Toxoplasma gondii in pork production : a stable to table approach

Filip Dámek

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Filip Dámek. Toxoplasma gondii in pork production : a stable to table approach. Human health and pathology. Université Paris-Est Créteil Val-de-Marne - Paris 12, 2023. English. ⟨NNT : 2023PA120024⟩. ⟨tel-05100577⟩

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A Stable to Table Approach

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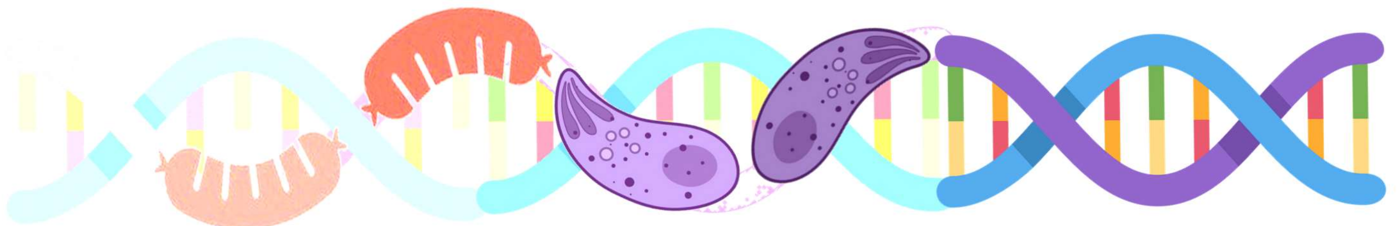

Toxoplasma gondii in Pork Production:

A Stable to Table Approach

Filip Dámek

Doctoral thesis in Pathology and clinical research was accredited by the University of Paris-Est Créteil, Faculty of Health, Doctoral School of Life and Health Sciences

The majority of the research was conducted at the Joint Research Unit of Bipar, Animal Health Laboratory of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) and the Biomedical Research Platform of the National Veterinary School of Alfort (EnvA), Maisons-Alfort, France. Parts of the work were done at the University of Veterinary and Pharmaceutical Sciences (UVPS), Brno, The Czech Republic, the Moredun Research Institute (MRI), Penicuik, Scotland, and National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands.

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Thesis directors:

Prof. Radu Blaga – The National veterinary school of Alfort (EnvA)

Dr. Delphine Le Roux – The National veterinary school of Alfort (EnvA)

One Health European Joint Project Supervisors:

Dr. Marieke Opsteegh - The National Institute for Public Health and the Environment (RIVM)

Dr. Arno Swart - The National Institute for Public Health and the Environment (RIVM)

Presented publicly on April 5th 2023

In front of a jury composed of:

1. Prof. Jean Dupouy-Camet, Professor Emeritus, Paris Cité University, President of the jury, Reporter
2. Prof. Arie Hendrik Havelaar, University of Florida, Reporter
3. Prof. Caroline Frey, University of Bern, Examinator
4. Prof. Břetislav Koudela, University of Veterinary Sciences Brno, Examinator
5. Dr. Marieke Opsteegh, RIVM, Co-supervisor
6. Dr. Arno Swart, RIVM, Co-supervisor
7. Dr. Delphine Le Roux, EnvA, Co-supervisor
8. Dr. Pascal Boireau, Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (Anses), Co-supervisor
9. Prof. Radu Blaga, EnvA, Thesis director

With love, to my amazing family who have always been there for me and encouraged me in pursuing my dreams. And to my wonderful partner, who has been my constant source of inspiration and unwavering support.

Děkuji!

Abstract

Toxoplasma gondii is a food-borne zoonotic parasite of significant concern for both human and animal health. Felids are the only known definite hosts but essentially all warm-blooded animals, including humans can be infected. To understand the role of the parasite in human *T. gondii* infections, a "stable-to-table" approach was followed. Meat is a major source of human *T. gondii* infections in Europe, with pork the most consumed meat. There is a need for a source attribution for human meat-borne *T. gondii* infections and the application of effective preventive measures.

For this purpose, several studies were carried out. An oral fluid-based ELISA kit was tested for the detection of anti-*T. gondii* antibodies. An extensive literature review was conducted and analysed using a novel Bayesian model, resulting in age-dependent regional prevalence estimates for various animal host species in Europe. The potential effects of isolate strain, parasite stage, infective dose, and infection route on distribution patterns and load of *T. gondii* in pig tissues were evaluated. Additionally, the presence of *T. gondii* in retail meat and meat products was screened. Inactivation of the parasite in the presence of salt, additives, and processing was assessed in traditional French dry sausage and processed pork.

The commercial ELISA kit was validated for use with oral fluid. The results of the modelling showed a rising seroprevalence in screened animal species from north to south and from west to east. The highest seroprevalence was estimated in outdoor-held sheep in eastern Europe, while the lowest was in indoor-reared pigs in western Europe. The detection of the parasite in pig tissues revealed individual variability and stage- and strain-driven distribution patterns and a load. Both the infection with tissue cysts and CZ-Šimková type III isolate resulted in a higher frequency of positive tissues and parasite load. *T. gondii* DNA was found in retail meat and meat products from various animal species, particularly in venison. The effect of salting was demonstrated by the inactivation of *T. gondii* within the first day with NaCl concentrations above 2%, regardless of the presence of other additives or processing.

In conclusion, all articles included in this thesis focused on different aspects influencing the potential of pork and pork products as sources of human *T. gondii* infections. The factors explored, including the prevalence of *T. gondii* in animals, distribution and concentration of parasites in their tissues, processing of meat and meat products, and associated inactivation, provide valuable information and a basis for further investigation of the meat-borne transmission pathway of *T. gondii*.

Résumé

Toxoplasma gondii est un parasite zoonotique d'origine alimentaire très préoccupant pour la santé humaine et animale. Les félidés sont les seuls hôtes définitifs connus, mais pratiquement tous les animaux à sang chaud, y compris les humains, peuvent être infectés. Pour comprendre le rôle du parasite dans les infections humaines à *T. gondii*, une approche " de l'étable à la table " a été suivie. La consommation de viande représente un risque majeur d'exposition et d'infection à *T. gondii* chez l'homme en Europe, le porc étant la viande la plus consommée. Il est donc nécessaire d'attribuer une source d'infections humaines à *T. gondii* transmises par la viande et d'appliquer des mesures préventives efficaces.

Pour cela, plusieurs études ont été réalisées. Un kit ELISA a été testé pour la détection d'anticorps anti-*T. gondii* à partir de prélèvements salivaires chez le porc. De plus, un état de l'art de la littérature a été effectué et analysée à l'aide d'un nouveau modèle bayésien, résultant en estimations de la prévalence régionale en fonction de l'âge chez diverses espèces animales hôtes en Europe. Les effets potentiels des souche isolée, de stade parasitaire, de dose infectieuse et de voie d'infection sur les schémas de distribution et la charge de *T. gondii* dans les tissus porcins ont été évalués. Enfin, la présence de *T. gondii* dans la viande et les produits carnés vendus au détail a été recherchée. L'inactivation du parasite en présence de sel, d'additifs et de traitement a été évaluée dans la saucisse sèche française traditionnelle et le porc transformé.

Le kit ELISA commercial a été validé pour une utilisation sur salive. Les résultats de la modélisation ont montré une augmentation de la séroprévalence dans les espèces animales examinées du nord au sud et de l'ouest à l'est. La séroprévalence la plus haute a été estimée chez les moutons élevés en plein air en Europe de l'est, tandis que la plus faible a été observée chez les porcs issus d'élevage hors-sol en Europe de l'ouest. La détection du parasite dans les tissus de porc a révélé une variabilité individuelle. Les schémas de distribution ainsi que la charge parasitaire varient en fonction des stades et des souches. L'infection par des kystes tissulaires et l'isolat CZ-Šimková de type III a entraîné une fréquence plus élevée de tissus positifs et de charge parasitaire. L'ADN de *T. gondii* a été trouvé dans la viande vendue au détail et dans les produits carnés de diverses espèces animales, en particulier dans la venaison. L'effet du salage a été démontré par l'inactivation de *T. gondii* dès le premier jour avec des concentrations en NaCl supérieures à 2%, indépendamment de la présence d'autres additifs ou traitements.

En conclusion, les articles présentés dans cette thèse visent à estimer le potentiel de la viande de porc et de ses produits dérivés comme sources d'infections humaines par *T. gondii*. Les facteurs étudiés, y compris la prévalence de *T. gondii* chez les animaux, la distribution et la concentration des parasites dans leurs tissus, la transformation de la viande et des produits carnés, et l'inactivation associée, fournissent des informations précieuses et une base pour une étude plus approfondie de la voie de transmission de *T. gondii* par la viande de porc.

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Chapter 1

Introduction

Toxoplasma gondii is an intracellular food-borne parasite capable of infecting virtually all warm blooded animals, including humans. With more than one third of the global human population estimated to be infected (Montoya, 2002), this zoonotic apicomplexan protozoan is one of the most successful parasites in the world, and one of a major importance to both the medical and veterinary field (Dubey, 2022). Definitive and intermediate hosts may get infected, mainly orally. The course of *T. gondii* infection is often subclinical but may manifest in the form of non-specific symptoms such as swollen lymphatic nodes, fever and muscle ache. If a woman acquires infection for the first time during pregnancy, the parasite may transmit to the fetus and can cause abnormalities in the nervous system of the fetus, or can cause chorioretinitis later in life (Gilbert, 2000). The most severe outcome is abortion or stillbirth (Gilbert et al., 1999; Hampton, 2015; Luft and Remington, 1992; SYROCOT (Systematic Review on Congenital Toxoplasmosis) study group et al., 2007). Even though toxoplasmosis is the leading cause of infectious uveitis and retinal disease in humans (Holland, 2003; Talabani et al., 2010), it is not recognized due to the subclinical manifestation in the majority of cases (Dunay et al., 2018). The unification of disease burden estimates using disability adjusted life years (DALYs) allows for comparisons of the burden of different diseases, which puts the burden due to *T. gondii* infections into the perspective. The major impact of toxoplasmosis was then acknowledged by a relatively high ranking in disease-burden estimates of food-borne hazards (Havelaar et al., 2015), and by leading positions in multicriteria-based rankings for risk management of food-borne parasites at national (Batz et al., 2012; Havelaar et al., 2012; Mangen et al., 2015; Scallan et al., 2015) and international level (Boireau et al., 2014; Bouwknegt et al., 2018; Torgerson and Mastroiacovo, 2013). Given the limited treatment options, focusing mostly on the acute phase of *T. gondii* infections, effective prevention measures are important. By implementing proper prevention measures at the farm, industrial processing and consumer levels, the incidence of meat-borne toxoplasmosis can be reduced, ultimately lowering the overall impact of *T. gondii* infections on public health.

