MANUSCRIPT

Title: Transmissibility of Anisakid allergenic peptides from animal feed to chicken meat: proof of concept

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Declarations of interest: none

Abstract

Background and aim:

The nematodes *Anisakis simplex* and *Pseudoterranova decipiens*, are zoonotic parasites infecting many marine fish and pose a substantial human health risk. Besides being causative agents for gastrointestinal disease after ingestion of a live larva, and an allergic reaction after consuming/handling infected fish, there is proof-of-principle for hidden allergic concerns. Several anisakid allergens are highly resistant, and in this way may be transmitted to meat by use of fishmeal as a feed component for livestock. To consolidate this hypothesis of transmissibility, a controlled chicken feeding trial using Anisakidae-contaminated feed was conducted.

Methods:

Anisakid larvae were collected from codfish and freeze-dried according to fishmeal manufacturing conditions. This larvaemeal was then administered to chickens, and after three weeks of exposure, blood and muscle samples were subjected to a targeted proteomic analysis aiming at detecting anisakid peptides.

Results and discussion:

Results demonstrated that peptides from at least six *A. simplex* allergens were transferred from the feed to the chicken meat and blood. If future experiments confirm a remaining allergenic potency of these peptides in humans, it would significantly change the importance of these zoonotic nematodes from originally a purely fishborne food risk to potentially a wider risk from several food sources.

Keywords: Anisakidae, allergens, fishmeal, chicken meat, peptide transmissibility

[1] **Abbreviations:** WHO/IUIS: World Health Organization and International Union of Immunological Sciences; LC-MS/MS: liquid chromatography tandem mass spectrometry; MRM: multiple reaction monitoring; PC: positive

control; NC: negative control; PBS: phosphate-buffered saline; MTPEK: Minute™ Total Protein Extraction Kit for Muscles; TPER; Tissue-Protein Extraction Reagent

1. Introduction

Worldwide, the most important fish parasites implicated in human disease are several nematodes from the family Anisakidae. These are globally present in a variety of commonly exploited marine fish species, with the main pathogenic anisakid species being *Anisakis simplex* sensu stricto, *A. pegreffii*, *Pseudoterranova decipiens, P. azarazi, P. cattani, P. krabbei,* and *Contracaecum osculatum* (Aibinu et al., 2019). After consumption of raw/ undercooked fish or seafood products containing a viable third-stage larva (L3), humans may acquire an acute, mostly transient, gastro-intestinal infection known as anisakidosis (Audicana & Kennedy, 2008; Hochberg & Hamer, 2010). In addition to abdominal symptoms, a series of allergens present in *A. simplex* and *A. pegreffii* may also compromise human health with symptoms ranging from urticaria, angioedema, asthma, conjunctivitis, to even a potential lethal anaphylactic shock (Nieuwenhuizen, 2016). Cases of *Anisakis* allergy are increasingly being reported worldwide, with up to 22% of the population in some countries being sensitized (i.e., *Anisakis*-specific IgE seropositivity) (Rahmati et al., 2020). Also, it was stated that the most frequent cause of an anaphylactic episode due to a hidden allergen, is fish or shellfish infected with *A. simplex* (Anibarro et al., 2007).

So far, 23 major and minor *A. simplex* allergens (Ani s) have been described, from which 15 are officially recognized by the World Health Organization and International Union of Immunological Sciences (WHO/IUIS) (Fæste et al., 2014a). Several new probable allergens have also been reported (Baird et al., 2016; Fæste et al., 2014a; Kochanowski et al., 2020a, Llorens et al., 2018). Some of these anisakid allergens are excretory-secretory products expulsed by the larval body during its migration through the fish flesh. As such, patients may be exposed to them when consuming fish, even though the larvae might have been removed during the quality control of the fish, and thus not only through the direct ingestion of viable/dead larvae (Ivanović et al., 2017). Since at least eight allergens of *A. simplex* (i.e., Ani s 1, Ani s 4, Ani s 5, Ani s 7, Ani s 8, Ani s 9, Ani s 11-like, Ani s 13) also retain their allergenic potential after freezing, heating and/or digestion, an allergic reaction can be elicited in sensitized persons by the accidental consumption of dead larvae in frozen/cooked

fish or traces present in highly processed fishery products (Mehrdana & Buchmann, 2017; Rahmati et al., 2020). Furthermore, there is increasing evidence that anisakid allergens may be transmitted further down the food chain through the use of fishmeal as a feed component in livestock (Faeste et al., 2015; Polimeno et al., 2021). This potentially exposes consumers to anisakid allergens not only in fish, but also in meat (Armentia et al., 2006).

