PhD THESIS

Epidemiological and experimental studies regarding *Trichinella* spp. infestations in sylvatic fauna (wild boars, mustelids) from Romania

(Abstract)

Ph.D. Student: **Zsolt Boros**

Scientific coordinator: **Prof. dr. Vasile Cozma**
Full member of Academy of Agricultural and Forestry Sciences Bucuresti

ABSTRACT

Trichinellosis, is a cosmopolitan zoonosis, caused by nematodes from the genus *Trichinella*, with an intestinal (adults) and muscular stage (larvae) in the body of the same host (Pozio, 2001). This parasitic infestation affects several animal species as well as humans. Aside humans, another 300 vertebrate species can become infested by *Trichinella spp.*, usually evolving asymptomatically (Pozio, 2001). The parasite has two types of lifecycles, namely, the sylvatic cycle and the domestic one. The former refers to infestations that occur in wild animal species with species of the genus *Trichinella*, for which the main reservoirs are carnivores (wolves, foxes, jackals, wild felines) (Pozio, 2005). On the other hand, the domestic cycle implies the occurrence of *Trichinella* spp. infestations in domestic animals, such as pigs, and horses (Mikkonen et al., 2005).

Trichinella spiralis is widely considered the most medically significant species of the genus. It was first discovered by James Paget a medical student, during an anatomic dissection of human muscle tissue in 1835. Sir Richard Owen was the one who gave the name *Trichina spiralis* for this nematode in 1835 (Gould, 1970). The Trichina name was previously attributed to a genus of flies. Railliet, in 1895, decided to rename this parasite into *Trichinella. spp*. After its life cycle and link to ingesting infested meat had been established, the logical step was to take preventive actions to ensure public safety (Gould, 1970).

A total of 13 species of *Trichinella* were classified in two major groups, namely capsulated and non-encapsulated. Members of the encapsulated group, are as follows: *Trichinella spiralis*, *Trichinella nativa*, *Trichinellla britovi*, *Trichinella murrelli*, *Trichinella nelsoni*, *Trichinella patagoniensis*, and 3 other genotypes arbitrarily designated Trichinella T6, T8 and T9 with unresolved taxonomies (Pozio and Zarlenga, 2013). A newly identified encapsulated species from Canada has been named *Trichinella chanchalensis* with some notable differences from the other 2 artic taxa (*Trichinella nativa* and T6), while exhibiting a freeze-resistant character and a phylogenetically semblance to *T. patagoniensis* from South America (Sharma et al., 2020). The non-encapsulated group consists of *Trichinella pseudospiralis*, *Trichinella papuae* and *Trichinella zimbabwensis* (Pozio and Zarlenga, 2013).

Major characteristics of these nematodes consist in their cylindrical bodies, the presence of both sexes, with females usually bigger than males, and the one-way digestive tract (Murrel et al., 2000b). Externally, *Trichinella spp.* adults are covered by a chitinous, elastic, and transparent cuticle. In the subcuticular layer, 2 wide lateral formations are present, with an additional two narrow chords – one dorsal and one

ventral, defined as lines. The chords divide the muscles in four areas, each consisting of 4-5 elongated cells placed next to each other (Despommier et al., 2000).

Trichinella infestations in humans were reported in the past decades. This zoonotic infestation was documented in more than 55 counties, on five continents. *Trichinellosis* is strongly correlated with some cultural and culinary habits of consuming raw or undercooked meat from free-range, or back yard pigs (as well as horses, bears, and wild boars) (Dupouy-Camet, 2000). Cases of trichinellosis in non-endemic areas result from non-international transfer of this parasite. A good example would be the transfer of meat or meat products from endemic regions to non-endemic regions (Pozio and Marucci, 2003). Trichinellosis is a serious public health concern, with several human infestations recorded annually in Romania (Blaga et al., 2007). The highest number of cases (3649) was recorded in 1993. In Romania, the human population can become infested with trichinellosis by consuming raw, smoked, and undercooked pork and wild boar meat. The meat from these animals is usually prepared accordingly to local habits and customs that not always inactivate the larvae (Cironeanu and Ispas, 2002).

