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Proteome profiling of the parasitic nematode *Anisakis simplex* s. s.

DOCTORAL THESIS

by

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Niniejszą pracę pragnę zadedykować moim wspaniałym Rodzicom ***Hannie i Jarosławowi Stryińskim****, dzięki którym miałem możliwość kształcić się i zdobywać cenną wiedzę.*

ABSTRACT

Anisakis simplex is a parasitic nematode that causes anisakiasis in humans. Consumption of fish containing live *A. simplex* larvae may pose a health risk because of their ability to penetrate the gastrointestinal mucosa, as may foods contaminated with parasite allergens, which can cause severe allergic reactions in humans. The increasing popularity of eating raw fish and the rapid increase in the population of this nematode, as well as the expansion of its range, are leading to an increase in the number of cases of anisakiasis. So far, the biology of this parasite does not seem to be fully understood. Therefore, it seems useful to thoroughly study the proteome of this nematode, as it has not been fully characterized so far, which may shed light on the molecular mechanisms of the parasite's growth and development, as well as host-parasite interactions.

The main objective of this study was to identify and characterize the proteome of the parasitic nematode *A. simplex* at global and tissue levels. As a result of the first experiment, the global proteomes of the two developmental stages, L3 and L4, of the *A. simplex* nematode were described. The most accurate and comprehensive proteome of this species was identified and characterized for the first time using ultra high-performance liquid chromatography coupled with high resolution tandem mass spectrometry (LC-MS/MS) and isobaric tandem mass tagging (TMT) of peptides for relative quantification of proteins. The second experiment sought to identify nematode proteins in the tissues responsible for host contact, i.e., intestine and cuticle. By using a similar proteomic workflow and complex analysis of proteomic data, comprehensive sets of proteins characteristic of the nematode tissues studied were identified.

The results on the global proteome and the tissue proteome of *A. simplex* may serve as a basis for the development of new drugs against anisakiasis and, in the future, alleviate the symptoms of this disease or eliminate it in humans. Moreover, using quantitative TMT-based proteomics, the presented methodology can serve as a universal tool for the analysis of global proteome dynamics in the stages and tissues of parasitic nematodes.

STRESZCZENIE

Anisakis simplex jest pasożytniczym nicieniem, powodującym u ludzi anisakiozę. Zagrożenie dla zdrowia mogą stanowić spożywane ryby zawierających żywe larwy *A. simplex*, ze względu na ich zdolność do penetracji błony śluzowej przewodu pokarmowego, jak i produkty żywnościowe skażone alergenami pasożyta, ze względu na występujące u ludzi ciężkie reakcje alergiczne. Wzrost popularności spożywania surowych ryb oraz gwałtowny wzrost liczebności populacji tego nicienia oraz poszerzenie zasięgów jego występowania przekłada się na zwiększenie liczby przypadków anisakiozy. Do tej pory biologia tego pasożyta wydaje się nie do końca poznana. Zasadnym wydaje się zatem dokładne zbadanie proteomu tego nicienia jako do tej pory niedokładnie scharakteryzowanego, który może dostarczyć informacji na temat molekularnych mechanizmów wzrostu i rozwoju pasożyta, jak również na temat interakcji pasożyt-żywiciel.

Głównym celem niniejszej pracy była identyfikacja i charakterystyka proteomu nicienia pasożytniczego *A. simplex* na poziomie globalnym i tankowym. W wyniku pierwszego eksperymentu opisano proteomy globalne dwóch stadiów rozwojowych, L3 i L4, nicienia *A. simplex*. Przy użyciu tandemowej spektrometrii mas sprzężonej z wysokoprzepustową chromatografią cieczową LC-MS/MS (ang. *ultra high-performance liquid chromatography coupled with high resolution tandem mass spectrometry*) i izobarycznego znakowania peptydów przy użyciu TMT (ang. *isobaric tandem mass tagging*) po raz pierwszy zidentyfikowano i scharakteryzowano jak dotąd najdokładniejszy i najobszerniejszy proteom tego gatunku. W wyniku drugiego eksperymentu, podjęto próbę identyfikacji białek nicienia w tkankach odpowiedzialnych za kontakt z gospodarzem, to jest w jelicie i kutikuli. W wyniku zastosowania podobnej metodyki oraz złożonej analizy danych proteomicznych uzyskano złożone zbiory białek charakterystycznych dla badanych tkanek nicienia.

Uzyskane wyniki dotyczące globalnego i tkankowego proteomu *A. simplex* mogą służyć jako baza do opracowania nowych leków przeciwko anisakiozie i w przyszłości złagodzenia objawów tej choroby lub jej wyeliminowania u ludzi.

Ponadto, prezentowana metodyka przy użyciu proteomiki ilościowej opartej na TMT może posłużyć jako uniwersalne narzędzie do analizy globalnej dynamiki proteomicznej w stadiach i tkankach pasożytniczych nicieni.

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ABBREVIATIONS

2-DE: two-dimensional gel electrophoresis

A. simplex: *Anisakis simplex* sensu stricto

ADC: activated dendritic cells

APCs: Antigen Presenting Cells

Arg, Lys, His, Pro: arginine, lysine, histidine, proline

AUC: area under the curve

BAT: basophil activation test

CE: crude extract

CID: collision-induced dissociation

Cox2: cytochrome oxidase subunit 2

Da: Dalton (unit of mass)

DAILYs: disability-adjusted life years

E/S: excretory/secretory

EA: ectopic anisakiasis

EC: European Commission

EFSA: European Food Safety Authority

ELISA: enzyme-linked immunosorbent assay

ES: electrospray

ESI: electrospray ionization

ETD: electron transfer dissociation

FA: formic acid

FAO: Food and Agriculture Organization of the United Nations

FT-ICR: Fourier transform ion cyclotron resonance

GA: gastric anisakiasis

GAA: gastrointestinal anisakiasis

GI: gastrointestinal

H&E: histological staining using hematoxylin and eosin

HCD: higher energy C-trap dissociation

HPLC: high performance liquid chromatography

IA: intestinal anisakiasis

iCAP: immunoCAP assay

ICAT: isotope-coded affinity tags

IFN-γ: interferon gamma

Ig: immunoglobulin

IL: interleukin

iTRAQ: isobaric tags for relative and absolute quantification

ITS: internal transcribed spacer region

LBQ: label-based quantification

LC-MS: liquid chromatography coupled with mass spectrometry

LC-MS/MS: liquid chromatography coupled with tandem mass spectrometry

LC: liquid chromatography

LFQ: label-free quantification

m/z: mass-to-charge ratio

MALDI: matrix-assisted laser desorption/ionization

MDC: mature dendritic cells

MS: mass spectrometry

MΦ: tissue macrophages

NDC: naïve dendritic cells

NO: nitrogen monoxide

NTDs: neglected tropical diseases

PCR: polymerase chain reaction

PMN: polymorphonuclear leukocytes

Q: quadrupole

RPLC: reversed-phase liquid chromatography

RT-PCR: real-time PCR

SILAC: stable isotope labeling by/with amino acids in cell culture

SSU rRNA: small subunit ribosomal RNA

TFA: trifluoroacetic acid

Th: T helper cells

TLRs: toll-like receptors

TMT: tandem mass tags

TNF-β: tumor necrosis factor-beta

TOF: time-of-flight

WB: Western blot

WHO: World Health Organization

WoRMS: World Register of Marine Species

ORIGINAL MANUSCRIPTS INCLUDED IN THE DOCTORAL THESIS

1. **Stryiński R.,** Mateos J., Pascual S., González Á. F., Gallardo J. M., Łopieńska-Biernat E., Medina I., Carrera M. Proteome profiling of L3 and L4 *Anisakis simplex* development stages by TMT-based quantitative proteomics. *Journal of Proteomics*. 2019; 201: 1-11, https://doi.org/10.1016/j.jprot.2019.04.006 (IF = 4.044; MEiN 100 points[1]).

2. **Stryiński R**., Mateos J., Carrera M., Jastrzębski J. P., Bogacka I., Łopieńska-Biernat E. Tandem Mass Tagging (TMT) reveals tissue-specific proteome of L4 larvae of *Anisakis simplex* s. s.: enzymes of energy and/or carbohydrate metabolism as potential drug targets in anisakiasis. *International Journal of Molecular Sciences*. 2022; 23(8): 4336. https://doi.org/10.3390/ijms23084336 (IF = 6.208; MEiN 140 points[1]).

[1] Points assigned to journals based on the current *List of scientific journals and peer-reviewed materials from international conferences* announced by the Ministry of Science and Higher Education (MEiN) in Poland

STATEMENT OF ORIGINALITY

1. In this manuscript, the identification of the global proteome of two developmental stages (L3 and L4) of *Anisakis simplex* was performed using a proteomic approach. In 2016, Carrera et al. (*Journal of Proteomics* (2016), 142 (1): 130-137) also reported the identification of 1,231 proteins from L3 stage worms. Our work extends these results by presenting 2,443 identified proteins for two developmental stages: L3 and L4, with the more abundant proteins identified and listed for L3 (330 proteins) and for L4 (571 proteins) during global quantification. After sequential application of the next quantification criteria, 8 proteins in L3 and 12 proteins in L4 were selected as proteomic signatures of these specific developmental stages of *A. simplex*. In addition, functional annotations were applied to the proteomes of the different stages of the parasite. To our knowledge, this is the largest dataset of *A. simplex* proteins for two developmental stages (L3 and L4) identified to date.

2. In this manuscript, proteomic analysis of L4 larval stage tissues was performed for the first time, such as the cuticle and intestine, which are fully developed and functional in contrast to the L3 stage. Thus, this work provides for the first time the tissue-specific proteome of *A. simplex* larvae. The presented dataset of 272 differentially regulated proteins, of which 133 proteins are more abundant in the cuticle and 139 proteins are more abundant in the intestine, identified in this parasite, together, with their functional annotation, expands current knowledge on the mechanisms leading to the development, survival, and host-parasite interactions of GI nematodes in their host. The identified tissue-specific proteins may be useful for the scientific community to initiate further molecular studies related to this parasite and targets for new drugs against anisakiasis.

AUTHOR'S CONTRIBUTIONS

The design and execution of experiments, as well as raw data analysis, visualization and writing of the manuscripts presented in this thesis were carried out by the author with the supervision of Elżbieta Łopieńska-Biernat, DSc, PhD, and Mónica Carrera Mouriño, PhD. In both manuscripts, Jesús Mateos, PhD, contributed with the proteomic analysis and analysis of the data. Additionally, Prof. S. Pascual S., Prof. Á. F. González, Prof. J. M Gallardo, and Prof. I. Medina contributed with the writing – review and editing of the first manuscript. In the second manuscript, Jan Paweł Jastrzębski, PhD, contributed with the data visualization of 3 D structures of selected proteins and Prof. I. Bogacka contributed with the review and editing and funding acquisition.

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INTERNATIONAL COOPERATION

International collaboration between the University of Warmia and Mazury in Olsztyn, Poland, and the Institute of Marine Research of the Spanish National Research Council in Vigo, Spain, began in 2017 when I completed a three-month internship at the Institute of Marine Research under the direction of Mónica Carrera, PhD. My next trip was in 2018 (2 months), another in 2019 (2 months), and the last in 2020 (2 weeks). The first three internships were funded by the Erasmus + programme and the last one by the International Scholarship Exchange of Doctoral Students and Academic Staff (PROM) programme funded by the Polish National Agency for Academic Exchange (NAWA).

During these internships, I learned how to use high throughput liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using TMT labeling on the LTQ-Orbitrap Elite mass spectrometer. At the same time, I was trained in the use of Proteome Discoverer software (Thermo Fisher Scientific) to process proteomic data. The collaboration has resulted in 6 publications, two of which are included in the doctoral thesis presented. At the same time, there are currently 2 international projects funded by the National Science Center, Poland, including one in which I am the principal investigator.

1. GENERAL INTRODUCTION

Gastrointestinal (GI) worm infections are parasitic diseases caused by helminths, including flukes (Trematoda), tapeworms (Cestoda), and roundworms (Nematoda), living in the human GI tract (Castro, 1996). Human GI nematode infections are amongst the most prevalent, and it is estimated that in 2019 there was ~1 billion cases worldwide posing a burden estimated at ~3 million disability-adjusted life years (DALYs), where one DALY represents the loss of the equivalent of one year of full health (Institute for Health Metrics and Evaluation, 2020; World Health Organization, 2020). Parasitic nematodes also cause substantial losses in livestock and companion animals and are responsible for $80 billion in annual crop damage worldwide (Barker et al., 1994). Globalization of food supply, increase in international trade, convenience of travel, increase in highly susceptible people (e.g.: elderly, malnutrition, human immunodeficiency virus infection), changes in cooking traditions and lifestyle habits, and advanced diagnostic tools are some of the reasons for the increase in parasitic diseases worldwide (Dorny et al., 2009; EFSA and ECDC, 2018; Murrell, 2013; Pozio, 2020; Stryiński et al., 2020). Depending on local geographic, environmental, and economic conditions, each human population in the world has its own unique set of parasites. Developments in animal husbandry, sanitary conditions, and diagnostic methods have undoubtedly led to the reduction or even elimination of certain parasite species in developed and some developing countries. However, the decline in case numbers does not apply to all parasite species, especially food-borne nematodes, and there are countries where the incidence of these infections in humans is still high (Crompton, 1999; Pozio, 2020). Moreover, the global estimates of GI nematode infections were calculated based on the species of greatest medical importance like *Ascaris lumbricoides* (roundworm), *Ancylostoma duodenale*, *Necator americanus* (hookworms), and *Trichuris trichiura* (whipworm) belonging to soil-transmitted helminths (Jourdan et al., 2018). This limitation, which did not consider water-, plant-, meat-, and seafood-borne species, along with continued population growth and awareness that still most human GI nematode infections are asymptomatic, may have dramatically increase the real number of infected individuals (Papaiakovou et al., 2022; Pullan et al., 2014; Stepek et al., 2006). This all may be a reason why GI nematode infections have been considered neglected tropical diseases (NTDs) in terms of public recognition and research funding.

Anisakis simplex sensu stricto (*A. simplex*) is one of the most important emerging parasitic nematodes in Europe, according to the Risk Management Ranking of Foodborne Parasites, prepared for recommendations by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) (Bouwknegt et al., 2018; Stryiński et al., 2020). The disease caused by the genus *Anisakis* is called anisakiasis and is dangerous and frequently described (Aibinu et al., 2019; Audicana et al., 2002; van Thiel, 1962). It is estimated that the incidence is 0.32 cases per 100,000 individuals/year worldwide (Prevalence of rare diseases, 2022). The aforementioned reasons, such as globalization, the development of diagnostic tools and better analytical methods, have led to significantly even more anisakiasis being reported. Before 2010, over 20,000 cases of anisakiasis were reported worldwide, with the highest prevalence (over 90%) in Japan (Baird et al., 2014; EFSA, 2010), where now 7,000 cases of the disease are reported annually (Suzuki et al., 2021; Yorimitsu et al., 2013). European countries where cases of anisakiasis have been reported include among others, Spain (Herrador et al., 2019), Italy (Guardone et al., 2018; Mattiucci et al., 2018), France (Yera et al., 2018), Croatia (Mladineo et al., 2015a), and Poland (Kołodziejczyk et al., 2020).

Although we do not yet fully understand the molecular mechanisms by which parasites protect themselves from host immune responses, it is generally known that parasitic nematodes are equipped in and release a variety of products, primarily proteins, to infect a host by penetrating tissue barriers, migrating through host tissues, and evading immune responses (Anthony et al., 2007; Cooper & Eleftherianos, 2016; Harder, 2016). Although the importance of proteins in the establishment and maintenance of host-parasite interactions is widely recognized, in the case of parasitic nematodes, much remains to be learned about the composition of their proteomes and the mechanisms by which parasitic proteins regulate the immune system. However, recent advances in biochemistry, molecular biology, and proteomic data acquisition have helped provide the first clues to some of the proteomes of parasitic helminths.

Therefore, to fill the gap described, it was decided to analyze and characterize the proteome of *A. simplex*, a parasite of great public health importance. In the central part of this work, we focused on the identification and characterization of the proteome of *A. simplex* in order to increase our knowledge of the biology of this organism and to find a way to understand how this parasite might interact with the host via protein-protein interactions.

2. LITERATURE REVIEV

2.1. Nematoda – overview

2.1.1. General information

To demonstrate the validity of the research objectives pursued in this thesis, the general overview on nematodes is briefly presented below.

The nematodes constitute the phylum Nematoda and are among the most ubiquitous organisms on earth. They include free-living forms, commensals, as well as parasites of plants, insects, humans, and other animals (Basyoni & Rizk, 2016). Nematodes are invertebrates that comprise a huge number of species. In phylum Nematoda, there are approximately 25,000 described species, but it is estimated that there are nearly 1 million species (Iqbal & Jones, 2017). Nematodes are widely distributed and have successfully adapted to almost any ecosystem. They have mastered all biotopes in which one can live, from the tropics to polar regions, deserts, hot springs, ocean depths, caves, and high mountains. Nematodes play an important role in many biocenoses. They are of great medical and economic importance (Blaxter & Denver, 2012). One-third of the genera occur as parasites of vertebrates; about 60 nematode species occur in humans. Parasitic species represent a whole range of adaptations to this way of life and, in particular, infest all kinds of plants and animals (refer to 2.1.3). There are known species that can survive a lifetime in anabiosis. Many forms can encyst, and their eggs can survive in the soil for several years. Most species of higher plants and animals have at least one nematode as a parasite. The approximately 2,271 genera are divided into 256 families (R. C. Anderson, 2000; Blaxter, 2016; Borgonie et al., 2011).

2.1.2. Anatomy – brief description

The representatives of the nematodes are characterized by a uniform structural plan. They are structurally cylindrical, and bilaterally symmetrical. The body is very elongated, narrowed at the ends, oval in cross-section and of various shapes: cylindrical, spindle-shaped or filiform. The body color of nematodes is predominantly milky white, but there are also species with a red, yellowish, green or orange body color. The nematode body wall is composed of a cuticle which the outer most layer is the epicuticle, and a single layer of longitudinal muscle cells. The cuticle is composed primarily of protein with trace

amounts of lipid and carbohydrates, and function as an interface connecting nematodes and their host, acting to protect the animal from the external environment and allowing it to remain homeostatic inside (Basyoni & Rizk, 2016). The buccal cavity, pharynx, rectum, excretory duct, and pore are chief openings of the nematode that are also cuticle lined. As a new cuticle is generated for each developmental stage, it is synthesized five times during the nematode lifecycle, with synthesis of the first cuticle beginning during late embryogenesis (Page et al., 2014). The assembly of cuticle, hypodermis and muscular layer composes the nematode body wall, and its main function is to act as hydrostatic-skeletal system (Harris & Crofton, 1957). The body cavity is a fluid-filled pseudocoelom (meaning that it is not lined by mesoderm) under high hydrostatic pressure. There is no circulatory system. Gender dimorphism is usually present: females are generally larger than males. Eutely (constant number of cells of the organism) is the rule and indicates that no repair is possible after injury (Lucius et al., 2017).

Nematodes have digestive, nervous, excretory-secretory, and reproductive systems. The nervous system of nematodes consists of anterior nervous tissue surrounding the pharynx, forming dorsal and ventral nerve cords that extend from one end to the other. The nerve ring is called the circum-pharyngeal commissure and surrounds the isthmus of the pharynx. In addition to the nerve ring, the central nervous system includes interneurons and associated ganglia, as well as the ventral nerve bundle. It is noteworthy that nematodes are enriched with a variety of sensilla (small sense organs) including cephalic and caudal papillae that represent chemoreceptors and mechanoreceptors concentrated in head and tail region (Bodri, 2022).

The digestive system has three parts. It is clearly divided into stomodaeum, mesenteron and proctodeum (Basyoni & Rizk, 2016; Bird & Bird, 1991). The stomodaeum (foregut) includes the mouth and lips, buccal cavity, and oesophagus. The mouth has either three or six lips, often bearing a row of teeth on their inner edges. The buccal cavity may be small or large. The pharynx is usually a muscular sucking organ with a triradiate lumen, sometimes with a valve at its distal end, which prevents regurgitation (Lucius et al., 2017). The intestine (midgut) is simple, hallow, straight non muscular tube consisting of a single layer of epithelial cells. One characteristic feature of nematode intestine is that it is anchored firmly to the oral cavity and to the anus (Bird & Bird, 1991). The proctodeum (hindgut) consists of rectum and anus in female. The rectum of the male receives from the testis and ejects the sperm and is thus considered a cloaca (Basyoni & Rizk, 2016; Lucius et al., 2017).

The excretory/secretory (E/S) system appears to have secretory and osmoregulatory functions. Two basic types of E/S systems exist: glandular and tubular. Most free-living nematodes (Adenophora) have the glandular type while the tubular type is commonly found in the parasitic nematodes (Secernentae) (Nelson et al., 1983). In overall, two excretory channels run along each side of the body, emptied by an excretory pore near the head (Lucius et al., 2017). In parasitic nematodes, E/S is closely related to the pathogenicity of the species, as its function involves the production, excretion, and secretion of a variety of bioactive excretory and secretory products. Some of these products, which exhibit hydrolytic activity, provide enzymatic degradation of host tissues, allowing larval entry and migration, and possibly feeding. Other important functions of E/S include antigenic interaction with the host immune system that elicits an allergic response, modulation of T helper and innate immunity, larval ecdysis, and, as recently suggested, antimicrobial activity in the host gastrointestinal system (Basyoni & Rizk, 2016; Mladineo et al., 2019).

Sex organs of nematodes are simple tubular structures that are continuous with their ducts so, neither sperms nor eggs are shed into the pseudocoelom (Bird & Bird, 1991). The male usually has a single gonad, starting with a testis. Then follows a seminal vesicle, the *vas deferens*, and the muscular ejaculatory duct. This opens into the cloaca together with the intestine. In addition, males possess accessory organs including spicules, copulatory bursa and genital alae. In secernenteans, there is only one testis (monorchic) compared to adenophoreans which possess two testes (diorchic) (Bird & Bird, 1991). Unlike that of the male, the female reproductive system is usually didelphic (Greek: *delphys* means uterus) so there are two ovaries, two oviducts and two uteri. There is only one vagina and gonopore. Those with one gonad are monodelphic (Basyoni & Rizk, 2016; Lucius et al., 2017).

2.1.3. Parasitic lifestyle

Humans are hosts to nearly 300 species of helminths and over 70 species of protozoa, some derived from our primate ancestors, and some acquired from the animals we have domesticated or come in contact with during our relatively short history on Earth. Of this number, about 60 nematode species occur in humans (Cox, 2002). Parasitism of both animals and plants seems to have arisen multiple times during nematode evolution, and

all major clades include parasites. On the basis of small subunit ribosomal RNA (SSU rRNA) phylogenetics, nematodes can be divided into three major clades: Dorylaimia (clade I), Enoplia (clade II) and Chromadorea (which includes Rhabditida, also known as Secernentea). Rhabditida can be further subdivided into Spirurina (clade III), Tylenchina (clade IV) and Rhabditina (clade V) (Blaxter et al., 1998; Parkinson et al., 2004) (Figure 1). This differs from recent results of phylum-wide analysis of phylogenesis, which divided the phylum Nematoda into 12 clades whose common ancestor is considered non-parasitic. According to this classification, parasitism has so far been found in clades 1, 2, 5, and 8 to 12 (Holterman et al., 2006; van Megen et al., 2009). With the current available molecular data, three origins of plant parasitism can be defined, ten of parasitism of a wide range of non-vertebrates and five of parasitism of vertebrate hosts (Blaxter & Koutsovoulos, 2015). The adaptations to the parasitic lifestyle of parasitic nematodes in animals and humans will be described in more detail, as they play an important role in this doctoral thesis. Only the parasitic GI nematodes will be considered.

The environment of the small intestine, for example, where nematodes and other parasites live, is characterized by a lack of oxygen, a high concentration of active digestive enzymes, high osmolarity, and host immune responses, among other factors. However, these harsh conditions are seemingly compensated by the great abundance of nutrients. Morphological, physiological, and other adaptations are required for long-term use of such a habitat. Such adaptations either existed prior to the ancestral transition from a free-living to a parasitic existence or evolved rapidly after the transition to parasitism through intense natural selection. These include morphological structures that allow attachment in the host intestine, a tough cuticle to resist immune attack, or a metabolism that can cope with low oxygen availability (Grencis & Harnett, 2011; Komuniecki & Tielens, 2003; Łopieńska-Biernat et al., 2019; Lucius et al., 2017; Page et al., 2014).

What is special about the coevolution of parasites and hosts is the fact that hosts respond to parasite infection by, for example, developing defense reactions. For this reason, parasites must not only adapt to an inhospitable environment, but also evade host defense responses. These strategies include both actively suppressing effector responses of cells such as B lymphocytes and mast cells and modulating the polarity of immune responses by acting on Antigen Presenting Cells (APCs), thereby inhibiting responses that are potentially protective of the host. Nematodes also induce an anti-inflammatory response (King & Li, 2018; McRae et al., 2015).

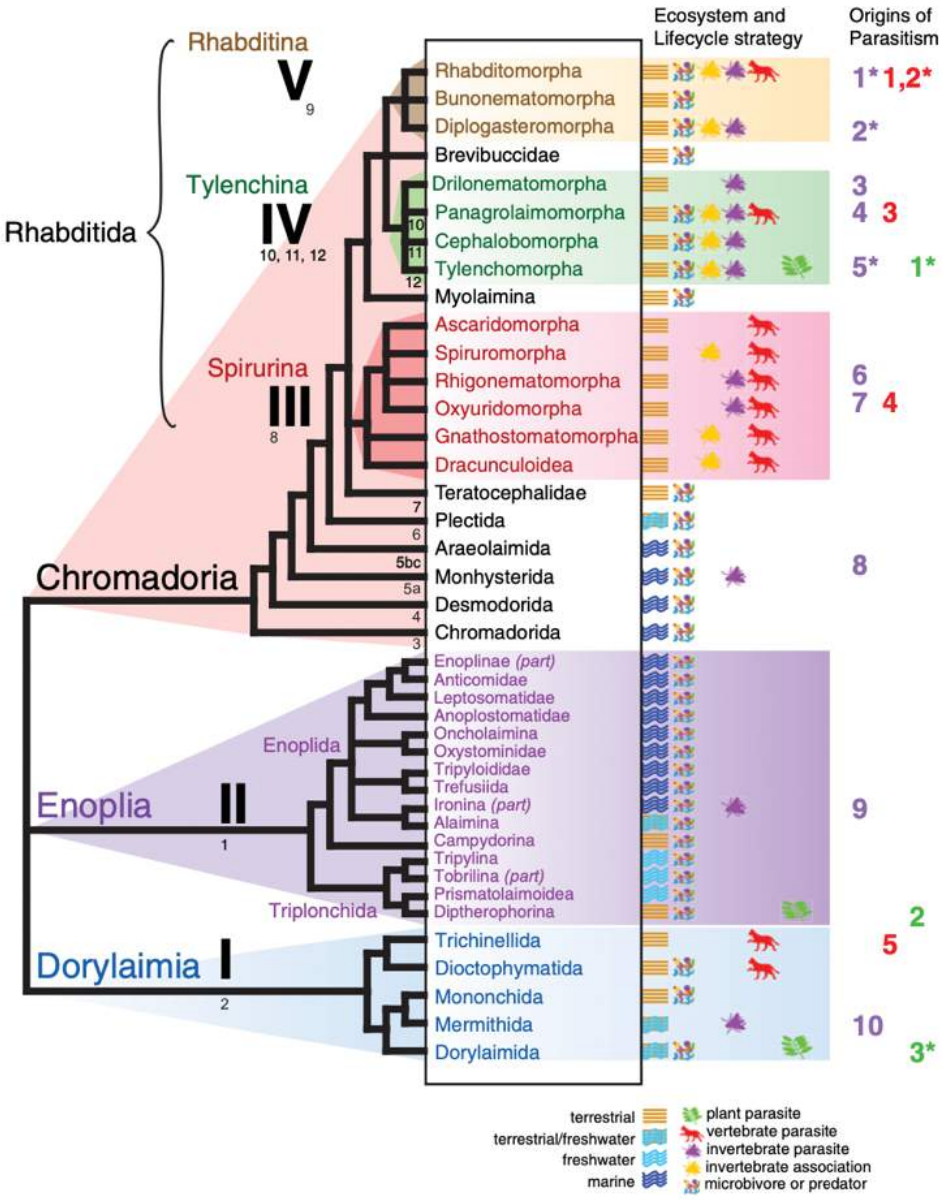


Figure 1. Phylogenetic relationship between nematode clades (I-V) based on the small subunit ribosomal RNA gene. Parasitic nematodes are present in all clades, and their hosts are indicated pictorially. Adapted from Blaxter & Koutsovoulos (2015). Helder and colleagues revised the numbering of clades (Holterman et al. 2006; van Megen et al. 2009), and their schema is given in smaller Arabic numerals beneath the relevant branches. * - multiple parasitism events

Unlike most viral and bacterial pathogens, which exploit their hosts only temporarily, typical eukaryotic parasites persist in their hosts for long periods of time. Intestinal nematodes, for example, often live in their hosts for several years and rarely cause disease as long as the worm burden is low (Grencis & Harnett, 2011; Lucius et al., 2017; Tritten et al., 2021). Therefore, the relationship between host and parasite must be stable and the

parasite must remain latent as long as possible. The metabolism products of parasitic nematodes e.g., E/S products play an important role here. Among them, proteins are an important group.