In the 'farm to fork' food safety management, increasing attention is being paid to safety assessment of feed components in food production (Ling & Wahab, 2020). Fishmeal, a major feed component with a global production of more than five million ton annually, is included mostly in compound foods for the aquaculture, pig, and poultry sector. Currently, almost 70 percent of the annual fishmeal production goes to the aquaculture sector where it serves as the main feed component. The remaining goes mainly to the pig and poultry sector in non-Europe countries (e.g., Brazil and Thailand), from which annually 743.000 MT chicken meat is being imported to Europe (EU, 2019). The pig and poultry sector in Europe on the other hand, are more reliant on fishmeal as a rather small supplement in plant-based feeds (5-10 %) (EC, 2021; FAO, 2020). Fishmeal is an easily digestible, protein-rich, flour-type material produced by cooking (95°-100°C), press drying and shredding of by-catch and offal left from fish processing (FAO, 2020). In this context, the carry-over potential of *A. simplex* proteins has been investigated preliminary, as several allergens of the nematode are highly resistant (Rodriguez-Mahillo et al., 2010; Vidacek et al., 2009). Indeed, in 2006, proof-of-principle was provided that *A. simplex* sensitized patients can react to the presence of allergens in meat derived from chickens that had been fed with fishmeal, a common practice in many parts of the world (Armentia et al., 2006; McVey et al., 2002). Furthermore, the transmissibility of *A. simplex* peptides by feeding fishmeal to zebrafish was demonstrated by Faeste et al. (2015), whereas Polimeno et al. (2021) reported an evident presence of *A. simplex* allergens in aquacultured fish fed with commercial fishmeal while no anisakid nematode was detected in the fish. These studies suggest that low levels of allergenic epitopes may be transferred from animal feed into food, including chicken, pork, and fish from aquaculture. The focus, however, was only on *A. simplex* allergens while the

phylogenetically closely related *P. decipiens* shares similar antigens and also has an allergenic activity in mice (Kochanowski et al., 2020a; Ludovisi et al., 2017; Saelens et al., 2022). Additionally, *P. decipiens*, like *A. simplex*, is highly present in offal from whitefish and a range of pelagic fish species which are typically converted into fishmeal (FAO, 2020; Mercken et al., 2020). Therefore, a systematic study involving a controlled feeding in a relevant food species, that focuses on feed with both *A. simplex* and *P. decipiens* antigens, is required to consolidate these findings. Consequently, the objective of the present study is to investigate the transmissibility of anisakid allergens through a chicken feeding trial with an Anisakidae contaminated feed. A multiple reaction monitoring (MRM)-based targeted proteomics assay was applied for the detection of anisakid allergens and three distinctive protein extraction systems were used to explore its effect on both the anisakid allergenic recovery as well as protein yield.

2. Material and methods

2.1 Collection of larvae and preparation of the larvae meal for the chicken feeding trial

Feed for the chickens was prepared using freeze-dried *A. simplex* and *P. decipiens* larvae (L3) processed in the laboratory of foodborne parasites. The larvae were collected weekly from highly infected codfish obtained from a major fish distributor in Belgium. Specifically, whole fish were digested for anisakid detection and collection with an acidified pepsin solution developed by CODEX (244-2004, 2004), after which species identification occurred based on microscopic *in-situ* appearance according to Petter and Maillard (1998). The larvae were stored at -80°C until a sufficient number was obtained to prepare the larvae meal. Two days prior to onset of the chicken feeding trial, and according to the manufacturing processes of commercial fishmeal, the collected L3 were covered with tap water, heated to 100°C, freeze-dried, and fine-grinded using an A11 basic analytical mill (IKA-Werke, Germany) (Fig. 1). Larvae meal portions per chicken per day were weighed, transferred into Eppendorf tubes, and stored at 5°C until use (Table 1). Additionally, an excess of 2 g larvae meal was prepared and analysed for the presence of *A. simplex* allergens using a previously developed

liquid chromatography tandem mass spectrometry (LC-MS/MS) method by targeted MRM (see section 2.4) (Saelens et al., 2022).