HISTORY OF THE PARASITE

The parasite *Toxoplasma gondii* was first observed in 1908, independently by French scientists Charles Jules Henri Nicolle and Louis Herbert Manceaux (Nicolle and Manceaux, 1908) during a research in Tunisia, and Italian Alfonso Splendore (Splendore, 1908) in Brazil. The name for the protozoan, derived from its morphological shape (from Greek "toxon" meaning "bow" and "plasma" meaning "shape" or "something formed") and the name of the rodent, *Ctenodactylus gundi*, from which the parasite was first extracted, was suggested by Nicolle in 1909 (Kennou, 1986). Several transmission routes were tested until an infection via consumption of *T. gondii*-contaminated tissue was proposed by Sabin and Olitsky in 1937 (Sabin and Olitsky, 1937). This hypothesis was later proved to be correct, and showcased by infection of children in a sanatorium due to consumption of infected undercooked meat in 1965 by Desmonts (Desmonts et al., 1965). The same year an infection via oral inoculation of winter-resistant forms (later named oocysts) (see Fig. 1 in "Parasitic stages") was first demonstrated in mice by Hutchinson (1965), explaining thus the previously observed high incidence in strict herbivores (Jacobs, 1963). The complete life cycle (see Fig. 2 in "Life cycle & transmission") of *T. gondii* was first proposed around the year 1970, when several teams simultaneously described different parts of the sexual and asexual development in small intestine of a cat (Dubey et al., 1970a, 1970b, 1970c; Frenkel et al., 1970; Hutchison, 1965; Hutchison et al., 1969; Overdulve, 1970a, 1970b; Piekarski et al., 1971; Sheffield and Melton, 1970; Weiland and Kühn, 1970).

PARASITIC STAGES

There are three potentially infective stages of *T. gondii* – tachyzoites, oocysts and tissue cysts. A depiction of the individual stages of *T. gondii* along the multiplication cycle in felids is shown in Fig. 1.

Tachyzoites are proliferative forms of *T. gondii*. The term tachyzoite, originating from Greek "tachos" meaning "speed" was first used by Frenkel, 1973 (Frenkel, 1973) as a reference to their rapid growth. Tachyzoites are crescent-shaped, sized approximately 2 by 6 µm, with

ultrastructures detailed by Dubey, 1998 (Dubey et al., 1998). Later *T. gondii* develops into tissue cysts containing bradyzoites as a mechanism to escape elimination by the immune system (Cerutti et al., 2020; Jeffers et al., 2018).

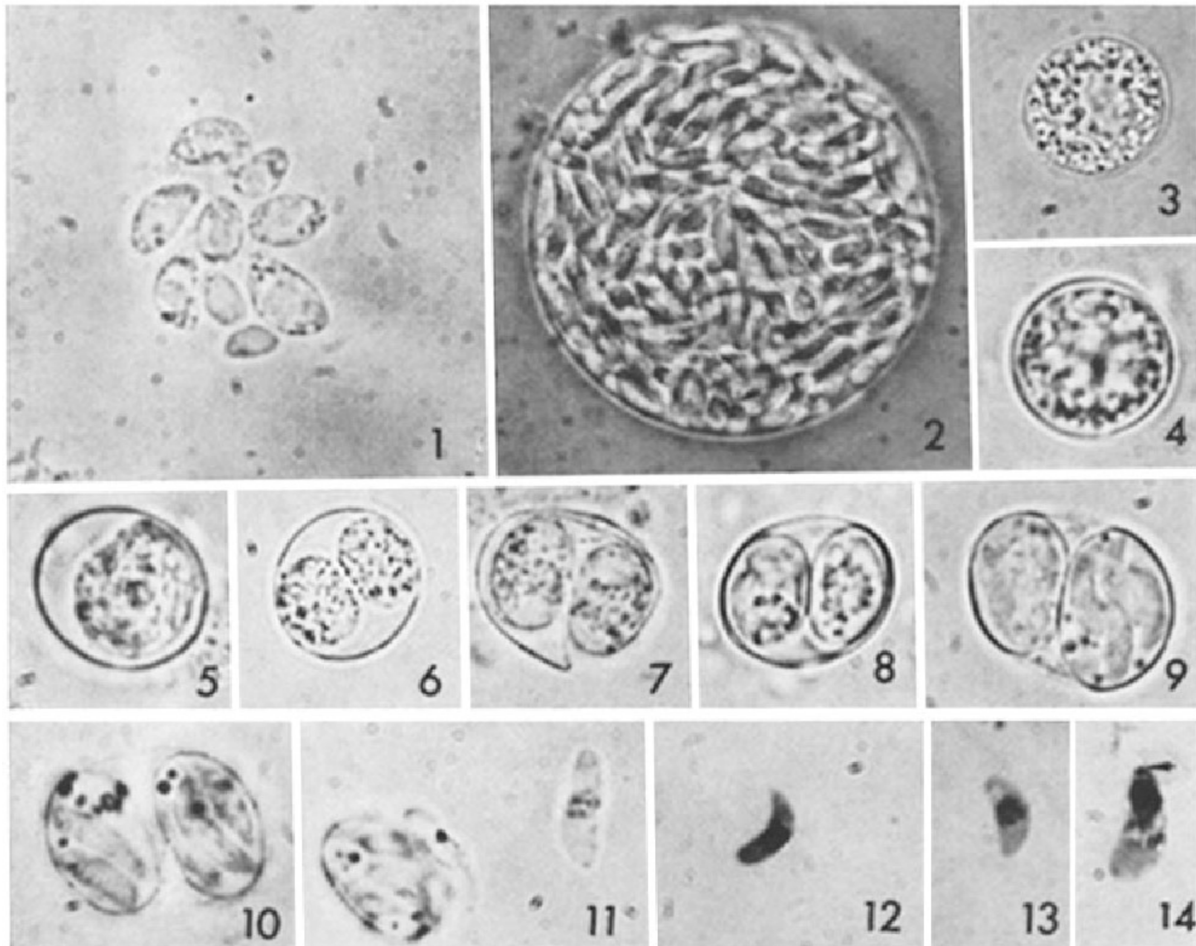

Figure 1 Photomicrographs of different stages of *Toxoplasma*. X 1600. Part 1. Trophozoites from peritoneal exudate of a mouse infected with RH strain. Part 2. Cyst from a mouse brain infected with M-7741 strain. The cyst wall enclosing the numerous bradyzoites is clearly shown. Part 3 and 4. Oocysts, unsporulated in freshly passed cat feces. The oocyst in Part 3 is slightly flattened. Part 5. Oocyst with contracted sporont after 9 hr aerobic development at room temperature. Part 6. Oocyst with two sporoblasts after 12 hr aerobic development at room temperature. Part 7. Oocyst with two sporocysts after 18 hr aerobic development at room temperature. Light areas interpreted as nuclei are seen at both ends of sporocysts. The oocyst wall has ruptured during preparation of the oocyst mount. Part 8. Oocyst sporulated after 24 hr aerobic development at room temperature. Sporocyst residua are in focus, appearing as only a few granules in one sporocyst and as a ball in the other. Part 9 and 10. Oocysts sporulated 30 min after treatment with 6% sodium hypochlorite solution. Note the thinness of the wall. The outer wall has been dissolved. Part 9. All four sporozoites are in focus in one sporocyst. Part 10. Sporocysts showing the arrangement of sporozoites. Part 11. Free sporozoite released from sporocyst by pressure on cover slip. Part 12-14. Sporozoite stained with Giemsa. Representative variation in shape, nuclear position, and staining at the tip are shown. Part 12. Elongated form with nucleus in the middle. Part 13. Crescentic form with nucleus in the middle. Part 14. Nucleus and a conoid at the anterior end. Parts 1-11 are unstained fresh preparations, Parts 12-14 are stained with Giemsa. Reprinted including the legend from Journal of Experimental Medicine 1970, 132(4), 636–662, Dubey, J. P., Miller, N. L., & Frenkel, J. K., "The *Toxoplasma gondii* oocyst from cat feces". Copyright© 1970.

Tissue cysts, a type of intracellular structure, were first described by Levaditi in 1928 (Levaditi et al., 1928). These cysts are characterized by a encapsulated structure that hosts slowly replicating stages known as bradyzoites. The term "bradyzoites" originates from the Greek word "bradys," meaning "slow," and was first introduced by Frenkel in 1973 (Frenkel, 1973). The name tissue cyst was proposed in 1988 by Dubey and Beattie (Dubey and Beattie, 1988) and the structures within, similar to those of tachyzoites, were later described and summarized by Dubey et al. in 1998. Tissue cysts vary in size between 5 and 100 µm and contain anywhere from two to hundreds of bradyzoites (Dubey et al., 1998).

Oocysts are environmental stages of *T. gondii* and the result of a multiplication of the parasite in the definitive host, felids. Sporulated oocyst are subspherical to ellipsoidal and are 11 by 13µm in diameter, containing a total of eight sporozoites, divided over two sporocysts comprising four sporozoites each, as detailed by Dubey et al. (1970a).

LIFE CYCLE & TRANSMISSION

By the early seventies, both the sexual and asexual multiplication phases of *T. gondii* life cycle have been described. The members of the *Felidae* family are the only known species where sexual multiplication occurs, making them effectively the sole definitive host species (Dubey, 2009a). Recently it was found that the excess of linoleic acid in cat intestine likely limits sexual multiplication of *T. gondii* to felids (Martorelli Di Genova et al., 2019), designating therefore all other mammals and birds as intermediate hosts (Tenter et al., 2000).

In the majority of cases, infection is acquired through the oral route following either carnivory of infected hosts containing viable parasites within tissue cysts in their tissues, ingestion of *T. gondii* sporulated oocysts from the environment, or drinking milk of an infected mother containing tachyzoites (Tenter et al., 2000). Naïve felids may acquire infection by ingestion of oocysts or tissue cysts, which are digested and sporozoites or bradyzoites released in the small intestine begin to actively enter intestinal epithelial cells (Barragan and David Sibley, 2003). Upon invading the cells, the asexual part of multiplication takes place and four types of schizonts and their merozoits, microgamonts and macrogamonts, develop within enterocytes. The merozoites can undergo a repeated cycle of asexual multiplication by invading other

epithelial cells or begin a sexual multiplication called gametogony by differentiating into one of the sexual stages, female gamete (macrogamont) or male gamete (microgamont) (Dubey and Frenkel, 1972). Following fertilization of the female gamete by the male gamete, an oocyst wall is formed around the zygote. Infected epithelial cells rupture and discharge newly formed oocysts into the intestinal lumen where they continue to develop by dividing its nucleus, creating two sporoblasts. As a result, millions of oocysts containing sporoblasts are shed with feces. The shedding of oocysts into environment occurs after a period of 3 to 10 days post-ingestion of bradyzoites, and after 14 or more days in case of oocysts, and lasts anywhere between 4 and 13 days in experimentally infected cats (Dubey, 1995). Oocysts shed by felids become infectious after sporulation in the environment. This process is dependent on the presence of oxygen and its duration can be influenced by various factors such as temperature and humidity, taking usually 48 to 72 hours (Dubey et al., 1970a; Frenkel et al., 1970).