Nematode parasites excrete/secrete proteins differ from the somatic protein group in class and/or abundance. Many of these proteins appear to be involved in the uptake and transport of nutrients, reflecting the strategies that these parasites employ to obtain and utilise energy sources from the host that are necessary for the establishment of chronic infection. Much attention has been given to their involvement in evasion/modulation of the immune system to prevent elimination and development of host immunity (Hewitson et al., 2013; Tritten et al., 2021). Thus, tissues in direct contact with the host, and those producing E/S products (proteins) can provide valuable information about the molecules and signaling pathways involved in host-parasite interactions.

From a parasitological perspective, the cuticle and intestine are the nematodes' tissues most responsible for host-parasite interactions (Basyoni & Rizk, 2016; Page et al., 2014). There is strong evidence that most secretion and excretion occurs through the parasite's organs, including the excretory gland, esophagus, intestine, and cuticle (Bird & Bird, 1991; Mehrdana & Buchmann, 2017; Mladineo et al., 2019). Enzymes that play a fundamental role in metabolism, such as proteases, nucleotidases, esterases, glycases, and dismutases, which are associated with parasite infectivity, immune defense, and pathogenicity, and thus play an extremely important role in the life cycle of parasitic nematodes, are produced in great manner by mentioned structures (Bahlool et al., 2013; Page et al., 2014; Wang et al., 2013; White & Artavanis-Tsakonas, 2012).

Based on the above premises, it seems very useful to study the proteome, i.e., protein composition, of the whole organism of parasitic nematodes and individual tissues, especially the intestine and cuticle, in order to increase our knowledge of parasite physiology and host-parasite interactions.

2.2. *Anisakis* spp. – characterization

2.2.1. Genus *Anisakis* – taxonomic classification

According to the World Register of Marine Species (WoRMS) (Horton et al., 2022), the genus *Anisakis* Dujardin, 1845, currently belongs to:

- (phylum) Nematoda

- (class) Chromadorea
- (subclass) Chromadoria
- (order) Rhabditida
- (superfamily) Ascaridoidea Baird, 1853
- (family) Anisakidae Railliet & Henry, 1912
- (subfamily) Anisakinae Railliet & Henry, 1912
- (genus) *Anisakis*

To date, this genus has already had the description of 34 distinct species, however currently there are only twelve valid species, nine of which have been molecularly confirmed by various phylogenetic analyses to proof the phylogenetic distance of these organisms. Most of the rest were relegated to synonyms of other species, most of them to synonyms of *A. simplex*, which is one of the most widely distributed species (Mattiucci et al., 2018; Santos et al., 2020). The nine species considered valid and molecularly identified are:

- *Anisakis berlandi* Mattiucci, Cipriani, Webb, Paoletti, Marcer, Bellisario, Gibson & Nascetti, 2014;
- *Anisakis brevispiculata* Dollfus, 1966;
- *Anisakis nascettii* Mattiucci, Paoletti & Webb, 2009;
- *Anisakis paggiae* Mattiucci, Nascetti, Dailey, Webb, Barros, Cianchi & Bullini, 2005;
- *Anisakis pegreffii* Campana-Rouget & Biocca, 1955;
- *Anisakis physeteris* Baylis, 1923;
- *Anisakis simplex* Rudolphi, 1809;
- *Anisakis typica* Diesing, 1860; Baylis, 1920;
- *Anisakis ziphidarum* Paggi, Nascetti, Webb, Mattiucci, Cianchi & Bullini, 1988.

Phylogenetical studies based on the molecular tools such as sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA, mitochondrial gene cytochrome oxidase subunit 2 (Cox2), allozyme analysis, and polymerase chain reaction (PCR) coupled with restriction fragment length polymorphism, grouped this nine *Anisakis* species into four distinct clades (Mattiucci et al., 2014; Mattiucci & Nascetti, 2006, 2008; Valentini et al., 2006). These distinct clades are also accompanied by different morphological characteristics of the adult individuals of each species group (Mattiucci et al., 2018; Santos et al., 2020):

- **Clade 1**: is formed by three species called together *A. simplex* sensu lato (s. l.) complex; it includes *A. berlandi*, *A. pegreffii*, and *A. simplex* (s. s.). They have identical adult morphology with a longer than wide ventricle, sigmoid in shape, and in males the two spicules are thin and of different length.
- **Clade 2**: is formed by two species: *A. ziphidarum* and *A. nascettii*. The morphology shows a long ventricle, not sigmoid in shape, and the male has two long, thin, and equal sized spicules.
- **Clade 3**: includes *A. physeteris*, *A. brevispiculata* and *A. paggiae*. The morphology presents a short ventricle, never sigmoid, wider than long, and the two male spicules are short, robust and of equal size.
- **Clade 4**: includes only *A. typica*. Morphologically, it presents a long ventricle, and the male spicules are long, thin and of different dimensions, where one is 3 times larger than the other.

Anisakis sp. 1, a newly identified gene pool, was genetically detected by both allozyme and mitochondrial DNA analysis, at the larval stage, as a parasite of the fish *Nemipterus japonicus*. This new taxon was found to be closely related to *A. typica* (Mattiucci & Nascetti, 2008), however its precise position from a phylogenetic point of view is yet to be resolved.

2.2.2. Genus *Anisakis* – life cycle

The life cycle of *Anisakis* nematodes follows the general nematode life cycle pattern, including four larval stages (L1–L4), within the eggs (L1) and subsequently in the intermediate or paratenic hosts (L2–L3), and as preadults (L4) and adults in the cetacean final hosts (Klimpel & Palm, 2011) (Figure 2). The nematode eggs are excreted with the feces by marine mammals and embryonate in seawater. The larvae are eaten by the intermediate host, crustaceans (copepods, isopods, amphipods, euphausiids) and mollusks (Baird et al., 2014). It is well established that the most important first intermediate host in the *Anisakis* life cycle are Euphasiids (krill) (Smith & Wootten, 1978). During ingestion by the crustacean first intermediate host (planktonic or semiplanctonic), the larvae are most probably released from the second stage cuticle by the action of the mouthparts. This allows the third stage larvae to penetrate the gut prior to establishing themselves in the haemocoel (Køie et al., 1995). Some authors suggest that larvae can proceed directly from the crustacean intermediate host to a marine

mammal definitive host, although they often use fish or cephalopod “transport” hosts (Audicana et al., 2002; Fiorenza et al., 2020; Hays et al., 1998; Measures, 2014). Larger invertebrates (mainly copepods) and smaller fish are thought to be important second intermediate hosts, and various predatory fish species and cephalopods serve as paratenic hosts. If small fish are preyed upon by larger piscivorous fish, the larvae can re-infect the latter without further molting. Consequently, piscivorous hosts may accumulate enormous numbers of larvae (Münster et al., 2015; Zuo et al., 2016). The *Anisakis* larvae encyst on the intestines and other visceral organs of these hosts. The parasite does not develop further and remains at the third stage of larvae development (L3) in these hosts. The life cycle comes to a completion when infected intermediate and paratenic hosts are eaten by cetaceans (marine mammals such as whales, seals, and dolphins). In these definitive hosts, the larvae grow to the L4 stage and subsequently to the adult stage. The nematode feeds, grows, mates, and then releases eggs via the host faces into the sea water to continue its life cycle (Aibinu et al., 2019). This complex life cycle involves a variety of hosts that are transferred through the marine food chain (Klimpel & Palm, 2011). Humans become accidental hosts when undercooked or raw fish and cephalopods, contaminated with the parasite are consumed. Once in the human GI tract, L3 of *A. simplex* can develop into L4, but only few exceptional cases have been described (Kagei & Isogaki, 1992; Kliks, 1983, 1986; Sohn et al., 2015; Weerasooriya et al., 1986).

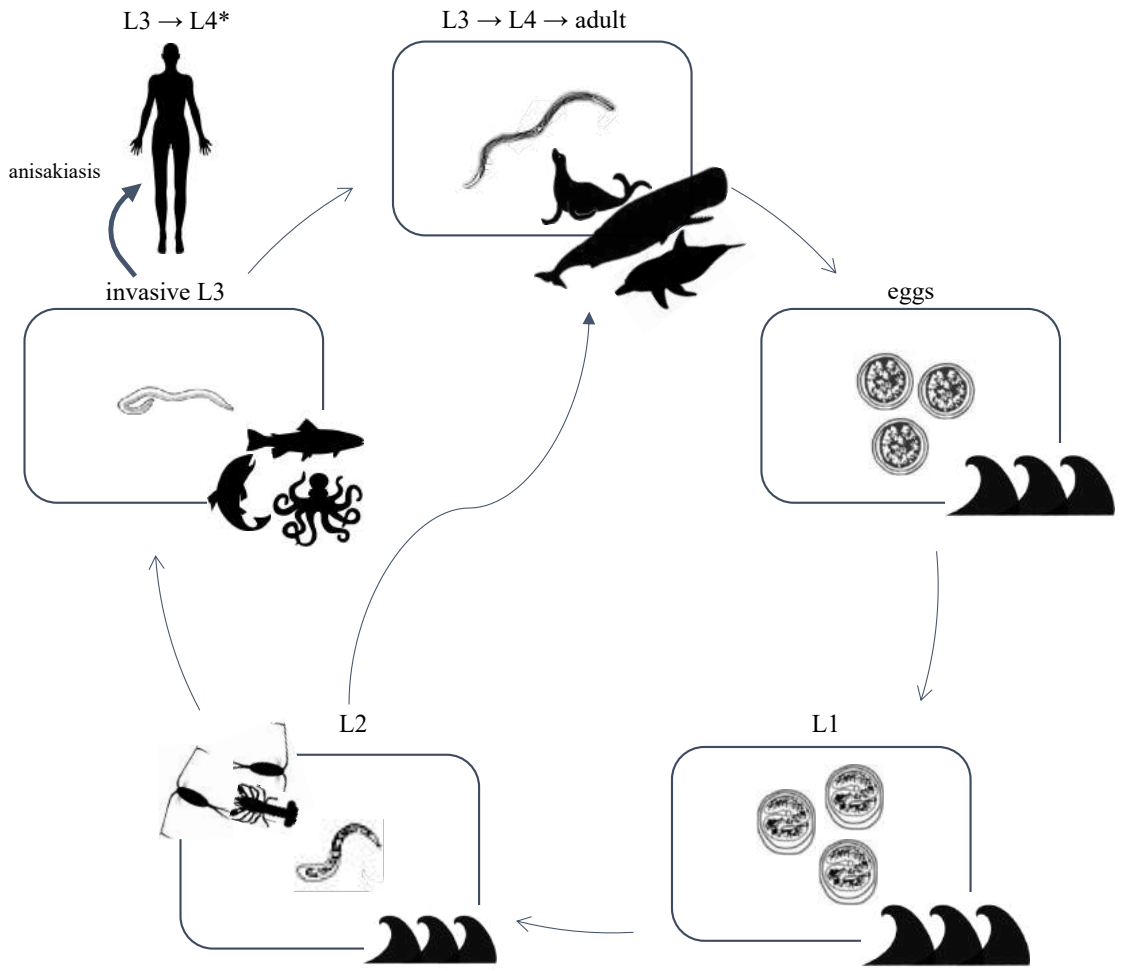


Figure 2. The life cycle of *Anisakis simplex*. Adapted with modifications from Stryiński (2018). Originally based on Audicana et al. (2002); Klimpel & Palm (2011) and Centers for Disease Control and Prevention (https://www.cdc.gov/dpdx/anisakiasis/index.html) *- molting from L3 to L4 is exceptional, but such cases have been described (refer to 2.2.4.).

2.2.3. Genus *Anisakis* – zoonotic potential

Nearly three decades ago, the most common fish and shellfish sources for *A. simplex* were spotted chub mackerel (*Scomber japanicus*) and Japanese flying squid (*Todarodes pacificus*) (Nagasawa & Moravec, 1995). However, a little over a decade later, Abollo and colleagues concluded that most species of cephalopods and fish can potentially harbor these parasitic marine nematodes (200 species of fish and 25 species of cephalopods have been identified as hosts for *Anisakis* spp.) (Abollo et al., 2001; Aibinu et al., 2019; Mattiucci et al., 2018). Moreover, *Anisakis* larvae are found in various crustacean, cephalopod, and especially fish species of commercial importance such as herring (*Clupea harengus*), hake (*Merluccius merluccius*), anchovy (*Engraulis encrasicolus*),

mackerel (*Scomber scombrus*), cod (*Gadus morhua*), sardines (*Sardina pilchardus*), and salmon (*Salmo salar)* (Stryiński et al., 2022).

According to the present knowledge not all among the *Anisakis* species have ability to provoke the seafood-borne zoonosis in humans, commonly known as anisakiasis (Van Thiel, 1962) (refer to 2.2.4.). While *A. simplex* and *A. pegreffii* have demonstrated their ability to cause anisakiasis in humans, it is not clear if any other *Anisakis* species can do the same (Mattiucci et al., 2018; Nieuwenhuizen & Lopata, 2014). This may be due to the fact that, the different *Anisakis* species seem to have preferential infection sites in their fish hosts, and tend to locate in the body cavity, encysted on visceral organs, often coiled up in tight spirals. In some fish species the larvae tend to be embedded in the liver, over the gonads, in the mesentery, or attached to the intestinal wall. The species most frequently found in the editable parts (fish musculature) of commercially important fish are as previously mentioned, *A. simplex* and *A. pegreffii* (Bao et al., 2017; Cipriani et al., 2016, 2018; Karl et al., 2011; Levsen & Lunestad, 2010; Pierce et al., 2018; Setyobudi et al., 2011). These studies described the localization of *Anisakis* larvae within the body of fish immediately after capture. What is most interesting, studies conducted after the death of the fish host, showed that larvae can migrate to the fish flesh from inedible parts (Cipriani et al., 2016; Suzuki et al., 2010). Recent study on motility and mobility showed that the temperature had significant effect on larval movements, with the highest migration observed at 22 ℃ for *A. simplex* larvae. The median migration of larvae was 10 cm at 22 ℃ (Guan et al., 2021). This study was performed in *in vitro* conditions and should be further validated in parasite-infected/spiked fish fillets, however this indicates an important aspect, which is the storage of fish-based food intended for consumption (refer to 2.2.4.).

The pathogenic role of remaining species belonging to the genus *Anisakis* is relatively low. The third species from clade 1, *A. berlandi* has never been associated with human disease (Mattiucci et al., 2014, 2018). As for the species of clade 2, i.e., *A. ziphidarum* and *A. nascettii*, these species are rarely found in commercial fish species, e.g., *M. merlucius*, and in all cases, the larvae were not found in fish musculature (Mattiucci et al., 2004). Finally, the species from clades 3 and 4 are very rarely reported to penetrate fish muscle, at least those of commercial importance (Mattiucci et al., 2018; Palm et al., 2008).

2.2.4. Anisakiasis

Epidemiology

The first case of human infection by a species of the Anisakidae family occurred in the 1960s, when Van Thiel of the Institute of Tropical Medicine in Leiden, the Netherlands, identified patients who suffered severe abdominal pain after eating fish (Van Thiel et al., 1960). In the original article, the authors incorrectly identified the helminth as *Eustoma rotundatum*. Later, in 1962, Van Thiel realized his mistake and identified the helminth as *Anisakis* spp. larva (Van Thiel, 1962). Since the 1960s, the term anisakiasis had been used for a human disease caused by the third-stage larvae (L3) of members of the family Anisakidae. In 1988, a standardized nomenclature recommended three different terms: (1) anisakidosis caused by any members of the family Anisakidae, (2) anisakiasis caused by members of the genus *Anisakis*, and (3) pseudoterranovosis caused by members of the genus *Pseudoterranova* (Audicana & Kennedy, 2008; Klimpel & Palm, 2011). Prior to 2010, over 20,000 cases of anisakiasis were reported worldwide, with the highest prevalence (over 90%) in Japan (Baird et al., 2014; EFSA, 2010), where 7,000 cases of the disease are currently reported annually (Suzuki et al., 2021; Yorimitsu et al., 2013). Recent data shown by Orphanet (Prevalence of rare diseases, 2022) as reported by Guardone and colleagues in a recent retrospective epidemiological study of human anisakiasis (Guardone et al., 2018), estimated a global incidence of 0.32 cases per 100,000 inhabitants/year. The worldwide introduction of a different cuisine, the development of better diagnostic tools, and a greater knowledge of *Anisakis* and its infection have resulted in significantly more cases of anisakiasis being reported. In addition to the Netherlands, Japan, and the aforementioned European countries (Spain, Italy, France, Croatia), where dishes made from raw fish are very commonly consumed, cases of anisakiasis have also been reported in other countries and continents, including China (Qin et al., 2013), Korea (Sohn et al., 2015), Malaysia (Amir et al., 2016), Taiwan (Li et al., 2015), Egypt, the United Kingdom, Germany (Audicana & Kennedy, 2008), Denmark (Andreassen & Jorring, 1970), Norway (Jacobsen & Berland, 1969), the United States of America (Kliks, 1983; Kojima et al., 2013), Australia (Shamsi & Butcher, 2011), South America (Eiras et al., 2018), and South Africa (Audicana & Kennedy, 2008), meaning that anisakiasis is present throughout the globe, with the exception of Antarctica (Aibinu et al., 2019). In addition, a recently published meta-regression analysis revealed

a significant 283-fold increase in the rate of *Anisakis* spp. infection in fish (average number of worms/fish) over a 53-year period from 1962 to 2015 (Fiorenza et al., 2020). This increase in the infection rate of *Anisakis* spp. in fish and broadly understood globalization may have implications for human health, marine mammals health, and fisheries profitability.

Transmission of the parasite is clearly associated with the consumption of raw or undercooked fish. In particular, Japanese sushi and sashimi, Dutch salted or smoked herring, Nordic gravlax (dry, cured salmon), Hawaiian lomi-lomi (raw salmon), German rollmops (rolled fillet of marinated/pickled herring), South American cebiche, and Spanish boquerones en vinagre (pickled anchovies) are regular routes of infection (Audicana et al., 2002; Ivanovic et al., 2015).

Symptoms and clinical manifestation

Infective L3 larvae of *Anisakis* spp. usually do not molt into L4 stage in humans. However, few cases of the presence of L4 stage larvae were confirmed. In 1986, Kliks (1986) identified "a partially molted adult male" *A. simplex in situ* attached to the duodenum of a patient suffering acute abdominal pain and vomiting of four days' duration. The L4 stage larvae of *A. simplex* (type I) was also described in human patients in the Ooiwa Gastroenterological Clinic in Fukuoka, Japan (Weerasooriya et al., 1986). In a case of a patient with multiple infections of *A. simplex* larvae, the presence of L4 stage was also confirmed (Kagei & Isogaki, 1992). The recent case study from Korea showed that among 32 *Anisakis* (type I) larvae detected, 7 were in the fourth stage (L4), which had molted in the stomach of the patients (Sohn et al., 2015).

Despite the stage of the larvae, based on gastrointestinal site reached by living larvae, the disease is often subdivided into gastric anisakiasis (GA), intestinal anisakiasis (IA), ectopic anisakiasis (EA), and gastroallergic anisakiasis (GAA). In addition, anisakiasis is clinically classified as 'acute' or 'moderate' and as 'invasive' or 'noninvasive', depending on the condition and vitality of the larvae. The 'noninvasive' form occurs when the larva cannot penetrate the gastric or intestinal mucosa and remains luminal. In this case, the patient may be asymptomatic, and the larva may be excreted in the stool and/or vomit. The 'invasive' form is characterized by several steps of parasite infection: (1) attachment to the gastric or intestinal mucosa and (2) active penetration of the larva into the tissues

of the gastrointestinal tract. In the case of 'invasive anisakiasis', severe gastrointestinal symptoms are described during the acute phase of infection, such as epigastralgia, nausea, vomiting, and abdominal pain. Chronic infection of anisakiasis is characterized by anisakis larvae penetrating deep into the gastric and intestinal walls causing direct tissue damage, ulcers, and eventually eosinophilic granulomas (Aibinu et al., 2019; Audicana et al., 2002; Audicana & Kennedy, 2008; Ivanovic et al., 2015; Mattiucci et al., 2018).

In GA, a preference for invasion of the greater curvature of the stomach has been suggested, with the larva embedding itself in the stomach walls. Usually, significant gastric mucosal oedema is observed endoscopically in the gastric mucosa around the area of penetration. The typical clinical presentation is usually acute severe epigastric pain occurring a few hours after consumption of infected fish, with symptoms developing within 12 hours. Cases of GA due to *A. pegreffii* described in Italy were characterized clinically by epigastric pain just after 2 hours following the ingestion of the raw seafood, vomiting, and other digestive symptoms (Mattiucci et al., 2013). In IA, nonspecific clinical features such as nausea, vomiting, or diarrhea occur within 5 days after consumption of infected fish. It is known that intestinal infection takes longer for anisakiasis symptoms to manifest than GA (Shimamura et al., 2016; Takabayashi et al., 2014). The results of Guardone et al., (2018) showed that the frequency of localization of *Anisakis* larvae in the stomach and intestine during infection was very similar, with only a slightly higher frequency of GA. Also, in a retrospective case series study conducted in Tokyo, Japan, 47% of patients had GA and 53% had IA (Takabayashi et al., 2014). However, other authors note that in Japan, the acute gastric form predominates (95%), whereas in Europe, the chronic intestinal form appears to be more common (Montalto et al., 2005; Shimamura et al., 2016). In addition, it has been recognised that patients with intestinal anisakiasis are often misdiagnosed with other diseases such as inflammatory bowel disease, intestinal obstruction, ulcers, acute appendicitis, diverticulitis, ileitis, or cholecystitis (Miura, 2010).

To invade the GI mucosa, the L3 of *A. simplex* probably use mechanical destruction of the tissue in conjunction with the release of potent proteolytic enzymes capable of degrading the extracellular matrix (Audicana & Kennedy, 2008). These proteases are likely produced by the dorsal esophageal excretory gland cell of the larva to be secreted through the excretory pore and mouth opening (Mladineo et al., 2019). The invasive ability of the larvae, together with the presence of anticoagulant substances in the products of E/S system, explains the existence of the multiple, well-defined, erosive

and/or hemorrhagic lesions usually detected near the main lesion in the gastric mucosa of patients with anisakiasis (Audicana & Kennedy, 2008).

Protective responses to intestinal nematodes are associated with the production of T helper-type (Th2) cytokines and the resulting mastocytosis, immunoglobulin E-mediated (IgE) response, and eosinophilia, all of which are typical of allergic reactions and immune responses to tissue-parasitic helminths (Anthony et al., 2007). However, human anisakiasis is special because this parasite is not adapted to life in humans and the infection is transient. Moreover, more than 90% of the cases described are caused by a single larva. Therefore, differences between the pathogenesis of *A. simplex* and that caused by other helminths in humans are to be expected (Audicana & Kennedy, 2008). In both acute and chronic anisakiasis, eosinophilic infiltration was noted in the tissues surrounding the parasite. The high concentration of eosinophilic cells in the infested areas seems to be related not only to the production of chemotactic factors released by T lymphocytes, mast cells, and basophils, but also to some chemotactic substances produced directly by the larvae of *Anisakis* spp. that attract these cells. Eosinophil infiltration is the most effective process in destroying larvae at the level of the gastrointestinal tract, as immune hypersensitivity in response to *Anisakis* infection (Nieuwenhuizen, 2016).

The immunopathology of anisakiasis is based together on innate and adaptive immune responses and was presented in detail by Audicana and Kennedy (2008). In brief, the human patient can be exposed to *A. simplex* antigens from several sources: E/S antigens from living larvae and somatic and cuticular antigens from dead and disintegrating larvae present in food. Epithelial cells are often the first responders to immune and inflammatory stimuli in the recruitment and activation of inflammatory cells in response to enteric parasites. Epithelial cells can secrete cytotoxic molecules such as nitrogen monoxide (NO) as well as chemokines and cytokines that attract polymorphonuclear leukocytes (PMN), tissue macrophages (MΦ), naïve dendritic cells (NDC), basophils, and eosinophils. Innate responses may also involve toll-like receptors (TLRs) from epithelial cells and activated dendritic cells (ADC). In the adaptive response, antigen presentation by mature dendritic cells (MDC) stimulates a dual Th1 and Th2 response. Other T cells may be recruited as T- regulatory cells and Th3. Th1 cytokines (IFN-γ, TNF-β, IL-2 and IL-3) induce IgG2a (Akao & Yoshimura, 1989), opsonizing and complement-fixing antibodies, macrophage activation, antibody-dependent cell-mediated cytotoxicity and delayed-type hypersensitivity. Th2 cytokines (IL-4, IL-5, IL-6, IL-9, IL-

10, and IL-13) promote IgG1 and IgA production, and stimulation of T lymphocytes produces antigen specific IgE and total/polyclonal IgE. Mastocytosis and eosinophilia are triggered by a Th2 response and chemoattractant cytokines and may be responsible for parasite expulsion. Basophils are critical for triggering a Th2 response (Audicana & Kennedy, 2008; Nieuwenhuizen, 2016; Nieuwenhuizen & Lopata, 2014).

Symptoms of GAA range from urticaria and angioedema to life-threatening anaphylactic shock, which is usually accompanied by gastrointestinal symptoms. Allergic reactions may occur after primary infection with *Anisakis* larvae and exposure to allergenic proteins in food. The potential for type I hypersensitivity reactions in GAA is evidenced by the fact that serum anti-*A. simplex* IgE levels rise rapidly from 2 or 3 hours to 2 or 3 days after consumption of infected fish and remain high for months or years (Audicana & Kennedy, 2008; Daschner et al., 2000; Ivanovic et al., 2015; Ivanović et al., 2017). It is noteworthy that >50% of *A. simplex*-allergic patients required emergency treatment, and five out of 64 were hospitalized because of respiratory arrest (one case), severe anaphylactic shock (two cases) and persistent angioedema (three cases) (Fernández de Corres et al., 1966). However, there are increasing cases of allergy to *A. simplex* larvae after consumption of fish that has undergone proper heat treatment (see 2.2.4 *Diagnosis, detection, and prevention*), as well as occupational allergies in persons employed in the fish processing industry. This is due to the thermoresistance of larval antigens and the lack of degradative effect of high or low temperatures on their sensitizing ability (Moneo et al., 2005; Rodríguez-Mahillo et al., 2010). Currently, 36 *A. simplex* antigens are described in the literature. They were divided into three groups: (1) excretory-secretory, secreted mainly during larval penetration of the host gastrointestinal tract wall, (2) cuticular, protecting the larva from digestive enzymes, and (3) somatic antigens present in the larval body (Baird et al., 2014, 2016; Fæste et al., 2014; Sakanari & McKerrow, 1990; Valls et al., 2005). However, the situation in which species hybridize may allow for the possible evolution of new adaptive traits to environmental conditions where such a hybrid exists. Llorens et al., (2018) obtained no less than 121 different allergens from hybrids compared to the parent individuals *A. simplex* (36 allergens) and *A. pegreffii* (39 allergens).

Because of the thermostable properties of some *A. simplex* allergens, some authors believe that dead larvae can cause allergic reactions. In a study by del Pozo et al., (1996) with *A. simplex*, extracts were prepared and exposed to temperature (40 °C for 10 minutes and 100 °C for 20 minutes) and then administered to patients, followed by a skin test,

which results were positive. The allergic properties of *A. simplex* extract were also confirmed by Rodríguez-Mahillo et al., (2010) using the basophil activation test (BAT). In addition, there are reports of *Anisakis* allergies in humans caused by the consumption of chicken meat fed with fish meal (Armentia et al., 2006).