2.2 Set-up of the chicken feeding trial

For the chicken feeding trial, ten female Brown Leghorn chickens were purchased from a commercial poultry store at the age of one day. The sample size was computed by comparison of two proportions (1-sided) and an ethical clearance (EC 2020-090) was obtained from the Ethical Committee from the Faculty of Veterinary Medicine (Ghent University) in accordance with the European legislation for ethics in animal research. The chicks were randomly allocated into two treatment groups (i.e., positive and negative control (PC and NC) group) of five chickens and given *ad libitum* access to water and a well-balanced soybean-based commercial diet (Farm 1 Mash) (Hobby First, Schoten, Belgium) without fishmeal (and hence negative for *A. simplex* and *P. decipiens* allergens) during the entire trial (Fig. 2). Housing consisted of a total confinement cage (5 animals per 3 m^2) with 14-16h of light under controlled temperatures and humidity, and each cage was equipped with nesting material, perches, and absorptive litter material. Body weight was measured weekly and a scoring grid with welfare indicators was implemented during the entire experiment. From the age of three weeks, the PC group received the pre-calculated larvae meal portion (Table 1) solubilized in water by oral administration twice per day using a 1.0-cc needless syringe. Meanwhile, both groups kept receiving the commercial soy-based feed *ad libitum* (Fig. 2). After three weeks of larvae meal administration, the 6-week-old chickens were euthanized by injecting an overdose of pentobarbital in the wing vein. This was six hours after they received a last portion of larvae meal. Blood (maximum 5 mL) was collected from the jugular vein instantly after euthanasia, and muscle tissues (i.e., chest and leg muscles) were gathered during dissection of the carcase on ice. Both blood and muscle samples were immediately subjected to protein extraction (see section 2.3).

2.3 Protein extractions from blood, muscle tissue, larvae meal, and commercial soy-based feed

Three distinctive protein extraction systems were applied to the different sample types (Table 2). First, two grams of larvae meal, commercial feed, and chest and muscle tissue from both groups were each homogenized with 10 mL sterile phosphate-buffered saline (PBS) pH 7.4 (Oxoid, Basingstoke, UK) containing protease inhibitor (Sigma-Aldrich, Missouri, USA). After protein extraction overnight at 4°C while shaking, the mixture was centrifuged (5000 *g*, 4°C) for 45 min. The supernatant was subsequently collected and concentrated by centrifugation (4,032 *g*, 4°C) for 120 min using Vivaspin® 15R centrifugal concentrators (molecular weight cut-off 2,000) (Vivaproducts, Littleton, USA). Secondly, for the protein extraction from the blood samples, 5 mL of blood was first left to settle overnight at 4°C. The collected serum was then heated to 95°C for 15 min and left to cool down before the addition of an equal volume of PBS (Oxoid, Basingstoke, UK) containing protease inhibitor (Sigma-Aldrich, Missouri, USA). This mixture was homogenized by stirring, incubated at 4°C for 4 h while shaking, and centrifuged (3220 *g*, 4°C) for one hour. Protein concentration of the supernatant using the Vivaspin® 15R occurred as above. Finally, two additional protein extraction protocols were applied for the protein extraction from the chest and leg muscles from the PC group (Table 2). In the first, two grams of muscle tissue was homogenized with 15 mL Tissue-Protein Extraction Reagent (TPER)™ (Thermofisher, Massachusetts, USA) containing protease inhibitor (Sigma-Aldrich) and centrifuged (5000 *g*, 4°C) for 45 min. Likewise, protein concentration of the supernatant using the Vivaspin® 15R occurred as described above. In the second protocol, the Minute™ Total Protein Extraction Kit for Muscles (Invent Biotechnologies, Plymouth, USA) was applied to 30 mg of muscles and performed according to manufacturer's instructions. All protein extracts prepared above were stored at -80°C until assayed using the LC-MS/MS (see section 2.4). Protein concentrations of all extracts were determined in triplicate using the Pierce Coomassie Bradford Assay kit (ThermoFisher, Massachusetts, USA).