Oocysts shed into the environment can be a source of infection for other naïve intermediate or definitive hosts (Dubey, 1996). Intermediate hosts can become infected in the same ways as definitive hosts. The oocyst wall or tissue cyst, depending on the infection source, is digested within a short period of time and accordingly sporozoites or bradyzoites are released into the gut of the host. Both sporozoites and bradyzoites behave similarly by actively invading the cells of lamina propria, transforming into tachyzoites and rapidly multiplying (Morisaki et al., 1995), undergoing a cycle every 6 to 8 hours (Halonen and Weiss, 2013). Upon the destruction of the maternal cell they invade cells in the surrounding or present leukocytes, through which they enter blood system, cross bodily barriers and disseminate into tissues (Courret et al., 2006; Unno et al., 2008). Even though tachyzoites show affinity to various tissues, they are found within lymph nodes, spleen and lungs in the early days post infection and with brain and heart in the later phase of the infection (Algaba et al., 2018; Opsteegh et al., 2010; Verhelst et al., 2011). Approximately two weeks following infection, induced by the developing immune response, tachyzoites slowly convert into bradyzoites and encyst within a tissue cyst (Cerutti et al., 2020; Jeffers et al., 2018). Tissue cysts are thought to last for life in many species, awaiting the potential predation by the definitive host (Rougier et al., 2017). Intact tissue cysts are thought to cause no harm to the host but an infection may reoccur from ruptured dormant tissue cysts in case immunity is weakened. It remains unknown whether

the immunity is generated by the response of T lymphocytes to tissue cysts (Gazzinelli et al., 1992; Yap et al., 2000) or rather by killing of tachyzoites which emerge after occasional re-differentiation of the latent stage and tissue cyst rupture (Ehmen and Lüder, 2019).

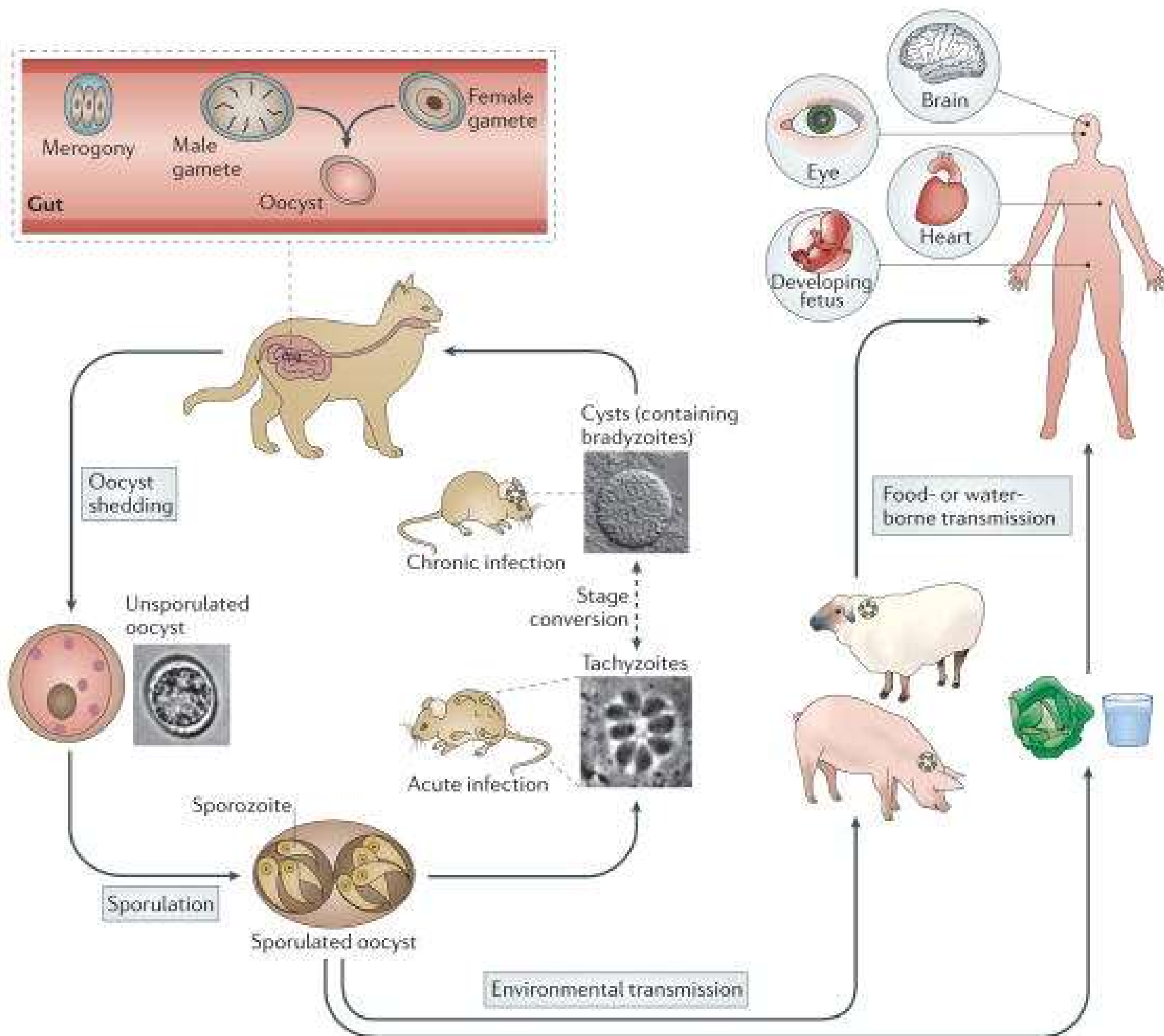

Figure 2 Life cycle of *T. gondii*. Reprinted from "Modulation of innate immunity by *Toxoplasma gondii* virulence effectors" by Hunter, C. A., & Sibley, L. D., (2012). Nature reviews. Microbiology, 10(11), 766–778., with permission from Springer Nature.

Differences were observed in the pathogenesis related to transmission by different parasitic stages. Generally, definitive hosts were shown to be more prone to an infection with tissue cysts, arguably due to the link to the carnivorous diet of felids. On the other hand, oocysts tend to be more infective and cause more severe clinical symptoms in intermediate hosts (Dubey and Beattie, 1988). Pigs are in particular highly susceptible to experimental infections by oocysts (Dubey et al., 1996) and the different aspects of the pathogenesis, immunogenesis,

tropism and burden of *T. gondii* in porcine tissues in correlation with the stage of the parasite were studied in detail in the Chapters 4, 5 and 7.

In addition to the horizontal transmission routes, *T. gondii* can be transmitted also vertically from mother to foetus. Congenital infection usually occurs when a naïve host acquires *T. gondii* infection during pregnancy, when tachyzoites in leukocytes cross the placental barrier. However, a chronic infection may also lead to vertical transmission in some species, as was observed in mice (Pezerico et al., 2009), sheep (Rodger et al., 2006) and minipigs (Jungersen et al., 2001). Congenital transmission of *T. gondii,* from an infected mother to her offspring, has been observed in various animal species. It has been demonstrated that the type of placenta can influence the transmission of the parasite. The epitheliochorial placenta found in ruminants, characterized by multiple layers separating maternal and foetal tissues, may serve as a barrier to the parasite compared to the endotheliochorial placenta in dogs and cats or the haemochorial placenta found in primates and humans, where the transmission of the parasite is more efficient (Liempi et al., 2020). In humans, congenital transmission of *T. gondii* is estimated to occur in approximately 30% of cases where the mother is primarily infected (SYROCOT (Systematic Review on Congenital Toxoplasmosis) study group et al., 2007). The probability of transmission increases with the duration of gestation but foetuses in early pregnancy are more likely to manifest more severe clinical signs of infection which may vary from negligible to neurological lesions, abortion, or stillbirth (Gilbert et al., 1999; Hampton, 2015; Luft and Remington, 1992; SYROCOT (Systematic Review on Congenital Toxoplasmosis) study group et al., 2007). Congenital toxoplasmosis is a major public health issue in humans and a cause of substantial economic losses in animal husbandry.

A relatively rare iatrogenic mode of transmission may occur (especially in immunosuppressed recipients) via blood transfusion containing infected leukocytes (Siegel et al., 1971), or with transplanted organs from a donor with chronic toxoplasmosis (Gallino et al., 1996). Even though the recipient may acquire toxoplasmosis during such transfusion or transplant, the major risk results from the reactivation of latent infection in previously seropositive recipients (Derouin and Pelloux, 2008; Štajner et al., 2022).

GENETIC BACKGROUND

With the growing phenotypical understanding of the parasite, the focus shifted towards genetic identification. The importance of genetic typing became evident with an early observation linking genotypes to variable levels of virulence and cytokine response in mice (Araujo and Slifer, 2003; Sibley and Boothroyd, 1992). This was later confirmed by proving significant variability in virulence of the different *T. gondii* strains across animal species (Calero-Bernal et al., 2022; Dubey et al., 2012).

Early attempts to distinguish *T. gondii* strains focused on targeting polymorphic enzymes using multi-locus restriction fragment length polymorphism (RFLP), revealing three major lineages (types I, II, and III) by screening samples predominantly from North America and Europe (Dardé et al., 1992; Howe et al., 1997; Howe and Sibley, 1995) (Fig. 3). These three clonal lineages probably originated in the last 10,000 years (Su et al., 2003) from a limited number of interactions between ancestral strains (Boyle et al., 2006).

Certain limitations of the RFLP method led to exploration of different approaches such as random amplification of polymorphic DNA (Ferreira et al., 2004; Guo and Johnson, 1995), a PCR-based priming from the site of polymorphism (Su et al., 2003), or focusing on short repeated segments of DNA called microsatellites (MS) (Ajzenberg et al., 2002a, 2002b). Both RFLP and MS typing are still popular owing to their cost-effectiveness, and are widely used as a tool for identification of *T. gondii* population structure in spite of underestimating the true rate of polymorphism (Sibley et al., 2009). These shortcomings can be solved by direct sequencing of genomic regions or whole genome, and detecting within them the previously used molecular markers such as single nucleotide polymorphism (SNPs) or indels (Bontell et al., 2009; Su et al., 2004; Yang et al., 2013). Particularly useful in this sense are centralized platforms with genomic information like ToxoDB (Amos et al., 2022). Such organized databases help to access, compare, and analyze genomic information, which can in turn lead to new discoveries and insights into the biology of the *T. gondii* and can also be used to develop new diagnostic tools.

Recent advancements led to identification of a fourth lineage (type 12) among the atypical strains in North America using sequenced-based phylogenetic and population analyses (Khan et al., 2011). Yet, the populations in the Northern hemisphere are largely clonal and dominated by the presence of type II strains (Ajzenberg et al., 2009, 2002a, 2002b; Dardé et al., 1992; Hosseini et al., 2020; Khan et al., 2007; Lorenzi et al., 2016; Shwab et al., 2014). In contrast, in South America, *T. gondii* is more diverse including less common genotypes that show greater evidence of recombination (Sibley and Ajioka, 2008), many of which were previously misclassified as type I or recombinant (Frazão-Teixeira et al., 2011).