The gold standard method for identifying the muscle larvae of *Trichinella* spp. is artificial digestion. According to European Community Regulation No 1375/2015, slaughtered animals for human consumption have to be tested in this method. Trichinellosis constitutes a real economic concern for pig farmers, the marketing sector, as well as food safety regulators. The focus, therefore, has shifted towards prevention, followed by the elimination of *Trichinella* from the food circuit (Gottstein et al., 2009).

Benzimidazole derivatives are the most commonly used classes for treating trichinellosis. There is more and more interest in developing new anthelmintic derivatives including those from medicinal plants and probiotics. This is due to the increasing evidence of parasitic resistance against current anthelmintic substances (Pozio et al. 2001).

This thesi is 138 pages long and is structured in two parts: the first part, the current state of knowledge, and the second part, the personal contribution.

The first part of this thesis aims to review the current literature on the biology (morphology), ecology, diagnostics, and alternative treatments for *Trichinella* spp. infestations. The treatments mostly focus on probiotics, especially *Lactobacillus* strains and different medicinal plant extracts at different concentrations.

The second part (II. Original research) consists of 5 original manuscripts and is divided in 4 parts. Chapter II.1 details a study on the presence of anti-*Trichinella* antibodies in wild boars, which previously tested negative using the golden standard method. Chapter II.2 focuses on two epidemiological studies conducted on European badgers and polecats from Romania, in which *T. britovi*, as well as a *T. spiralis* and *T. britovi* co-infestation were identified. Chapter II.3 describes an *in vitro* experimental

protocol performed on *T. spiralis* and *T. britovi* larvae, which were exposed to four different alcoholic plant extracts. Chapter II.4 covers and experimental *in vivo* protocol conducted on laboratory mice with *T. britovi* larvae and two probiotics (*Lactobacillus*) strains. At the end of the thesis, we summarized our conclusions and lastly, we included the references listed from our studies.

In the first chapter of the second part (II.1), we aimed to assess the presence and prevalence of *Trichinella* spp. in wild boars from Bihor County, Romania, by using the golden standard and serological methods.

Eighty-four plasma and diaphragm samples, collected from wild boars, were included in this study. Artificial digestion, ELISA and Western blot were performed on these specimens. For statistical analysis chi-squared independence test was used.

All diaphragm samples were negative for *Trichinella* larvae in artificial digestion, while in ELISA, 54 (64.2 %) plasma samples were positive and 6 (7.1 %) plasma samples were doubtful. Western blot was performed on 26 plasma samples chosen randomly, from which only 6 (23.0 %) had a positive result. No inter-rated agreement between the methods chosen for diagnosis was observed.

Serological evidences indicate the presence of *Trichinella* spp. in wild boars from western Romania. Therefore, human consumers might be at risk to ingest *Trichinella* larvae, even in low numbers.

The second chapter contains two manuscripts regarding the epidemiology of *Trichinella* spp. in wild mustelids from Romania. The first manuscript (II. 2.1) aimed to investigate the occurrence of these parasites in European badgers from Romania and to identify the involved *Trichinella* species with the help of molecular biology.

In total 61 badgers (32 males, 29 females; 47 adults, 14 young) originating from 14 counties were examined by trichinoscopy and artificial digestion. For species determination, the positive muscle samples, and the larvae obtained from the artificial digestion method were used for DNA isolation, and further processed by multiplex PCR.

A single female badger, originating from Sibiu County, Central Romania, was found positive for *Trichinella* spp. infestation. Five cysts were identified using trichineloscopy: four in the diaphragm and one in the foreleg muscles. Artificial digestion revealed an infestation rate of 70 larvae/100 g of muscle. The PCR indicated the occurrence of *T. britovi*.

Although *T. britovi* has previously been reported in Romania, this represents the first report of its occurrence in the European badger in the country. However, the low prevalence indicates a minor reservoir role of this animal species.

The second manuscript of this chapter (II.2.2) aimed to investigate the occurrence of *Trichinella* spp. in European polecats from Romania and to identify the parasite species by molecular tools.

In total of 75 wild European polecats were examined by trichineloscopy and artificial digestion. The positive muscle samples and the larvae recovered from artificial digestion were collected for DNA isolation and further processed by means of Multiplex PCR. Prevalence and 95 % Confidence Interval (95 % CI) were calculated using EpiInfo 7 software (CDC, USA).