2.4 *In-silico* peptide selection in Skyline and MRM method creation

For the detection of *A. simplex* allergens during liquid chromatography-mass spectrometric MRM analysis, a target list of unique peptides for each allergen was constructed as described earlier (Saelens et al., 2022). Briefly, precursor peptides and their fragment ions specific for each allergen were predicted by *in-silico* digestion using the Skyline application (ver. 20.6, 64-bit, Seattle, USA, www.skyline.ms) (MacLean et al., 2010). The sequences from WHO/IUIS-recognized allergens (Ani s 1, Ani s 2, Ani s 3, Ani s 4, Ani s 5, Ani s 6, Ani s 7, Ani s 8, Ani s 9, Ani s 10, Ani s 11, Ani s 11-like, Ani s 12, Ani s 13, and Ani s 14) were obtained from Universal Protein Resource (UniProt) (http://uniprot.org), and retention times for each precursor peptide were imported in MassLynx software (ver. 4.2, Waters Corporation, Massachusetts, USA) for method creation (scheduled MRM).

2.5 Targeted proteomics by LC-MS/MS analysis for the detection of *Anisakis simplex* allergens

The protein samples (i.e., chicken meat and blood, larvae meal, and plant-based feed) were prepared for the analysis by LC-MS/MS as described earlier (Saelens et al., 2022). Briefly, 10 µg of the protein sample extracts were reduced, alkylated, and digested with trypsin overnight. Digested samples were then acidified, purified using Bond Elut OMIX C18 pipette tips (Crawford Scientific Ltd, Lanarkshire, UK), and dried by SpeedVac centrifugation. Prior to reduction, bovine serum albumin (ThermoFisher, Massachusetts, USA) (MS-grade protein standard) was spiked into the samples (1:250 concentration ratio to protein extract).

Equal amounts of trypsin digested protein extracts were analyzed by LC-MS/MS in MRM mode using a high-resolution Waters NanoAcquity M-Class UPLC and an IonKey source connected to a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation, Massachusetts, USA). First, a phosphorylase B standard solution was added to each dried sample (10 fmol/µL sample, phosphorylase B diluted in ultrapure H_2O/ACN/HCOOH (97:3:0.1, v/v/v) for UPLC-MS/MS performance evaluation. Each peptide solution was then transferred to an MS-vial from which five

µL (0.5 µg) was injected and trapped (2 min, 15 µL/min, 3% ACN/0.1% HCOOH) onto a 300 µm × 50 mm, 5 µm, 100 Å Acquity UPLC M-Class Symmetry C18 Trap Colum (Waters Corporation, Massachusetts, USA) before separation on the iKey using a 3 to 50% ACN gradient for 10 min at a 2 µL/min flow rate. The outlet of the analytical column containing the separated peptides was subsequently introduced into the Waters Xevo TQ-S mass spectrometer after passing the positive electrospray ionization source (Waters Nanolockspray, Massachusetts, USA) with a capillary voltage of 3.6 kV, a cone voltage of 35 V and source temperature of 120 °C. Data were acquired with the previously developed MRM mode targeting *A. simplex* peptides (see section 2.4) and uploaded into Skyline for data analysis. Finally, chromatograms with transition peaks for each precursor peptide from each allergen were manually verified and checked for co-elution, overlapping of fragment ions, and symmetry in order to determine presence or absence of a specific allergen in the protein extract. To confirm that detected peptides indeed derived from the allergens, proteotypicity of each detected precursor peptide was verified using Unipept (www.unipept.ugent.be) (Mesuere et al., 2016).