Diagnosis, detection, and prevention

The available diagnostic methodologies currently used to confirm human anisakiasis are presented below. Endoscopy is commonly used to remove larvae of *Anisakis* spp. from the stomach and intestines. However, the very limited specific diagnostic characteristics of individual *Anisakis* spp. larvae available on the basis of morphological examination allow identification only at the genus level. If the removed larva is spoiled or fragmented so that it is unusable for microscopy, and identification may not be possible. Eosinophilic granulomas surgically removed from gastric and intestinal areas may show the presence of worms with the characteristic morphological features of larvae of *Anisakis* spp. Histological preparations (H&E) can reveal relevant features using conventional light microscopy, but only if the nematode in the nodule is in a good state of preservation (Mattiucci et al., 2018; Mladineo et al., 2015b). Molecular diagnostics allowed rapid and accurate identification of the causative agent of human anisakiasis. Paoletti et al., (2018) recently developed a RT-PCR DNA probe test, that identified *A. pegreffi* from an eosinophilic granuloma in the intestine (Mattiucci, Paoletti, et al., 2017). Serodiagnostic methods are based on human IgE response, using *Anisakis* spp. specific E/S products and/or crude extract (CE). Serodiagnosis currently is based on immunoCAP (iCAP) assay, immunoblotting by Western blot (WB), and enzyme-linked immunosorbent assay (ELISA), however, the iCAP assay detection specificity is overestimated (Mattiucci et al., 2017).

The European Union has introduced regulations for some food-borne parasites, such *Trichinella* spp., *Taenia* spp. and *Anisakis* spp. (European Commission, 2004a, 2004b, 2015). Annex III, Section VIII, Chapter III, point D of Regulation (EC) No 853/2004 stipulates the fishery products to be consumed raw or almost raw must be frozen at a temperature of not more than - 20 °C in all parts of the product for not less than 24 hours. Freezing also applies if they are to undergo a cold smoking process in which the internal temperature of the fishery product is not more than 60 °C (herring, mackerel, sprat, wild

Atlantic and Pacific salmon) and marinated and/or salted fishery products if the processing is insufficient to destroy nematode larvae. Chapter V, point D of the same regulation provides that:

'Food business operators must ensure that fishery products have been subjected to a visual examination for the purpose of detecting visible parasites before being placed on the market. They must not place fishery products that are obviously contaminated with parasites on the market for human consumption.'

Annex II, Section I, of Commission Regulation (EC) No 2074/2005 lays down detailed rules relating to visual inspections to detect parasites in fishery products. However, due to the low overall efficacy of candling, recently published study recommends further assessment of its cost–benefit and impact on consumers' health risk (Mercken et al., 2022). Lastly, Annex III, Chapter II, point F of Regulation (EC) No 854/2004 provides that the authorities of a Member State are to carry out random testing to verify compliance with Community legislation on parasites. According to the EU regulations, cooking or pre-freezing are the most effective ways of killing the larvae that may be present in the flesh of the fish. However, the GAA in term of thermoresistant antigens continues to be a serious problem. In addition, recent studies show that the regulations adopted so far by the EU should be evaluated and updated (Łopieńska-Biernat et al., 2020).

Molecular methods for *Anisakis* spp. detection in fish and fishery products are limited to the PCR reaction and the method most often used to detect *Anisakis* spp. proteins, which is ELISA. Unfortunately, there are no commercial enzyme immunoassays capable of detecting *Anisakis* spp. antigens in fish and fish products intended directly for the consumers (Kochanowski et al., 2020). Other methods have been developed to detect the presence of *Anisakis* spp. in fish products (Carrera et al., 2016; Carrera, Pazos, et al., 2020; Fæste et al., 2014, 2015, 2016). However, so far, they have not been widely used. Among these methods, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) deserves attention. It is a technique that allows the identification and quantification of chemical compounds. Despite its high sensitivity, this technique is recommended by the authors as a method for confirming the presence of *Anisakis* spp. proteins in a sample in advanced research units due to its high cost and equipment requirements. This topic will be discussed in more detail in the second part of the thesis on proteomics and its applications in studies on Anisakidae (refer to 2.3.).

2.3. Proteomics

2.3.1. Short history and definition

The most general definition of proteomics states that it is the large-scale study of proteins and proteomes. In the broadest sense, the proteome of biological systems (e.g., cell lines, tissues, or body fluids) describes the totality of all expressed proteins, including all protein isoforms and modifications, as well as the interactions between individual proteins at a given time and under given conditions. The proteome is not constant. It differs from cell to cell and changes over time, making it more difficult to determine (Anderson & Anderson, 1998; Blackstock & Weir, 1999).

The term "proteome" was first coined and used by Marc R. Wilkins in 1994, which founded the first dedicated proteomics laboratory in 1995 (Wasinger et al., 1995). However, the first studies of proteins that could be called proteomics began as early as 1975, after the introduction of two-dimensional gel electrophoresis (2-DE) (orthogonal separation by isoelectric point and molecular mass) and the mapping of proteins from the bacterium *Escherichia coli* (O'Farrell, 1975). Since then, proteomics has undergone a very rapid and spectacular development.

The rapid development of proteomics was mainly based on the advances in mass spectrometry (MS) initiated by the development of soft ionization methods, i.e., matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), which contributed to the improvement of protein identification methods, an essential stage of proteomics research, as well as to advances in protein separation techniques and analysis of the obtained results (Fenn et al., 1989; Hillenkamp et al., 1991). However, it was not until electrospray was coupled on-line with liquid chromatography (LC) and algorithms were developed to identify digested peptides based on their fragmentation spectra and comparison with theoretical spectra from in sequence databases (a technique called bottom-up or shotgun proteomics) that a new direction in the development of proteomics began. Many further advances in mass spectrometry and related fields were necessary to enable large-scale studies of peptides and proteins and to create the field of mass spectrometry (MS)-based proteomics (Aebersold & Mann, 2003; Beck, 2016; Link et al., 1999; Tabet & Rebuffat, 2003).

2.3.2. Mass spectrometry-based proteomics

Mass spectrometric measurements are performed in the vacuum of ionized analytes. By definition, a mass spectrometer consists of an ion source, a mass analyzer that measures the mass-to-charge ratio (*m/z*) of the ionized analytes, and a detector that records the number of ions at each *m/z* value (Aebersold & Mann, 2003). Since the invention of techniques that allow peptides and proteins to be ionized without destroying them, i.e., the aforementioned ESI and laser desorption, proteomics methods based on MS have evolved greatly, especially since high-performance liquid chromatography (HPLC) has been used to separate peptide mixtures.

In the proteomic field two main approaches: top-down and bottom-up were distinguished. In the top-down approach, intact proteins and fragments produced from them are analysed in a mass spectrometer (Catherman et al., 2014; McLafferty et al., 2007). In principle, this method can cover the entire protein sequence and detect all different protein isoforms and modifications. However, the analysis of complex intact protein mixtures or very large proteins with LC-MS is extremely difficult because proteins are often insoluble, difficult to separate chromatographically, and have many other charge states. Therefore, the bottom-up approach also called shotgun proteomics, based on the analysis of peptides from a crude proteome digest, is much simpler and by far the most commonly used method (Aebersold & Mann, 2003; Cravatt et al., 2007; Yates et al., 2009) (Figure 3).

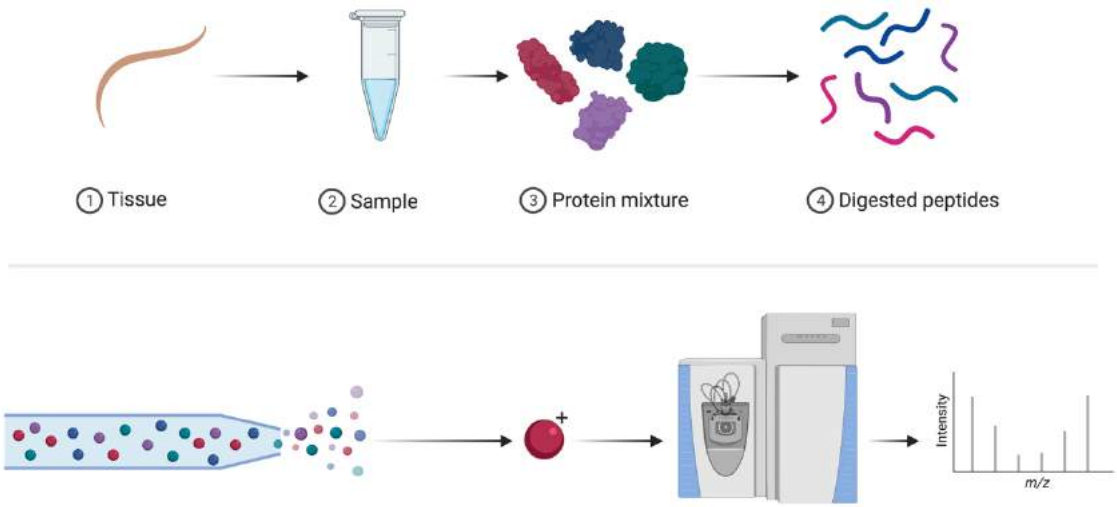


Figure 3. Typical bottom-up/shotgun MS-based proteomics workflow. Proteins derived from cells, tissues, body fluids are digested, and the resulting peptides are then separated chromatographically. Peptides then are ionized and analysed in the mass spectrometer. Created with BioRender.com.

In the first step of the standard bottom-up workflow, proteins are extracted from the source of interest, such as cells, tissues, or whole organisms. This involves lysis of the samples and direct isolation of the proteins, followed by digestion of the protein mixture. This is usually done with a sequence-specific enzyme, such as trypsin, which cleaves the C-terminal arginine or lysine (Olsen et al., 2004). The mixture is then usually purified and therefore separated by reversed-phase liquid chromatography (RPLC) depending on its hydrophobicity. For proteomics, a chromatography column with C_{18} material (reversed phase C_{18} silica beads) is almost always used. Peptide mixtures are acidified by addition of an amphiphilic acid (FA or TFA), which protonate the basic residues of the peptides (Arg, Lys, His, Pro) and for that acquire single or multiple charges according to the number of basic residues in the sequence. They are eluted from the column by increasing the percentage of organic phase in the mobile phase, which is achieved by gradually changing the mixing ratio of aqueous and organic buffer solvent in the buffer (Neverova & van Eyk, 2005). Peptides eluting from the column are then ionized by electrospray ionization, generally leaving them with at least two charges (at the basic N-terminus and at the C-terminal of arginine or lysine residue due to the trypsin cleavage), or more charges for larger peptides according to the number of basic residues, before entering the mass spectrometer. Very complex peptide mixtures benefit greatly from reducing sample complexity through fractionation, resulting in more identifications after LC-MS/MS analysis. As the next step in the bottom-up MS-based proteomics workflow, ions generated by ES enter the mass spectrometer, where mass analysis takes place. Various mass spectrometer configurations and scan modes are available for the analysis of complex peptide mixtures (Beck, 2016).

2.3.3. Tandem mass spectrometry

Tandem mass spectrometry (MS/MS, MS2) involves two stages of MS (McLafferty, 1981). Molecules of a given sample (i.e., mixture of peptides) are ionized, and then analyzed in the mass spectrometer in a first stage (MS1) that separates ions according to their *m/z* ratio. Ions from the MS1 full scan with a certain *m/z* ratio are selected and then fragmented in a collision cell mainly with He molecules into smaller product ions. These ions are then sent to the second analyzer (MS2), to measure their *m/z* ratio. The

fragmentation step makes it possible to identify and separate ions with very similar *m/z* ratios. Tandem mass spectrometry can be classified into the two categories: "tandem in space" and "tandem in time" (Johnson et al., 1990). In "tandem in time" mass spectrometers the analysis of parent ions and product ions is performed consecutively in the same mass analyzer but at different times. In contrast "tandem in space" instruments record the mass of parent ions and product ions in different mass analyzers or spaces.

There are many methods used to fragment the ions and these can result in different types of fragmentation and thus different information about the structure and composition of the molecule. In MS/MS-based proteomics the most used are collision-induced dissociation (CID), higher energy C-trap dissociation (HCD), or electron transfer dissociation (ETD). While CID and HCD are very effective for the fragmentation of tryptic peptides and peptides with stable modifications, ETD may be preferable for intact proteins and longer peptides as well as for peptides carrying labile modifications (Mikesh et al., 2006).

2.3.4. The Orbitrap mass analyzer

Various mass analyzers can be divided into two major groups based on their principle of operation: beam type analyzers that continuously scan ions (time-of-flight (TOF) and quadrupole (Q)), and trap-based analyzers that capture ions of interest for a specific time to acquire a mass spectrum (Fourier transform ion cyclotron resonance (FT-ICR), ion trap, and orbitrap) (Figure 3). TOF instruments can be connected to a MALDI source to perform pulsed analysis. In contrast, trapping mass analyzers are typically coupled to an ESI source. All devices have unique characteristics and therefore differ in resolution, mass accuracy, scan speed, *m/z* range, dynamic range, and sensitivity. All have seen dramatic improvements in these parameters in recent years (Beck, 2016). For the purposes of this thesis, the Orbitrap mass analyzer is discussed in more detail.

The establishment of the Orbitrap analyzer by Alexander Makarov in 2000 and commercial introduction in 2005 led the way to further advancements and applications in proteomics (Hu et al., 2005; Makarov, 2000; Makarov et al., 2006). The device consists of an outer barrel-shaped electrode and an inner spindle-shaped electrode. Ions entering the mass analyzer are trapped as they travel around (orbit) and along the center electrode. The oscillation along the field axis is specific to the *m/z* value of the ion and can be measured with high accuracy using image current detection at the segmented outer

electrode. The recorded image stream is Fourier Transform (FT) processed to convert the information into a mass spectrum. The Orbitrap mass analyzer is characterized by the continuous improvement in mass resolution and mass accuracy over the past 10 years, as well as a high dynamic range. Following the first hybrid Orbitrap mass spectrometer, the LTQ Orbitrap (Makarov et al., 2006), Thermo Fisher Scientific introduced the LTQ-Orbitrap Elite, which features a dual linear ion trap and a higher resolution Orbitrap analyzer (Michalski et al., 2012). The Q Exactive introduced was the quadrupole-orbitrap combination (Michalski et al., 2011); the latest version of this configuration is called Q Exactive HF, with a high-resolution orbitrap mass analyzer and an improved Fourier transformation algorithm (Scheltema et al., 2014). Due to the combination of high sequencing rates and very high resolution and mass accuracy achieved by hybrid Orbitrap mass spectrometers, they have become the workhorse of most proteomics laboratories, especially for shotgun proteomics (Makarov, 2019).

2.3.5. Quantitative proteomics

Quantitative proteomics is used to determine the relative or absolute amount of proteins in a sample. Over the years, various approaches to relative and absolute quantification have been developed and can be divided into label-free quantification (LFQ) and label-based quantification (LBQ). Depending on the quantification approach, samples are combined at different stages of sample preparation, resulting in differences in the accuracy and robustness of quantification. In addition, depending on the strategy, quantitative information is extracted at either the MS1 or MS2 level (MS/MS) (Bantscheff et al., 2007).

Label-free quantification can be divided into two different groups depending on the method used for data extraction. On the one hand, quantification can be derived by counting the number of peptides or spectra associated with a given protein and are therefore commonly referred to as spectral counting methods. On the other hand, when coupling liquid chromatography with mass spectrometry, quantitative values can be measured by extracting the area of the chromatographic peaks of the precursor ions – the area under the curve (AUC) or MS1 signal intensity methods. However, the different samples are not mixed, but each sample is measured in separate MS runs. Since each sample is treated separately until data analysis, artifactual quantitative differences may

occur during sample processing. Therefore, the LFQ may be less accurate compared to label-based techniques (J. Cox et al., 2014).

Label-based quantification approaches can be divided into two main categories: metabolic labeling, and chemical labeling. In metabolic labeling, the metabolism of living cells or organisms is used to introduce stable isotopes by feeding them with heavy-isotope-modified amino acids. In stable isotope labeling with amino acids in cell culture (SILAC) heavy isotope substituted forms of Arg and/or Lys are used (Ong & Mann, 2007). Another approach to metabolic labeling is chemical labeling, which can be applied to any sample. In chemical labeling methods, stable isotopes are introduced late in the sample preparation workflow. This can be done at the protein or peptide level. Several chemical labeling strategies have been developed, including isotope-coded affinity tags (ICAT), isobaric tags for relative and absolute quantification (iTRAQ), and tandem mass tags (TMT). TMT is described in more detail because it plays an important role in this thesis.

Tandem mass tags

Tandem mass tagging (TMT)-based shotgun proteomics enables multiplexed relative quantification and identification of proteins. An example of a TMT-based analysis protocol is shown in Figure 4. Each mass tagging reagent has the same nominal mass (isobaric) and a chemical structure consisting of an amino-reactive NHS ester group, a spacer arm, a mass reporter, and a mass normalizer (Figure 4). The chemical structures of all the tags are identical, but each contains substituted isotopes at different positions, so that the mass reporter and mass normalizer regions in each marker have different molecular masses. When fragmenting in MS/MS mode, sequence information is obtained from fragmentation of the peptide backbone and quantification data based on the relative intensities of the reporter ions specific to the labels (McAlister et al., 2012; Rauniyar & Yates, 2014; Thompson et al., 2003).

Different TMT reagents are currently available (TMT-duplex, TMT 6-plex, TMT 10-plex, TMT 16-plex) and their choice depends on the experiment we are conducting. Each of the TMT labels has a different structure and isotopic position. TMT labels have different numbers and combinations of 13C and 15N isotopes in the mass reporter, resulting in each peptide in a studied sample having a monoisotopic mass difference on a

scale, i.e., TMT 10-plex, from 126 to 131 Da (126, 127C/N, 128C/N, 129C/N, 130C/N, 131) (Stryiński et al., 2021).

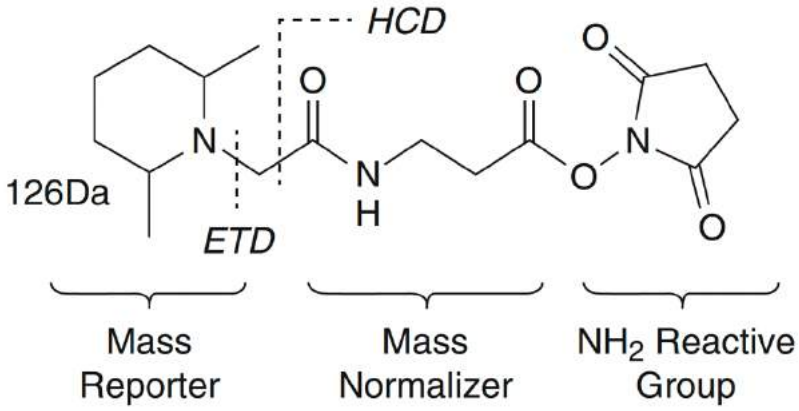


Figure 4. Chemical structure of the tandem mass tag. MS/MS fragmentation sites marked in the picture as higher energy collision dissociation (HCD) site and electron transfer dissociation site (ETD). Adapted from Stryiński et al. (2021).

Currently, only Orbitrap instruments can achieve the resolution required to resolve the 6 mDa differences needed to measure all 10 channels of the TMT 10-plex. Advantages of isobaric quantification strategies at the MS2 level include no dilution effect at the MS1 level due to multiple samples, no redundancy in MS/MS scan events due to multiple precursor ion species and bypassing the capacity limit of the C-trap by not storing the entire mass range for quantification. The major drawback is the expensive reagents for TMT-based sample preparation in the proteomic workflow (Beck, 2016). Typical TMT-based analysis protocol is presented in the Figure 5.

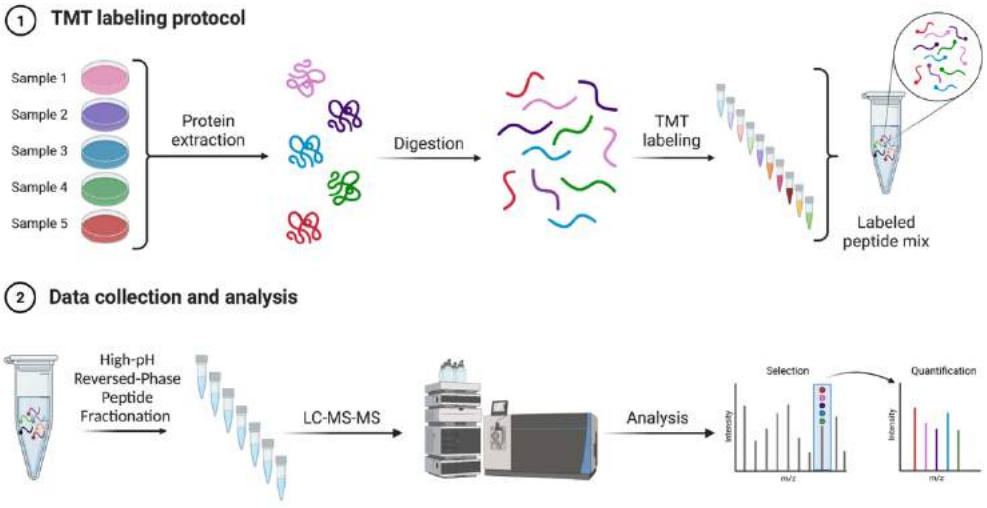


Figure 5. TMT-based analysis protocol. Created with BioRender.com

2.3.6. Discovery and targeted proteomics in parasitology

In the last two decades, proteomics has become a powerful tool for describing dynamically changing biological systems. Currently, the practical applications of proteomics have made two proteomics strategies very clearly visible. Scientists distinguished two characteristic approaches using proteomic methods, i.e., discovery and targeted workflows (Aebersold et al., 2016; Aebersold & Mann, 2003; Borràs & Sabidó, 2017; Carrera, Piñeiro, et al., 2020; Gallardo et al., 2013; Martinez et al., 2020; Stryiński et al., 2020, 2022) (Figure 6). Discovery proteomics is applied to identify and characterize the proteins in the sample (e.g., global proteomes, cellular, subcellular, or E/S proteomes), while targeted proteomics is monitoring the previously identified and selected protein biomarkers (single or multiple peptides) in analyzed samples. The techniques that proteomics offers are one of those most interesting new tools and approaches used for health management, epidemiology, disease research, and in food science (Ahmed et al., 2019; Cifuentes, 2013; Herrero et al., 2012; Moreira et al., 2021; Rodrigues et al., 2012, 2016).

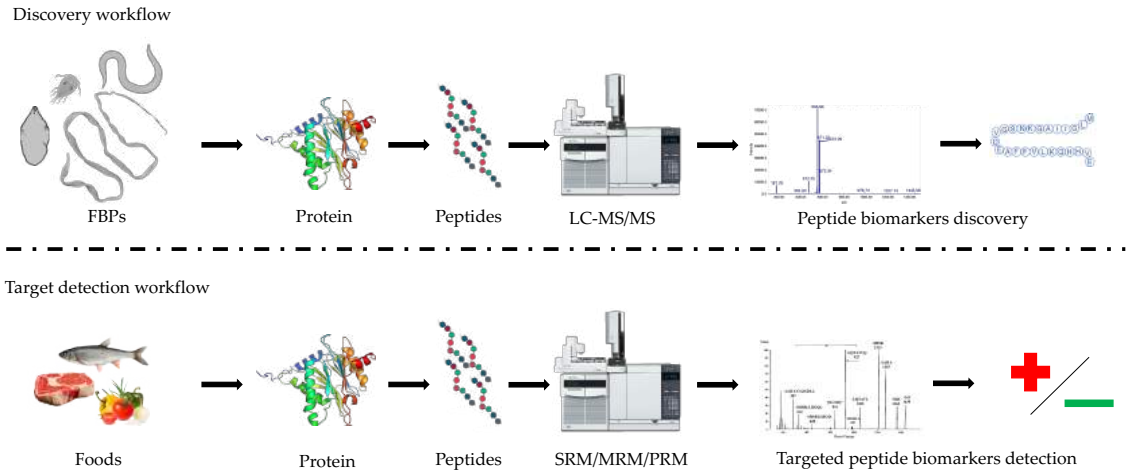


Figure 6. Schematic representation of two proteomic strategies in the studies of foodborne parasites (FBPs): discovery and targeted workflows. Red plus—positive detection (sample contaminated); green minus—negative detection (sample free); LC-MS/MS—liquid chromatography tandem mass spectrometry; SRM/MRM/PRM—selected/multiple/parallel—reaction monitoring. Adapted from Stryiński et al. (2020).

It is therefore not surprising that applications of proteomics have gained recognition in diverse branches of science such as animal physiology, pathology, widely recognized parasitology, and the more specific characterization of host-pathogen interactions. The reviews on the application of proteomics in parasitology were prepared by the author of

this thesis. Therefore, the reader is advised to review the more comprehensive information cited here (Stryiński et al., 2020, 2022).

3. OBJECTIVES

Anisakis simplex is a cosmopolitan parasitic nematode of marine organisms characterized by a complex life cycle. Massive infestations of *A. simplex* larvae have been observed in many commercially important fish species such as herring and cod. Consumption of fish containing larvae can pose a serious health risk, as these parasites are capable of penetrating the mucous membranes of the gastrointestinal tract, causing damage to the stomach and intestinal walls and triggering severe allergic reactions. According to the European Food Safety Authority (EFSA), a panel of experts has classified *A. simplex* as a biohazardous organism.

The factors that allow the parasites to survive in the host gastrointestinal tract need to be identified in order to develop effective methods of treating anisakiasis as well as methods of neutralizing the larvae.

The invasive L3 stage of the nematode does not usually develop into the L4 stage in humans, but cases have been reported in the literature where the L4 stage has occurred in humans. Therefore, this developmental stage should also be investigated. In addition, direct contact of the parasite with the host via the cuticle and the excretory and secretory products of the nematode intestine are largely responsible for host-parasite interactions. In *A. simplex*, the intestinal lumen of L3 larvae is shrunken, and intestinal functionality and permeability are restored at the L4 stage of development. It is thought to intestine "takes over" selected nutritional functions from the cuticle - the outer layer of the nematode body - during development.

The differences observed so far at the morphological and molecular levels have led us to formulate the following research hypotheses:

1. L3 and L4 larvae of *A. simplex* nematodes differ in their proteomic profiles at the global level;

2. the tissues, the fully functional intestine of the L4 stage of *A. simplex*, in contrast to the L3 stage, and the cuticle are characterized by specific proteins responsible for growth, development and contact with the host.

Therefore, our goal was to study the global and tissue proteome of *A. simplex* (refer to section 4.).

4. METHODOLOGY AND RESULTS

4.1. Global proteome of human-infecting developmental stages of *Anisakis simplex*

Manuscript I

Proteome profiling of L3 and L4 *Anisakis simplex* development stages by TMT-based quantitative proteomics.

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In 2017, due to the lack of data in the literature on the proteomic analyzes of *A. simplex* and the characteristics of the proteomes of its developmental stages, as a parasite of public health importance, together with my supervisor at the time, Elżbieta Łopieńska-Biernat, PhD, we started a close collaboration with specialists in the field of proteomics and spectrometry, such as Mónica Carrera Mouriño, PhD, from Department of Food Technology, Institute of Marine Research, Spanish National Research Council in Vigo, Spain.

The hypothesis that accompanied us was that the proteomes of the L3 and L4 stages of *A. simplex* are different and that these differences could contribute to the understanding of the pathogenicity of this parasite. Therefore, our aim was to investigate the global proteome of the L3 and L4 stages of *A. simplex* using LC-MS/MS analysis in an LTQ-Orbitrap Elite mass spectrometer.

Using TMT labeling and high pH reverse phase fractionation of peptides from each developmental stage of the nematode, we have identified approximately 2,500 proteins in *A. simplex*, the largest proteome coverage reported to date for this organism. In addition, the obtained results were subjected to statistical and bioinformatic analysis, the results of which allowed a global proteomic comparison of the *A. simplex* stages studied.

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Proteome profiling of L3 and L4 *Anisakis simplex* development stages by TMT-based quantitative proteomics

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ABSTRACT

Anisakis simplex is a parasitic nematode that can cause anisakiosis and/or allergic reactions in humans. The presence of invasive third-stage larvae (L3) in many different consumed fish species and the fourth-stage larvae (L4) in marine mammals, where L3 can accidentally affect to humans and develop as far as stage L4. World Health Organization and food safety authorities aim to control and prevent this emerging health problem. In the present work, using Tandem Mass Tag (TMT)-based quantitative proteomics we analyzed for the first time the global proteome of two *A. simplex* development stages, L3 and L4. The strategy was divided into four steps: (a) protein extraction of L3 and L4 development stages, (b) high intensity focused ultrasound (HIFU)-assisted trypsin digestion, (c) TMT-isobaric mass tag labeling following by high-pH reversed-phase fractionation, and (d) LC-MS/MS analysis in a LTQ-Orbitrap Elite mass spectrometer. A total of 2443 different proteins of *A. simplex* were identified. Analysis of the modulated proteins provided the specific proteomic signature of L3 (i.e. pseudocoelomic globin, endochitinase 1, paramyosin) and L4 (i.e. neprilysin-2, glutamate dehydrogenase, aminopeptidase N). To our knowledge, this is the most comprehensive dataset of proteins of *A. simplex* for two development stages (L3 and L4) identified to date.

