinary practices of eating raw or undercooked fish compromise effectiveness of prophylaxis measures which become a critical control point.

Otherwise, as it was already previously demonstrated by Pastoriza, Sampedro, Herrera, and Cabo (1998) carbon dioxide atmosphere can extend the shelf-life of packaged fish up to 2 weeks. MAPs have thus improve the shelf-life of refrigerated fish, but unfortunately this technical achievement in the fish-processing industry run counter to the presence of visible active worms in

the commercial display. Parasites can therefore reduce the commercial value of infected MAP-stored fish as a result of the anaesthetic problem perceived by retailers and consumers. The problem could be worse for those fish products coming from heavily infected stocks, namely pelagic species with short shelf-life (Abollo et al., 2001).

To solve this gap specific proactive inspection should be done by fishery operators to eliminate highly *Anisakis*-contaminated MAP fishes. This appointment needs a low cost, easy and reliable validated method for fish inspection of edible parts which could be incorporated into an HACCP program. In this context, a detection limit for an allowable number of larvae and/or amount of DNA-antigen traces in fresh fish musculature should be provided by sanitary authorities to makes work much easier during inspections, for implementing adequate correction measures and enhancing consumer confidence.

Further experimental work on the role of different MAP conditions on *Anisakis* ES (excretory–secretory) products remaining in fish musculature are required to increase our understanding of the physiological trade-off of parasites infecting MAP fishes and the sanitary implications of infected commercial fishes.

References

- Abollo, E., Gestal, C., & Pascual, S. (2001). *Anisakis* infestation in marine fish and cephalopods from Galician waters: An updated review. *Parasitology Research*, 87(6), 492–499.
- Berland, B. (1961). Nematodos from some Norwegian marine fishes. *Sarsia*, 2, 1–50.
- Caballero, M. L., & Moneo, I. (2004). Several allergens from *Anisakis simplex* are highly resistant to heat and pepsin treatments. *Parasitology Research*, 93, 248–251.
- Cabo, M. L., Herrera, J. R., Sampedro, G., & Pastoriza, L. (2005). Application of nisin, CO₂ and permeabilizing agents in the preservation of refrigerated blue whiting (*Micromesistius potassou*). *Journal of the Science of Food and Agriculture*, 85, 1733–1740.
- Downes, P. F. (2001). *Compendium of methods for the microbiological examination of foods*. Washington: American Public Health Association.
- Pastoriza, L., Sampedro, G., Herrera, J. R., & Cabo, M. L. (1996a). Effect of carbon dioxide atmosphere on microbial growth and quality of salmon slices. *Journal of the Science of Food and Agriculture*, 72, 348–352.
- Pastoriza, L., Sampedro, G., Herrera, J. R., & Cabo, M. L. (1996b). Effect of modified atmosphere packaging on shelf-life of iced fresh hake slices. *Journal of the Science of Food and Agriculture*, 71, 541–547.
- Pastoriza, L., Sampedro, G., Herrera, J. R., & Cabo, M. L. (1998). Influence of sodium chloride and modified atmosphere packaging on microbiological, chemical and sensorial properties in ice storage of slices of hake (*Merluccius merluccius*). *Food Chemistry*, 61(1/2), 23–28.
- Petronijevic, T., & Rogers, W. P. (1987). Undissociated bases as the stimulus for development of the early parasitic stages of nematodes. *International Journal for Parasitology*, 17, 911–915.
- Schäfer, H. D., & von Jagow, G. (1987). Tricine–sodium dodecyl sulphate–polyacrylamide gel electrophoresis for the separation of proteins in the range of 1–100 kDa. *Analytical Chemistry*, 166, 368–379.
- Sivertsvik, M., Jeksrud, W. K., & Rosnes, T. (2002). A review of modified atmosphere packaging of fish and fishery products–significance of microbial growth, activities and safety. *International Journal of Food Science & Technology*, 37, 107–127.
- Wang, M. Y., & Brown, W. O. (1983). Effect of elevated CO₂ atmosphere on storage of freshwater crayfish (*Pacifastacus leniusculus*). *Journal of Food Science*, 48, 158–159.