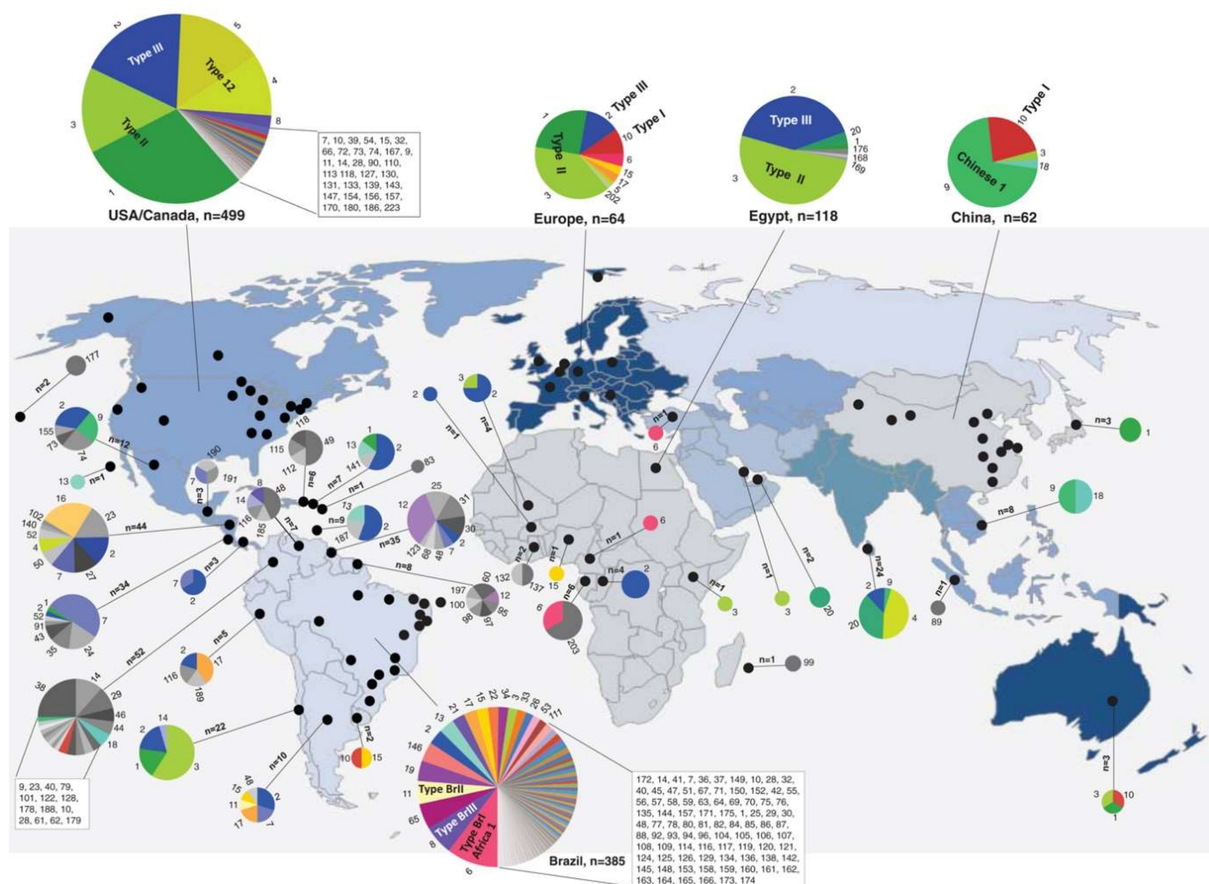

Figure 3 Geographical distribution of *T. gondii* genotypes. Black dots indicate locations from which *T. gondii* isolates were obtained and genotyped using the PCR-RFLP method. The numbers around pie chart edges indicate ToxoDB PCR-RFLP genotypes. Sizes of pie charts correlate with total number of isolates (n), and colours indicate different genotypes. The figure and legend were reprinted from publication: (Shwab et al., 2014) “Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping” in Parasitology , Volume 141 , Issue 4 , April 2014 , pp. 453 - 461, Copyright © Cambridge University Press 2013.

The advances in the field brought however the need for a robust system for describing and interpreting evolutionary subdivisions in the prevalent pathogen. This novel system, using three independent sets of polymorphic DNA markers, clustered existing genotypes into 15 haplogroups defining six major clades (Su et al., 2012). The whole haploid genome of *T. gondii* is spread over 14 chromosomes and is 65Mbp long (Khan et al., 2005). Understanding the genetic background of the six clusters should ease comparative genomics and identification of genes that control important biological phenotypes including pathogenesis and transmission (Galal et al., 2019; Su et al., 2012).

DETECTION METHODS

Detecting the pathogen was always the first step in understanding its epidemiology and since the first microscopical observation of *T. gondii*, more efficient ways of detection were looked for. Indirect methods were successfully applied across a wide spectrum of pathogens, and since specific anti-*T. gondii* antibodies post-infection are expected to be long-lasting, if not lifelong, Sabin and Feldman appeared to be on the right track with the development of their dye test (Sabin and Feldman, 1948). This otherwise successful and highly specific method, which became the standard for diagnosis of toxoplasmosis, had a downside of requiring live tachyzoites for the reaction (Dubey et al., 1985; Singh, 2003). This inconvenience prompted the development of the direct detection test (DAT) of *Toxoplasma*-specific antibodies by Desmonts & Remington (Desmonts and Remington, 1980), later followed by the modified agglutination test (MAT) by Dubey & Desmonts (Dubey and Desmonts, 1987). This latter method uses formalin-fixed parasites in the agglutination, acting as an antigen upon encountering the potential specific IgG antibodies in serum or other bodily fluid. The fact that MAT works on a variety of matrices, supported by its relative simplicity and that it can be readily applied to any species (Blaga et al., 2015; Villena et al., 2012), makes it one of the most commonly used tests in detection of *T. gondii*-specific antibodies across the species spectrum, including humans (Djokic et al., 2016a; Dubey et al., 2016; Halos et al., 2010; Macrì et al., 2009; Santos et al., 2009; Villena et al., 1998). This method has still certain disadvantages including the subjective evaluation of the positivity, and relatively low sensitivity and specificity (Sharma

et al., 2019). Since then, several indirect methods, based on the detection of surface protein markers (hemagglutination test - IHAT, indirect fluorescence test - IFAT, latex agglutination test – LAT, enzyme-linked immunosorbent assay – ELISA, immunoblot – IB, and others) were developed in hopes of acquiring an increased sensitivity and specificity for *T. gondii*-specific antibodies. According to the numerous comparisons between the methods, a set of advantages and disadvantages were observed for each of them (Casartelli-Alves et al., 2014; Hirvelä-Koski, 1990; Sharma et al., 2019; Singh, 2003). While easy to use, IHAT and LAT lack sensitivity and cannot distinguish between the individual classes of immunoglobulins. Highly sensitive and specific methods like IFAT and IB, which in case of IFAT can also be partially automated, are laborious and rely on a subjective interpretation of results and IFAT requires the use of a fluorescence microscope (Robert-Gangneux et al., 1999; Shaapan et al., 2008). Overall, ELISA represents a fast, sensitive and specific detection commercially available tool, applicable without advanced laboratory equipment. Although ELISA is commonly used for testing blood sera, other methods such as MAT, IFAT, and IB can also be employed as complementary tools. These tests may help enhance the results of the ELISA tests, particularly when ELISA is found to be lacking sensitivity in other matrices (Forbes et al., 2012).

Regarding the matrices, alternatives to blood sera were especially proposed for large-scale applications in animal farms where blood collection represents a costly and time-consuming procedure due to the individual sampling. Milk was shown as a promising alternative matrix for bulk-testing for *T. gondii*-specific antibody detection in milk-producing small ruminants and cattle (Gazzonis et al., 2018; Liu et al., 2022, 2021; da Silva et al., 2015). Similarly, a relatively high specificity of *T. gondii* antibody detection was shown in oral fluids of pigs (Campero et al., 2020) providing the possibility of testing all mammals, regardless of the lactating status. Moreover, obtaining oral fluids is non-invasive while chewing ropes and other sample collection tools provide welcomed welfare-enriching elements. Validation of a commercial ELISA kit for a farm-scale screening using oral fluids was performed as a part of this thesis and is detailed in Chapter 2.

Antibody presence does not always correlate with the presence of *T. gondii* in the tissues of cattle (Opsteegh et al., 2011c, 2019) and potentially other animal species like horse (Klun et

al., 2017; Opsteegh et al., 2016a), as well as in immunodeficient or immunocompromised individuals (Dupont et al., 2021). For this reason, the direct detection of the parasite presents an alternative detection tool to the immunological methods. Animal bioassay has been a golden standard in the direct detection of the parasite while testing its viability at the same time. However, the ethically questionable, labor-intensive and time-consuming process of bioassays raised the demand for an alternative direct detection tool. The discovery of Taq DNA polymerase in 1976 was the first step towards the development of molecular detection techniques based on targeted amplification of DNA. Polymerase chain reaction (PCR), discovered by Kary Mullis, appeared to be a promising tool also for *T. gondii* detection. First applied in 1989 for the detection of *T. gondii* DNA using the 35-fold repetitive B1 gene (Burg et al., 1989), molecular detection immediately proved to be highly specific and sensitive. The sensitivity of *T. gondii* PCR detection further improved over time using the novel 529 bp fragment as PCR target (Homan et al., 2000). Using this element, repeated 200 to 300-fold in the *T. gondii* genome, compared to the approximately ten times less present B1 gene, and concentrations as low as four tachyzoites per PCR reaction were detectable (Homan et al., 2000).

The lack of sensitivity in *T. gondii* detection from tissues was the main disadvantage of PCR and its modifications (Garcia et al., 2006; Dolores E. Hill et al., 2006) when compared to bioassay. The issue of sensitivity was not in the PCR method itself but rather in the low amount of DNA extracted from the digested sample which was then input in the PCR reaction, compared to the larger quantity of digested sample fed to animals during bioassay. Concentrating the largest possible amount of tested matrix prior to molecular analysis in order to increase the chances of detecting the parasite from the estimated non-homogenous distribution of tissue cysts in tissues can be achieved by enzymatic digestion of the sample. Pepsin digestion, imitating human stomach environment, has the longest tradition in the digestion-based concentration of tissues. The properties of this method were explored in the isolation of *T. gondii* from meat samples from retail and subsequent genotyping of the recovered parasites in Chapter 6. Trypsin digestion is another method for isolating *T. gondii* from tissues, offering the advantage of not needing an acidic environment for enzyme activation and a faster tissue digestion compared to acid-pepsin digestion. Additionally, the

market availability and ease of application make trypsin the most commonly used proteases for tissue digestion. In this thesis (Chapters 5, 6 and 7), the standard protocol of trypsin digestion (Opsteegh et al., 2016a) was selected for its benefits of the overall robustness, reproducibility and relative cost-effectiveness to isolate *T. gondii* from naturally and experimentally infected pigs. Moreover, this method is often used for isolating the parasite from tissues that contain low levels of tissue cysts. Due to this advantage, the possibility of using this method for parasite burden estimation in the tested tissues was assessed (Chapters 5 and 6). The main disadvantage of using trypsin digestion for *T. gondii* isolation is that it may not be as effective as acid-pepsin digestion for breaking down tissues and releasing the tissue cysts. However, limitations in digesting certain tissues in comparison with acid-pepsin digestion and potential handling hazards when using powder forms due to inhalation. This led to the development of improved modifications such as introduction of stable liquid trypsin solutions, stabilization using reductive methylation or purification by benzamidine-sepharose (Chamrád et al., 2011; Rice et al., 1977). The possibility to use a larger input sample for PCR was achieved by Opsteegh et al. (2010), using proteinase-K digestion, followed by magnetic capture, targeting the specific DNA sequence of *T. gondii*, applied in order to isolate this DNA from the potentially disruptive host DNA background. Thanks to its high specificity and sensitivity, the method was frequently used (Deng et al., 2021a; Gomez-Samblas et al., 2015; Juránková et al., 2013a, 2014a, 2014b, 2015; Opsteegh et al., 2010) and further slightly modified to reach a 99% limit of detection of 65.4 parasites per 100 grams of meat (Gisbert Algaba et al., 2017). Therefore, the magnetic capture protocol offers an increased sensitivity and precision in the estimation of the parasite burden in sampled tissues. In order to assess the previously reported qualities of this method relative to its two other alternatives, a closer look at the magnetic capture protocol was applied in Chapter 7 of this thesis, where the obtained results were compared to trypsin digestion protocol. All three commonly used DNA extraction protocols were assessed in this thesis. Acid-pepsin digestion was applied in a combination with an in-house DNA extraction, trypsin digestion was followed by DNA extraction using a commercial kit and proteinase K was applied in magnetic capture DNA extraction.