Significance: A. simplex is a fish-borne parasite responsible for the human anisakiosis and allergic reactions around the world. The work describes for the first-time the comparison of the proteome of two *A. simplex* stages (L3 and L4). The strategy is based on four steps: (i) protein extraction, (ii) ultra-fast trypsin digestion under High-Intensity Focused Ultrasound (HIFU), (iii) TMT-isobaric mass tag labeling followed by high-pH reversed-phase fractionation and (iv) peptide analysis using a LTQ-Orbitrap Elite mass spectrometer. The workflow allows to select the most modulated proteins as proteomic signature of those specific development stages (L3 and L4) of *A. simplex*. Obtained stage-specific proteins, could be used as targets to control/eliminate this parasite and in future eradicate the anisakiosis disease.

1. Introduction

Anisakis simplex (*A. simplex*) is a fish-borne parasitic nematode that can causes anisakiosis and/or allergic reactions in humans [1]. It is characterized by a complex life cycle involving several hosts, where adult stages live in marine mammals. Its paratenic hosts are sea fish or cephalopods [1,2]. *Anisakis*, particularly third-stage larvae (L3) from cold-blooded fish, can accidentally affects to humans and develop to L4 stage [3,4]. The entire development cycle of *A. simplex* depends on the temperature of the water [5]. Due to continuous climate change and rising temperatures, *A. simplex* extends its range, resulting appearance of this species in the seas and oceans where it has not previously been found before. That might have a direct effect on the growing number of many other fish species infections [6–8]. The risk of anisakiosis is mainly originate by the consumption of raw or undercooked marine fish or cephalopods parasitized with L3 larvae. Occasionally, L3 moults into L4 larvae in humans, but they do not progress to the adult stage [9,10]. These larvae can elicit a parasitic infection of the digestive tract and can occasionally affect other organs, causing erosive and hemorrhagic lesions, ascites, and perforations leading to granulomas. To date, the only

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Table 1
Number of identified and characterized proteins for *Anisakis simplex* using TMT10-plex labeling.

	Identified in this study	Characterized to date among Identified *	New characterized in this study	Still uncharacterized among Identified *
L3	330	40	241	49
L4	571	51	356	164
common	1542	202	1075	265
Total	2443	293	1672	478
		SUM: 2443		

*According to UniProt/TrEMBL database for *Anisakis simplex* released 2017_08; 528.050 entries.

effective treatment for anisakiosis is the endoscopic removal of live larvae and the symptomatic treatment. Additionally, in sensitive humans, *A. simplex* larvae can cause allergic reactions [11,12]. The symptomatology of this allergy arises within 60 min of ingestion and comprise rash, abdominal pain, vomiting, diarrhea and respiratory distress [13]. In the most severe cases, anaphylaxis shock can be potentially fatal [14,15]. In the cases of allergenic patients, a fish free diet is the unique recommended treatment.

The number of reported cases of anisakiosis continues to increase. A total of 20,000 new cases of the disease were reported in 2010 worldwide [16]. According to the quantitative risk assessment model, the prevalence of anisakiosis in Europe will increase to 7500–8500 cases per year [17]. Anisakiosis is also an economic problem for fisheries due to the negative impact on consumer confidence resulting decrease in demand on the market for potentially parasitized fish species [10,18,19]. Based on Scientific opinion of the European Food Safety Authority (EFSA) [16], there are not Anisakis free zone, thus the European Union (EU) promulgated rules (Regulations 853/2004, 1276/2011) to control this risk [20]. In this sense, to avoid problems of anisakiosis fish products have to be frozen at −20 °C for 24 h or at −35 °C for not < 15 h or heating at temperatures > 60 °C for 1 min to kill any live nematodes in the foodstuffs [16,20]. As a food control method, a new targeted proteomics strategy published by our group that is based on the monitoring of several anisakids peptide biomarkers using mass spectrometry (MS) achieves the fast detection of anisakids in any food product in less than two hours [21].

In the last years, Proteomics methods have gained more acceptance among the food scientific community [22–24]. Carrera et al. [21,25] has provided new applications in the food control area using targeted proteomics methodologies for the rapid detection of Anisakidae in fishery products. However, still there is a lack of data and information regarding to those proteins, which have a crucial role in the developmental stages (L3 and L4) of this parasitic nematode.

According to that, we chose high-resolution tandem mass tag (TMT)-based quantitative proteomics to analyze global proteomic dynamics in *A. simplex*, between two developmental stages L3 and L4. Multiplex quantification using TMT 10-plex increasing the confidence in both the identification and quantification of the proteins. The numbers of identified peptides and proteins in shotgun proteomics experiments using TMT 10-plex is highly satisfactory and the precision on the level of peptide–spectrum matches and protein level dynamic range are more acceptable than in other isobaric mass tags [25–27]. Each mass-tagging reagent has the same nominal mass (isobaric) and chemical structure composed of an amine-reactive NHS-ester group, a spacer arm and a mass reporter. For each sample, a unique reporter mass in the low mass region of the MS/MS spectrum is used to measure relative protein expression levels during peptide fragmentation. Each of 10 TMT labels has different structure and isotopes position. TMT labels have different numbers and combinations of ^{13}C and ^{15}N isotopes in the mass reporter through what each peptide in tested sample have monoisotopic mass differences on a scale of 126 to 131 Da (126, 127C/N, 128C/N, 129C/N, 130C/N, 131) [28]. Those mass differences in the reporter can be detected using high resolution mass spectrometry. Benefits of the TMT 10-plex comprise increased sample multiplexing for relative quantitation, better sample throughput and fewer missing quantitative channels among samples [29]. However, the use of TMT-based quantitative proteomics for Anisakidae investigations has not yet been applied.

Therefore, in this work we presented for the first time the proteome profiling of L3 and L4 *A. simplex* larvae by TMT-based quantitative proteomics. The methodology is based on the following steps: (a) protein extraction of L3 and L4 larvae, (b) HIFU-assisted trypsin digestion, (c) TMT-isobaric mass tag labeling following high-pH reversed-phase fractionation, and (d) LC-MS/MS analysis in a LTQ-Orbitrap Elite mass spectrometer.

The results from this study will provide a significant novel protein repository of L3 and L4 *A. simplex* larvae that will be very useful for the comparison of L3 and L4 development stages and for further anisakiosis investigations.

2. Materials and methods

2.1. Anisakis simplex

Parasitic nematodes were collected from the Biobank platform implemented for the PARASITE project (www.parasite-project.eu) at IIM-CSIC, Vigo, Spain. The experiments were performed on the L3 and L4 larval stages of *A. simplex* which were isolated: L3 from hake (*Merluccius merluccius*) and L4 from striped dolphin (*Stenella coeruleoalba*). All used larvae were taxonomically identified using conventional PCR to amplify the ITS region and Cox2 gene [30].

2.2. Protein extracts

Protein extracts were obtained from ten L3 and three L4 *A. simplex* larvae. For that, parasites were crushed manually with a sterile plastic pestle in 2 mL centrifuge tubes. Then, protein extraction was performed by homogenizing 0.2 g of sample in 1.5 mL of lysis buffer (60 mM Tris-HCl pH 7.5, 1% lauryl-maltoside, 5 mM PMFS and 1% DTT) on ice for 3 cycles of 30 s pulses in a sonicator device (IKA-Werke, Staufen, Germany). Proteins extracts were centrifuged at 16,000*g* for 30 min at 4 °C in a J221-M centrifuge (Beckman, CA, USA). The supernatant proteins were quantified using the bicinchoninic acid method (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, San Jose, CA, USA). All analyses were performed in triplicate.

2.3. SDS-polyacrylamide gel electrophoresis

Protein extracts were separated and evaluated on 12% (*v*/v) polyacrylamide gels (acrylamide/N, N′-ethylene-bis-acrylamide, 200:1) with a stacking gel of 4% polyacrylamide. A total of 25 μg of proteins in Laemmli buffer were boiled for 5 min at 100 °C and separated per well in a Mini-PROTEAN 3 cell (Bio-Rad, Hercules, CA, USA). The running

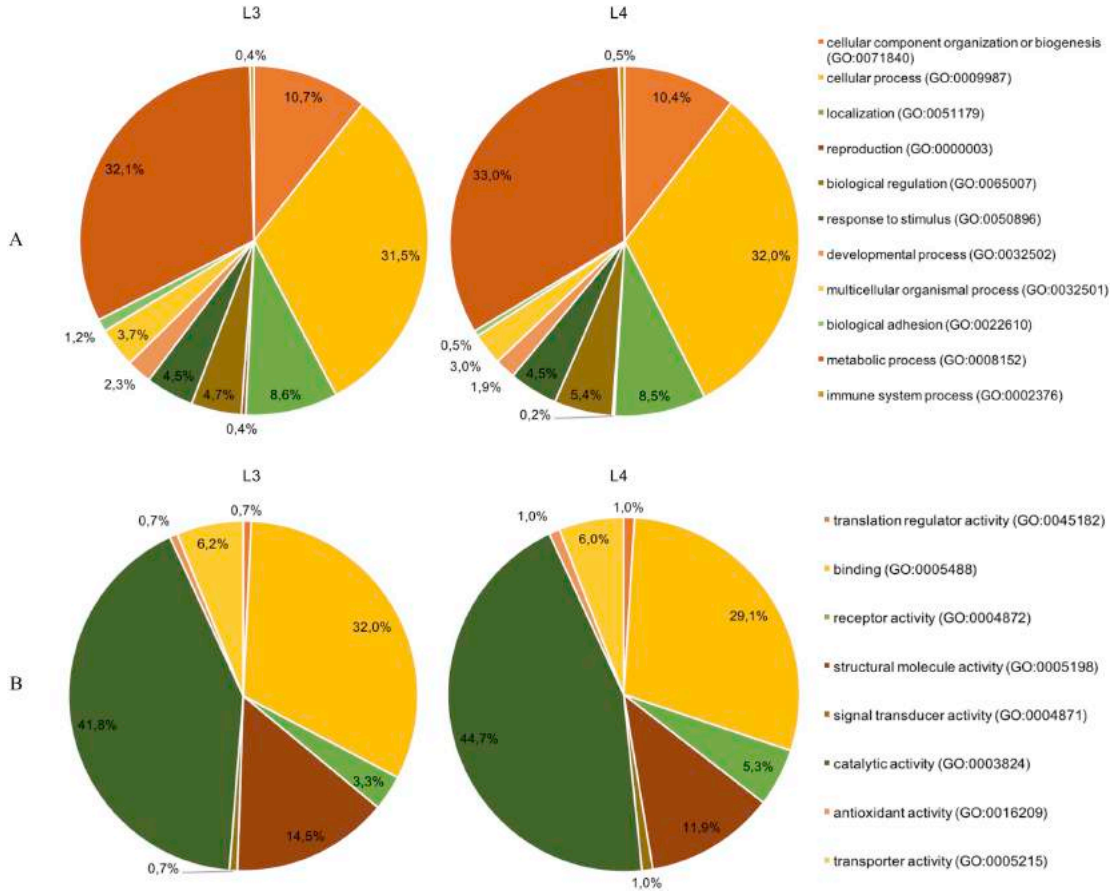


Fig. 1. Biological processes (A) and molecular functions (B) of L3 and L4 *Anisakis simplex* development stages classified by PANTHER. Analysis performed on proteins obtained after global quantification.

buffer consisted of an aqueous solution, composed by 1.44% (*w/v*) glycine, 0.67% Tris-base, and 0.1% SDS. Running conditions were 80 V for the first 20 min and then 120 V until the end of the electrophoresis. Gels were silver stained using the Pierce Silver Stain for Mass Spectrometry kit (Thermo Fisher Scientific) (Supplementary Fig. S1).

2.4. Tryptic digestion by HIFU

Protein supernatants (100 µg) were ultrafast in-solution digested with 2.5 µg of trypsin (Promega, WI, USA) in 25 mM ammonium bicarbonate pH 8, with the simultaneous application of HIFU [31]. The trypsin digestion was carried out as described before by Carrera et al. [32].

2.5. TMT peptide labeling

TMT 10-plex isobaric label reagents (0.8 mg Thermo Fisher Scientific) were re-suspended in 41 µL of anhydrous acetonitrile and added to 100 µg of protein digest dissolved in 100 µL of 0.1 M triethylammonium bicarbonate (TEAB) buffer solution. A total of two different complete experiments of Tandem Mass Tag (TMT) 10plex labelings (Thermo Fisher Scientific) with different biological replicates were performed. Within each experiment, samples were labeled with TMT10-plex in quadruplicate (L3 larvae: 126, 127 N, 127C, 128 N; L4 larvae: 128C, 129 N, 129C, 130 N). Additionally, a standard sample resulting of mixing equal amount of proteins for the eight samples was included (channel 131). Reaction was carried out by 1 h at room temperature. To quench the reaction 8 µL of 5% hydroxylamine was added to each sample and incubated for 15 min. Samples were combined in a new tube at equal amounts. TMT-labeled peptide concentration was measured using the Pierce Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific).

2.6. High-pH reversed-phase peptide fractionation

To increase the number of peptide identifications, labeled samples were fractionated using Pierce High-pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific) following manufacturer's instructions. After conditioning of the spin columns, a total of 100 µg of the total labeled and digested sample was dissolved in 300 µL of 0.1% TFA solution. Sample solution was loaded onto the spin column and centrifuged at 3000 ×*g* for 2 min. Eluate was retained as "flow-through" fraction. The same was performed with Milli-Q water to retain the "wash" fraction. TMT-labeled samples require an additional column

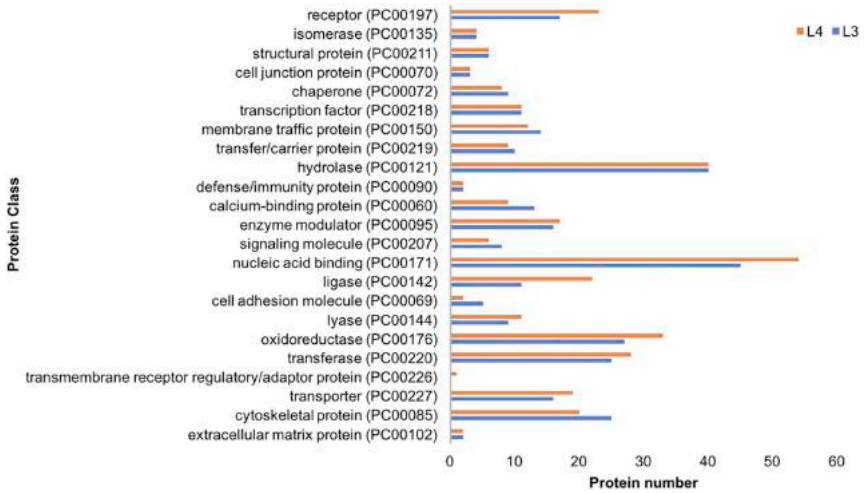


Fig. 2. Classes of proteins categorized per each L3 and L4 *Anisakis simplex* development stage by PANTHER. Analysis performed on proteins obtained after global quantification.

wash with 300 µL of 5% ACN, 0.1% trimethylamine (TEA) to remove unreacted TMT reagent. Fractionation of six different sample fractions were performed by stepwise using the appropriate elution solutions according to manufacturer's instructions. Peptide content of each fraction was determined by colorimetric analysis using the Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific) and evaporated to dryness using vacuum centrifugation (SpeedVac concentrator, Thermo Fisher Scientific). The samples (6 fractions plus the wash and flow-throughput) were stored at −80 °C for further analysis.

2.7. LC-MS/MS analysis

Peptide samples were acidified with 0.1% formic acid, cleaned on a C_{18} MicroSpin™ column (The Nest Group, South-borough, MA, USA) and analyzed by LC-MS/MS using a Proxeon EASY-nLC II liquid chromatography system (Thermo Fisher Scientific) coupled to a LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Peptide separation (1 µg) was done on a RP column (EASY-Spray column, 50 cm × 75 µm ID, PepMap C18, 2 µm particles, 100 Å pore size, Thermo Fisher Scientific) with a 10 mm pre-column (Accucore XL C18, Thermo Fisher Scientific) using 0.1% formic acid and 98% ACN with 0.1% formic acid as mobile phases A and B, respectively. The 240 min of linear gradient from 5 to 35% B, at a flow of 300 nL/min was used. A spray voltage of 1.95 kV and a capillary temperature of 275 °C were used for ionization. The peptides were analyzed in positive mode from 400 to 1600 amu (1 µscan), followed by 10 data-dependent HCD MS/MS scans (1 µscans), using a normalized collision energy of 38% and an isolation width of 1.5 amu. Dynamic exclusion for 30s after the second fragmentation event was applied and unassigned charged ions were excluded from the analysis.

2.8. Processing of the mass spectrometry data

All the MS/MS spectra acquired were analyzed using SEQUEST-HT (Proteome Discoverer 2.1 package, Thermo Fisher Scientific) against a custom-made database containing *A. simplex* plus *Ascaris suum, Toxocara canis, Brugia malayi, Loa loa* and *Caenorhabditis elegans* UniProt/TrEMBL protein entries (released 2017_08; 528.050 entries). These organisms were selected according to phylogenetic similarity. The following restrictions were used: full tryptic cleavage with up to 2 missed cleavage sites and tolerances of 7 ppm Da for-parent ions and 0.6 Da for MS/MS fragment ions. TMT-labeling (lysine and peptide n-terminus) and carbamidomethylation of cysteine were set as fixed modifications. The permissible variable modifications were methionine oxidation and acetylation of the N-terminus of the protein. The results were subjected to statistical analysis to determine the peptide false discovery rate (FDR) using a decoy database and the Target Decoy PSM Validator algorithm [33]. The FDR was kept below 1%.

2.9. Modulated proteins and statistical analysis

Relative quantification was performed using the Quantification Module and normalization against total peptide amount (Proteome Discoverer 2.1 package, Thermo Fisher Scientific). The comparison of the abundance of proteins in L3 or in L4 was performed according to Abundance Ratio: L3/L4 (Supplemental Data 1). Log2 of the Abundance ratio: L3/L4 was calculated. Negative values ($\leq$-1) mean that protein is more abundant in L4, and positive values ($\geq$1) in L3. Those extreme ratios let us classified all the proteins as equally abundant in both stages (Log2 Abundance ratio: L3/L4 $\leq -1;1 >$), and those more abundant in one of the stage. After global quantification several filters were applied to the global quantification results to obtain the final list of the most modulated proteins: a) proteins quantified with at least two unique peptides, b) at least a 2 fold change in L3/L4 normalized ratios, c) proteins quantified in the two TMTs and d) after Kruskal-Wallis statistical test using the R commander software. Differences in the modulation were considered significant when p-value $\leq$.01 (Supplemental Data 4).

2.10. Functional analysis

To analyze functions and the involvement in the common biological processes, the final list of non-redundant protein IDs obtained after global quantification was classified into three different categories of Gene Ontology (GO): biological processes, classes of proteins and molecular functions. Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to ascribe identified proteins to particular biological mechanisms and cellular pathways (the established criteria: p adjusted < 0.05). GO and KEGG enrichment analysis were performed using PANTHER Classification System (Protein ANalysis THrough

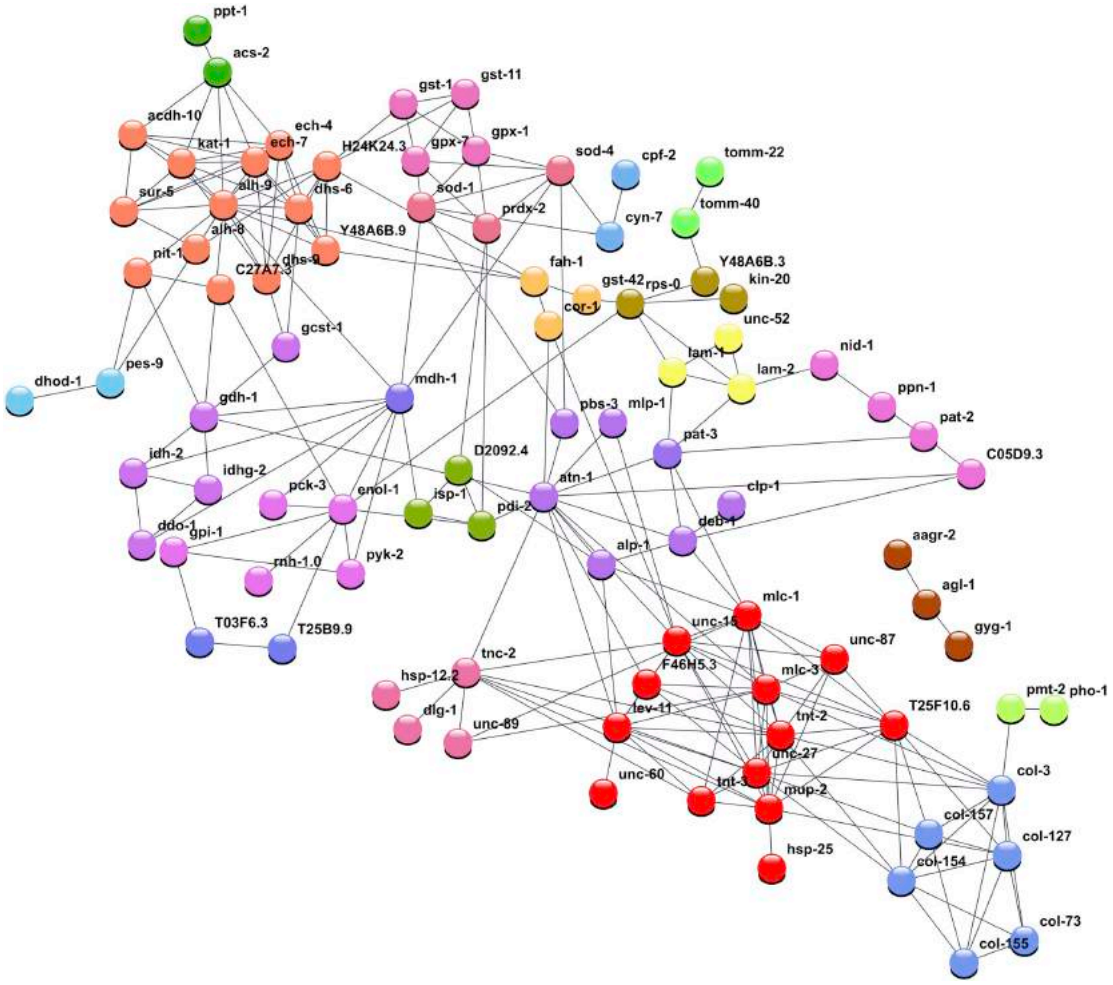


Fig. 3. Protein-protein interaction network analysis of L3 *Anisakis simplex* development stage using STRING. Analysis performed on proteins obtained after global quantification.

Evolutionary Relationships, http://pantherdb.org) and DAVID 6.8 (Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov/home.jsp), respectively. The significantly enriched functional GO categories and KEGG pathways were reported by comparing the input data with the background of GO/KEGG annotations in the *Caenorhabditis elegans* genome, as the phylogenetically closest nematode available in both databases. GO categorization applied PANTHER overrepresentation test controlled with a Bonferroni correction test to correct the smallest *p*-value of GO terms.

2.11. Network analysis

Network analysis was performed submitting both protein datasets, L3 and L4 development stages of *A. simplex*, obtained after global quantification, to the STRING (Search Tool for the Retrieval of Interacting Genes) software (v10.5) (https://string-db.org/). Interactions have been identified through comparing the input data with the background of the *Caenorhabditis elegans* genome, as the phylogenetically closest nematode, available in the STRING software. Proteins were represented with nodes and the interactions with continuous lines to represent direct interactions (physical), while indirect ones (functional) were presented by interrupted lines. Cluster networks were created using the MCL inflation parameter, which is included in STRING website and a value of 3 was selected for all the analyses.

3. Results and discussion

3.1. Global proteome of A. simplex L3 and L4 development stages

In this study, the TMT-based quantitative proteomics experiments were used to create a reference proteome dataset for each of the two development stages of *A. simplex*, L3 (n = 80 samples) and L4 (n = 24) (Supplemental Data 1).

A total of 11,412 non-redundant peptides corresponding to 2443 different proteins were identified (Table 1). According to the Abundance L3/L4 ratios during global quantification more abundant

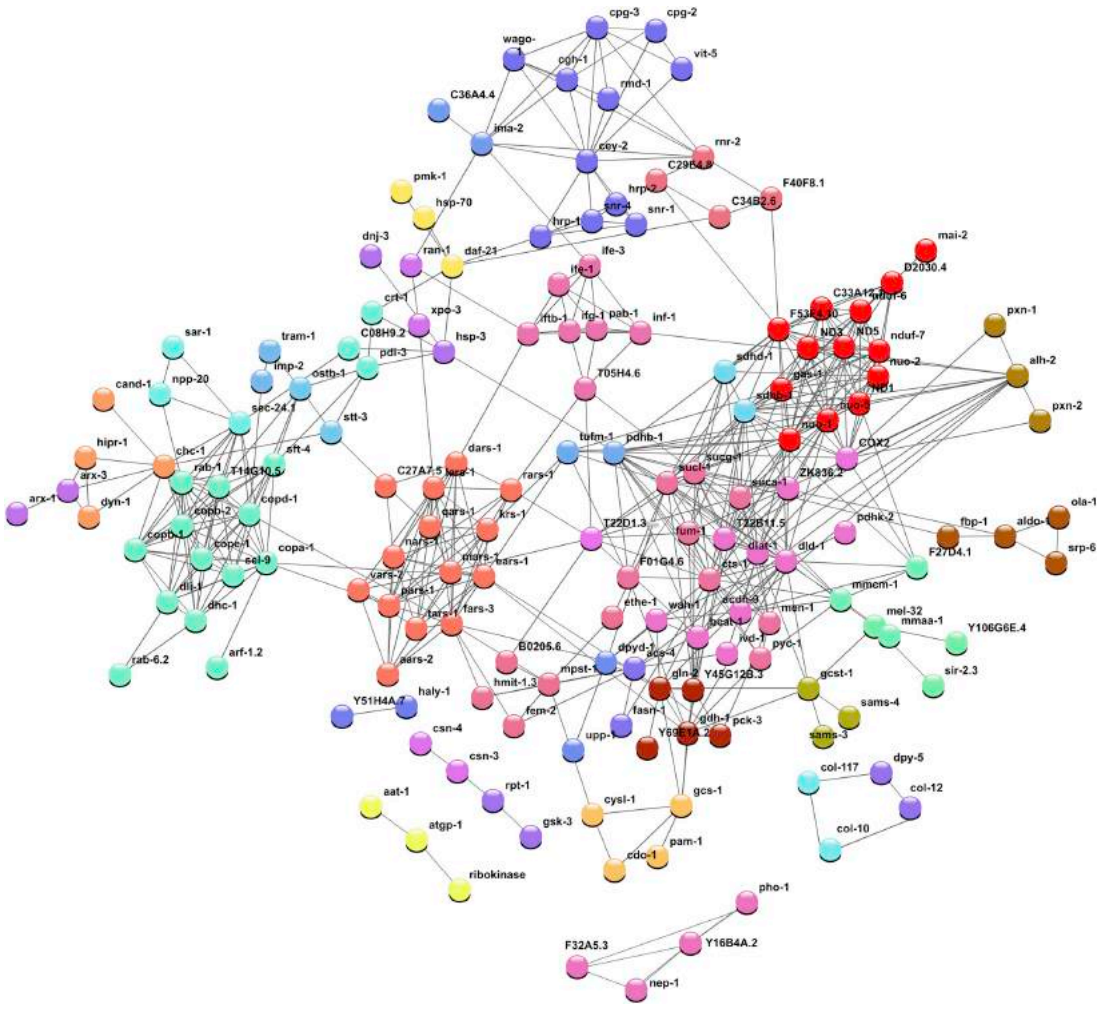


Fig. 4. Protein-protein interaction network analysis of L4 *Anisakis simplex* development stage using STRING. Analysis performed on proteins obtained after global quantification.

proteins for L3 (330 proteins) and for L4 (571 proteins) were identified and listed (Supplemental Data 1). Additionally, the results showed a high degree of overlap (equally abundant 1542 proteins) between L3 and L4 developmental stages (Table 1). To our knowledge, this is the major dataset of proteins of *A. simplex* for two development stages (L3 and L4) identified to date.

The final global datasets were subsequently investigated by gene ontology and network analysis to gather more functional insights.

3.2. Functional analysis

To understand molecular function and biological processes of the identified proteins that they are involved in, gene ontology (GO) term was performed by PANTHER classification system (Supplemental Data 2). Among biological processes (Fig. 1a) the smallest differences are related to cellular and metabolic processes (GO:0009987, GO:0008152). The biggest differences between L3 and L4 are related to the biological adhesion (GO:0022610), multicellular organismal processes (GO:0032501), developmental processes (GO:0032502) and biological regulation (GO:0065007). In all cases, except biological regulation, L3 has highest number of proteins related to that biological process (Fig. 1a) (Supplemental Data 2).