2.6 Statistics and figures

Differences in protein concentrations from the different sample types (blood, chest muscle, and leg muscle) obtained by the different extraction systems, as well as differences in protein concentrations between samples from the PC and NC group, were explored using a one-way ANOVA with Tukey's multiple comparison. A p-value of < 0.05 was considered as significant. All statistics and figure designing were conducted using RStudio (Version 1.3.1093) and Microsoft PowerPoint (Version 16.58).

3. Results

3.1 Detection of anisakid allergens in protein sample extracts using the LC-MS/MS in MRM mode

The larvae meal, plant-based feed, and chicken meat and blood from both the NC and PC group were analyzed using the LC-MS/MS in MRM mode. No anisakid peptides were detected in the plant-based diet whereas the LC-MS/MS analysis of the larvae meal revealed the presence of 41 precursor peptides accounting for 13 *A. simplex* allergens (Ani s 1, Ani s 2, Ani s 3, Ani s 5, Ani s 7, Ani s 8, Ani s 9, Ani s 10, Ani s 11, Ani s 11-like, Ani s 12, Ani s 13, and Ani s 14) (Supplementary Table 1). Likewise, precursor peptides from in total six *A. simplex* allergens (Ani s 2, Ani s 3, Ani s 5, Ani s 7, Ani s 8, and Ani s 13) were detected in the meat and/or blood from chickens receiving the larvae meal in their diet (Figure 3). These precursor peptides were identical as those detected in the larvae meal. Specifically, for Ani s 5 and Ani s 7, one precursor peptide (TDPEIEK and YGIEFCNR, respectively) was detected in the muscles from all chickens receiving the larvae meal. Similarly, the precursor peptides DAFAALAQTFK and/or FLDGADQATK from Ani s 8 were detected in the muscles from all chickens from the PC group. Lastly, one precursor peptide from Ani s 2 (ADLSVQLIALTDR or LLQDDFESER) and Ani s 3 (SLEVSEEK or VQEAEAEVAALNR) was detected in one and four chickens from the PC group, respectively. Nevertheless, the detected precursor peptides from Ani s 2 and Ani s 3 were not proteotypic to the *Anisakis* genus, and hence the presence of the other peptides of Ani s 2 and Ani s 3 could not be linked conclusively to the larvae meal. Surprisingly, when analyzing the blood samples from the PC group, considerably less precursor peptides from only two allergens were detected. One precursor peptide from Ani s 13 (LFAEYLDQK) was detected in three blood samples, and one precursor peptide from Ani s 7 (YGADFCK) was detected in one blood sample. No precursor peptides were detected in the blood and muscle tissue from the NC group, with the exception of one precursor peptide from Ani s 3 (SLEVSEEK). Similarly as in the muscle samples, this peptide was not proteotypic to the *Anisakis* genus after homology searching using Unipept and is present in hundreds of other nematodes, crustaceans, insects, and bacteria.

A list of the target proteins with their selected proteotypic precursor peptides is provided in Supplementary Table 2.

3.2 Comparative analysis of different extraction systems in protein recovery

In this study, three distinctive protein extraction systems were compared in terms of protein yield and anisakid allergen recovery. Results from the Bradford assay revealed that the highest concentrations were obtained with PBS and TPER, whereas the total protein yield obtained with MTPEK was significantly lower ($p = < 0.005$) (Figure 4, left panel). Additionally, when using the PBS buffer, a significantly ($p = < 0.005$) lower concentration was observed in the blood compared to both muscle tissues, as well as a significant ($p = < 0.05$) lower yield in the leg muscles compared to the chest muscles. No difference was observed for the protein concentration of blood and muscles between the PC and NC group when using PBS (Figure 4, right panel).

Lastly, there was a significant difference in protein yield between the chest and leg muscles using PBS ($p = < 0.005$), while this was not the case for MTPEK and TPER (Figure 4).

Considering the difference in anisakid peptide recovery between the three extraction systems, results showed that both PBS and TPER are effective with respect to *A. simplex* extraction and recovery, whereas *A. simplex* peptides were not detected in any of the MTPEK-based extracts (Table 3). Precursor peptides from Ani s 2, Ani s 3, and Ani s 8 were detected using either PBS, TPER, or both. Precursor peptides from Ani s 5 and Ani s 7 were detected by both PBS and TPER.