T. gondii INFECTION IN HUMANS AND OTHER ANIMALS

Toxoplasmosis is a global zoonosis, with the ubiquitous presence of the parasite showcased by the detection of specific antibodies in fauna on all continents, even in Antarctica (Jensen et al., 2012; Rengifo-Herrera et al., 2012), and oceans (Ahmadpour et al., 2022; Fung et al., 2021). As one of the most successful parasites in the world, *T. gondii* is estimated to have infected around a third of the world human population (Montoya and Liesenfeld, 2004). Regional human prevalence estimates are incoherent but suggesting the highest estimated prevalence in South America and the lowest in the Western Pacific region (Bigna et al., 2020; Molan et al., 2019; Rostami et al., 2020) (Figure 4), reflecting to some extent the socioeconomic status (Mareze et al., 2019; Yasodhara et al., 2004) rather than the effect of temperature. Toxoplasmosis was associated with certain risk factors, including unemployment, illiteracy, and a lack of household water storage, which underscores the significance of maintaining proper hygiene.

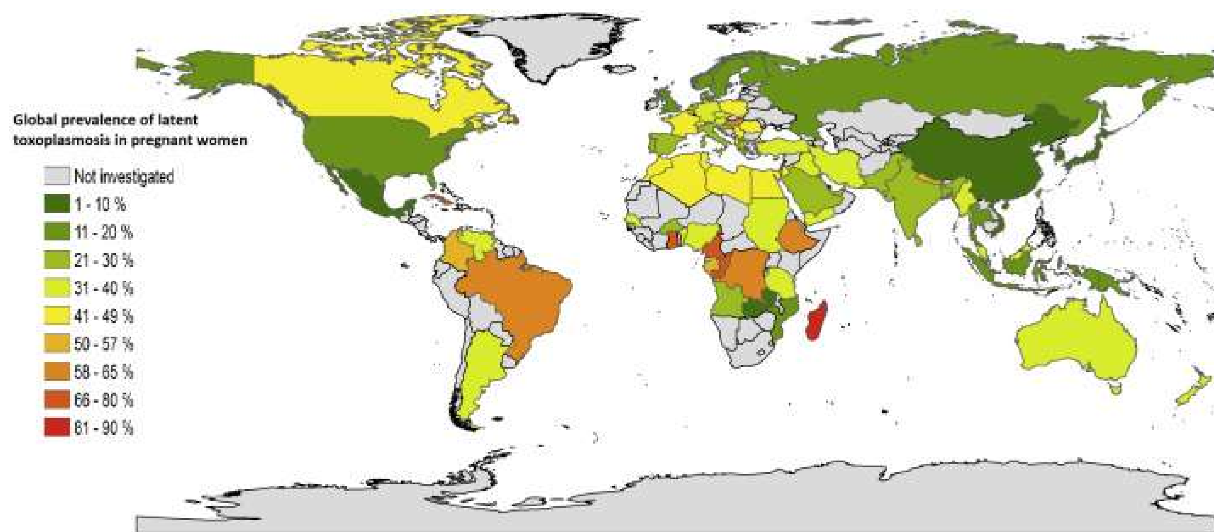

Figure 4 Fig. Prevalence of latent toxoplasmosis in pregnant women in different countries using geographic information system (GIS). Reprinted from Clinical Microbiology and Infection, Volume 26, Issue 6, 2020, Pages 673-683, *(Rostami et al., 2020)*, Global prevalence of latent toxoplasmosis in pregnant women: a systematic review and meta-analysis, Copyright (2020), with permission from Elsevier.

In animals, on the other hand, an increasing trend was observed in prevalence from north to south in the Northern Hemisphere (Gamarra et al., 2008; Jokelainen et al., 2010; Malmsten et al., 2011; Uggla and Hjort, 1984), regardless of the animal species. Similarly, animal husbandry

systems and outdoor access play a role in the prevalence (EFSA, 2011; Eppink et al., 2022; Olsen et al., 2020; Stelzer et al., 2019; Swanenburg et al., 2019; Wallander et al., 2016), with lower rates observable in intensive pig farms and higher prevalences in organic farms and wild boars (Bier et al., 2020; Djokic et al., 2016a; Eppink et al., 2022; Kijlstra et al., 2004; Puchalska et al., 2021). Moreover, seroprevalence of *T. gondii* was proven to be age-dependent (Berger-Schoch et al., 2011; Burrells et al., 2016; Djokic et al., 2016a; Jokelainen et al., 2017; Katzer et al., 2011; Opsteegh et al., 2011b; Wilking et al., 2016; Xu et al., 2015) making age an important factor to consider when comparing the prevalence in different groups or populations. These data indicate existence of risk factors for acquiring *T. gondii* infection in humans. The most recent European studies on *T. gondii* prevalence in animals were screened and the data were used as an input for an ambitious novel age-dependent model for *T. gondii* prevalence in livestock, wildlife and felids. The model incorporated the effects of animal species, European region, type of detection method, sample matrix, and age and outdoor access of the animal in order to estimate the prevalence of *T. gondii*. The individual steps and outcomes of the systematic review and meta-analysis are detailed in Chapter 3.

T. gondii infects a variety of warm-blooded hosts, including humans (Dubey, 2022). The parasite is most frequently described in tissues of pigs, goats and sheep while being less frequently observed in poultry, horses, rabbits, dogs, and cattle (Tenter et al., 2000) and there exists a clear interspecies variability of pathogenesis. Some of the pigeon breeds, New World Monkeys (such as howler monkeys), Australian marsupials, and Pallas cats are all known to manifest more severe clinical symptoms than most other intermediate hosts, and the infection often prove to be fatal (Canfield et al., 1990, p. 2002; Dubey, 2002; Epiphanio et al., 2003; Innes, 1997; Kenny et al., 2002). Different strains were similarly observed to induce variable immune response and clinical symptoms. Of the three most explored lineages, type I isolates (of haplogroup1) and mostly South American atypical strains (of haplogroups 4 – 10) are overall considered highly virulent, whereas type II strains (of haplogroup 2) are of intermediate virulence, and type III isolates (of haplogroup 3) are often avirulent in mice (Calero-Bernal et al., 2022; Dubey et al., 2012; Grigg and Suzuki, 2003; Khan et al., 2007; Sibley and Boothroyd, 1992). In case of clinical signs, toxoplasmosis often manifests with non-specific symptoms such as fever, headache, swollen lymph nodes, and muscle weakness (Calero-

Bernal and Gennari, 2019; Dubey, 2022). Toxoplasmosis is also the most common opportunistic infection of the central nervous system in immunocompromised people (Porter and Sande, 1992) with encephalitis the leading manifestation (Robert-Gangneux and Dardé, 2012). Moreover, it is the leading cause of retinal infections even in immunocompetent humans (Holland, 1999; Jones et al., 2006; Maenz et al., 2014; Silveira et al., 2001). Similar symptoms were recorded in other animal species (Calero-Bernal and Gennari, 2019; Davidson and English, 1998; Escoffier et al., 2010; Kreuder et al., 2003). Chronic infections are asymptomatic in most cases but may result in reactivation upon severe immunosuppression. In addition, studies have associated the infection with an increased risk of brain disorders like Alzheimer's disease (Kusbeci et al., 2011), Parkinson's disease (Miman et al., 2010), schizophrenia and bipolar disorder (Hinze-Selch et al., 2010; Torrey and Yolken, 2007), and epilepsy (Palmer, 2007; Stommel et al., 2001), perhaps due to the parasite's affinity towards neural tissues. However, these associations have not been confirmed and causal relation yet remains to be shown. In mice and rats a reduced response to cat urine and overall reduced fear of cats has been observed (Abdulai-Saiku and Vyas, 2017; Lamberton et al., 2008; Vyas et al., 2007; Webster, 2001), although these behavioural changes and the presence of tissue cysts within the host brain was challenged as merely incidental (Abdulai-Saiku et al., 2021). Primary infection is particularly important in pregnant individuals, as congenital infection may lead to encephalitis, chorioretinitis and even neonatal mortality (Ajzenberg et al., 2002b; Gilbert, 2000; Havelaar et al., 2007; Lindsay and Dubey, 2011; McAuley, 2014). Congenital toxoplasmosis present in animal husbandry could be responsible for considerable economic losses (Stelzer et al., 2019).

Toxoplasmosis is undisputedly a food-borne disease as well, with outbreaks in humans associated to both food- and water-borne sources (Almeria and Dubey, 2021). Epidemiological data from several case-control studies attribute human toxoplasmosis to various sources within the two primary pathways, meat-borne and environmental (Cook, 2000; Jones et al., 2001; Jones and Dubey, 2010). In Europe, meat was estimated to contribute 30-63% of *T. gondii* infections, depending on the country, whereas 6-17% of infections could be attributed to soil contact (Cook, 2000). With regards to the fact that majority of infections are meat-

borne, and the nature of the disease which leaves treatment options limited, prevention strategies and food safety play an important role in public health.