Identified proteins were also divided according to their molecular function (Fig. 3b). Analysis showed that in both stages, binding (GO:0005488) and catalytic activity (GO:0003824) are the most common molecular functions of the analyzed proteins. The most significant differences occurred between the L3 and L4 stages in terms of receptor activity (GO:0004872) and structural molecule activity (GO:005198). In L4 number of proteins which have receptor activity is higher than in L3, respectively 5.3% to 3.3% from all identified proteins. Different situation is with structural molecule activity proteins, where L3 has higher number of that type of proteins than L4, respectively 14.5% to 11.9% from all identified proteins (Fig. 1b). Moreover, the identified proteins when divided into two *A. simplex* development

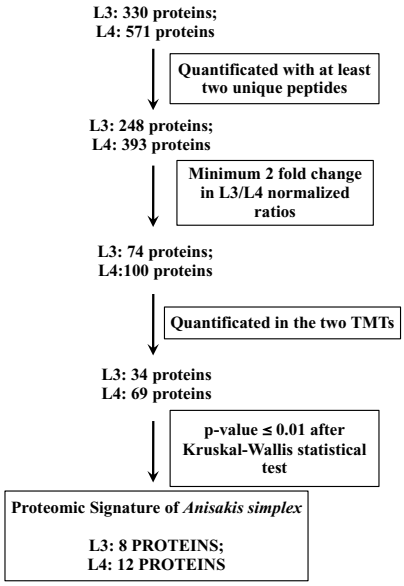


Fig. 5. Summary of the filtering process during the selection of the most modulated proteins in L3 and L4 *Anisakis simplex* larvae.

Table 2
The list of quantitative modulated proteins in *Anisakis simplex* specific for L3 and L4 larvae.

Development stage	Protein name	UniProtKB/TrEMBL ID for Anisakis simplex	Gene
L3	Pseudocoelomic globin	A0A0M3JEL6	*glb-1*
	Phosphoenolpyruvate carboxykinase GTP	A0A0M3KCB9	*PEPCK*
	Endochitinase 1	A0A0M3K6E2	*CHIA1*
	Paramyosin	A0A158PP35	*unc-15*
	Putative leucine-rich repeat containing protein	A0A0M3J2S6	N/A
	Uncharacterized protein	A0A0M3K064	N/A
	Uncharacterized protein	A0A0M3J3L5	N/A
	Uncharacterized protein	A0A0M3K1G1	N/A
L4	Glutamate dehydrogenase	A0A0M3JI05	*gdh-1*
	Neprilysin-2	A0A0M3JXL8	*nep-2*
	Aminopeptidase N	A0A0M3K8U2	*ANPEP*
	C-type lectin protein	A0A0M3KDB5	*clec-64*
	Prolylcarboxy peptidase like protein 5	A0A0M3K3Q4	*pcp-5*
	L-threonine 3-dehydrogenase, mit.	A0A0M3K9T7	*tdh*
	Phosphotranferase	A0A0M3J2N1	N/A
	Glyoxylate reductase/ hydroxypyruvate reductase	A0A0M3KK68	*grhpr*
	Uncharacterized protein	A0A0M3JCR4	N/A
	Uncharacterized protein	A0A0M3K6J1	N/A
	Uncharacterized protein	A0A0M3K2A5	N/A
	Uncharacterized protein	A0A0M3IYI5	N/A

Note. N/A- not available, mit.- mitochondrial.

stages, were assigned into specific protein classes according to the annotations of the proteins (Fig. 4) (Supplemental Data 2).

According to PANTHER, 23 protein classes with smallest *p*-value ($p < 0,05$) were taken under consideration. In L4 stage was more proteins classified to ligases (PC00142), lyases (PC00144), oxidoreductases (PC00176) and transferases (PC00220) than in L3. However, in L3 more proteins were assigned to calcium-binding proteins (PC00060), signaling molecules (PC00207), call adhesion molecules (PC00069) and cytoskeletal proteins (PC00085) (Fig. 2) (Supplemental Data 2).

KEGG pathway analysis by comparing the input data with the background of the *C. elegans* genome by DAVID 6.8 (https://david.ncifcrf.gov/home.jsp) showed that most of identified proteins in both stages: L3 and L4 were involved in main metabolic pathways (cel01100), ribosome (cel03010), biosynthesis of antibiotics (cel01130), carbon metabolism (cel01200) and oxidative phosphorylation (cel00190) (Supplemental Data 2).

3.3. Network analysis

To gain information on protein interaction network after global quantification of L3 and L4 development stages of *A. simplex*, possible interactions were analyzed using the STRING v10.0 software. Two separate network maps have been made for each *A. simplex* development stage (Fig. 3 and Fig. 4). A total of 92 proteins for L3 and 157 proteins for L4 constituted very complex and strongly interactive networks, which were clustered to a specified MCL inflation parameter (MCL = 3). According to MCL clustering 21 nodes in L3 stage and 29 nodes in L4 stage were obtained, each of them depicted as a group of different color circles. Most of all analyzed proteins were mutual interactive or interactive with other proteins in those complex protein-protein interaction networks (Fig. 3 and Fig. 4; Supplemental Data 3). Among them, 236 interactions in L3 and 594 interactions were shown in connection with co-expression, co-occurrence and due to the appearance of any information on the interactions between those proteins in different databases (Fig. 3 and Fig. 4; Supplemental Data 3). The most complex nodes of those interaction networks in L3 were those related to energy metabolism (orange circles), regulation of muscle contraction (red circles), protein catabolic processes (dark purple circles), oxidative metabolism (light pink circles) and cuticle creation (dark blue circles) (Fig. 3, Supplemental Data 3). In contrast, in the L4 stage most complex nodes were those related to citrate cycle (light pink circles), aminoacyl-tRNA biosynthesis (peach circles), mitochondrial membrane respiratory chain (red circles) and vesicle-mediated transport (turquoise circles) (Fig. 4, Supplemental Data 3).

Global proteome quantification analysis of L3 and L4 development stages of *Anisakis simplex* showed that in those two stages most of the proteins are involved in very important pathways like those related to energy metabolism, oxidation, cellular transport, and signaling pathways.

3.4. Quantitative modulated proteins

A total of 2443 different proteins of *A. simplex* were identified. Proteins were divided between L3 and L4 stages, where 1542 proteins were classified for both of them. The global quantification results showed 330 and 571 more abundant proteins, identified with at least one unique peptide, for L3 and for L4 development stage, respectively. From them, 248 and 393 were quantified with at least two unique peptides. After sequentially applying the next three criteria (Fig. 5), 8 proteins in L3 and 12 proteins in L4 were selected as proteomic signature of those specific development stages of *A. simplex*. The list of those proteins was shown in Table 2.

A detailed representation of the relative abundance of all quantified proteins is depicted in the scatter plot (Fig. 6). When comparing normalized TMT abundances values from all the L3 and L4 samples for each protein it is shown that L3 and L4 larvae differs in protein composition and proportion. The proteins selected as the proteomic signature of each *A. simplex* development stage, were the most modulated proteins occurred in both development stages of the parasite, but in transition L3/L4, they were the most statistically significantly (*p*-value $\leq .01$) abundant in one of the stage (Fig. 7; Supplemental Data 4).

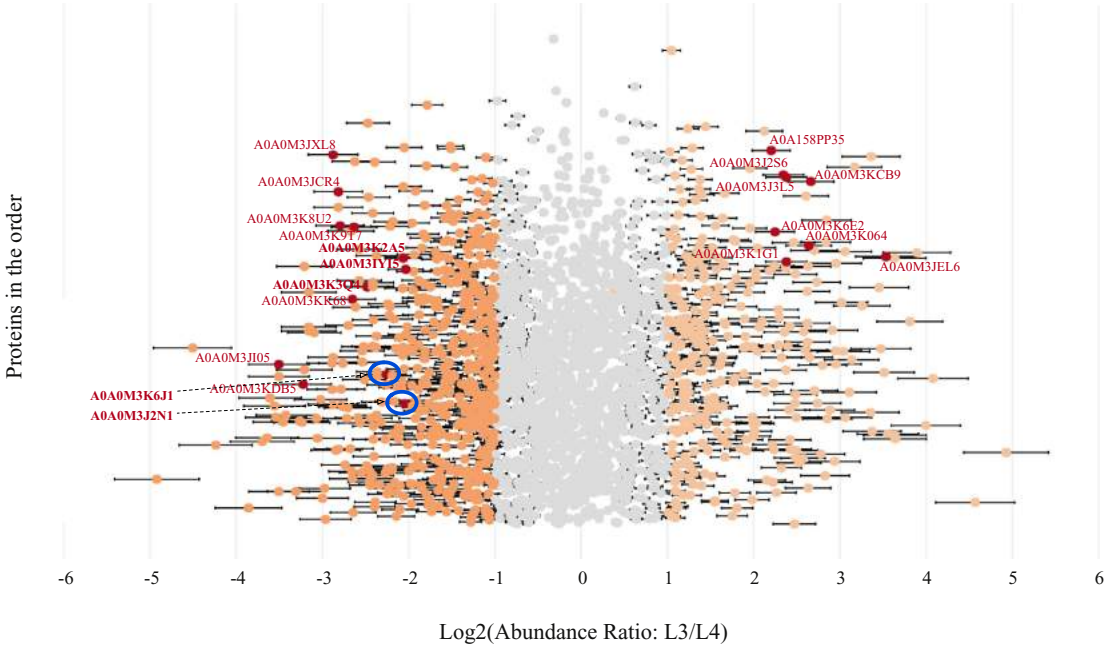


Fig. 6. Scatter plot with detailed representation of the relative abundance of all quantified proteins. Dots representing the modulated proteins in L3 and L4 *Anisakis simplex* marked with red with protein number. For the legend see Table 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Most of all modulated proteins belong to hydrolases with particular regard to peptidases (Table 2). A number of specific proteins for each development stage have been recognized as allergens and registered in the Allergome database [34]. Among these is known protein such a somatic paramyosin (Ani s 2). Endochitinase, a glycoside hydrolase from the chitinase class III group was also assigned as an allergen of *A. simplex* [8] (Table 2).

Considering proteins that were homologous to known allergens, according to Allergome database, 3 of quantified modulated proteins could be potential novel allergens of *A. simplex*. The pseudocoelomic globin (AF009, Aller-gome database) assigned to L3 larvae, prolylcarboxy peptidase like protein 5 (AF061, Aller-gome database), and L-threonine 3-dehydrogenase, mit. (AF029, Aller-gome database), selected as one of the L4 specific proteins. First protein, pseudocoelomic globin, represents a group of globins found in nematode worms. Globins are heme-containing proteins involved in binding and/or transport of oxygen. The major groups of globins are hemoglobins and myoglobins from vertebrates, invertebrate globins, leghemoglobins from plants, and flavohemoproteins from bacteria, where invertebrates and vertebrates globins causes allergic reactions [35–37]. Second protein belongs to prolyl oligopeptidase family. All allergens belonging to this family are dipeptidyl peptidases. They were identified in several species of the dermatophytic fungus *Trichophyton* genus [38] and in bee and wasp venoms [39]. Third one protein, L-threonine 3-dehydrogenase, is a zinc-dependent dehydrogenase. It is closely related to zinc alcohol dehydrogenases, which are found in bacteria, mammals, plants, and in fungi [40], where one of the allergens from *Candida albicans* is an alcohol dehydrogenase [41].

Analyzing only modulated proteins in STRING v10.0 software (Fig. 8) after applying MCL clustering, expected number of edges was 4, where current set of proteins showed 9. This means that these proteins have more interactions among themselves than what would be expected for a random set of proteins of similar size, drawn from the genome. Such an enrichment indicates that the proteins are partially biologically connected, as a group what is shown in the Fig. 8. This means that function blocking or silencing of appropriate genes for these proteins, especially those with the highest number of protein-protein interactions (i.e. glutamate dehydrogenase; A0A0M3JI05 or neprylisin-2; A0A0M3JXL8) could be the basis for combating *Anisakis simplex* L3 and L4 larvae, due to disruption of metabolically important processes for both stages.

Proteins which are not connected with any or not shown have not been studied very much and that their interactions might not yet be known to STRING. On the other hand, interactions have been identified through comparing the input data with the background of the *C. elegans* genome, the phylogenetically closest nematode to *A. simplex* available in the STRING software. However, it is free-living species, not parasitic one.

Obtained stage-specific proteins, could be used as targets to control/eliminate this parasite and in future eradicate the anisakiosis disease. Further detailed in vitro studies on the silencing of expression or blocking of activity, as well as post-translational modifications of these proteins, due to their high participation in important metabolic processes in Nematoda, are needed. Their accurate knowledge and description of the mechanism of action could be used in research on antiparasitic substances and give an answer to the question how to neutralize invasive L3 larvae and thus help in the fight with anisakiosis.

In a next step, it will be advantageous to verify our findings by using fractionated or recombinant proteins for the immunoblotting experiments, and to study the allergenicity of selected allergen candidates in more detail. On the other hand, determination of marker peptides of selected modulated *A. simplex* proteins may be used for the classification of food and feed contaminants.

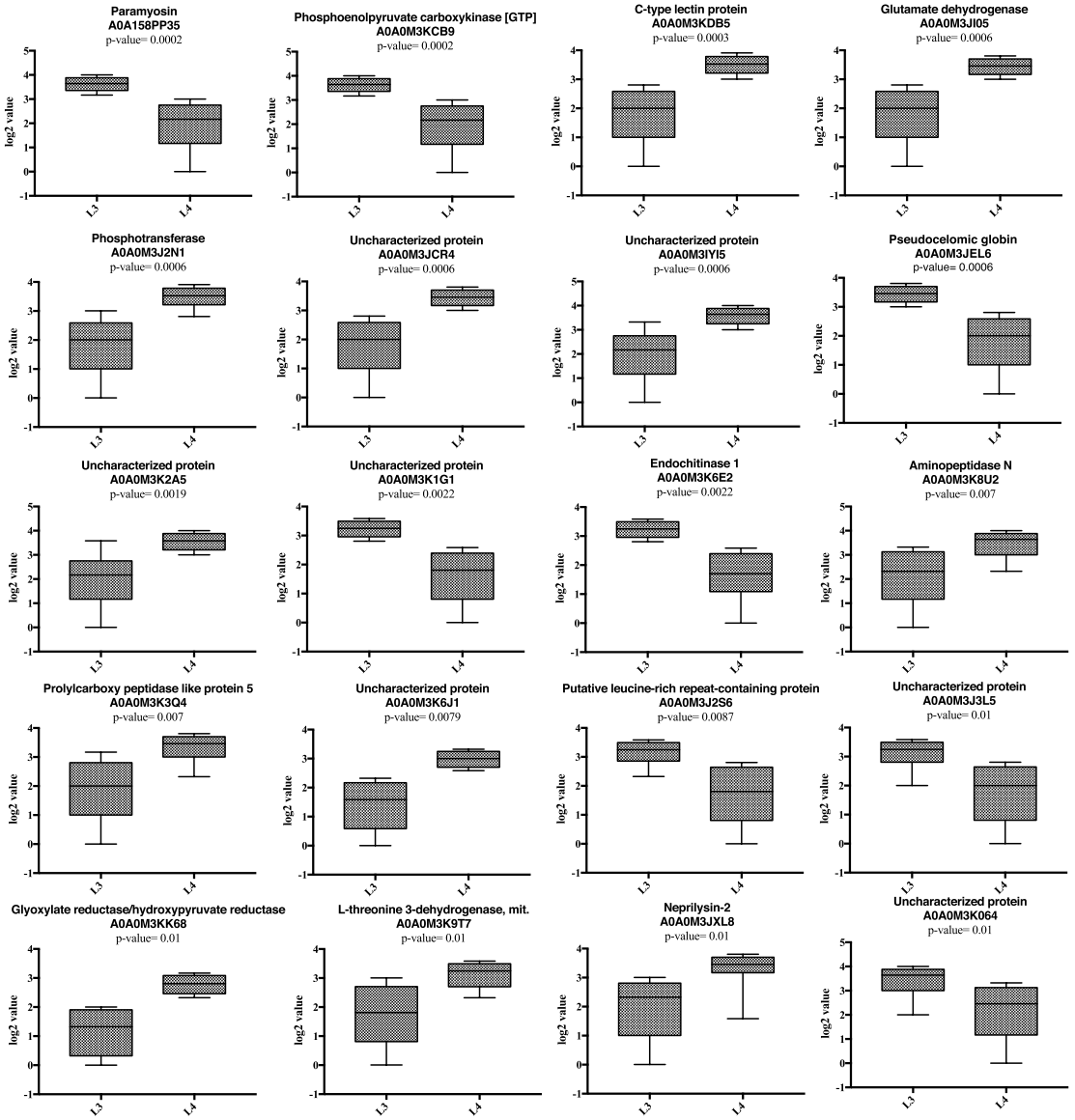


Fig. 7. The modulated proteins selected as the proteomic signature of each *A. simplex* development stage. Each protein L3/L4 transition is showed. Box-plots representing ranks calculated for normalized TMT values for all L3 and L4 samples transformed into log2 values. Differences in the modulation were considered significant when p-value ≤ 0.01.

4. Conclusions

In this study, we performed comparative quantitative proteomics analyses employing L3 and L4 *Anisakis simplex* development stages to reveal global proteomes of those two. The proteomes were analyzed using TMT label-based multiplex quantification analysis technique, which was based on the following steps: (a) protein extraction, (b) HIFU-assisted trypsin digestion, (c) TMT-isobaric mass tag labeling, and (d) global proteomic comparison analysis between two developmental stages of *A. simplex* using a LTQ-Orbitrap Elite mass spectrometer. Interestingly, 2443 different proteins were identified, where the results showed a high degree of overlap (1542 different proteins) among L3 and L4 of *A. simplex* and high amount of proteins more abundant for L3 (330) or L4 (571). The final list of modulated proteins consists 8 proteins in L3 and 12 proteins in L4 selected as proteomic signature of those specific development stages of *A. simplex*.

To our knowledge, the current study represents the most extensive analysis of global proteomes for *A. simplex* two development stages: L3

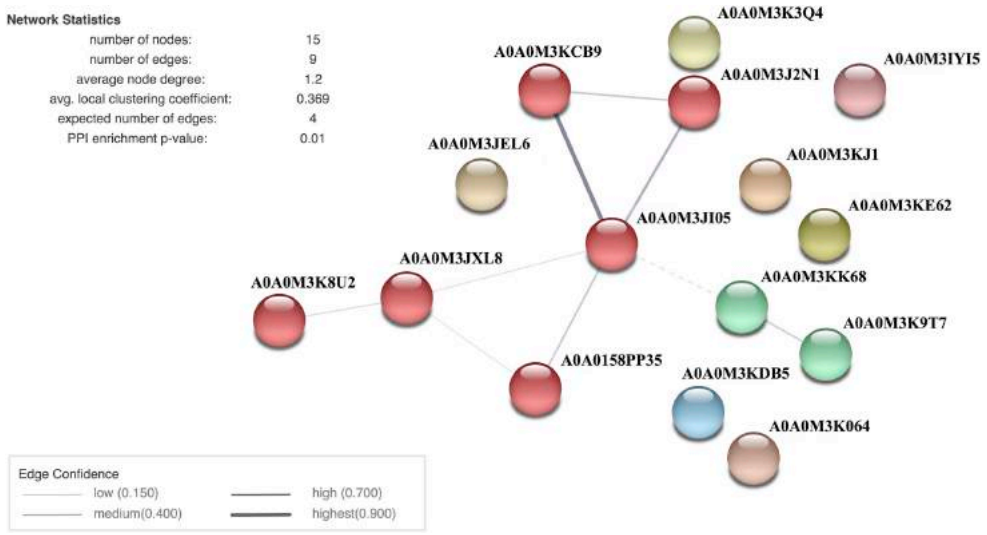


Fig. 8. Protein-protein interaction network analysis of *Anisakis simplex* the most modulated proteins using STRING. Edge confidence explained in the figure. For the legend see Table 2.

and L4, provided to date and might be very useful for further investigations under anisakiosis, including the determination of marker peptides of selected modulated *A. simplex* proteins what may be used for the classification of food and feed contaminants.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2019.04.006.

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4.2. Tissue-specific proteome of L4 larvae of *Anisakis simplex*

Manuscript II

Tandem mass magging (TMT) reveals tissue-specific proteome of L4 larvae of *Anisakis simplex* s. s.: enzymes of energy and/or carbohydrate metabolism as potential drug targets in anisakiasis

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In 2021, we teamed up with a group of animal physiologists and bioinformaticians from the Faculty of Biology and Biotechnology at the University of Warmia and Mazury in Olsztyn in the second project to fully investigate the tissue-specific proteome of the L4 stage of *A. simplex*.

The hypothesis that accompanied us was that the tissues, the fully functional intestine of the L4 stage of *A. simplex*, in contrast to the L3 stage, and the cuticle are characterized by specific proteins responsible for growth, development and contact with the host. We therefore aimed to study the tissue-specific proteome of the L4 stage of *A. simplex* using the shotgun proteomics method based on isobaric tandem mass labeling (TMT) with a combination of nano-high performance liquid chromatography (nLC) coupled to an LTQ-Orbitrap Elite mass spectrometer.

We identified 272 differentially regulated proteins, of which 133 proteins were more abundant in the cuticle and 139 proteins were more abundant in the intestine of the L4 stage of *A. simplex*. Functional annotation of these proteins, and host-parasite interactions were also investigated.

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Article

Tandem Mass Tagging (TMT) Reveals Tissue-Specific Proteome of L4 Larvae of *Anisakis simplex* s. s.: Enzymes of Energy and/or Carbohydrate Metabolism as Potential Drug Targets in Anisakiasis

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Abstract: *Anisakis simplex* s. s. is a parasitic nematode of marine mammals and causative agent of anisakiasis in humans. The cuticle and intestine of the larvae are the tissues most responsible for direct and indirect contact, respectively, of the parasite with the host. At the L4 larval stage, tissues, such as the cuticle and intestine, are fully developed and functional, in contrast to the L3 stage. As such, this work provides for the first time the tissue-specific proteome of *A. simplex* s. s. larvae in the L4 stage. Statistical analysis (FC $\geq$ 2; p-value $\leq$ 0.01) showed that 107 proteins were differentially regulated (DRPs) between the cuticle and the rest of the larval body. In the comparison between the intestine and the rest of the larval body at the L4 stage, 123 proteins were identified as DRPs. Comparison of the individual tissues examined revealed a total of 272 DRPs, with 133 proteins more abundant in the cuticle and 139 proteins more abundant in the intestine. Detailed functional analysis of the identified proteins was performed using bioinformatics tools. Glycolysis and the tricarboxylic acid cycle were the most enriched metabolic pathways by cuticular and intestinal proteins, respectively, in the L4 stage of *A. simplex* s. s. The presence of two proteins, folliculin (FLCN) and oxoglutarate dehydrogenase (OGDH), was confirmed by Western blot, and their tertiary structure was predicted and compared with other species. In addition, host–pathogen interactions were identified, and potential new allergens were predicted. The result of this manuscript shows the largest number of protein identifications to our knowledge using proteomics tools for different tissues of L4 larvae of *A. simplex* s. s. The identified tissue-specific proteins could serve as targets for new drugs against anisakiasis.

Keywords: *Anisakis simplex*; L4 stage larvae; intestine; cuticle; folliculin; oxoglutarate dehydrogenase; proteomics; LC-MS/MS

1. Introduction

Anisakis simplex s. s. is one of the most important emerging parasitic nematodes in Europe, according to the ranking for risk management of foodborne parasites prepared for recommendations of the Food and Agriculture Organization of the United Nations

(FAO) and the World Health Organization (WHO) [1–3]. The disease caused by the *Anisakis* genus is called anisakiasis [4–6] and is dangerous, well-known, common, and frequently described. It is estimated that the incidence is 0.32/100,000 worldwide [7]. The global introduction of a different cuisine, the development of better diagnostic tools, and a greater knowledge of *Anisakis* and its infection have led to significantly more anisakiasis being reported [5]. Prior to 2010, over 20,000 cases of anisakiasis were reported worldwide, with the highest prevalence (over 90%) in Japan [8,9], where 2000–3000 cases of the disease are reported annually [10]. The European countries in which cases of anisakiasis have been reported include Spain [11], Italy [12,13], France, Germany [4], Croatia [14], and Poland [15]. In addition, a recently published meta-regression analysis revealed a significant 283-fold increase in infection level of *Anisakis* spp. (average number of worms/fish) over a 53-year period from 1962 to 2015 [16]. This increase in infection level of *Anisakis* spp. may have implications for human health, marine mammal health, and fisheries profitability.

The life cycle of *Anisakis* nematodes follows the general pattern of the life cycle of nematodes, including the four larval stages L1–L4: in eggs (L1–L2) and then in intermediate or paratenic hosts (L3), and as preadults (L4) and adults in cetacean final hosts [17]. Nematode eggs are excreted in feces and embryonate in seawater. During ingestion by the first crustacean intermediate host, L2 larvae are most likely freed from the cuticle of the second stage by the action of the mouthparts and mature into L3. This allows the third stage (L3) larvae to enter the gut before settling in the hemocoel [18]. Larger invertebrates (especially copepods, euphausiids) and smaller fish are considered important second intermediate hosts, and various predatory fish species and cephalopods serve as paratenic hosts (crustaceans infected with the L3 are eaten). Whales and dolphins ingest the nematodes by eating the intermediate hosts. This complex life cycle involves a variety of hosts that are transmitted through the marine food chain [17,19]. Humans become accidentally infected by eating raw or inadequately processed L3-contaminated fish or squid [4,17,20]. Once in the human gastrointestinal tract, L3 of *A. simplex* can develop into L4, but only in exceptional cases is the immature adult stage reached [21–25].

As is common among parasitic nematodes with complex life cycles, the morphology of *A. simplex* varies with different stages and infected hosts. In general, these differences result from changes in diet and mode of feeding. In L3 larvae of *A. simplex*, the intestinal lumen is shrunken, and data from the literature suggest that the cuticle, a complex outer matrix covering the body of the nematode, is involved in the uptake of energy compounds (saccharides) [26,27]. After the third molt, in L4 larvae, the intestine becomes clear and begins to function [28]. Probably, after its purification, it takes over the nutritional functions from the cuticle [27]. These phenomena are very interesting and require further directional research. Moreover, from the parasitological point of view, the cuticle and the intestine are the tissues most responsible for the contact of the parasite with the host.

The cuticle and intestine are composed of and release proteins, including enzymes, that play a critical role in the introduction and development of parasitic nematodes in their hosts. To date, little is known about the proteomic composition of these specific tissues of *A. simplex*, particularly larval stage L4, which is less studied by the scientific community. Given recent developments in proteomic methods used to identify and quantify the protein composition of cells, cell secretomes, and tissue sections and whole organisms [3,29], it is not surprising that proteomic applications have gained recognition in a rapidly evolving scientific discipline such as parasitology.

Therefore, in this work, we present for the first time quantitative proteomic profiling of the specific tissues of *A. simplex* s. s. L4 larvae: cuticle (CUT) and intestine (INT), compared to the rest of the body (REST; the remains of the larval body after isolation of the cuticle and intestine), using the shotgun proteomics method based on isobaric mass labeling (TMT) with a combination of nano-high performance liquid chromatography (nLC) coupled to an LTQ-Orbitrap Elite mass spectrometer.

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2. Results

As a result of LC-MS/MS analysis, we identified a total of 3435 proteins. These data were further processed according to selected criteria: (a) proteins classified as master proteins (2902 proteins remaining), and (b) proteins with at least 2 unique peptides (1831 proteins remaining). We used a total of 1831 proteins for further analysis (File S1). There were 1277 characterized proteins in the obtained cohort. This is the largest repository of proteins identified to our knowledge for specific tissues of L4 larvae of *A. simplex* s. s.

2.1. Overview of Differentially Regulated Proteins

After relative quantification, several filters were next applied to obtain the final list of differentially regulated proteins (DRPs): (a) at least a 2-fold change (FC $\geq$ 2) in normalized ratios (CUT vs. REST, INT vs. REST, CUT vs. INT), (b) ANOVA on ranks and Tukey HSD post-hoc test (p-value $\leq$ 0.01) (File S1). The volcano plot representations of DRPs are shown in Figure 1A–C.