4. Discussion

In the last few years, the clinical and epidemiological interest in the thermostable and pepsin-resistant characteristics of *A. simplex* allergens has increased with more and more studies examining highly processed fish products for the presence of anisakid proteins, peptides, and DNA (Fæste et al., 2014b; Kochanowski et al., 2020b). While of importance, less attention has been paid to the potential transmissibility of anisakid allergens from fishmeal as a feed component further down the food chain. With the results from the present study, an evident transmissibility of peptides was proven with peptides from six anisakid allergens being detected in chickens that had been fed with anisakid larvae

meal. Proof-of-principle for such transmission in fish had already been provided by Faeste et al. (2015) who revealed the presence of one precursor peptides from Ani s 13 (LFAEYLDQK) in the meat from zebrafish fed with commercial zebrafish feed spiked with 20 percent freeze-dried *A. simplex* larvae. In the present study, a plant-based diet as a basic feed was preferred to ensure the absence of any anisakid traces in the feed. Considering that the chickens ate approximately 28, 33, and 37 g per day during week 1, 2 and 3 from the feeding trial, respectively, the percentage of larvae meal administered here was only 2.8 – 3.8 %. Despite such a considerably lower dose compared to the zebrafish feeding trial by Faeste et al. (2015), anisakid peptides were still transferred from the animal feed to the chicken muscles and blood. Given that the poultry and pig sector in Europe are more reliant on fishmeal as a small supplement in plant-based feeds (5-10 %) (EC, 2021; FAO, 2020), the next step is thus to examine whether or not such ratio is also sufficient to transfer anisakid peptides.

While the transfer from anisakid peptides from animal feed to food was evidently proven, the larvae meal processing (i.e., submitting the larvae to 100°C) presumably affected the peptide detection in this way that non-thermostable allergenic peptides would not be detected. Additionally, for most of the allergens, only one precursor peptide was detected (Figure 3). Ideally, a minimum of two peptides per protein with minimum three fragment ions per peptide (and hence a total of six transitions per protein) are detected to claim protein detection (Mustafa et al., 2015). A protein with matches to only a single peptide sequence is exposed to a higher false positive discovery rate. This limitation, however, has to be accepted when only one proteotypic peptide could be predicted or when the peptide detectability is affected by for instance matrix interference, abundance of other proteins, the choice of protein digestion enzyme, trypsin digestion time, etc. Also, in terms of food safety, it is advisable to opt for a zero-risk approach and assume the presence of an allergen in the case of already one peptide match because of the potential serious consequences of the adverse reactions in terms of

298 mortality and morbidity. Evidently, the remaining allergenic potency of the chicken meat in humans
299 still needs to be confirmed in future experiments using a risk-based quantitative approach.
300 The detection of more and different peptides (3-5) in the chest and leg muscles compared to the blood
301 (0-1) was also remarkable. Since the chickens were exsanguinated immediately during euthanasia
302 and hence little traces of blood remained behind in the muscle tissue, the detected peptides in the
303 breast and legs most likely derive from the chicken muscle tissue itself. In fact, it seems that the
304 peptides are deposited *post-prandial* rather fast from the blood to the meat since only one (or even
305 no) peptide could be detected in the blood. It could, however, also be that the protein extraction
306 system for blood was less efficient in terms of allergen recovery. Blood is a different matrix compared
307 to muscle tissue and the extraction protocol differed to some extent from that of the muscle samples
308 starting with a heating step of 15 min to 95°C. This could have had an influence and either way only
309 selects for the thermostable peptides during peptide detection. The blood samples were also left to
310 settle overnight, potentially allowing some allergens to passively attach to red blood cells and
311 precipitate with them. One allergen, Ani s 13 specifically, was only detected in the blood and hence
312 was present at too low concentrations to be detected, or probably never built in the skeletal muscles.
313 The latter could be because native Ani s 13 haemoglobin is present in an octamer form, giving it a
314 large molecular weight (MW) of 294 kDa instead of the theoretical 36 kDa when present in a
315 monomer (González-Fernández et al., 2017). This in comparison to the other detected allergens from
316 which the MW ranges between 15 and 139 kDa. Lastly, several allergens detected in the larvae meal
317 (Ani s 1, Ani s 9, Ani s 10, Ani s 11, Ani s 11-like, Ani s 12, and Ani s 14) that had withstood the
318 thermal processes during preparation, were nowhere detected in the muscle tissue or blood. Either
319 these never crossed the gut mucosa-blood barrier, either they were present below the detection limit
320 (see above).
321 A final remark is the presence of one precursor peptide from Ani s 3 (SLEVSEEK) in not only the
322 meat from the PC group, but also in the majority of the muscle samples from the NC group. As
323 described earlier (Saelens et al., 2022), many precursor peptides from Ani s 3 (also referred to as