Two polecats (2.7 %; 95 % CI 0.32 – 9.3 %) from southern Romania tested positive for *Trichinella* spp. infestation. During trichineloscopy examination, 48 (in a polecat from Giurgiu County) and 78 (in a polecat from Ialomița County) cysts were found in the tested (56 samples/animal) muscle samples. Artificial digestion indicated a infestation with 2466 larvae/100 g of muscle in the polecat from Ialomița and 254/100 g in the polecat from Giurgiu. The Multiplex PCR confirmed the presence of *Trichinella spiralis* in the polecat from Giurgiu and a co-infestation with *T. spiralis* and *T. britovi* in the polecat from Ialomița.

The current study confirms through molecular biology, the occurrence of *T. spiralis* and *T. britovi*, as well as the occurrence of co-infestation with these two *Trichinella species* in European polecats from Romania.

In third chapter of the second part (II.3), we aimed to observe and compare the anti-parasitic effects of *Artemisia absinthium* (wormwood), *Allium sativum* (garlic), *Cucurbita pepo* (pumpkin), and *Coriandrum sativum* (coriander) against *T. spiralis* and *T. britovi* larvae through an experiment performed *in vitro.*

Control groups were established by using 100 larvae of *T. spiralis* (9 groups) and *T. britovi* (9 groups), each group placed in cell culture plates of 3 mlalong with 1ml of ethyl alcohol of 3 different concentrations (70%, 35%, and 17.5%). Beside these, six additional control groups containing 100 larvae of *T. spiralis* (3 groups) and *T. britovi* (3 groups) on an RPMI 1640 medium were established. The experimental groups were built similarly, by using 100 larvae of *T. spiralis* and *T. britovi* on 1 ml of RPMI 1640 medium; 1 ml of alcoholic extract from the abovementioned plants were added at different concentrations (10%, 5%, 2.5%) to eachof the experimental groups. All groups were then incubated at 37°C for the duration of the experiment. The study lasted for a total of 48 hours, during which both *T. spiralis* and *T. britovi* larvae were examined under a stereomicroscope at 1, 2, 16, 24, and 48 hours.

The alcoholic extracts of *A. sativum, A. absinthium* (conc 10%; 5%) and *C. pepo*, with *C. sativum* (conc. 10%; 5%; 2.5%) had similar effects to the alcoholic solutions used in the control groups, completely inhibiting the mobility of *T. spiralis* and *T. britovi*

larvae. Alcoholic extracts of *A. sativum* and *A. absinthium* with a concentration of 2.5% had a lower efficiencyin inhibiting the mobility of *T. britovi*, although some movements were still observed after 48 hours. *A. absinthium, C. pepo*, and *C. sativum* did not cause any lesions in neither parasite species. *A. sativum* alcoholic extracts induced lesions in the larvae at concentrations of 10% (*T. britovi* with lesions 6%), 5% (*T. spirali* with lesions 3%), and 2.5% (*T. spiralis* with lesions 6%) after 48 hours.

More studies with different concentrations are necessary to determine the effects and mechanism of these plants against *Trichinella* spp.

In the last chapter (II.4) we aimed to evaluate the antiparasitic effect of *Lactobacillus casei* ATCC 393 (original) and *L. paracasei* CNCM in CD-1 mice experimentally infested with *Trichinella britovi*.

Four groups of 20 mice (10 females and 10 males/group) were used, with two control groups and two experimental groups, in which each animal received a daily oral dose of 100 µl of 10^5 CFU/ml probiotics in Ringer solution. On day 7, all mice (except negative control) were infested orally with *Trichinella* (100 larvae/animal) beside the two probiotics. On day 9 post-infestation (p.i.), 10 mice/group were euthanized, and the presence of adult parasites in the intestinal content and wall was tested. On day 32 p.i., 10 mice/group were euthanized, then trichineloscopy and artificial digestion were performed to assess the muscle infestation with *T. britovi*.

On day 9 p.i., the experimental group pretreated with *L. casei* ATCC 393 (6.3±3.03) showed a significantly lower number of adult parasites in the intestinal wall compared with the positive control group (24.6±4.78). A significantly lower adult parasite count in the intestinal wall was registered in female mice pretreated with *L. paracasei* CNCM (7.4±4.71), and *L. casei* ATCC 393 (4.8±1.53) compared to female mice from positive control (29.0±5.17). No statistically relevant results were obtained concerning the male mice.