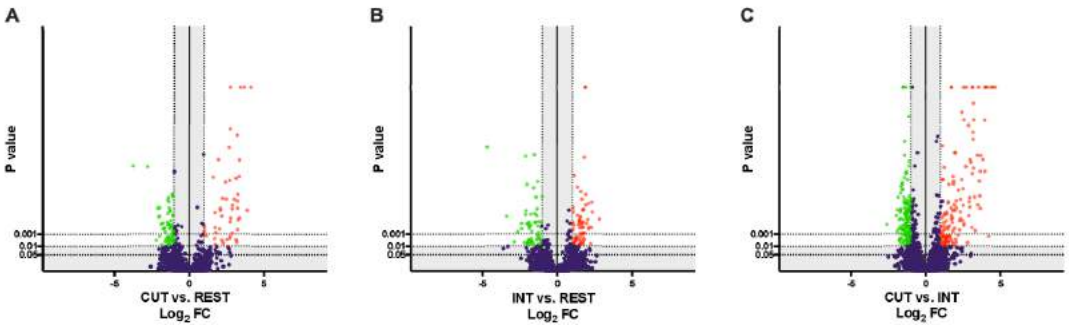


Figure 1. Volcano-plot representations of tissue-specific proteome of the L4 developmental stage of *Anisakis simplex* s. s. The following comparisons between the groups were visualized: (**A**) cuticle vs. the rest of the body of the L4 stage larvae, (**B**) intestine vs. the rest of the body of the L4 stage larvae, and (**C**) cuticle vs. intestine (FC $\geq$ 2, p-value $\leq$ 0.01). The detailed list of DRPs in each of the compared groups is presented in File S1.

Statistical analysis (FC $\geq$ 2; p-value $\leq$ 0.01) showed that 107 proteins were differentially regulated between the cuticle and the rest of the larval body at the L4 stage, of which 49 were more abundant in the cuticle and 58 in the rest of the nematode body (Figure 1A, File S1). Comparing the intestine and the rest of the body of L4 stage larvae, 123 proteins were identified as DRPs, with 67 proteins being more abundant in the intestine and 56 in the rest of the body of *A. simplex* s. s. (Figure 1B, File S1). Comparison of the individual tissues examined revealed a total of 272 DRPs, with 133 proteins more abundant in the cuticle and 139 proteins more abundant in the intestine (Figure 1C, File S1).

Additional graphical representation of the abundance differences in DRPs between the cuticle, intestine, and the rest of the body of *A. simplex* s. s. is shown in Figure 2.

Among the proteins found in the intestine (File S1), we identified those with catalytic activity, including carboxypeptidase (A0A0M3K6G9), glutamate dehydrogenase (A0A0M3JI05), fructose-bisphosphate aldolase (A0A0M3JYW9), alanine-glyoxylate aminotransferase (A0A0M3K6B4), or aminopeptidase (A0A3P6SUT5), and proteins that involved both oxidative phosphorylation and the tricarboxylic acid cycle (the processes that generate energy), e.g., oxoglutarate dehydrogenase (A0A0M3JPF6) and succinate dehydrogenase (A0A0M3JRB2).

In the cohort of proteins more abundant in the cuticle of the parasite (File S1), we noticed proteins which take part in the immune response, e.g., galectin (A0A0M3JA06) or twitchin (A0A158PN23); proteins directly involved in the cuticle formation, e.g., the N-terminal region of nematode cuticle collagens (A0A0M3JZM9, A0A0M3IYS8, A0A0M3JZM7) or extracellular

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matrix proteins, e.g., papilin (A0A0M3KE06) or ADAMTS-like protease (A0A0M3K300); and proteins involved in glycolysis/gluconeogenesis, e.g., cofactor-independent phosphoglycerate mutase (A0A0M3JK61), PEPCK_N domain-containing protein (A0A0M3J768), or pyruvate dehydrogenase E1 component subunit beta (A0A0M3KJM6).

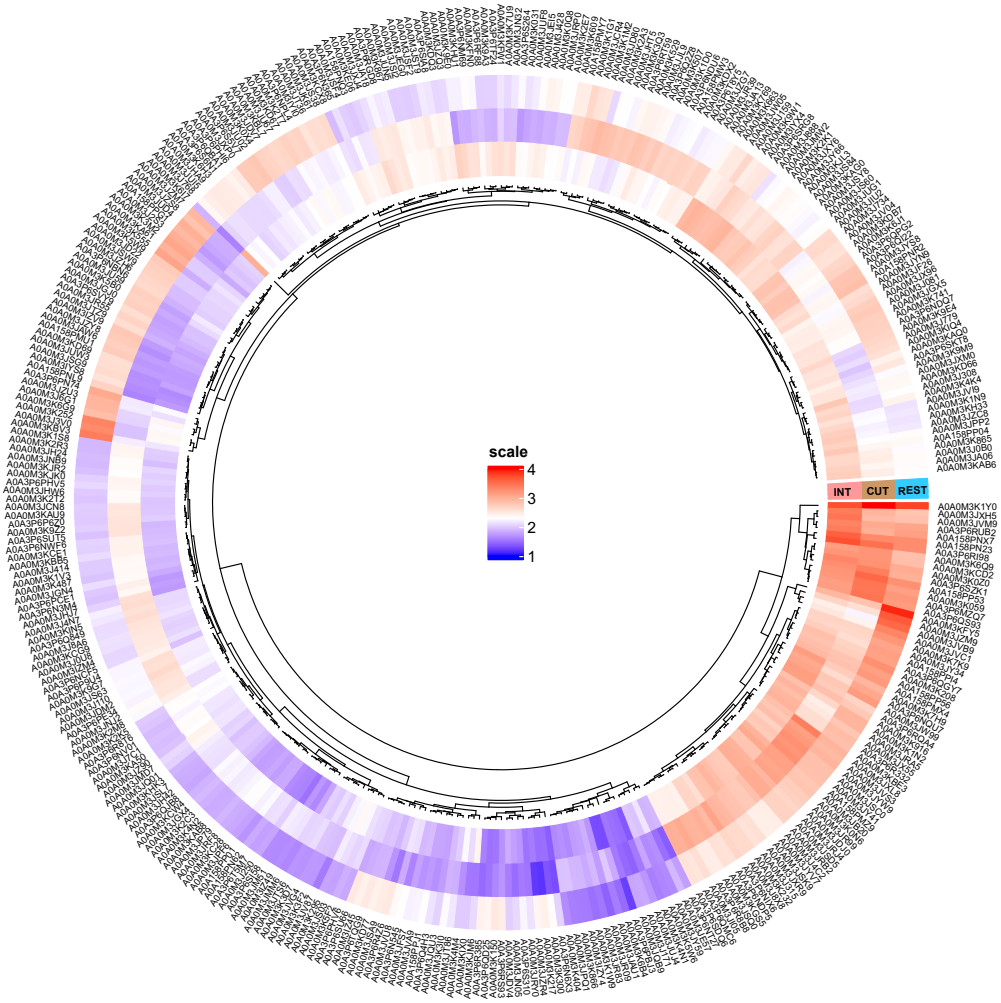


Figure 2. Visualization of the differentially regulated proteins (FC $\geq$ 2, *p*-value $\leq$ 0.01) between 3 tested groups: cuticle (CUT), intestine (INT), and the rest of the body of *A. simplex* s. s. (REST). Red and blue colors describe an increased and decreased abundance in the compared groups, respectively. The detailed list of DRPs in each of the compared groups is presented in File S1.

2.2. Functional Enrichment Analysis and Pathway Identification

2.2.1. Gene Ontology (GO) Analysis

Gene ontology analysis assigned all the obtained proteins (n = 1831) into 3 different categories: molecular function (MF, 53 different functions), biological processes (BP, 126 different processes), and cellular components (CC, 77 different components) (Figure 3). Detailed annotation can be found in the Supplementary Materials File S2.

Two cohorts of proteins, those more abundant in the intestine and cuticle, were additionally assigned into the GO categories (Figure 3). The top subcategories (a = 0.05) assigned to each of the 3 main GO annotations for the protein cohorts obtained for the intestine and cuticle are shown in Table 1.

Proteins more abundant in the intestine were annotated to the molecular functions (MF category), such as *serine hydrolase activity, serine-type peptidase activity,* and *peptidase activity* (GO:0017171, GO:0008236, and GO:0008233, respectively). Moreover, proteins with *oxidoreductase activity* (GO:0016491) were also found in the intestine of *A. simplex* s. s. L4 larvae (Table 1). In the BP category, most of the proteins were involved in the *tricarboxylic acid cycle* (GO:0006099) and *cellular respiration* (GO:0045333). In the cellular component category, most of the proteins found in the intestine were assigned, among others, to the *mitochondrion*, *mitochondrial membrane,* and *mitochondrial envelope* (GO:0005739, GO:0031966, GO: 0005740).

Table 1. The top subcategories from each GO category obtained in g:Profiler. The adjusted enrichment *p*-values with the number of proteins assigned to each subcategory are presented. For graphical representation, see Figure 4. Detailed annotation of all proteins submitted to the analysis can be found in Files S1 and S2.

Term Category *	Term Name	Term ID	Adjusted *p*-Value	Term Size	Input Data Size
		INTESTINE			
MF	serine hydrolase activity	GO:0017171	1.495×10^{-9}	115	15
MF	serine-type peptidase activity	GO:0008236	1.495×10^{-9}	115	15
MF	peptidase activity	GO:0008233	1.038×10^{-6}	417	22
MF	oxidoreductase activity	GO:0016491	3.416×10^{-4}	488	20
MF	calcium-dependent phospholipid binding	GO:0005544	4.901×10^{-4}	19	5
BP	tricarboxylic acid cycle	GO: 0006099	3.291×10^{-7}	18	7
BP	cellular respiration	GO:0045333	1.083×10^{-6}	32	8
BP	energy derivation by oxidation of organic compounds	GO:0015980	1.338×10^{-6}	43	8
BP	aerobic respiration	GO:0009060	1.446×10^{-5}	29	7
BP	proteolysis	GO:0006508	1.596×10^{-4}	598	23
CC	mitochondrial membrane	GO:0031966	6.598×10^{-6}	64	8
CC	mitochondrial envelope	GO:0005740	1.882×10^{-5}	73	8
CC	mitochondrion	GO:0005739	1.402×10^{-4}	165	10
CC	organelle envelope	GO:0031967	1.713×10^{-4}	97	8
CC	envelope	GO:0031975	1.713×10^{-4}	97	8
		CUTICLE			
MF	structural constituent of cuticle	GO:0042302	6.155×10^{-15}	103	16
MF	structural molecule activity	GO:0005198	1.010×10^{-7}	296	16
MF	hexokinase activity	GO:0004396	1.472×10^{-3}	6	3
MF	glucose binding	GO:0005536	1.472×10^{-3}	6	3
MF	glutathione hydrolase activity	GO:0036374	2.562×10^{-3}	7	3
BP	cellular glucose homeostasis	GO:0001678	8.682×10^{-4}	6	3
BP	nucleobase-containing small molecule metabolic process	GO:0055086	9.613×10^{-4}	173	8
BP	glucose homeostasis	GO:0042593	1.513×10^{-3}	7	3
BP	carbohydrate homeostasis	GO:0033500	1.513×10^{-3}	7	3
BP	small molecule metabolic process	GO:0044281	2.182×10^{-3}	497	12
CC	extracellular region	GO:0005576	3.644×10^{-2}	130	5
CC	extracellular space	GO:0005615	1.324×10^{-2}	56	4

* MF—molecular function, BP—biological processes, CC—cellular component.

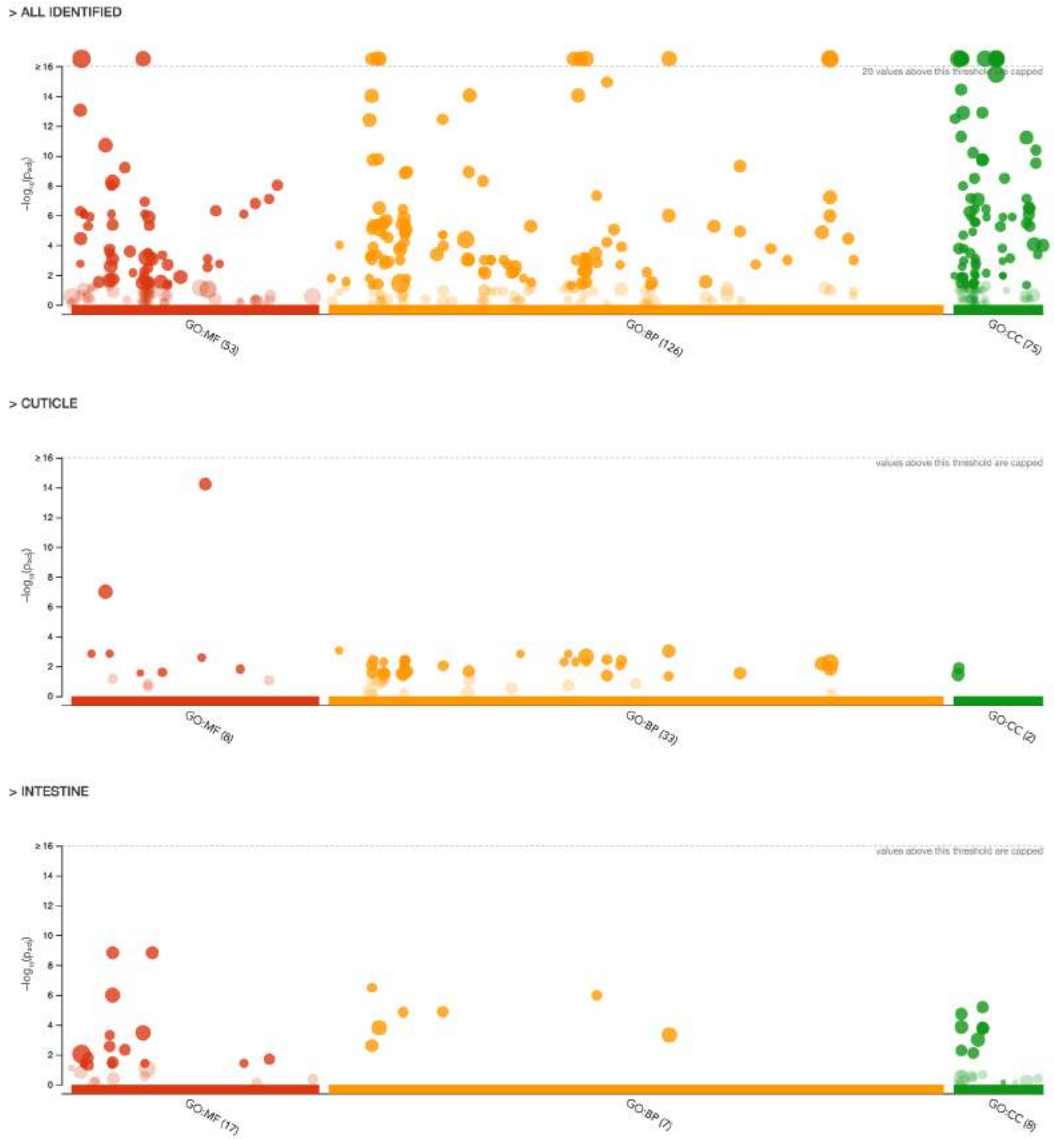


Figure 3. Manhattan plot illustrating the results of the GO analysis. The functional terms are grouped and color-coded by data sources, i.e., molecular function (MF; in red), biological processes (BP; in orange), and cellular components (CC; in green). The five top subcategories from each category are described in Table 1. Detailed representation and annotation of all proteins submitted to the analysis can be found in Files S1 and S2.

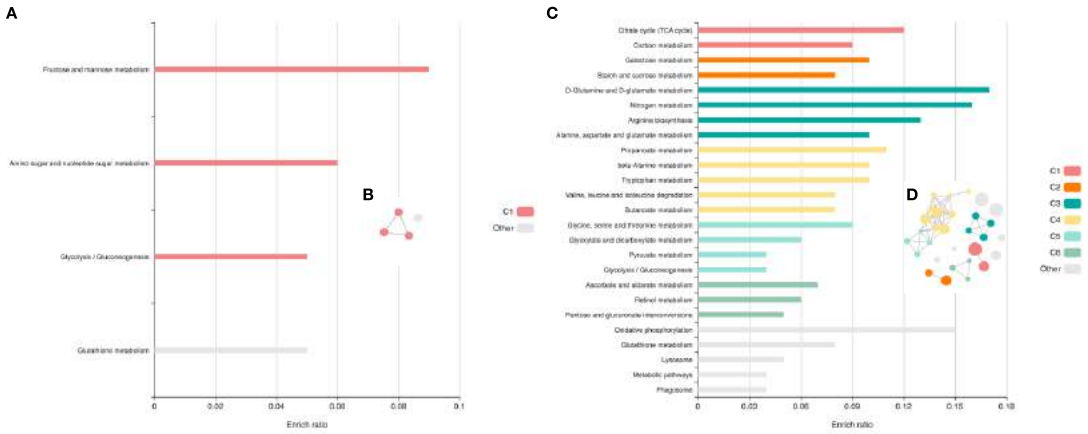


Figure 4. Pathways statistically significantly associated with proteins identified in the cuticle (**A**,**B**) and intestine (**C**,**D**) of the L4 development stage of *Anisakis simplex* s. s. Enriched terms for the cuticle and intestine are visualized in bar plots (**A**,**C**), respectively. Each row represents an enriched function, and the length of the bar represents the enrich ratio, which is calculated as input protein number/background protein number. The color of the bars represents different clusters. For each cluster, if there are more than 5 terms, the top 5 with the highest enrich ratio is shown. Panels (**B**,**D**) are circular network views (cirFunMaps) for the cuticle and intestine, respectively. The node color represents different clusters. Each node represents an enriched term, and node size represents 6 levels of *p*-value, node size from small to large: [0.05, 1], [0.01, 0.05], [0.001, 0.01], [0.0001, 0.001], $[1 \times 10^{-10}, 0.0001]$, $[0, 1 \times 10^{-10}]$; the edge represents interaction larger than the user-defined threshold (0.35). Detailed description of the analysis results can be found in File S3. C1–C6—clusters from 1 to 6.

The molecular functions (MF category) assigned to the proteins characteristic of the cuticle were *structural constituent of cuticle* and *structural molecule activity* (GO:0042303 and GO:0005198), *hexokinase activity* and *glucose binding* (GO:0004398 and GO:0005536), and *glutathione hydrolase activity* (GO:0036374) (Table 1). In the BP category, most of the proteins characteristic of the cuticle were involved in the *cellular glucose homeostasis* (GO:0001678), *glucose homeostasis* (GO:0042593), and *carbohydrate homeostasis* (GO:0033500), and in the *nucleobase-containing small molecule metabolic process* and *small molecule metabolic process* (GO:0055086 and GO:0044281) (Figure 3, Table 1). The distribution of the identified proteins according to their abundance in the cellular components was associated with the *extracellular region* (GO:0005576) and *extracellular space* (GO:0005615) (Figure 3, Table 1) (detailed annotation in File S2).

2.2.2. Pathway Identification

When metabolic pathways were constructed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG), the most abundant proteins in the cuticle were assigned to three metabolic pathways (Figure 4A). The most enriched pathways included *fructose and mannose metabolism* (enrichment ratio = 0.09375, *p*-value = 0.002) and *glycolysis/gluconeogenesis* (enrichment ratio = 0.04672, *p*-value = 0.001). In the cohort of intestinal proteins, analysis revealed involvement in 31 signaling pathways. Those most enriched are shown in Figure 5. Those with the lowest p-value included the *citrate cycle (TCA cycle)* (*p*-value = 6.49×10^{-9}) and *carbon metabolism* (*p*-value = 1.92×10^{-11}). The detailed results of the analysis can be found in File S3. Circular network views (cirFunMaps) for the cuticle and intestine are shown in Figure 5, respectively. Each node represents an enriched term, and the color of the node represents different clusters. The maps show the interactions

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between the clusters and the enriched terms. The *p*-value of the clusters and the size of the clusters can be found in File S3.

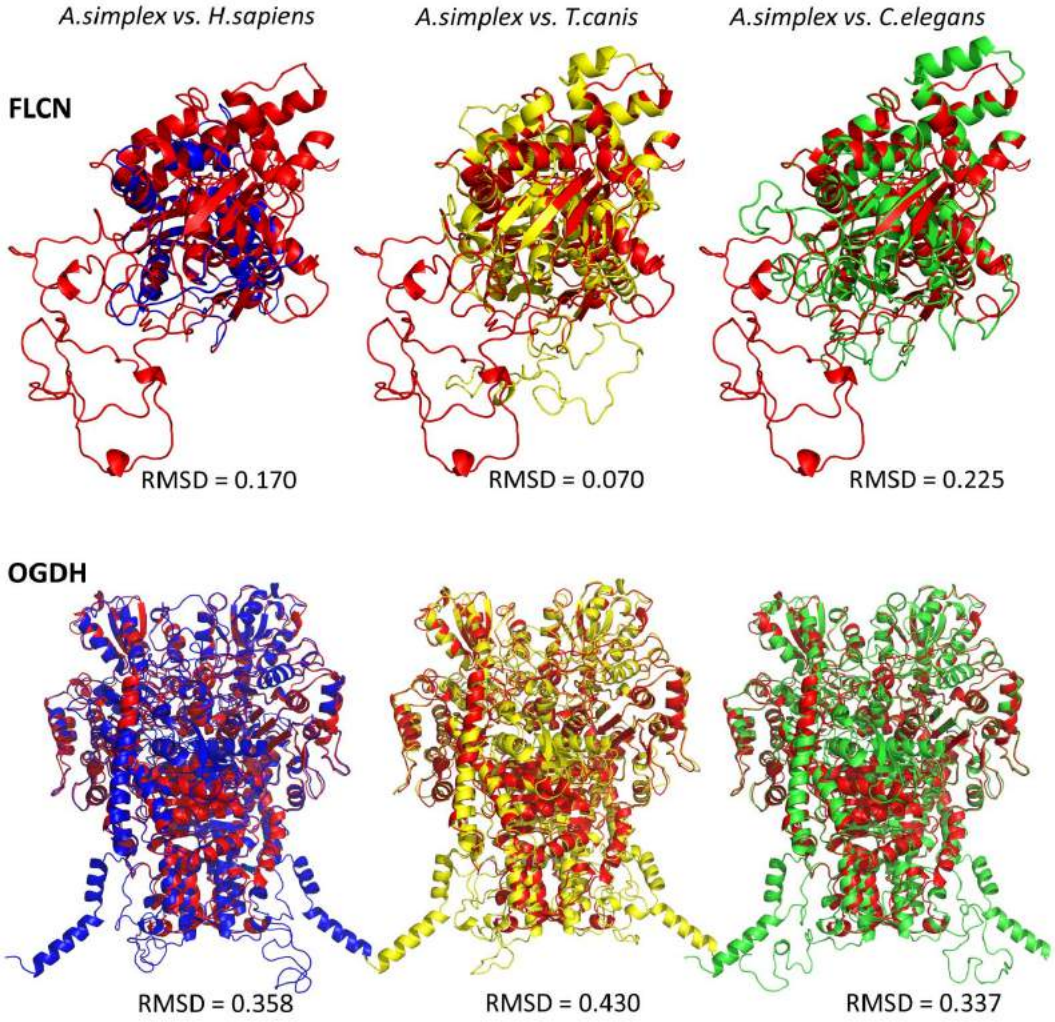


Figure 5. Predicted tertiary structures of *A. simplex* s. s. oxoglutarate dehydrogenase (OGDH) and folliculin (FLCN). The structures are color-coded (structural alignment) for the respective species: *A. simplex* s. s.—red, *H. sapiens*—blue, *T. canis*—yellow, *C. elegans*—green. The root mean square deviation (RMSD) value indicates the average deviation between the corresponding atoms of two proteins: the smaller the RMSD, the more similar the two structures are.

2.3. Enzymes Identification

The InterPro protein family database was used to identify tissue-specific enzymes from *A. simplex* s. s. L4 larvae. In total, 32 cuticular proteins were assigned to 5 enzyme classes and 66 intestinal proteins were assigned to 6 enzyme classes. The most abundant enzyme class was hydrolases (10 proteins in CUT, 28 in INT), and the less represented classes were oxidoreductases, translocases, and transferases in INT, whereas oxidoreductases, transferases, and lyases in CUT. No proteins belonging to the class of isomerases were

detected in INT, and no ligases and translocases were detected in CUT. The distribution of the number of proteins in each enzyme class is shown in File S4.

2.4. Tertiary Structure Protein Modeling and Its Detection with Western Blot

Multiple sequence alignment (MSA) was performed to compare the identity of the sequences of the proteins of interest from *A. simplex* s. s., oxoglutarate dehydrogenase (OGDH) and folliculin (FLCN), with the same proteins from *H. sapiens*, *T. canis*, and *C. elegans* (Figure S1; selection criteria explained in the discussion). The structural models of OGDH and FLCN were built for the A0A3P6SZK1 and A0A0M3JT48 sequences, respectively.

The predicted tertiary structures of *A. simplex* s. s. OGDH and FLCN and their structural similarity with the same proteins of other species are shown in Figure 5. The root mean square deviation (RMSD) value indicates the average deviation between the corresponding atoms of two proteins: the smaller the RMSD, the more similar the two structures are. The FLCN of *A. simplex* was structurally most homologous to the FLCN of *T. canis* (RMSD 0.07), less homologous to the human FLCN (RMSD 0.17), and least homologous to the FLCN of *C. elegans* (RMSD 0.225). The 3D structure of OGDH was structurally similarly homologous to human and *C. elegans*, where the RMSD was 0.358 and 0.337, respectively. The OGDH of *A. simplex* s. s. was least homologous to the OGDH of *T. canis* (RMSD 0.430) (Figure 5). Detailed characteristics of all protein sequences subjected to analysis can be found in File S5.

To confirm the presence of OGDH and FLCN in a complex mixture of *A. simplex* s. s. proteins and to validate the 3D structural modelling and LC-MS/MS, Western blot analysis was performed (Figure S2). Two extracts were prepared from two *A. simplex* s. s. stages (L3 and L4). Due to the high sequence similarity of OGDH and FLCN with other species, specific antibodies recognizing endogenous levels of total OGDH and FLCN proteins were used for detection in *A. simplex* s. s. larvae. The same analysis was performed using a porcine protein extract as a positive control. Western blot analyses revealed the presence of an immunoreactive band at 70 and 100 kDa, corresponding to calculated molecular masses of FLCN and OGDH of 72.5 and 90.2 kDa, respectively (Figure S2).

2.5. Protein–Protein Interactions (PPIs)

2.5.1. Global PPI Network

The network of protein interactions was performed by submitting only DRPs to the STRING database (v. 11.5.) [30]. All interactions were indicated in the context of co-expression, co-occurrence, and based on the emergence of information about the interactions between these proteins in different databases. The analysis revealed that a total of 136 proteins formed a very complex and highly interactive network (653 interactions) (Figure 6). The proteins characterized for the tissues studied (cuticle and intestine) and the rest of the body of *A. simplex* s. s. were additionally grouped (Figure 6). There were 38 proteins in the cuticle group (233 interactions), 63 proteins in the intestine (496 interactions), and 38 proteins (362 interactions) were observed in the group characterized for the rest of the larval body at the L4 stage (Figure 6). The remaining input proteins, not shown in Figure 6, were excluded. This primarily reflects the fact that these interactions were identified on a nematode *Toxocara canis* background (*A. simplex* not available in the STRING database) and that many of the input proteins were uncharacterized in the Universal Protein Resource (UniProt) [31]. A detailed description of all proteins submitted to the analysis can be found in File S6.