tropomyosin) are not proteotypic to the *Anisakis* genus and during homology searching in Unipept, it was found that the detected precursor peptide has matches with proteins from several bacteria (Actinobacteria, Proteobacteria), plants (Araceae) and Metazoa (Arthropoda, Chordata, Nematoda, Mollusca, etc.). This most likely explains its presence in the NC control group, yet also means its presence in the PC group should not be evidently linked to the *Anisakis* allergen. The same accounts for the two detected precursor peptides from Ani s 2 (LLQDDFESER and ADLSVQLIALTDR) in the chest and legs from one chicken in the PC group. On the other hand, as these peptides from Ani s 2 are only shared within the phylum of the Nematoda, and were not detected in the NC group, their presence could be linked with more certainness to the larvae meal. The aforementioned precursor peptides from Ani s 2 and Ani s 3 should, nevertheless, not be used for specific detection of *A. simplex* allergens during food quality control.

Food products are a very diverse group, and often present a difficult matrix to work with. Different protein extraction systems were therefore investigated on the muscle tissues, determining the protein concentration and allergen detection. All of them were based on the combination of mechanical extraction and chemical lysis. The Minute™ Total Protein Extraction Kit for Muscles (MTPEK, Invent Biotechnologies) was selected since the denaturing buffer and extraction powder were specifically tailored for muscle tissues, and for its short turn over time, as the whole procedure takes less than 10-15 minutes. The results however, revealed a significant lower concentration of proteins extracted compared to the Tissue-Protein Extraction Reagent (TPER)™ (Thermofisher) and PBS, and no anisakid peptides were detected in the extracts. This might have been due to the mechanical disruption step which was performed using a small pestle on only 30 mg of tissue, whereas this was done more rigorous by use of a hand mixer on 2 grams of tissue for the other two systems. A second extraction system selected, was the TPER designed for all tissues and based on a mild proprietary detergent in 25 mM bicine and 150 mM sodium chloride (pH 7.6) that enhances more efficient extraction from cellular compartments. When comparing this with PBS, both buffers were equally

effective in terms of total protein yield and allergen recovery. On the other hand, it was occasionally noticed that certain allergenic peptides were detected using PBS and not with the TPER, and *vice versa*. PBS had already been used in previous studies for the protein extraction from seafood and zebrafish and demonstrated to have a high efficiency for total protein extraction (Fæste et al., 2014b, 2015; Werner et al., 2011). Nonetheless, the results of the present study indicate the usefulness of including a second system to optimize allergen recovery. The exact influence of the commercial buffers could not be determined since their content was not provided by the manufacturers.