Pigs are one of the most frequently farmed animals in the world with an estimated 339 million tons of pig meat produced. As a result, pork is also one of the most consumed meats in the world, projected to increase by 17% by 2031 to 129 Mt, accounting for a third of the total increase in meat consumption. Even though stagnating in the recent years, pork is and will remain over the coming decade the most eaten meat in the European Union (OECD and Food and Agriculture Organization of the United Nations, 2022). Despite the observed drop in the seroprevalence in pigs between years 1989 and 2009, as reported by Dubey (Dubey, 2009b), pork and pork products are still recognized as a potential source of human toxoplasmosis (Batz et al., 2012; Condoleo et al., 2018; Deng et al., 2020; Djurković-Djaković et al., 2013; Dubey, 2009b; Hofhuis et al., 2011; Opsteegh et al., 2011a; Scientific Opinion on the public health hazards to be covered by inspection of meat (swine), n.d.; Sroka et al., 2020; Suijkerbuijk et al., 2019). Pork is likely to remain an important source of potential human *T. gondii* infections, as recognized by recent quantitative microbial risk assessments (QMRA) in Europe (Belluco et al., 2018; Deng et al., 2020; Opsteegh et al., 2011a), and a leading risk for acquiring *T. gondii* infections for consumers in the USA and China (Deng et al., 2020; Dubey, 2009b; Dubey et al., 2020). Europe has a long tradition of preparation and consumption of pork and pork products in form of charcuterie (Vitale et al., 2014). Consequently, a variety of recipes using pork have emerged, many of which include undercooked or raw pork. Due to these consumption preferences and the potential risk arising from the consumption of raw or undercooked meat (Ducrocq et al., 2021; Guo et al., 2015b), preventive measures and processing techniques have been developed to address this issue. The most influential preventive measures will be detailed throughout this thesis, and in Chapter 7 in particular.

Similarly, game meat is considered an important source of food-borne transmission for humans, due to its rising popularity and common undercooked consumption of this high-quality but relatively frequently *T. gondii*-positive meat (Ballash et al., 2015; England et al., 2019; Jones and Dubey, 2012; Ross et al., 2001). Game meat was repeatedly identified as the source of *T. gondii* infection (Conrady et al., 2022; Ross et al., 2001; Sacks et al., 1983).

RISK FACTORS & PREVENTION ALONG THE MEAT-BORNE PATHWAY

The "stable to table" concept represents the holistic approach embracing all elements, which may have an impact on the safety of food, at every level of the food chain from stable to the table. Applied to *T. gondii,* this approach emphasises the systematic need for interaction between all participants in the entire food chain, from the animal feed manufacturer down to the individual consumer (Henke et al., 2021). However, it's worth noting that *T. gondii* is not exclusively related to how the animal was raised. Proper food handling, cooking and hygiene practices are also important to prevent *T. gondii* infection and consequently toxoplasmosis. Risk factors and prevention measures can be categorized into two groups, pre-harvest and post-harvest measures (Kijlstra and Jongert, 2008), based on their place within the stable to table process.

Pre-harvest measures

The first group of measures focuses on the pre-harvest measures which mainly take place at the beginning of the pork supply chain, at a farm or a slaughterhouse. The prevalence of *T. gondii* in modern intensive pig farms is minimal as the higher seroprevalence in pigs is linked to the outdoor access of the animals (EFSA, 2011; Olsen et al., 2020; Swanenburg et al., 2019) and remains predominantly an issue of smaller farms with insufficient sanitary measures (Djokic et al., 2016a; Herrero et al., 2016; Limon et al., 2017). Intervention studies with pre-harvest intervention strategies in finishing pig farms in the Netherlands proved that cat-neutering and feed coverage were associated with reduction in seroprevalence (67% and 96% respectively) while rodent control was not (Eppink et al., 2021). Similar findings, linking the presence of cats, rodents, their access to feed and poor hygiene to the higher seroprevalence, were reported also from pig farms in the USA, the UK, Italy and Spain (García-Bocanegra et al., 2010; Herrero et al., 2016; Dolores E. Hill et al., 2006; Limon et al., 2017; Tenter et al., 2000; Villari et al., 2009). An impressive large-scale serological screening of slaughter pigs was done between 2012 and 2016 in the Netherlands, revealing a low seroprevalence of 1.4% to 2.8%, with an interesting seroprevalence peak in winter. Moreover, the study further proved the link between organic farming and higher seroprevalence of *T. gondii* (Swanenburg et al., 2019).

Post-harvest measures

Post-harvest measures take place after the slaughter of the animals. Important division in this category is between the processing in industry and at consumer level. Consumption of raw or undercooked meat is known to be a major risk for human *T. gondii* infections (Bobić et al., 2007, 1998; Cook, 2000; Kapperud et al., 1996). Majority of *T. gondii* infections in the US and Europe are attributed to meat (Batz et al., 2012; Cook, 2000). Several studies discovered viable *T. gondii* tissue cysts in fresh meat (Bayarri et al., 2012; Dubey et al., 2005; J.P. Dubey et al., 2011; Rani et al., 2020; Wang et al., 2012), proving once again the potential risks of the meat for the consumers.

General prevention methods such as application of freezing and heating were shown to be effective in inactivation of *T. gondii* parasites within infected pig tissues (Condoleo et al., 2018; Dubey, 1988, 1974; Dubey et al., 1990; Hill et al., 2004). Freezing at below -12°C (Kotula et al., 1991) or -20 °C for at least three days, especially when followed by a slow thawing and then salting and curing was shown to inactivate all *T. gondii* parasites (Gomez-Samblas et al., 2016). At the same time heating above 67°C inactivates effectively all *T. gondii* parasites (Dubey et al., 1990). Additives and ingredients as well as factors such as pH, water activity, smoking, vacuuming, and fermentation were all observed to influence the viability of *T. gondii* in meat products (Alves et al., 2020; Deng et al., 2020; Fredericks et al., 2020, 2019; Guo et al., 2015a; Lundén and Uggla, 1992; Mirza Alizadeh et al., 2018; Neumayerová et al., 2014; Pott et al., 2013).

Salting of meat products was historically associated with limited pathogen growth and raised therefore an auspicious potential of yielding inactivating effect on *T. gondii* in meat. A detailed testing of the effect of salting using concentrations of 0.85-6.00% NaCl in brine, stable temperature between 4°C and 20°C, and time by Dubey et al. (1997) provided an interesting observation of the relationship between these factors. Concentrations of NaCl of 0.85-6.0% in temperatures between 4°C and 20°C provided the following outcomes: at 4°C viable tissue cysts were still detectable for at least 56 days in 0.85% NaCl, for 49 days in 2.0% NaCl, and for 21 days in 3.3% NaCl solutions. The additional effect of temperature showed that tissue cysts survived at 10°C and 0.85%, 2.0%, and 3.3% NaCl solutions for at least 21 days. At 15°C the

inactivation process advanced more rapidly, especially in higher salt concentrations, with tissue cysts surviving for at least 21 days in 0.85% NaCl but only 14 days in 2.0%, and 3.3% NaCl solutions. Finally, at 20°C, tissue cysts survived for 14 days in 0.85% NaCl, 7 days in 2.0% NaCl, and just 3 days in 3.3% NaCl solutions. Tissue cysts generally did not survive in 6.0% NaCl solution at any temperature. The effect of salting was subsequently tested in many different applications known in the modern meat product industry.

The effect of dry curing was tested in hams of naturally infected pigs using commercial curing salt mix, showing viable *T. gondii* in pork meat after 7 months but no longer at 14 months (Bayarri et al., 2010). The same salting method was tested on Serrano hams, finding 8.8% of the samples to be positive by qPCR and more than half of these were found viable by mouse bioassay (Gomez-Samblas et al., 2015). In a subsequent experiment, hams and legs were treated comparably to a production of Serrano ham and no viable parasites were found after 7 months of curing (Gomez-Samblas et al., 2016). Similarly, Parma ham was used for the parasite viability testing and was left curing for 12, 14 or 16 months, however, with no viable parasites were recovered at any of the time-points (Genchi et al., 2017). Obtaining more details on the effect on salt in dry curing process remained a focal point of the subsequent studies. Dry curing of hams of naturally infected pigs at around 3% NaCl concentration for 9 or 12 months had a significant effect on the loss of viability as opposed to fresh ham but viable parasites could still be found at both sampled time-points (Herrero et al., 2017). In a recent study, no viable parasites were retrieved from dry hams during the first sampling point at 33 days with the NaCl concentration in the final product above 4% (Fredericks et al., 2020). Studies showed variable effects of dry salting and curing on *T. gondii* viability based on the previous observations, giving us an approximation of the effectivity of NaCl treatment. Using salting methods other than dry curing, NaCl concentrations above 2.0% for more than 8 days proved to be effective in inactivating bradyzoites in tissue cysts (Deng et al., 2020; Fredericks et al., 2019; Neumayerová et al., 2014; Pott et al., 2013).

Fermentation, smoking and some additives on their own or in combination with NaCl were shown to inactivate *T. gondii* tissue cysts (Christieans et al., 2018; Pott et al., 2013; van Sprang, 1984). Fermentation, as a process commonly used in charcuterie, comprises among other

factors of decrease of pH in the final product. However, the effect of pH alone is not sufficient for inactivation of tissue cysts as those are relatively resistant to low pH, surviving for up to 26 days at pH = 5 (Pott et al., 2013). This fact was highlighted in a study testing various pH conditions in combination with NaCl in dry cured pork sausage, resulting in a viable-*T. gondii* free dry sausages after more than 6 hours at 1.3% NaCl concentration minimum, regardless the pH of the final product (Fredericks et al., 2019; Hill et al., 2018).

The work presented in Chapter 7 aimed to address the limited knowledge of the various processes involved in the production of dry pork sausage, a viable representative of fermented processed pork products, which might be influencing viability of *T. gondii* tissue cysts in this type of charcuterie.

Contrary to the protective effects of NaCl, freezing and heating, the vacuum treatment actually seems to increase the survival of tissue cysts in goat meat (Neumayerová et al., 2014). On the contrary, dry aging of pork, a modification of vacuum treatment of meat, inactivates the parasite efficiently after 21 days of 0°C aging for up to 28 days in vacuum-wrapped pork meat (Alves et al., 2020). Perhaps surprisingly, fresh non-heated pork sausages were shown to bare low but not negligible risks for consumers (Abdulmawjood et al., 2014; Dias et al., 2005).

Prevention guidelines and recommendations for “high-risk” groups

Considering the two major transmission routes for *T. gondii* in humans, the environmental and meat-borne, both general and *T. gondii*-specific prevention measures can be applied to lower the risk of acquiring new infections in humans. General safety advices comprise of steps as following: washing hands and cooking surfaces after a contact with soil or unwashed fresh produce, washing and/or peeling these products thoroughly before consumption, or cleaning all cutting boards and utensils with soap and warm water after each use. Prevention measures targeted specifically at *T. gondii* can be split into two categories with the first being the pre-harvest measures, focusing mostly on limiting exposure of animals and produce to *T. gondii* by monitoring of cat presence. The post-harvest measures aim at inactivation of the parasite, applicable both at the producer (e.g. pre-washing ready-to-eat produce, salting, smoking,

fermentation, industrial freezing and heating) and consumer (heating, freezing) (Kijlstra and Jongert, 2008).

A series of recommendations aimed at handling toxoplasmosis was published by various responsible entities. In relation to the pre-harvest part of the pork production, European Food Safety Authority (EFSA) recommended a set of measures aiming to help reduce *T. gondii* infections (EFSA, 2011). Similarly, Codex Alimentarius Commission (CAC) referred to both the pre-harvest and post-harvest practices aimed at preventing, reducing, inactivating, or otherwise controlling foodborne parasites, including *T. gondii* (CAC, 2016). A standardized approach to *T. gondii* diagnosis was published in Terrestrial Manual of the World Organisation for Animal Health (WOAH, previously OIE) (Terrestrial Manual Online Access, 2018), which was followed shortly by an overview of the etiology, epidemiology, potential impacts, diagnosis and prevention of *T. gondii* infection was published by WOAH (2019). The Center for Disease Control and Prevention (CDC) provided consumer guidelines for risk reduction of acquiring toxoplasmosis from food and the environment (Prevention, 2019). In addition, FAO published a guide for meat inspectors in developing countries, describing pathological signs of *T. gondii* (Herenda and Chambers, 1994).