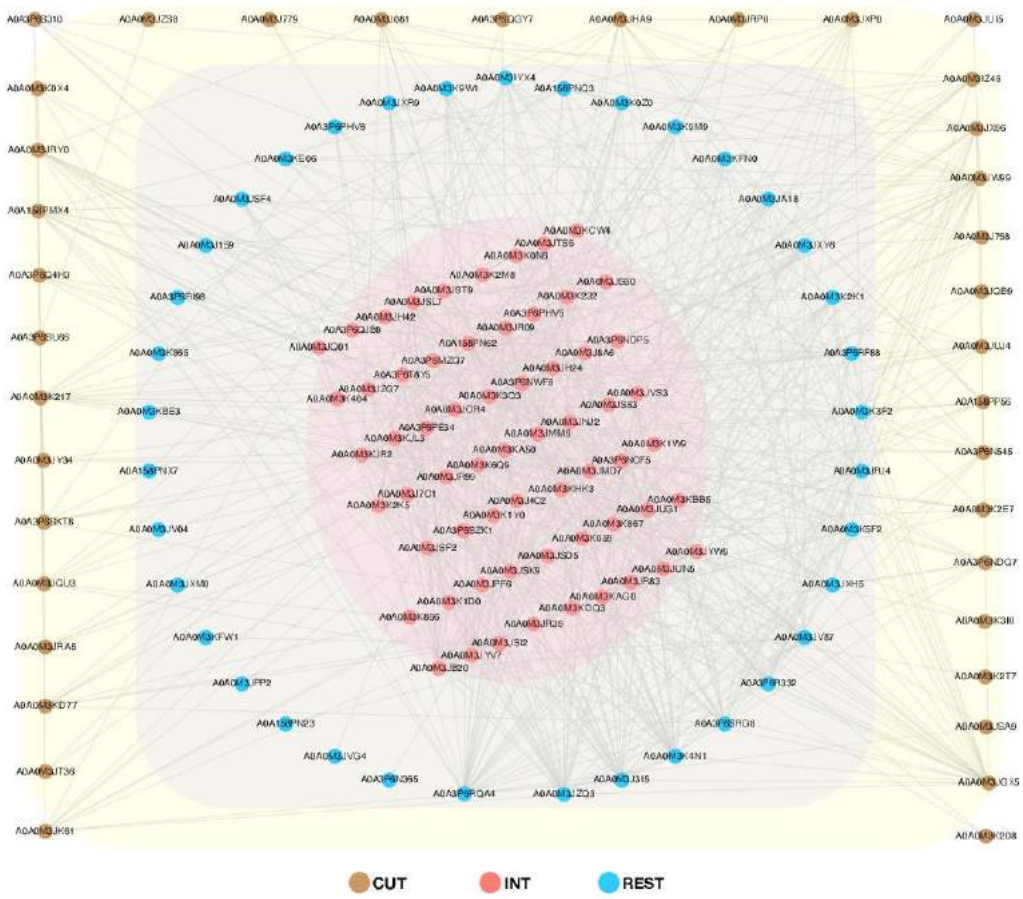


Figure 6. Protein–protein interaction network analysis of differentially regulated proteins between the cuticle, intestine, and rest of the body of the L4 development stage of *Anisakis simplex* s. s. Visualization was performed in Cytoscape v. 3.9.1. A detailed description of all proteins submitted to the analysis can be found in File S6.

2.5.2. Host–Pathogen Protein Interactions

The HPIDB 3.0 server was used to predict host–parasite interactions between the *A. simplex* s. s. tissue proteins and both human and dolphin proteins. In total, 39 proteins of human and 4 *Anisakis* cuticular proteins were identified in the host–parasite interaction network (Figure 7A). These proteins formed the four main clusters in which the *Anisakis* proteins formed their core: cofactor-independent phosphoglycerate mutase (A0A0M3JK61, 22 interactions), serine/threonine-protein phosphatase (A0A0M3KIX0, 11 interactions), and 2 peptidase M1 domain-containing proteins (A0AP6PHI6 and A0A3P6NPL4, 7 interactions each). Intestinal proteins of *A. simplex* larvae together with human proteins constituted three clusters, among which two parasite proteins formed their core: oxoglutarate dehydrogenase (succinyl-transferring; A0A0M3JPF6, 12 interactions), and succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial (A0A0M3JRB2, 4 interactions). One of the formed clusters had as its core a human protein: polyubiquitin-C (P0CG48, 11 interactions) (Figure 7B).

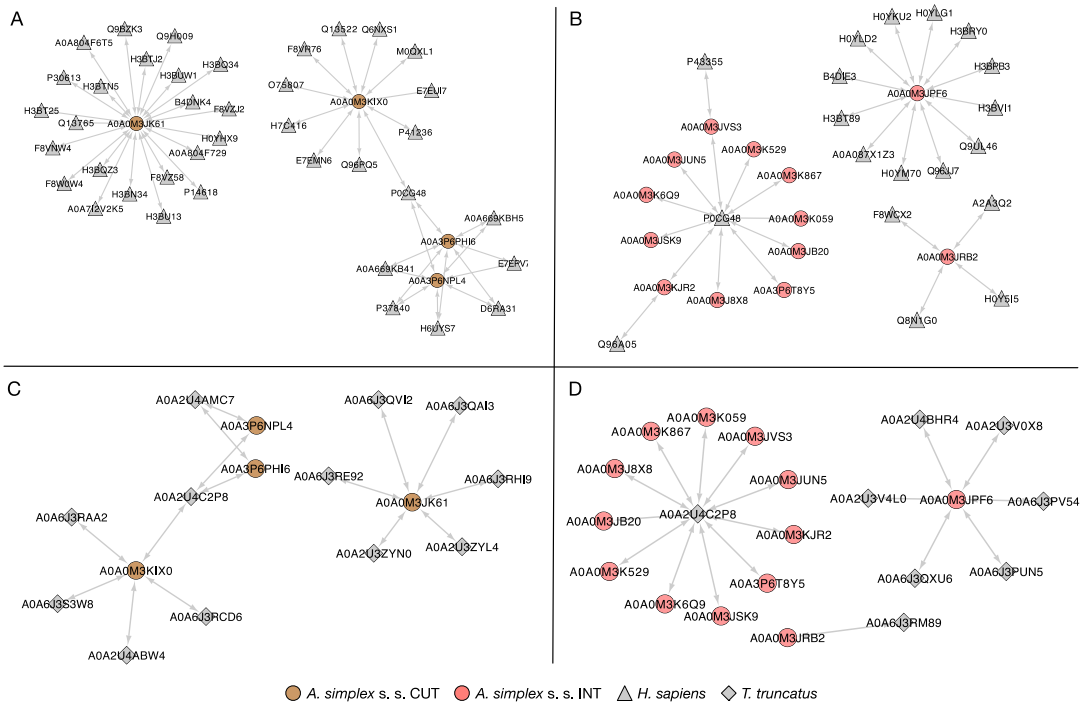


Figure 7. Host–parasite interactions between specific tissues of the L4 development stage of *Anisakis simplex* s. s. and selected hosts. Interactions with the human (*Homo sapiens*) proteome and proteins identified in the cuticle and intestine of *A. simplex* s. s. are presented in part (**A**,**B**), respectively. Interactions with the Atlantic bottlenose dolphin (*T. truncatus*) proteome and proteins identified in the cuticle and intestine of *A. simplex* s. s. are presented in part (**C**,**D**), respectively. Visualization was performed in Cytoscape v. 3.9.1. A detailed description of all proteins submitted to the analysis can be found in File S7.

All *Anisakis* tissue-specific proteins identified in this interactomes (Figure 7A,B) were also identified in the dolphin–parasite interaction networks (Figure 7C,D). The same four proteins of the larvae identified in the cuticle were involved in the interactions with proteins of the Atlantic bottlenose dolphin (*Tursiops truncatus*) (Figure 7C); however, the network was less complex (15 interactions in total, compared to 47 in the human–parasite network). The highest number of interactions showed one of the dolphin proteins, polyubiquitin-B isoform X1 (A0A2U4C2P8, 11 interactions). The detailed results of the identification of proteins involved in potential host–parasite interactions are presented in File S7.

2.6. Allergens Identification

The AllerCatPro server determined the allergenic properties of cuticular proteins, where 28 had strong evidence and 7 had weak evidence. Among the proteins identified in the intestine, 10 proteins were predicted with strong evidence and 10 with weak evidence to have allergenic potential (File S8). Most of the detected potentially allergic proteins showed high similarity to the proteins of the American house dust mite, *Dermatophagoides farina*. The other detected putative allergens showed similarity to allergens of *Bos taurus*, *Oncorhynchus mykiss*, *Salmo salar*, *Trichostrongylus colubriformis*, and *Aspergillus fumigatus*. The representatives of potential allergens found in the *A. simplex* s. s. tissue-specific proteomes are presented in Table 2.

Table 2. Five top strong evidence results of the identification of potential allergens in the tissue-specific proteome of *A. simplex* s. s. L4 larvae. Detailed annotation of all proteins submitted to the analysis can be found in File S8.

Anisakis Tissue-Proteome			*AllerCatPro Prediction*		
Tissue	**UniProt Accession**	**Protein Name**	**UniProt/NCBI Accession**	**Protein Name**	**Organism**
Intestine	A0A3P6Q2Z8	Uncharacterized protein	L7UZ85	Der f 24	*Dermatophagoides farinae*
Intestine	A0A0M3JS03	60S acidic ribosomal protein P2	Q9UUZ6	Asp f 8	*Aspergillus fumigatus*
Intestine	A0A0M3K232	Voltage-dependent anion-selective channel protein 3	Q1HR57	Aed a 6	*Aedes aegypti*
Intestine	A0A0M3JYW9	Fructose-bisphosphate aldolase	B5DGM7	Sal s 3	*Salmo salar*
Intestine	A0A0M3KBB5	Gelsolin-like protein 1	Q8MVU3	Der f 16	*Dermatophagoides farinae*
Cuticle	A0A0M3K208	Uncharacterized protein	G1FMP3	Ani s 24 kD	*Anisakis simplex*
Cuticle	A0A3P6N545	Uncharacterized protein	P59704	Aspartyl protease inhibitor	*Trichostrongylus colubriformis*
Cuticle	A0A158PPI4	Uncharacterized protein	E1BI98	Bos d Collagen 140 kD	*Bos taurus*
Cuticle	A0A0M3JZM7	Col_cuticle_N domain-containing protein	P02465	Bos d alpha2I	*Bos taurus*
Cuticle	A0A0M3JZS8	Uncharacterized protein	O93484	Onc m alpha2I	*Oncorhynchus mykiss*

3. Discussion

In accordance with the increasing incidence of anisakiasis, the molecular mechanisms responsible for the pathogenicity of *Anisakis* spp. remain unknown [32]. From a parasitological perspective, the cuticle and intestine are the tissues most responsible for parasite–host contact [33,34] and have long been thought to be involved in such mechanisms through their proteomic composition and, in particular, through the release of excretory-secretory proteins [35–38]. Moreover, most enzymes important for parasite growth and development are downregulated from the L1 to L3 developmental stages, including enzymes involved in carbohydrate, lipid, and energy metabolism, but many of them are upregulated in L4 and adult nematodes [39,40].

Therefore, in this study, we described for the first time the proteomic analysis of 2 tissues of the L4 developmental stage of *A. simplex* s. s. In the L4 stage, tissues, such as the cuticle and intestine, are fully developed and functional [27–29]. In addition, previous proteomic studies of *A. simplex* did not focus on comparative analysis of nematode tissues [41–51]. This work provides new insights into the tissue-specific proteome of *A. simplex* s. s., which was not previously possible. Our knowledge has been limited because it is difficult to isolate individual tissues, especially when the tissue is limited, as in small nematode larvae, and to characterize specific proteome profiles. To identify tissue-specific proteomes in *A. simplex* s. s., we used liquid chromatography-tandem mass spectrometry with tandem mass tag (TMT) labeling, a method that is accurate and capable of measuring small abundance differences in biological samples [52].

The cuticle of nematodes serves as the primary interface with the environment [33], and the proteome composition of the cuticle changes as the nematode organism develops [34,53,54]. The surface of the cuticle of nematodes contains many thermally stable allergens and other catalytic proteins, the presence of which is essential for nematode survival and development in the host [33,34]. The intestine of nematodes is a simple, hallow, straight, non-muscular tube consisting of a single layer of epithelial cells [34]. There is strong evidence that most secretion and excretion occurs through the parasite's organs, including the excretory gland, esophagus, intestine, and cuticle [54–56]. Enzymes that play a fundamental role in metabolism, such as proteases, nucleotidases, esterases,

glycases, and dismutases, are associated with infectivity, immune defense, and pathogenicity in parasites, thus playing an extremely important role in the life cycle of parasitic nematodes [33,37,38,53].

Although various approaches have been used to study the biology of nematodes and their interactions with the host, the proteomics of these two tissues—cuticle and intestine at the L4 stage—has not yet been studied. TMT-based LC-MS/MS analysis of L4 larvae of *A. simplex* s. s. allowed the identification of 107 DRPs between the cuticle and the rest of the larval body at the L4 stage, of which 49 were more abundant in the cuticle and 58 in the rest of the nematode body. When comparing the intestine and the rest of the larval body at the L4 stage, 123 proteins were identified as DRPs, with 67 proteins being more abundant in the intestine and 56 in the rest of the *A. simplex* s. s. body. Comparison of the individual tissues examined revealed a total of 272 DRPs, with 133 proteins more abundant in the cuticle and 139 proteins more abundant in the intestine (see Figures 1 and 2, Files S1,4–6). This is currently the largest proteomic dataset of *A. simplex* s. s. tissue-specific proteins. In addition, we again compared the proteomes of L3 and L4 larvae with the most recent reference proteome of *A. simplex* from the UniProt/TrEMBL database (downloaded November 2021; proteome ID: UP000267096). Compared to the results obtained previously by Stryński et al. [43], 142 DRPs were identified between the L3 and L4 larval stages (see File S9 and Figure S3), with 43 proteins being more abundant at the L3 and 99 proteins at the L4 larval stage. These results complement and update the previously published *A. simplex* proteome [43].

In general, the identified tissue-specific proteins of *A. simplex* s. s. have multiple functions, as shown by analysis of GO. On average, 43 and 32 GO terms were detected for the cuticle and intestine, respectively, for all proteins in each cohort subjected to analysis (see Figure 3, File S2). Annotation enrichment analysis provided interesting results. Of the enriched annotations, the most common and most enriched GO terms among cuticular proteins in general were those related to glucose and carbohydrate homeostasis and cuticle development. These annotations cover functions important for parasite metabolism and survival under anaerobic conditions in the host organism [57]. Of the GO terms annotated for intestinal proteins, most were related to enzymatic activity (hydrolases, peptidases, oxidoreductases) and proteolysis. This is consistent with a previously published study on L4 larvae of *A. simplex*, which found that ligases, lyases, oxidoreductases, and transferases are overrepresented in L4 stage larvae [43]. In addition, enzyme classes associated with cuticular and intestinal proteins, such as hydrolases, and the less represented classes, such as oxidoreductases, translocases, and transferases, in the intestine while oxidoreductases, transferases, and lyases in the cuticle are critical for host–parasite interactions, such as host invasion, migration through host tissues, molting, degradation of hemoglobin and other blood proteins, digestion of nutrients, evasion of the immune system, and activation of inflammation [58–60]. Therefore, inhibition of enzymes in these metabolic pathways represents a potential therapeutic strategy to combat parasite infections. However, the high degree of similarity between host and parasite enzymes makes this strategy potentially difficult. Nevertheless, there are several drugs for the treatment of helminth infections that target metabolic enzymes [61,62]. Of particular note are the enzymes involved in glycolysis and the tricarboxylic acid cycle (TCA cycle), as these metabolic pathways were most enriched by cuticular and intestinal proteins of *A. simplex* s. s. L4 stage, respectively (see Figure 4, File S3). Glycolytic and oxidative metabolism in the larvae of *Anisakis* spp. had already been studied by Hamajima et al. but in 1969 [63]. Therefore, it was decided to investigate the obtained results also with regard to the proteins of energy and/or carbohydrate metabolism in *A. simplex* s. s. L4 larvae.

The first step of glycolysis is catalyzed by hexokinase (EC 2.7.1.1). In the group of proteins more abundant in the cuticle, 3 phosphotransferases (A0A3P6NT27, A0A0M3JT86, A0A3P6S264) were found to possess hexokinase activity (see File S4). Studies on *Trypanosoma brucei* have validated this enzyme as a drug target [64]. In this parasitic kinetoplastid, a 60% reduction in cellular hexokinase activity results in parasite death. The

hexokinase of *B. malayi*, unlike the monomeric and dimeric forms found in mammals, is tetrameric and has different kinetic parameters than the human enzyme [65,66]. Modified monosaccharides have been proposed as general inhibitors or antimetabolites targeting hexokinase [67]; however, little action has been taken to utilize this proposal with hexokinases of helminth parasites [61]. Phosphoglycerate mutase (PGM) is an enzyme that catalyzes step 8 of glycolysis. These enzymes are classified into 2 different classes: either cofactor-dependent (EC 5.4.2.11, dPGM) or cofactor-independent (EC 5.4.2.12; iPGM) [68]. In most eukaryotes, PGM requires a tightly bound 2,3-bisphospho-D-glycerate cofactor. However, nematodes have an iPGM [69], and such an iPGM was detected in the cuticle of L4 larvae of *A. simplex* (A0A0M3JK61) in this study (see File S4). In addition to the requirement for cofactors, the two types of PGMs also differ in sequence, length, and catalytic mechanisms. For this reason, iPGM has attracted the interest of scientists as a potential drug target. Several potential inhibitors have been identified using computational chemistry methods but have not yet been tested in vitro or in vivo [70]. Recently, studies in *Caenorhabditis elegans* have shown that iPGM is essential for viability [69]. However, a last screening study has shown that iPGM has limited use as a drug [71]. Nevertheless, the anthelmintic Clorsulone can successfully and selectively inhibit the nematode enzyme in *Fasciola hepatica*, which has a host-like dPGM. This suggests that the search for novel molecules that can inhibit the iPGM of *A. simplex* may be the basis for further exploration of methods to control anisakiasis.

The final reaction of glycolysis leading to the second generation of ATP may differ greatly in different helminths [57]. Pyruvate kinase (EC 2.7.1.40; PK) can convert phosphoenolpyruvate (PEP) to pyruvate, which can then be reduced to lactate or ethanol in the cytosol or translocated to the mitochondrion for further oxidation by the TCA cycle. In contrast, phosphoenolpyruvate carboxykinase (EC 4.1.1.32; PEPCK) can carboxylate PEP to oxaloacetate (OAA), an intermediate of the TCA cycle [57,72,73]. This pathway is common in many parasites, especially lumen-dwelling helminths, such as *Ascaris suum* [57]. PK (A0A0M3JXT3) and 3 of 4 PEPCK proteins (A0A0M3IZF7, A0A0M3IZU8, A0A0M3J768) were detected in *A. simplex* tissues in this study but were not found to be statistically significant between the tissues examined (see File S1.4–S6). The fourth PEPCK protein (PEPCK_N domain-containing protein, A0A0M3J768) was more abundant in the cuticle of L4 larvae of *A. simplex* s. s. (see File S1.4–S6). Despite the lack of statistical significance, the abundance of PK ranged from 66 [a.u.] in REST to 188 [a.u.] in INT, where PEPCK levels were approximately 2000–2500 [a.u.] in all examined tissues (see S1.4–6). This is consistent with previous studies on *A. simplex*, where no activity was detected for PK and high activity of PEPCK was observed [74,75]. Moreover, activity of PEPCK increased following molting to the L4 stage [74,75].

If PEP is used to directly generate OAA, which is subsequently reduced to malate (MA) in the life stages of the parasite under anaerobic conditions [61,76,77], the reaction catalyzed by PK is omitted and helminth PKs have been relatively understudied [61]. Thus, since PK is unlikely to be active for the anaerobic steps of the parasitic nematode life cycle, its potential as a drug target may be limited [78,79]. Furthermore, pyruvate can be directly carboxylated to OAA by pyruvate carboxylase (EC 6.4.1.1; PC) [72,73]. This enzyme was detected in the intestine of *A. simplex* s. s. L4 larvae (A0A0M3K059) in this study, which is statistically significant compared to the cuticle (see Files S1.6 and S4). Accordingly, regulation of the PK/PEPCK branch point is potentially important in many helminths and further studies on this topic in *A. simplex* may be based on the results presented.

Glycolysis and the TCA cycle are linked by a reaction catalyzed by pyruvate dehydrogenase complex (PDC), a complex of three enzymes: pyruvate dehydrogenase (EC 1.2.4.1; PDH), dihydrolipoyl transacetylase (EC 2.3.1.12; DLAT), and dihydrolipoyl dehydrogenase (EC 1.8.1.4; DLD), and which converts pyruvate to acetyl-CoA by a process called pyruvate decarboxylation. Acetyl-CoA can then be used in the TCA cycle to carry out cellular respiration [72,73]. In the present study, the abundance of pyruvate dehydrogenase E1 component subunit beta (PDH, A0A0M3KJM6) was found to be much higher in the

cuticle than in the intestine (see Files S1.6 and S4). The whole PDC has been studied in detail in *A. suum* [80–83]. It has been shown that a single phosphorylation catalyzed by pyruvate dehydrogenase kinase (EC 2.7.11.2) can inhibit activation of the PDC in higher eukaryotes, but a greater degree of phosphorylation is required for inactivation of the PCD in *A. suum* [84] Consequently, the PCD remains active to some degree. Nevertheless, to the authors' knowledge, the PDC has never been studied in *A. simplex*, and this issue requires further investigation. Compared to glycolytic enzymes, TCA enzymes have, generally, received less attention in parasitic nematodes. However, Rahman et al. [85] showed that in *C. elegans* embryos, downregulation of the components of the TCA cycle inhibits entry into the first mitotic division and particularly, knockdown of citrate synthase (EC 2.3.3.1; CS) selectively inhibits the TCA cycle and blocks mitosis [85]. This revealed a previously unknown link between the TCA cycle and cell cycle progression. In the present study, the first enzyme of the TCA cycle, the CS (A0A0M3K529), was detected at significantly higher levels in the intestine than in the cuticle of L4 larvae of *A. simplex* s. s. (see Files S1.6 and S4).

Like the PDC, the oxoglutarate dehydrogenase complex (OGDC), known primarily for its role in the TCA cycle, has also been studied in *Fasciola hepatica* [86]. The OGDC consists of three enzymes: oxoglutarate dehydrogenase (EC 1.2.4.2; OGDH), dihydrolipoyl succinyltransferase (EC 2.3.1.61; DLST), and the previously mentioned DLD (EC 1.8.1.4). Diaz and Komuniecki [86] showed that the enzyme from *F. hepatica* is not upregulated by calcium ions, in contrast to the mammalian OGDH [87,88]. This issue also needs further investigation from the point of view of the effect of inhibitory substances on OGDH to kill helminths. In this study, we found statistically greater amounts of all 3 OGDC enzymes (OGDH, A0A3P6SZK1; DLST, A0A3P6T8Y5; DLD, A0A0M3JB20) in the intestinal tissue of *A. simplex* s. s. compared with the cuticle (see Files S1.6 and S4). OGDH was present in the DRPs cohort under 2 different UniProt IDs (A0A0M3JPF6, A0A3P6SZK1). In addition, we were able to detect the presence of OGDH in *A. simplex* L3 and L4 larvae for the first time using Western blot analysis (see Figure S2). This also prompted us to select OGDH for 3D structural modelling, which is described below. Chin et al. [89] reported that supplementation of α-ketoglutarate, an intermediate of the TCA cycle produced by oxidative decarboxylation from isocitrate and converted to succinyl-CoA by OGDC, delays ageing and prolongs the lifespan of adult *C. elegans*. In addition, increasing the α-ketoglutarate level by RNA interference of *ogdh* also prolongs the lifespan of worms [89]. This may suggest that increasing the activity of OGDC and thus decreasing the α-ketoglutarate level may be lethal to parasites. In addition, α-ketoglutarate can also be produced anaplerotically from glutamate by oxidative deamination using glutamate dehydrogenase (EC 1.4.1.2; GDH) [73]. This enzyme was detected in the intestine of L4 stage larvae of *A. simplex* (A0A0M3JCR4) in this study (see Files S1.6 and S4). The combination of increased OGDC activity and GDH inhibition could potentially lead to the expected results in terms of nematode larval death, but targeted research in this area is needed.

Adult parasitic helminths are not capable of gluconeogenesis. In contrast, gluconeogenesis can function in aerobic larval stages that use substrates other than carbohydrates for energy [57]. However, because L4 larvae are more similar to adults than L3 larvae, it was decided not to discuss gluconeogenesis.

In the current study, we also decided to take a closer look at folliculin (FLCN) as an inhibitor of lactate dehydrogenase A (EC 1.1.1.27; LHDA) and as regulator of the Warburg effect [90]. FLCN was selected for further study based on the PPI analysis results (see File S6). The sequence of the thioredoxin domain-containing protein (A0A0M3JRA5) of *A. simplex* s. s. (very abundant in the cuticle) resembled the FLCN of *T. canis* (A0A0B2VPH2), whose proteome formed the background for the PPI analysis. After searching the UniProt database, it was found that the FLCN protein for *A. simplex* s. s. was already annotated (A0A0M3JT48). However, LC-MS/MS analysis did not reveal the presence of this protein in the tissues studied but only the similar protein containing a thioredoxin domain (A0A0M3JRA5). Therefore, it was decided to compare the 2 *A. simplex* s. s. sequences

(A0A0M3JRA5, A0A0M3JT48) and, like in case of OGDH, build a 3D model of the FLCN protein of *A. simplex* s. s. on this basis.

FLCN acts as a binding partner and non-competitive inhibitor of LDHA [90]. LDHA converts pyruvate, the end product of glycolysis, to lactate in the absence of oxygen [72]. The presence of LDH was noted in many helminth species as early as 1967 [91]. LDH activity is common in parasitic nematodes and removes pyruvate produced by PK that is not passed to the mitochondria. It is also thought to be involved in the regulation of the redox balance in the cytosol of helminth cells [92]. In the current study, the 2 forms of lactate dehydrogenase (LDHD and LDHL), in which L-lactate dehydrogenase plays a key role in the production of lactate from pyruvate, were identified in *A. simplex* s. s. L4 larvae (A0A0M3KCR5 and A0A3P6PI01) (see File S1.2). In addition, we confirmed for the first time the presence of FLCN in *A. simplex* s. s. L3 and L4 larvae by Western blot analysis (see Figure S2).

A flexible loop in the amino terminus of FLCN controls the movement of the loop in the active site of LDHA, regulating enzyme activity and consequently metabolic homeostasis in normal cells. Cancer cells in which the Warburg effect occurs show dissociation of FLCN from LDHA. Treatment of these cells with a decapeptide from the FLCN loop region results in cell death. The glycolytic shift of cancer cells appears to be the result of FLCN inactivation or dissociation from LDHA [90]. Although the Warburg effect has not been studied in the context of helminth metabolism often, it is worth mentioning with respect to FLCN and lactate production.

The Warburg effect is a metabolic response to an increase in glycolytic flux due to hypoxia or a strictly anaerobic environment [93]. It is also referred to as aerobic glycolysis and is rather inefficient regarding ATP production (~4 mol ATP/mol glucose) when compared to the TCA cycle (~36 mol ATP/mol glucose) [94], which is the primary aerobic metabolism of parasitic larvae (L1-L3) [57]. However, anaerobic glycolysis produces at most ~2 mol ATP/mol glucose, which could explain the metabolic advantage of the larval Warburg effect during the transition to L4 or adult. This postulate has also been mentioned in metabolomic studies of *H. contortus* [95]. In addition, Łopieńska-Biernat et al. [96,97] indicate that the trehalose and glycogen synthesis and degradation pathways play a crucial role in parasitic nematodes' development and that genes related to these pathways are upregulated in L4 larvae of *A. simplex* s. l. Interestingly, the major pathway of lactate production is thought to be directly linked to trehalose synthesis and degradation [98–100]. Consistent with all this, FLCN-mediated inhibition of LDHA hyperactivity during the Warburg effect provides a new paradigm for controlling parasite survival. However, further evidence is needed to investigate such hypotheses in gastrointestinal nematodes and to shed light on the potential role of this alternative metabolic pathway.

The results discussed open new possibilities in the study of enzymes of energy and carbohydrate metabolism as potential drug targets in anisakiasis. Therefore, in vitro or in vivo experiments followed by activity assays are needed to verify the inhibition of the key proteins described in this study (Figure 8).

In addition, the current study provided new information on 3D models of OGDH and FLCN as proteins that may be potential candidates for controlling *A. simplex* survival. The models for OGDH and FLCN were predicted based on the sequences A0A03PSZK1 and A0A0M3JT48, respectively, and the obtained 3D structures were compared with those of other species (see Figure 5). As expected, the tertiary structure of FLCN was very similar to the structure of FLCN from *T. canis*, a parasitic nematode of canids. In the case of OGDH, the situation was reversed. The model showed the greatest structural similarity to OGDH from *C. elegans*. Importantly, there were differences in the structure of *A. simplex* and human proteins (see Figure 6). This could be the basis for further research to select an efficient method to kill nematodes without harming the host (Figure 8).

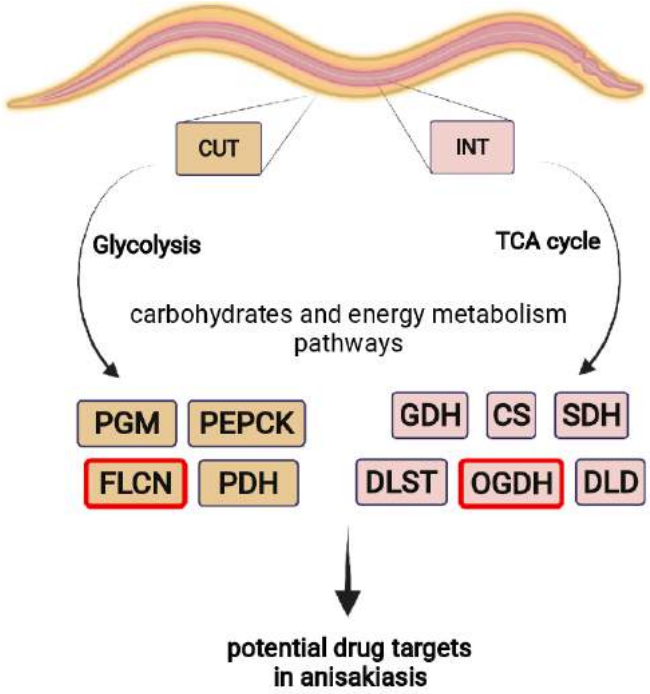


Figure 8. Summary of the results discussed. The possibility of using enzymes of energy and carbohydrate metabolism as potential drug targets in anisakiasis.

