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Fish feed as source of potentially allergenic peptides from the fish parasite *Anisakis simplex* (s.l.)

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ABSTRACT

The carry-over of certain feed components into animal products can be of concern for human health. The safety assessment of chemical contaminants including natural toxins, agrochemicals, veterinary drugs, and environmental pollutants is a key element of the "farm-to-fork" ("One Health") approach. The transmissibility of proteinaceous feed constituents such as enzymes, proteins from genetically engineered crops, and infectious prions in animal meal has also become of interest; but the transfer of proteins with allergic potential is little studied. In the present study, an exploratory zebrafish feeding trial using feed containing 20 percent of processed larvae of the marine fish parasite *Anisakis simplex* was performed as a proof-of-principle experiment. After a 2-week exposure period, anisakid peptides were detected in zebrafish tissue by high-resolution liquid-chromatography Orbitrap mass spectrometry and immunostaining using specific polyclonal antibodies or sera from patients with confirmed allergy to *A. simplex*. Since fishmeal produced from marine pelagic fish is an important feed component in the culture of Atlantic salmon and in the poultry industry, it should be considered as a source of potentially allergenic peptides in the final products. Furthermore, the substitution of fishmeal with plant proteins would not eliminate the potential health risk by allergen carry-over since crops of high nutritional value such as legumes also contain important food allergens. If our preliminary results from the present zebrafish feeding trial should be confirmed in necessary follow-up experiments, the question of labeling information on fish and animal food products raised on feed containing potentially allergenic ingredients could arise in order to minimize the exposure risk of allergic consumers.

1. Introduction

The larvae of the marine fish parasite *Anisakis simplex*, commonly occurring in popular food fish such as mackerel, herring, wild salmon, and cod, may adversely affect consumer health through direct infection (anisakiasis) and/or by eliciting allergic

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reactions including urticaria, angioderma, anaphylaxis, and asthma (Deardorff et al., 1991; Pravettoni et al., 2012). Anisakiasis always assumes consumption of raw or undercooked, previously unfrozen seafood (Sakanari and McKerrow, 1989; Daschner et al., 2002; Abe and Teramoto, 2014). However, allergic reactions to *A. simplex* proteins can also be elicited in sensitized persons by the accidental consumption of dead larvae or molecular traces thereof in strongly processed fishery products and fish containing anisakid proteins (Audicana et al., 1995, 2002; Daschner et al., 2000, 2002; Nieuwenhuizen et al., 2006). Additionally, cases of *A. simplex* allergy due to occupational exposure by fish-based feed have been reported (Mazzucco et al., 2012).

Several food allergens have been found to be heat-stable and relatively trypsin/pepsin tolerant. At least one of the major allergens of *A. simplex* appears to be highly resistant to freezing, heating, and digestion (Caballero and Moneo, 2004; Moneo et al., 2005; Rodríguez-Mahillo et al., 2010; Vidaček et al., 2009, 2011). Evidentially, allergenic peptides containing intact IgE-binding epitopes resistant to gastrointestinal hydrolysis, cytosolic and systemic peptidases can also be transported by carriers across the enterocytes into the blood circulation (Webb et al., 1992; Seal and Parker, 1992). Thus, a small portion of dietary proteins can cross the epithelium barrier (Kaminogawa et al., 1999) and unfold their biological activities, e.g. the stimulation of allergen specific effector cells. It has also been reported that allergic patients have increased antigen permeability of the gut mucosa (Majamaa and Isolauri, 1996). After systemic uptake, allergenic peptides can even cross the mammalian placenta or be transported into breast milk (Frank et al., 1999; Vadas et al., 2001).