The present study also had its limitations starting with the fact that both *A. simplex* and *P. decipiens* were mixed in the larvae meal and were not administered separately. While this was done to better reflect the authentic fishmeal composition, the mixed larvae meal makes it challenging to discern which of the detected anisakid peptides derive from which anisakid species. In previous studies, it was already revealed that at least six WHO/IUIS-recognized *A. simplex* allergens (Ani s 2, Ani s 5, Ani s 7, Ani s 8, Ani s 9, and Ani s 13) are shared with *P. decipiens* (Kochanowski et al., 2020a; Saelens et al., 2022). As peptides from all six were detected in the muscle tissues and/or blood, and since a substantially larger weight of the larvae meal was attributable to *P. decipiens* larvae, it could be assumed that the majority of the detected peptides comes from this nematode. However, only a controlled study design using *A. simplex* and *P. decipiens* larvae meal separately may truly determine which nematode transfers the majority of the peptides. A second limitation was the dose administration of the larvae meal which was based on the maximum predicted number of anisakid larvae present in commercial fishmeal and the average consumption of chicks at the age of 3 till 6 weeks old. Specifically, this was calculated according to the number of fish needed for 1 kg of fishmeal, the common fish species used for fishmeal, and the prevalence and intensity of anisakid infection in the respective fish species. However, numerous prevalence studies on Anisakidae in fish have been reported with large variations in occurrence, and inevitable geographical variations, making the exact composition of fishmeal in terms of Anisakidae alter with each production. As also

limited information was available on the exact fish species and their proportions in commercial fishmeal, only a thorough estimation of the number of Anisakidae in the latter could be calculated. Still, the approach in the present study is valid to provide proof of concept for anisakid peptide transmission to chicken meat.

Finally, reproducible quantification of the anisakid peptides in the present study could not be performed as this was complicated by interference of the complex matrix. While this might be overcome in the future by working with isotope-labelled peptides as internal standards, altering the timing of addition of the internal standard (i.e., before or after tryptic digestion), and/or optimizing the selected protein extraction and purification system, obtaining a reliable quantification of allergens in complex matrices is a well-known issue and explains why quantitative proteomic approaches applied to study food allergens are still in a nascent stage (Ahsan et al., 2016). The influence of the aforementioned parameters in combination with the lack of substantiated information on threshold doses in allergenic individuals, has made the industry grasp for precautionary allergen labelling (e.g., statements such as 'may contain traces of') rather than implementing a validated quantification system.

The present study demonstrates that certain anisakid peptides can not only withstand feed manufacturing procedures and gastrointestinal peptidases, but can also be transported across the gut mucosa towards the blood circulation and therefrom to muscle tissues. These findings may thus significantly change the importance of these zoonotic nematodes from originally a purely fishborne food risk to potentially a much wider risk from several food sources (chicken meat, pork meat, aquacultured fish, etc.). Nevertheless, clarification on the transmissibility of anisakid allergens requires a step-by-step approach of which the present study is a first one. It justifies moving to further in-vitro and in-vivo immunological investigations that are definitely required to confirm the remaining allergenic potency of the chicken meat before a potentially wrongful alarmism against the use of fishmeal is created.

Research data

Apart from the MS/MS data, all data generated or analyzed during this study is included in this published article (and its Supplementary Information files). A summary of the MS/MS data and the MS/MS transition list is provided in Figure 3, Table 3, and Supplementary Table 1. Raw MS/MS data are available from the corresponding author upon reasonable request.

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Figure captions

Figure 1. Preparation of the anisakid larvae meal and set-up of the chicken feeding trial. Larvae from *Anisakis simplex* and *Pseudoterranova decipiens* were collected (A), covered with tap water before heating to 100°C (B), freeze-dried and fine-grinded to obtain the larvae meal (C & D). The transmissibility from anisakid allergens was investigated by setting up a chicken feeding trial (E) in which the positive control group orally received the prepared larvae meal (F).

Figure 2. Timeline of the chicken feeding trial.

Figure 3. Transition peaks on Skyline for precursor peptides from Ani s 2, Ani s 3, Ani s 5, Ani s 7, Ani s 8, and Ani s 13 detected in muscle and blood samples from chickens fed with Anisakidae larvae meal.

Figure 4. Protein concentrations (µg/mL) from the chest and leg muscle tissue from positive control group determined using three different protein extraction systems: Minute™ Total Protein Extraction Kit for Muscles (MTPEK), phosphate-buffered saline (PBS), and Tissue-Protein Extraction Reagent (TPER) (left panel). Protein concentrations from the blood, legs, and chest from chickens in the positive and negative control group using a phosphate-buffered saline (PBS)-based extraction (right panel).