The probiotics used in the current study, could potentially impact the development of the intestinal stage of *T. britovi*, although the exact mechanism behind this process needs further research. The results of the present study also indicated that *L. casei* ATCC 393 may be more efficient in reducing the number of *T. britovi* adults in female mice than *L. paracasei* CNCM.

General conclusions:

The main aim of this PhD thesis was to investigate the presence of *Trichinella* spp. in wild boars and mustelids in Romania. But also to bring new information regarding the anti-parasitic effects of some medicinal plant extracts and probiotics against this parasite.

Regarding wild animals in Romania the current thesis concluded that wild boars from Bihor County, are still exposed to *Trichinella* spp. infestations, confirmed by serological evidence, although all of the animals were negative in the golden standard method. This thesis also proved that several mustelid species like, European badgers and polecats from Romania can act as hosts for *Trichinella* spp. thus helping to maintain the sylvatic life cycle. Both *T. spiralis* and *T. britovi* were found in polecats, but only *T. britovi* was found in European badgers.

Regarding alternative plant treatments against this parasite, the current thesis concluded that *C. pepo* and *C. sativum* at 2.5% concentration had a strong effect against *T. spiralis* and *T. britovi* larvae, but *A. sativum* and *A. absinthium* (2.5%) were less efficient against *T. britovi* larvae. The results showed that *T. britovi* was more resistent to lower concentrations of *A. sativum* and *A. absinthium* than *T. spiralis*.

The last chapter showed that probiotics (*L. casei* and *L paracasei*) can affect the intestinal stage of *T. britovi* infestation in CD-1 mice. The results of the present study also indicated that *L. casei* ATCC 393 may be more efficient in reducing the number of *T. britovi* adults in female mice than *L. paracasei* CNCM. In male mice no statistiacally significant results were obtained.

References:

1. BLAGA, R., B., S., DURAND, C., ANTONIU, C.M., GHERMAN, V., CREŃU, V., COZMA, P., BOIREAU, 2007, A dramatic increase in the incidence of human Trichinellosis in Romania over the past 25 years: impact of political changes and regional food habits. American Journal of Tropical Medicine and Hygiene, 76(5), 983- 986.
2. CIRONEANU, I., A.T., ISPAS, 2002, Totul despre trichineloza. [All about trichinellosis]. Bucharest: MAST Publishing House, Bucharest, Romania, 9-64.
3. DESPOMMIER, D.D., R.W., GWADZ, P.J., HOTEZ, C.A., KNIRSCH, 2000, *Trichinella spiralis*: Parasitic Diseases. Apple Trees Productions, LLC. Pub., New York, 125-132.
4. DUPOUY-CAMET, J., 2000, Trichinellosis: a worldwide zoonosis, Veterinary Parasitology, 93, 191-200.
5. GOULD, S. E, 1970, Trichinosis in man and animals. Trichinosis in man and animals.
6. GOTTSTEIN, B., E., POZIO, K., NÖCKLER, 2009, Epidemiology, diagnosis, treatment, and control of trichinellosis, Clinical Microbiology Review, 22(1): 127-145.
7. MIKKONEN, T., J., VALKAMA, H., WIHIMAN, A., SUKURA, 2005, Spatial variation of *Trichinella* prevalence in rats in Finnish waste disposal sites, Journal of Parasitology, 91: 210-213.
8. MURREL, K. D., J. R., LICHTENFELS, D. S., ZARLENGA, E., POZIO, 2000b, The sistematics of the genus *Trichinella* with a key to species, Veterinary Parasitology, 93: 293-307.
9. POZIO, E., 2001, New patterns of *Trichinella* infections, Veterinary Parasitology, 98: 133–148.
10. POZIO, E., 2005, The broad spectrum of Trichinella hosts: from cold- to warmblooded animals, Veterinary Parasitology, 132: 3–11.
11. POZIO, E., G., MARUCCI, 2003, Trichinella-infected pork products: a dangerous gift, Trends in Parasitology, 19: 338.
12. POZIO, E., D. S., ZARLENGA, 2013, New pieces of the Trichinella puzzle, International Journal of Parasitology, 43: 983-997.