In addition to the results already discussed (Figure 8), analyses of protein–protein interactions were also performed in the present work. The global PPI network was visualized using the DRPs between the tissues tested (see Figure 6, File S6). The proteins in the presented network interact with an average of 10 other proteins. For example, OGDH interacts with 11 other proteins. Such connections in the case of gene silencing or direct blocking of the activity of a particular protein offer the chance of promising results, as they affect many metabolic pathways simultaneously, which has been discussed previously [41–43]. In addition, for the first time for *A. simplex* s. s., the tissue-specific host–pathogen interactions were investigated (see Figure 7, File S7). Interestingly, the parasitic proteins characterized in detail above, such as OGDH, DLST, DLD, PC, or CS, were directly involved in the interactions with the human host. OGDH from the parasite was particularly interactive, and polyubiquitin-C (P0CG48) was the major identified target interacting with *A. simplex* proteins. Ubiquitin is known to modulate host–pathogen interactions, with a particular focus on host innate immune defenses and pathogen immune evasion [101]. This is consistent with the findings of Kochanowski et al. [51], who studied the excretory-secretory proteins of *A. simplex* larvae. The number of proteins involved in the human–parasite interaction network is about twice the number of proteins in the dolphin–parasite interactome. This result could be mainly due to the fact that the available database for non-human host–pathogen interactions is much more limited than for human–pathogen interactions, which was also highlighted by Kochanowski et al. [51]. Cuticular proteins of *A. simplex* interacting with the human or dolphin host included serine/threonine protein phosphatase (A0A0M3KIX0; EC 3.1.3.16; STP), 2 peptidase M1 N-domain-containing proteins (A0A3P6NPL4 and A0A3P6PHI6), and the aforementioned iPGM (A0A0M3JK61) (see Figure 7, File S7). It is worth noting that STPs are integral components of parasitic excretory/secretory proteomes that are thought to be released during host–parasite interactions. Recent studies on *Haemonchus contortus* have shown

that STP 1 is actively involved in suppressive regulation of the immune functions of goat peripheral blood mononuclear cells [94]. A group of intestinal proteins that interact with the hosts studied includes the iron-sulphur subunit of succinate dehydrogenase [ubiquinone] (A0A0M3JRB2; EC 1.3.5.1; SDH-IP) (see Figure 7, File S7). Iron-sulphur protein (IP) is an essential component of the mitochondrial complex II (succinate dehydrogenase, SDH), which is a functional enzyme of both the TCA cycle and the respiratory electron transport chain (ETC). SHD-IP is responsible for the transfer of electrons from succinate to ubiquinone (UQ) in normoxia (normal oxygen conditions). Under hypoxia, helminths use fumarate instead of oxygen as the final electron acceptor of ETC [102,103]. This is achieved by an alternative ETC in which rhodoquinone (RQ) serves as an electron carrier [61,102–104]. RQ is a redox-active quinone that is structurally similar to conventional UQ [105]. This change confers RQ a lower redox potential than UQ, which allows RQ to accept electrons from reduced nicotinamide adenine dinucleotide (NADH) via complex I and to reduce fumarate to succinate through complex II. In this alternative ETC, complex II functions as a fumarate reductase (FRD), in reverse to the conventional ETC, in which complex II functions as an SDH [104,106]. Mitochondria, especially in the aerobic developmental stages, such as L2 and L3, contain mainly UQ. In contrast, the mitochondria of adult worms living in a low-oxygen environment contain only RQ [107–109].

The association of an alternative complex II with RQ is well established in *A. suum* and has become a paradigm in helminth biochemistry. However, the results presented by Otero et al. [106] show that *A. suum* is the exception rather than the rule. ETC complexes appear to have evolved to adapt to different stages of the helminth life cycle, to environmental conditions, or to specific cells or tissues within the worms [106]. To the authors' knowledge, no studies have been performed on the SDH properties of L4 larvae of *A. simplex*. Moreover, the involvement of UQ vs. RQ at ETC in *A. simplex* has never been directly tested. Perhaps this is why the results discussed above refer to typically anaerobic conditions in L4 larvae and the occurrence of SDH oxidizing succinate to fumarate and reducing UQ may be an unexplored hypothesis so far.

Studies have shown that the parasite SDH enzyme could be a potential target for selective chemotherapeutic agents [110,111]. However, its potential use in *A. simplex* remains to be investigated.

The last analysis performed on proteins characteristic of the intestine and cuticle of *A. simplex* L4 larvae was able to identify allergens and potential allergens. Of the 39 *A. simplex* allergens listed in the ALLERGOME database (available online: https://www.allergome.org, accessed on 17 February 2022), only Ani s 24 kD resembled one of the uncharacterized proteins identified in the cuticle of *A. simplex* in the present study (see Table 2; File S8). In addition, nine potential allergens were also identified in this study (see Table 2; File S8). One of these, fructose-bisphosphate aldolase (A0A0M3JYW9) was also identified as a potential allergen in the secretome of *A. simplex* by Kochanowski et al. [51]. In addition, many of the potential allergens detected in *Anisakis* tissues show similarity to the potential allergens identified by other authors in the whole proteome and transcriptome of *Anisakis* larvae [9,43,45,48,50].

To sum up, the tissue-specific proteomes of the intestine and cuticle of L4 stage larvae of *A. simplex* s. s. were characterized for the first time. Comparison of the individual tissues revealed a total of 272 DRPs, with 133 proteins being more abundant in the cuticle and 139 proteins in the intestine. In general, the identified tissue-specific proteins of *A. simplex* s. s. have multiple functions, as shown by the analyses of GO and KEGG. In addition, host–pathogen interactions were identified, and potential new allergens were predicted. Of particular note are enzymes involved in glycolysis and the TCA cycle, as these metabolic pathways were most enriched by cuticular and intestinal proteins of *A. simplex* s. s. L4, respectively. Therefore, key enzymes and other proteins in the above metabolic pathways were discussed. Beyond iPGM, PK, PEPCK, PDH, CS, DLST, DLD, or GDH, two proteins, OGDH and FLCN, were the focus of our attention. Their presence

in L3 and L4 larvae was also confirmed by Western blot, and their tertiary structure was modulated and compared with other species.

The identified tissue-specific proteins, i.e., PGM, PEPCK, GDH, CS, SDH, DLST, DLD, OGDH, and FLCN, could serve as targets for new drugs against anisakiasis. However, it should be noted that the functional analysis of the identified tissue-specific proteins was performed using an omic approach. Therefore, in vitro or in vivo experiments and subsequent activity assays are required to verify the inhibition of the key proteins described in this study. Such a combination of global omic analysis with classical biochemical methods and molecular biology will provide a clear picture of the active metabolic pathways in the biological systems under investigation.

4. Materials and Methods

4.1. Anisakis Simplex

The experimental setup and workflow for comparison of tissue-specific proteomes of *Anisakis simplex* s. s. larvae at the L4 stage is presented in Figure 9. All experiments were performed on the L4 larvae of *A. simplex* s. s. isolated from striped dolphin (*Stenella coeruleoalba*). All larvae were obtained from the biobank of the PARASITE project [112] and were previously taxonomically identified using conventional polymerase chain reaction (PCR) to amplify the mitochondrial cytochrome c oxidase subunit II (mtDNA *cox2*) gene, and the elongation factor *EF1* α-1 nuclear DNA gene as described before by Levsen et al. [113] and using microscopical observation of morphological features characteristic of L4 stage larvae.

4.2. Sample Preparation

Twelve *A. simplex* s. s. L4 larvae were used in the study. Three whole larvae were placed in sterile test tube and stored at −80 °C until further analysis. The longitudinal incision of the next 9 larvae using a surgical scalpel was performed under the microscope (magnitude × 100). Then, using microscopic tweezers, the outer layer of the larvae—the cuticle—was separated from the body. The cuticle separated in this way was placed in sterile test tubes. A cuticle from three larvae was used for one sample (three samples in total). Then, the intestine of the larvae was isolated, cleaned of any other tissue residues, and transferred to sterile test tubes. An intestine from three larvae was used for one sample (three samples in total). The remains of the body (after cuticle and intestine isolation) of all used larvae were collected in the test tube and further processed. Additionally, 5 L3 stage larvae isolated from hake (*Merluccius merluccius*) were used to compare the global proteomes of L3 and L4 stages (File S3). The taxonomical identification of L3 larvae was performed as described in Section 4.1.

4.3. Protein Extraction, Preparation, and LC-MS/MS Analysis

4.3.1. Protein Extraction

Proteins were extracted as described before by Stryiński et al. [114]. The whole larvae and isolated tissues (L3, L4, cuticle sample further as CUT, intestine sample, abbreviated further as INT, and the remains of the rest of the body abbreviated further as REST) were crushed manually with a sterile plastic pestle in 2 mL centrifuge tubes. Then, protein extraction was performed in 1.5 mL of lysis buffer: 60 mM Tris-HCl pH 7.5, 1% laurylmaltoside (cat. no 89902, Sigma Aldrich, Poznań, Poland), 5 mM PMFS (cat. no 36978, Thermo Fisher Scientific, Waltham, MA, USA), and 1% DTT (cat. no D9779, Sigma Aldrich, Poznań, Poland). The protein concentration was quantified using the bicinchoninic acid method (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. A total of 100 µg of the protein from each sample (9 samples in total) were transferred into a new tube and methanol/chloroform precipitation was performed as described by Carrera et al. [115]. Then, ultrafast tryptic digestion with the simultaneous application of high-intensity focused ultrasound (HIFU) was carried out, as described previously by Stryiński et al. [43,114].

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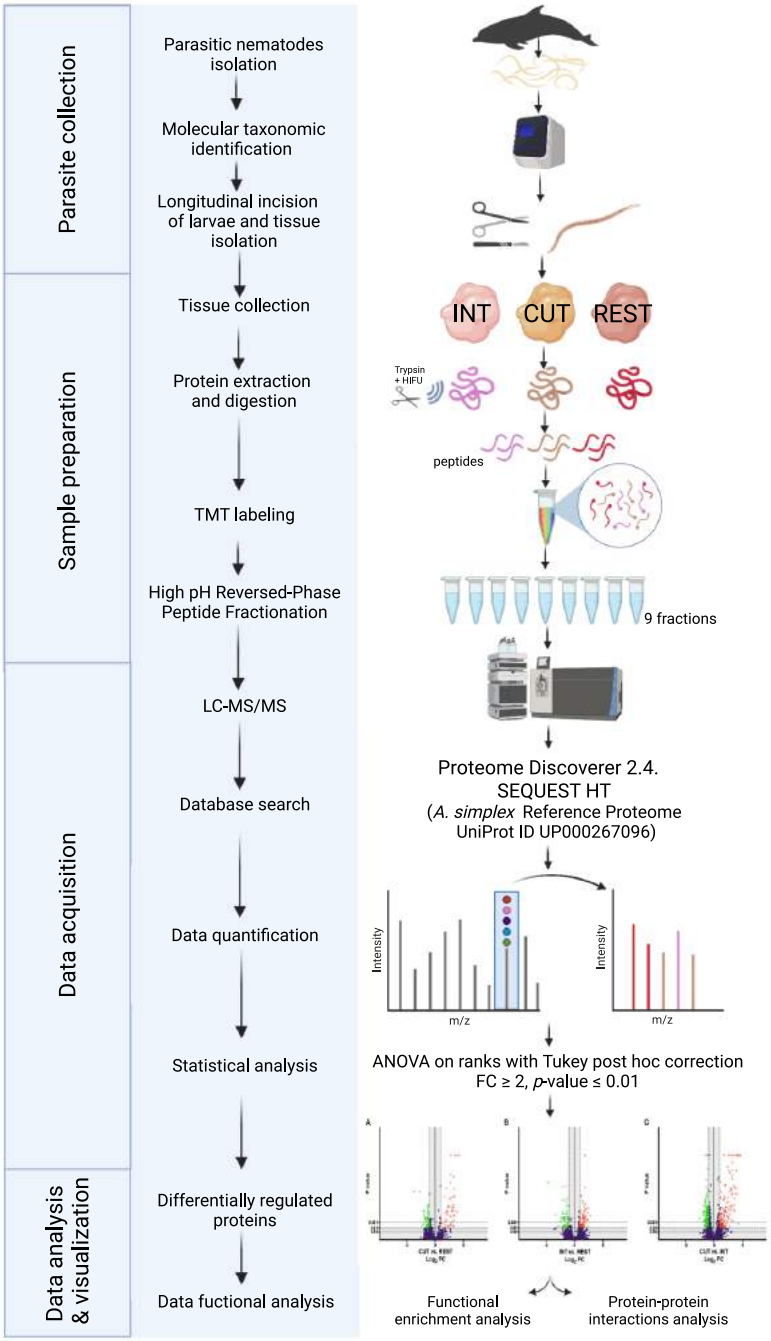


Figure 9. Experimental setup and workflow for comparison of tissue-specific proteomes of *Anisakis simplex* s. s. larvae at the L4 stage. In total, 12 *A. simplex* s. s L4 stage larvae were isolated from striped dolphin (*Stenella coeruleoalba*). Samples of the cuticle, intestine, and remaining body of the L4 larvae

were collected; proteins extracted; and subjected to trypsin digestion stimulated with high-intensity focused ultrasound (HIFU). Peptides from each sample were labeled with tandem mass tags (TMTs). After tagging, samples were pooled and fractionated using the high pH reversed phase fractionation method. Tandem mass spectrometry data were acquired using an LTQ-Orbitrap Elite spectrometer and proteins were identified using Proteome Discoverer 2.4. The identified proteins were subjected to statistical analysis and obtained results were visualized. Created with BioRender.com.

4.3.2. TMT Labeling and Reversed-Phase Fractionation

The TMT 10-plex isobaric label reagents (0.8 mg, Thermo Fisher Scientific, Waltham, MA, USA) were resuspended in 41 µL of anhydrous acetonitrile and added to 100 µg of protein digest, as described by Stryiński et al. [114]. Within the experiment, samples were labeled with TMT10-plex as follows (INT: 127C, 128N, 128C; CUT: 129N, 129C, 130N; REST: 130C). Additionally, whole L3 and L4 larvae were labeled (L3: 126; L4: 127N) and submitted to analysis. Samples were combined in a new tube at equal amounts according to the manufacturer's instructions. The TMT-labeled peptide concentration was measured using a Pierce Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. To increase the number of peptide identifications, eliminate the interference from co-isolated ions, and achieve results comparable to the MS3-based methods [42,116], the combined sample was fractionated using a Pierce High-pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The peptide concentration in each fraction was determined by colorimetric analysis using the Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Then, fractions were evaporated to dryness using vacuum centrifugation (SpeedVac concentrator, Thermo Fisher Scientific, Waltham, MA, USA). The samples (8 fractions) were stored at −80 °C until further analysis.

4.3.3. LC-MS/MS Analysis and Data Processing

Peptide fractions were acidified with 0.1% formic acid and analyzed by LC-MS/MS using a Proxeon EASY-nLC II liquid chromatography system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Peptide separation (1 µg) was performed as described by Stryiński et al. [43,114]. All acquired MS/MS spectra were analyzed using SEQUEST-HT (Proteome Discoverer 2.4 package; Thermo Fisher Scientific, Waltham, MA, USA) against a reference proteome of *A. simplex* available in the UniProt/TrEMBL database (downloaded November 2021; proteome ID: UP000267096; # of entries 20,779). The following restrictions were used: full tryptic cleavage with up to 2 missed cleavage sites and tolerances of 10 ppm for parent ions and 0.06 Da for MS/MS fragment ions. TMT labeling (+229.163 Da on N-termini and lysine residues) and carbamidomethylation of cysteine (+57.021 Da) were set as fixed modifications. The permissible variable modifications were methionine oxidation (+15.994 Da), acetylation (+42.011 Da) of the N terminus of the protein, and deamidation (+0.984 Da) of asparagine and glutamine. Moreover, searching parameters included four maximal dynamic modification sites.

4.3.4. Statistical Analysis

The results were subjected to statistical analysis to determine the peptide false discovery rate (FDR) using a decoy database and the Target Decoy PSM Validator algorithm [117]. The FDR was kept below 1%, and for further analysis, only proteins meeting selected criteria were submitted: (a) proteins classified as master proteins, (b) proteins with at least 2 unique peptides, and (c) characterized proteins. Relative quantification was performed using the Quantification Mode and normalization against the total peptide amount (Proteome Discoverer 2.4 package, Thermo Fisher Scientific, Waltham, MA, USA). After relative quantification, several filters were applied to obtain the final list of differentially regulated

proteins (DRPs): (a) at least a 2-fold change (FC $\geq$ 2) in normalized ratios, (b) 1-way ANOVA on ranks and Tukey HSD post-hoc test (p-value $\leq$ 0.01).

4.4. Results Analysis

4.4.1. Functional Enrichment Analysis and Pathway Identification

To perform functional enrichment analysis, the proteins identified in the cuticle, intestine, and rest of the body of L4 stage larvae of *A. simplex* s. s. were classified into 3 different categories of Gene Ontology (GO): biological processes, cell components, and molecular functions. GO analysis was performed using g:GOSt, the core of the g:Profiler (ELIXIR, Hinxton, Cambridgeshire, UK) that performs statistical enrichment analysis [118] (https://biit.cs.ut.ee/gprofiler/gost, accessed on 22 January 2022). The g:GOSt web-based tool applied an overrepresentation test controlled with the g:SCS algorithm. The significantly enriched functional GO categories were reported by comparing the input data with the background of GO annotations for parasite-specific data from WormBase ParaSite (*Anisakis simplex* PRJEB496).

KEGG pathways in which proteins of each tissue were involved were detected using the KOBAS 3.0 server (http://kobas.cbi.pku.edu.cn/, accessed on 19 February 2022) [119]. Enriched KEGG pathways were identified by Fisher's exact test using the *Brugia malayi* proteome as a reference and the whole *A. simplex* proteome as background. In this case, a p-value less than 0.05 with Benjamini and Hochberg correction was considered to indicate significance.

4.4.2. Enzymes Identification

InterPro protein family classification and enzyme identification were performed using OmicsBox with the Blast2GO algorithm (ver. 1.4.12; BioBam Bioinformatics SL, Valencia, Spain) with the default settings [120].

4.4.3. 3D Structures Modulation

The alignment of sequences of interest was performed using the MUSCLE v. 3.8.425 [121]. To approximate an accurate tertiary model of 2 selected *A. simplex* proteins: OGDH and FLCN, we used several protein structure prediction servers, namely FOLDpro [122], I-TASSER [123], 3DJigsaw [124], LOOPP [125], Phyre2 [126], and SwissModel [127]. To assess the quality of the output models and to choose the top candidates, we used Resprox [128], Qmean [126], and ModFOLD [129]. We then manually inspected the top three models of each protein to determine any unresolved secondary structures (i.e., α-helices) and optimize the structures (e.g., by removing steric clashes). The top model structures with an optimized structure were presented. Root mean square deviation (RMSD) was used to measure the average distances between atoms from two protein structures: one based on the *A. simplex* s. s. sequence and the second based on the sequence of *H. sapiens*, *T. canis*, and *C. elegans*, respectively.

4.4.4. Western Blot of Selected Proteins

To confirm the results of the analysis of 3D structures, we performed Western blot analysis for OGDH and FLCN proteins. Samples (L3 and L4 larvae) for Western blot analysis were homogenized on ice with T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA) enriched with protease inhibitors (Sigma Aldrich, Poznań, Poland). Lysates were clarified by centrifugation (10,000$\times$ g, 5 min, 4 °C). The protein concentration was quantified using the bicinchoninic acid method (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Western blot analysis was performed according to Smolinska et al. [130]. In total, 30 µg of total protein isolates were dissolved in sample buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 200 mM dithiothreitol, pH = 6.8) and incubated at 99.9 °C for 5 min. Samples were then separated by SDS-PAGE electrophoresis in a 12.5% polyacrylamide gel (100 V) and transferred to a polyvinylidene fluoride (PVDF) membrane (Roche Diagnostics, Poland). Then, 5% bovine serum albumin (BSA) in Tris-buffered saline

containing 0.1% Tween 20 detergent (TBST) was used to block nonspecific binding. Membranes were then incubated at 4 °C overnight with specific primary antibodies: OGDH (1:1000; cat. no 26865; Cell Signaling Technology, Danvers, MA, USA) and FLCN (1:1000; cat. no 3697; Cell Signaling Technology, Danvers, MA, USA). After incubation, the membranes were washed 3 times for 5 min in TBST buffer and then incubated for 1.5 h at room temperature with goat anti-rabbit IgG secondary antibody (AP156P; Merck Millipore, Kenilworth, NJ, USA) conjugated with horseradish peroxidase (HRP). Immunocomplexes were visualized using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Kenilworth, NJ, USA) according to the manufacturer's protocol and were visualized using the Azure 280 Imaging System (Azure Biosystems, Dublin, CA, USA).

4.4.5. Protein–Protein Interactions Analysis

The PPI analysis was performed by submitting the DRPs dataset to STRING v. 11.5 (ELIXIR, Hinxton, Cambridgeshire, UK, accessed on 22 January 2022) [30,131] and then visualized with the use of Cytoscape v. 3.9.1 (NIGMS, Bethesda, MD, USA), a software for visualizing complex networks [132]. Interactions were identified through comparison of the input data with the background of the *Toxocara canis*, the phylogenetically closest parasitic nematode with the highest number of proteins available in the STRING database. The network was limited only to the proteins that had at least one interaction with other proteins submitted to the analysis.

The host–parasite protein interactions were predicted using the HPIDB 3.0 server (https://hpidb.igbb.msstate.edu/hpi30_index.html, accessed on 21 January 2022) [133,134], which was run with the default setting using *A. simplex* s. s. tissue-specific proteins searched against human (*Homo sapiens*, UniProt proteome ID: UP000005640; # of entries 79,052) and Atlantic bottle-nosed dolphin (*Tursiops truncatus*, UniProt proteome ID: UP000245320; # of entries 45,130) proteomes. Interactome networks were visualized and analyzed using Cytoscape v. 3.9.1 (NIGMS, Bethesda, MD, USA) [132].

4.4.6. Allergen Identification

The protein allergenicity potential prediction was performed with the use of the AllerCatPro server with default settings (v. 2.0; https://allercatpro.bii.a-star.edu.sg/, accessed on 17 February 2022) [135].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23084336/s1.

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Data Availability Statement: The raw data files from LC-MS/MS (Tissue-specific proteome of L4 larvae of *Anisakis simplex* s. s.; #MSV000088491) are in the public domain and freely available through the MassIVE Repository (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp, accessed on 24 March 2022). Other data results are available upon reasonable request to the corresponding author.

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5. CONCLUSIONS

In this thesis high-throughput proteomic methods were used in the study of the protein composition of the parasitic nematode *A. simplex*.

In the first project, we applied high-resolution quantitative proteomics based on isobaric tandem mass tags (TMT) to analyze global proteome dynamics in *A. simplex* between the two developmental stages L3 and L4. A total of 11,412 non-redundant peptides corresponding to 2,443 different proteins were identified. To our knowledge, this is the largest dataset of *A. simplex* proteins for two developmental stages (L3 and L4) identified to date. The final global datasets were then examined using gene ontology and network analysis to gain further functional insights. After sequentially applying the next criteria of rigorous statistical analysis to the stage-specific proteomic data, we selected 8 proteins in L3 and 12 proteins in L4 as proteomic signatures for these specific developmental stages of *A. simplex*. Considering the proteins that were homologous to known allergens, three of the quantified stage-specific proteins could be potential novel allergens of *A. simplex* (pseudocoelomic globin, which was assigned to L3 larvae, prolylcarboxy peptidase like protein 5, and L-threonine-3-dehydrogenase, which was selected as one of the L4-specific proteins).

The obtained stage-specific proteins could be used as targets for control/elimination of this parasite. Further detailed *in vitro* or *in vivo* studies on suppression of expression or blocking of activity as well as post-translational modifications of these proteins are required due to their strong involvement in important metabolic processes. In a next step, it will be beneficial to verify our results by using fractionated or recombinant proteins for immunoblotting experiments and to investigate the allergenicity of selected allergen candidates in more detail. On the other hand, the determination of marker peptides of selected modulated *A. simplex* proteins can be used for the classification of food and feed contaminants.

Motivated by the positive outcome of the first project, we decided to deepen the biology of *A. simplex* and to study the protein composition of the tissues most responsible for host-parasite interactions, namely the cuticle and the intestine. At the L4 stage, the above tissues are fully developed and functional, so we decided to describe their proteomic composition at this particular stage of development. Moreover, previous proteomic studies of *A. simplex* did not focus on comparative analysis of nematode tissues. To identify tissue-specific proteomes in *A. simplex*, we used the shotgun

proteomics method based on isobaric tandem mass labeling (TMT) with a combination of nano-high performance liquid chromatography (nLC) coupled to an LTQ-Orbitrap Elite mass spectrometer, a method that is accurate and can measure small abundance differences in biological samples.

As a result of LC-MS /MS analysis, we identified a total of 3,435 proteins. These data were further processed, and 1,277 characterized proteins were found in the obtained cohort. This is the largest repository of proteins identified to date for specific tissues from L4 larvae of *A. simplex*. Comparison of the individual tissues examined revealed a total of 272 differentially regulated proteins, with 133 proteins more abundant in the cuticle and 139 proteins in the intestine. Of particular note are the enzymes involved in glycolysis and the tricarboxylic acid cycle, as these metabolic pathways were most enriched by cuticle and intestinal proteins, respectively, in the L4 stage of *A. simplex*. In addition to the key enzymes of the aforementioned metabolic pathways, two proteins, oxoglutarate dehydrogenase (OGDH) and folliculin (FLCN), were the focus of our interest because they may be potential candidates for controlling the survival of *A. simplex*. Their presence in L3 and L4 larval homogenates was further confirmed by Western blot, and their tertiary structure was modulated and compared with other species.

In addition, tissue-specific host-parasite interactions were studied for the first time for *A. simplex*, examining human and common bottlenose dolphin (*Tursiops truncatus*). OGDH from the parasite intestine was particularly interactive, and polyubiquitin-C was the major identified host target interacting with *A. simplex* proteins. Cuticular proteins of *A. simplex* interacting with the human or dolphin host included serine/threonine protein phosphatase, 2 N-domain-containing peptidase M1 proteins, and cofactor-independent phosphoglycerate mutase. In addition, nine potential allergens were also identified in this study.

Furthermore, in the second project, we again compared the proteomes of L3 and L4 larvae with the latest reference proteome of *A. simplex* from the UniProt/TrEMBL database (downloaded November 2021; proteome ID: UP000267096). Compared to the previously obtained results, 142 differentially regulated proteins were identified between the L3 and L4 larval stages, with 43 proteins more abundant at the L3 stage and 99 proteins more abundant at the L4 larval stage. These results complement and update the previously published *A. simplex* proteome.

The characterized global and tissue-specific proteomes of *A. simplex* could be used in antiparasitic research and provide an answer to the question of how larvae can be

neutralized and thus help control anisakiasis. The identified tissue-specific proteins could serve as targets for new drugs against anisakiasis. However, it should be noted that the functional analysis of the identified tissue-specific proteins was performed using an -omic approach. Therefore, *in vitro,* or *in vivo* experiments are required for their verification.

Moreover, the results described in this thesis clearly demonstrate the potential of proteomics and TMT-based LC-MS/MS, especially as a universal tool for analyzing global proteomic dynamics in parasitic nematode stages and specific tissues. In addition, host-parasite interactions *via* extracellular vesicles, which are currently under intense investigation in many biological systems, could be studied using the proteomic analysis scheme presented in this work. The combination of discovery and targeted proteomic workflows in parasitological research could have great benefits for the diagnosis and treatment of parasitoses as well as for food safety.

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Statement

We declare that Mr. Robert Stryiński (M. Sc.) was the main author of the scientific concept of research, experimental design, writing the manuscript and one of the investigators of experiments, data processing and analysis of results published in the work:

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Statement

We declare that Mr. Robert Stryiński (M. Sc.) was the main author of the scientific concept of research and experimental design, the investigator of experiments, data processing and analysis of results, as well as he wrote the manuscript and prepared it for publication:

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