Variable recommendation are provided also to pregnant women who are at particular risk. These advices may range by country from total discouragement from consumption of fresh unheated food to more reasonable recommendations regarding heating or freezing of meat and meat products prior to consumption, thorough washing of fresh produce and limited contact with soil and animals, especially cats. Additionally, several countries have active (Austria, Belgium, France, Slovakia and Slovenia) surveillance of congenital toxoplasmosis cases with compulsory screening program during pregnancy. Additional four countries (Bulgaria, the Czech Republic, Germany and Hungary) take part in the voluntary surveillance. The most thorough and advanced prenatal screening system is presently in France, which enabled the reporting of the highest numbers of congenital toxoplasmosis (ECDC Congenital toxoplasmosis Annual Epidemiological Report for 2017, 2017).

Despite the fact that consumption of undercooked meat is a significant risk factor for the acquisition of toxoplasmosis in humans (FERG, 2015), there is currently no international (EU

or other) regulation that sets specific requirements and standards concerning the control of *T. gondii* in meat. Moreover, with the traditional meat inspection tools incapable of detecting reliably *T. gondii* infection in animals at slaughterhouse at an individual level, there is currently no available cost-effective alternative (Alban et al., 2020).

AIMS AND OBJECTIVES OF THE THESIS

The work in this thesis focuses on the meat-borne transmission pathway, which is estimated to act as the main source of human *T. gondii* infections. More specifically, the thesis addresses the risks related to the consumption of pork and meat products originating from pig tissues, as pork is the most consumed meat in Europe and worldwide. The results obtained as a part of this thesis provide a crucial contribution to the risk assessment of meatborne *T. gondii* infections in Europe.

In order to achieve this goal, we set the following objectives:

- To validate a test for welfare-friendly farm-level screening of *T. gondii* antibodies in pigs.
- To provide age-related regional *T. gondii* prevalence estimates for a selection of its key animal host species in Europe.
- To compare the distribution patterns related to *T. gondii* infection (natural or experimental) with different parasite stages (oocyst or tissue cysts) and isolates of different strains (type II or type III).
- To rank a wide selection of individual pig organs and muscles based on the estimated parasite burdens in these tissues.
- To determine the prevalence of *T. gondii* in meat and meat products available in retail.
- To assess the inactivation capabilities of salt, nitrites, nitrates and processing steps in pork production, represented on an example of traditional French dry sausage.

The framework of this thesis consists of a detailed introduction to *T. gondii* in pork production according to the "stable to table" approach to food safety (Chapter 1) and of the following chapters:

Chapter 2

In this section, the validation of a cost-effective and welfare-friendly diagnostic tool for screening of animals for antibodies against *T. gondii* on a farm level was explored. The suitability of a commercial kit for the detection of specific antibodies to *T. gondii* in oral fluid samples from experimentally inoculated and naturally infected pigs was assessed, and compared to the serological results and the validatory immunoblot test.

Chapter 3

This chapter summarizes the latest epidemiological findings regarding the prevalence of *T. gondii* infection in animal species that can be relevant as sources of human infection. Collected as a part of an extensive literature review, the data were subsequently used to assemble a novel model embracing Bayesian principles to provide precise species-specific age-dependent regional prevalence estimates. The design of the model allows to borrow strengths from geographical regions with sufficient data and extrapolate the model in order to fill the gaps in regions with little or no prevalence data.

Chapter 4

The aim was to study the influence of parasite stage and type on the clinical outcome of toxoplasmosis in pigs and to collect information on the anatomical distribution and concentration of parasites in the commonly consumed butcher meat cuts and organs. The course of infection in pigs that were experimentally inoculated with oocysts and tissue cysts of a type II (ToxoDB #3) and a type III (ToxoDB #2) *T. gondii* isolate was evaluated, assessing the clinical signs, histopathological changes, and the evolution of the specific humoral response in the different inoculation groups. In addition tropism of the parasite was explored within the tested tissues of these experimentally infected pigs, and tissues were ranked based on the estimated parasite load per gram of tissue.

Chapter 5

Parasite distribution in both naturally and experimentally infected pigs was explored in detail in this part, in order to provide insight in the distribution patterns in pig tissues. Furthermore, differences between two infective stages of *T. gondii*, bradyzoites and sporozoites, were compared in regards to the anatomical distribution in individual muscles and tissues and ranked according to the parasite burden, providing thus, in combination with the results of previous chapter, a more complete picture of the effect of parasite stage on infection in pigs.

Chapter 6

Pork is one of the most consumed meats globally, however game meat is rapidly rising in popularity due to higher valued taste, and perceived health-related benefits. Higher consumption frequency in combination with lower level of heating applied for reasons of culinary preference lead to an increased risk to consumers. The presence of *T. gondii* DNA in different meat products available for human consumption, including venison, was screened in samples from retail in Scotland.

Chapter 7

This chapter explores the parasite burden in heart and butcher meat cuts traditionally used for the production of French pork delicacies. Meat of experimentally infected pigs was used for production of dry sausages and non-encased sausage batter. Impact of different concentrations and combinations of salt, nitrates, and nitrites as well as processing steps (ripening, drying and storage) on the viability of *T. gondii* in these pork products was assessed, and detailed measurements of the whole process analysed in order to provide valuable data which could be used as a model for similar products.

All studies described in this thesis provide new insight on several aspects of *T. gondii* in animals and meat products, especially pork. In the last chapter the implications of these findings for the risk of human infection will be discussed.

Chapter 2

Detection of *Toxoplasma gondii*-specific antibodies in pigs using an oral fluid-based commercial ELISA: Advantages and limitations

Johanna Kauter [a], Filip Damek [b], Gereon Schares [c], Radu Blaga [b], Franziska Schott [d], Peter Deplazes [e], Xaver Sidler [d], Walter Basso [a,⥣]

[a] Institute of Parasitology, Vetsuisse-Faculty, University of Bern, Länggassstrasse 122, CH-3012 Bern, Switzerland
[b] Anses, INRAE, Ecole Nationale Vétérinaire d'Alfort, Laboratoire de Santé Animale, BIPAR, Maisons-Alfort F-94700, France
[c] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Epidemiology, Südufer 10, 17493 Greifswald Insel Riems, Germany
[d] Department of Farm Animals, Division of Swine Medicine, Vetsuisse-Faculty, University of Zurich, Winterthurerstrasse 260, CH-8057 Zurich, Switzerland
[e] Institute of Parasitology, Vetsuisse-Faculty, University of Zurich, Winterthurerstrasse 266a, CH-8057 Zurich, Switzerland

⥣ Corresponding author at: Institute of Parasitology, Vetsuisse-Faculty, University of Bern, Länggassstrasse 122, CH-3012 Bern, Switzerland. E-mail address: walter.basso@unibe.ch (W. Basso).

Article: https://doi.org/10.1016/j.ijpara.2022.11.0030020-7519/_ 2022, supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2022.11.003.

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Toxoplasma gondii is a major food-borne parasite and undercooked meat of infected pigs represents an important source of infection for humans. Since infections in pigs are mostly subclinical, adequate diagnostic tests for use at the farm level are pursued. Oral fluid (OF) was shown to be a promising matrix for direct and indirect detection of infections with various pathogens in pigs. The objective of this study was to assess whether *T. gondii* infections in pigs could be diagnosed using an indirect ELISA kit adapted for OF samples (OF-ELISA). Routine serology and OF-immunoblot (IB) were used as standards for the comparison. For this, serial OF samples from sows (n = 8) and fatteners (n = 3) experimentally inoculated with *T. gondii* oocysts, individual field samples from potentially exposed sows (n = 9) and pooled OF samples from potentially exposed group-housed fatteners (n = 195 pig groups, including 2,248 animals) were analysed for antibodies against *T. gondii* by ELISA. For individual animals, OF-ELISA exhibited a relative diagnostic specificity of 97.3% and a relative diagnostic sensitivity of 78.8%. In experimentally infected animals, positive OF-ELISA results were observed from 1.5 weeks post inoculation (pi) until the end of the experimental setup (8 to 30 weeks pi); however, values below the estimated cut-off were occasion- ally observed in some animals despite constant seropositivity. In potentially exposed individual animals, OF- and serum-ELISA results showed 100% agreement. In group-housed fatteners, antibodies against *T. gondii* could be reliably detected by OF-ELISA in groups in which at least 25% of the animals were seropositive. This OF-ELISA, based on a commercially available serum-ELISA, may represent an interesting non-invasive screening tool for detecting pig groups with a high exposure to *T. gondii* at the farm level. The OF-ELISA may need further adjustments to consistently detect individual infected pigs, probably due to variations in OF antibody concentration over time.

INTRODUCTION

Toxoplasma gondii is a worldwide distributed zoonotic proto- zoan parasite belonging to the family Sarcocystidae. This parasite has a facultative indirect life cycle with cats and other felids as the only definitive hosts, in which the parasite undergoes a sexual multiplication leading to production of oocysts, which are shed through their faeces. All warm-blooded species (mammals and birds) may act as intermediate hosts, with development of tissue cysts in several organs (e.g., CNS, heart and skeletal musculature) after asexual multiplication of the parasite (Deplazes et al., 2012). Natural infections in pigs may occur by ingestion of infected intermediate hosts such as rodents or birds carrying tissue cysts but more often through oral uptake of fodder or water contaminated with oocysts from cat faeces (Stelzer et al., 2019). Undercooked meat containing tissue cysts represents an important infection source for humans (Dubey, 2021) and pork is considered to be one of the major meat sources associated with human *T. gondii* infections (Guo et al., 2015b). Previous studies in Switzerland, based on meat juice analyses by *T. gondii* P30 (TgSAG1) ELISA, showed seroprevalences of 14%, 13% and 36% in finishing pigs (~6 months old), free-ranging pigs (~6 months old) and adult animals (~3-4 years old), respectively (Berger-Schoch et al., 2011). A very recent study investigating the seroprevalence of anti-*T. gondii* antibodies from healthy pigs at slaughter in Switzerland showed a decrease in the seroprevalence to only 1.3% with higher seropositivity in free-range pigs (2.9%) than in indoor pigs (0.4%) (Kelbert et al., 2021). It is estimated that approximately one-third of the global human population is infected with *T. gondii* (Montoya and Liesenfeld, 2004). The parasite is prioritised as the second most important food-borne parasite in Europe after *Echinococcus multilocularis* (Bouwknegt et al., 2018). Infections often remain asymptomatic in healthy adults, but it is of high clinical relevance in immunocompromised patients (e.g., AIDS patients, patients under immunosuppressive treatments) and in cases of primary infection during pregnancy, as *T. gondii* may cause severe foetal damage (e.g., hydrocephalus, microcephalus, intracerebral calcifications and chorioretinitis) leading to abortion, stillbirth, or to the birth of asymptomatic children, which may develop learning and visual disabilities or severe life-threatening infections later in life (Deplazes et al., 2012; EFSA Panel on Biological Hazards (BIOHAZ) et al., 2018). In addition, ocular toxoplasmosis was described both in immunocompetent and immunocompromised patients after congenital and