The transmissibility of peptides and small extremely resistant proteinaceous infectious particles (prions) from feed or food to various tissues of the final host organisms in a still bioactive stage can sometimes have devastating effects, e.g. in bovine spongiform encephalopathy (BSE) (Colchester and Colchester, 2005). Several animal models have been established to assess prion transmissibility and convertibility and zebrafish are frequently used as a model for prion pathobiology (Málaga-Trillo et al., 2011). There is also evidence that allergenic peptides can carry-over from animal feed into food products causing symptoms in sensitized consumers (Armentia et al., 2006). Comparably, fragments of plant DNA have been detected in pig and poultry organs and meat (Klotz et al., 2002; Chesson and Flachowsky, 2003).

Increasing attention has been paid to feed quality in food production. The safety assessment of feed components is a key element of the "farm-to-fork" ("One Health") approach (Mantovani et al., 2009). Commonly, this evaluation considers chemical residues in feed including natural toxins, agrochemicals, veterinary drugs, and environmental contaminants. However, the experiences with the BSE epidemic, the addition of enzymes to animal feed (Pariza and Cook, 2010), and the introduction of genetically engineered crops into feed and food (Goodman et al., 2005) have led to the inclusion of peptides into the list of transmissible compounds of possible health concern.

In this context, *A. simplex* is an interesting source for the study of peptides with carry-over potential. The detection of *A. simplex* peptides in the sera of chickens that had been fed with fishmeal-containing feed indicates considerable peptide transmissibility (Armentia et al., 2006). Furthermore, eight patients with high sensitization to *A. simplex* experienced allergic symptoms after having consumed raw meat from those chickens suggesting that allergenic *A. simplex* peptides had passed over from the feed and had at least partly retained their biological activity.

In a recent study, the presence of *A. simplex*-related peptides in the belly flap musculature of freshly harvested, net pen-reared Atlantic salmon was demonstrated (Fæste et al., 2014b). Since there was no concurrent infection with *A. simplex* larvae, or any sign of previous infections, the parasite-related peptides may have reached the muscle tissue, or its vascular network, through the fish feed. Generally, farmed fish are fed processed feed only and considered to be free of parasites (EFSA, 2010). However, products of pelagic fish (fishmeal, fish oil, silage) are important components in feed for domestic animals (including farmed fish), and e.g. feeding stuffs for chicken, turkey, or suckling piglets contain up to 4, 6, or 12 percent fishmeal, respectively (data from the Norwegian Food Safety Authority and Norwegian feed manufacturers). Our analysis of commercial feed samples for salmon and poultry farming using a specific ELISA method for the detection of *A. simplex* (Werner et al., 2011) resulted in maximum contents of 40 and 60 mg/kg, respectively (unpublished data).

Based on these findings, we have therefore conducted a pilot feeding trial using laboratory-raised zebrafish (*Danio rerio*) and fish feed containing processed *A. simplex* larvae, in order to investigate if or to what extent, *A. simplex*-related peptides may be transferred from the feed into the zebrafish tissue or its percolating blood.

2. Materials and methods

2.1. Preparation of feed for the zebrafish trial

Four days prior to trial onset, three types of feed were prepared (Table 1), composed of basic commercial zebrafish feed (Aqua Schwarz GmbH, Göttingen, Germany) and 12 percent gelatin, and in addition either freeze-dried *A. simplex* larvae (F1), fish meal (F2) that had been exclusively produced from Atlantic herring (*Clupea harengus*) for research purposes (NOFIMA AS, Bergen, Norway), or without further supplements (F3).

The *A. simplex* larvae used in the trial feed preparation (F1) were collected fresh from the visceral organs of Blue Whiting (*Micromesistius poutassou*) caught 8 months pre-trial in northeastern Atlantic waters (N58°16′W09°36′). After removing the host-induced capsule, each larva was morphologically identified to genus-level (*Anisakis* ssp.) based on *in-situ* appearance (coil-shaped), and the presence of both a caudal mucron and an esophageal ventricle without caeci. After repeated washing in physiological saltwater (0.9 percent), the larvae were deep-frozen (−20 °C) in bulk before further use. Subsamples of