post-natal infections, and is considered the most common form of infectious posterior uveitis, which can lead to blindness (Maenz et al., 2014). In pigs, *T. gondii* infections are mostly asymptomatic (Dubey et al., 2020; Stelzer et al., 2019). Nevertheless, *T. gondii* has been reported to be a cause of reproductive failure in sows (Basso et al., 2015) and occasionally of severe illness with respiratory, digestive and/or neurological signs and even death in different age categories (Dubey, 2009b; Dubey et al., 2020; Klein et al., 2010; Li et al., 2010; Olinda et al., 2016; Stelzer et al., 2019). In order to reduce human exposure (Djurković-Djaković et al., 2019) and secondly to improve pig health, control of *T. gondii* is necessary. Therefore, adequate diagnostic tests are needed. Currently, serology is the most commonly used method to detect *T. gondii* infections in pigs (Basso et al., 2013) and commercial ELISA kits are confirmed to be a useful tool in the detection of antibodies to Toxoplasma in serum or meat juice in pigs (Basso et al., 2013; Liyanage et al., 2021; Macaluso et al., 2019). Detection of *T. gondii* antibodies in oral fluid (OF) might serve as an alternative to standard serology, aiding in the identification of farms with high exposure to *T. gondii* (Campero et al., 2020). Compared with blood sampling, collection of OF represents a non-invasive, animal welfare friendly method with less discomfort or stress for the animals (Henao-Diaz et al., 2020). OF is not equivalent to saliva. It can be defined as the fluid obtained during the placement of absorbent tissue in the oral cavity (Henao-Diaz et al., 2020). It consists of a combination of saliva and serum transudates from capillaries in the oral mucosa and gingival tissues. In particular, the gingival crevice (space between teeth and gingiva) allows access to systemic immune factors from blood into the oral cavity via crevicular fluid (Challacombe et al., 2015). Therefore, OF contains antibodies deriving from the systemic immune system (from the passage of serum antibodies) but also locally produced antibodies from the secretory immune system in the salivary glands. There are reports of the diagnostic use of OF in swine dating back to 1976 (Prickett and Zimmerman, 2010). So far, OF has been used as matrix for direct and indirect detection of numerous viral and bacterial agents causing disease in pigs (Campero et al., 2020). Recently, Henao-Diaz et al. (2020) published in their ‘‘Guidelines for oral fluid-based surveillance of viral pathogens in swine” a list with more than 23 swine viral pathogens that have been successfully detected with OF. OF has also been used in pigs as a matrix for the detection of hormones (Colson et al., 2012) and recently, for the detection of antibodies

against *T. gondii* (Campero et al., 2020). As Campero et al. (2020) showed in their study, antibodies to *T. gondii* can be detected in OF from infected pigs by immunoblot (IB). Their results suggested that IgA-antibodies present in OF would represent a better target than IgG, as a higher correlation with the serological status from individual animals was observed. In pooled samples from groups of pigs, positive IB results were obtained only in herds with a high proportion of seropositive pigs. No false positive results were observed in the study, which is particularly important when used as a surveillance strategy (Henao-Diaz et al., 2020). As Campero et al. (2020) suggested, their preliminary results may serve as a basis to develop further serological assays. Indirect ELISA kits designed to detect antibodies in serum samples have been adapted in several studies to detect antibodies against different swine pathogens in OF (Henao-Diaz et al., 2020; Prickett and Zimmerman, 2010; Rotolo et al., 2017). Changes in incubation times or temperature, as well as in the concentration of OF or conjugate, lead to suitable results. Besides, the use of commercial ELISA kits has significant advantages over IB techniques. ELISA kits are easily available to practitioners, are generally cost-effective, time-saving due to a higher throughput, and easy to use. The results are also less biased (i. e., measurable as OD) and hence easier to standardize compared with results from IB.

The objective of this study was therefore to determine if a commercial ELISA kit can represent a suitable tool for the detection of antibodies against *T. gondii* in OF samples from experimentally inoculated and naturally infected pigs, to compare the results with standard serology as well as with IB, and to assess its potential as a screening method at the farm level.

MATERIALS AND METHODS

Animals (OF and serum samples)

OF and serum samples were simultaneously collected from pigs experimentally inoculated with *T. gondii* oocysts (at several time points after inoculation), as well as from potentially exposed pigs (at one time point) as previously described (Campero et al., 2020).

Experimentally inoculated pigs

This sample set included serial OF and serum samples from eight sows experimentally inoculated with 10^4 *T. gondii* oocysts (CZ isolate, clone H3, type II) in Switzerland, and from three negative control sows (collected at 1, 1.5, 2, 3, 4, 8, 12, 13 and 30 weeks post inoculation (pi)), which derived from previous studies (Basso et al., 2017; Campero et al., 2020) and had been earlier tested for antibodies against *T. gondii* in serum and OF by ELISA and IB, respectively. In addition, new serial OF and serum samples from three Large White fattening pigs (weight 47–58kg) inoculated with 1.4×10^3 *T. gondii* oocysts (ME49 strain, type II) in France, (collected at 0, 1, 4, 5, 6, 7 and 8 weeks pi), and from three further non-inoculated fatteners from the same experimental setup were included in the study.

Field samples from potentially exposed pigs

Pooled OF samples were obtained from potentially exposed group-housed fattening pigs in Switzerland by hanging one or two (in cases of groups with up to 20 or >20 animals, respectively) cotton ropes in the pens for 45–60 min for the animals to chew on, as previously described by Campero et al. (2020). When two ropes were used, both OF samples were pooled after collection. Pooled OF samples from a total of 195 pig groups (2–40 pigs/group; mean 12 pigs/group; 2,248 pigs in total) deriving from 22 Swiss pig farms were included in the study. Thirty-nine of these OF samples derived from a previous study and had been earlier tested for *T. gondii* antibodies by IB techniques (Campero et al., 2020). Also, blood samples were taken in parallel from each of the group-housed fatteners.

In addition, individual OF and serum samples were taken from nine potentially exposed sows aged 8–34 months from one further farm.

All animal experiments were authorized either by the Cantonal Veterinary Offices of Zurich, (permission no. ZH 216/2013) and Luzern (permission no. LU 03/2014) in Switzerland or by the APAFIS N° 14363-2018032908554996v3 in France, and complied with Swiss and French Animal Welfare guidelines, respectively.

Toxoplasma gondii serum-ELISA

To assess the serostatus of all pigs included in the study, individual serum samples of all animals were tested for anti-*T. gondii* antibodies with commercial ELISA kits. Either the ELISA PrioCHECK™ Porcine Toxoplasma Ab Kit, Thermo Fisher Scientific, Schlieren, Switzerland (for testing of the experimentally infected sows and group-housed pigs), which was validated for detecting antibodies in sera and meat juice from swine (Basso et al., 2013), or the ID Screen® Toxoplasmosis Indirect Multi-Species Test, ID.vet, Grabels, France (for testing of the experimentally infected fatteners and individual sows) were used. The analyses were performed as described by the manufacturers. Results of the ELISA assays were measured as O.D. at 450 nm and the values were normalized by calculating a sample/positive control ratio (SP; $\mathrm{SP\%} = \frac{\mathrm{OD\ sample} - \mathrm{O\ \ \ negative\ control}}{\mathrm{OD\ positive\ control} - \mathrm{OD\ negative\ control}} * 100$). According to the manufacturer, animals with S/P% ≤ 40% were considered negative, inconclusive if 40% < S/P% > 50% and positive if S/P% ≥ 50%.

TgSAG1 (P30) immunoblot (IB)

As it was previously shown that antibodies against *T. gondii* could be detected in OF from infected pigs by IB techniques, OF samples were tested in parallel with a *T. gondii* tachyzoite surface antigen TgSAG1 (P30)-based IB to detect specific antibodies (IgG and IgA) against *T. gondii* as a standard of comparison. In order to achieve the highest possible comparability, all samples were treated in exactly the same way as described by Campero et al. (2020). The reaction between antibody and the immunodominant antigen TgSAG1, visible as a sole band of a relative molecular mass of 30kDa, was recorded in the following way: strong band = high positive (++); clear band = positive (+); weak band = weak positive (+/-); no band = negative (-) (Campero et al., 2020). For evaluation in this study, weak positive, positive, and high positive reactions of IgA or IgG were considered as positive IB reactions.

Toxoplasma gondii OF-ELISA

OF samples were tested for antibodies against *T. gondii* with a commercial indirect ELISA kit (ID Screen® Toxoplasmosis Indirect Multi-Species Test, IDvet, Grabels, France), which was

validated for its use in serum and meat juice of pigs. To assess the most adequate conditions for testing OF, a small sample set including known positive and negative OF samples was first tested under different incubation conditions (temperature and time of incubation) and using different sample and conjugate concentrations (see Supplementary data). Based on these preliminary results, OF samples were tested under the following conditions: sample dilution 1:2 in sample buffer (serum samples were tested at a 1:10 concentration), sample incubation with the antigen at 4 °C overnight (16 h +/- 1 h) and conjugate dilution 1:2 in conjugate buffer (double con- centration as used with serum samples). Except for these modifications, the ELISA was carried out as described by the manufacturer (TOXO-MS ver. 1014 DE, Stand 08.2018). ELISA values were measured as O.D. at 450 nm and normalized by calculating a sample : positive control ratio (SP; $SP\% = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD positive control} - \text{OD negative control}} * 100$).

Two separate cut-offs were calculated: one cut-off for the group of pooled OF samples, and another for samples from individual animals.

For the calculation of the cut-off for pooled samples, ELISA results from OF samples from pens in which all individual animals in the group tested serologically negative in serum-ELISA were considered (n = 167 samples). The S.D. as well as mean value (MV) were calculated, and the cut-off was defined as follows: cut-off = MV + 3*SD (Classen et al., 1987; Lardeux et al., 2016).

For the calculation of the cut-off-value for individual animals, only individual negative animals from an experimental setting were included: serial samples from negative control sows (28 samples, three animals), and single samples before inoculation (five samples, five animals) (n total = 33 samples). Calculations were performed the same way as described above for pooled samples (cut-off = MV + 3*SD).

The sensitivity, specificity as well as predictive values for the OF-ELISA were calculated in relation to a reference standard of comparison. An animal was regarded as reference standard-positive if it tested serum ELISA- or OF-IB IgG- and/or IgA- positive. All remaining animals were regarded as reference standard-negative.

RESULTS

Analyses of samples from individual animals

Serum-ELISA

Serum-ELISA results from the eight experimentally inoculated sows included in this study were previously described (Basso et al., 2017). Briefly, all sows seroconverted between two and 3 weeks pi and remained seropositive until the end of OF sampling (30 weeks pi) or until euthanasia (between eight and 22 weeks pi).

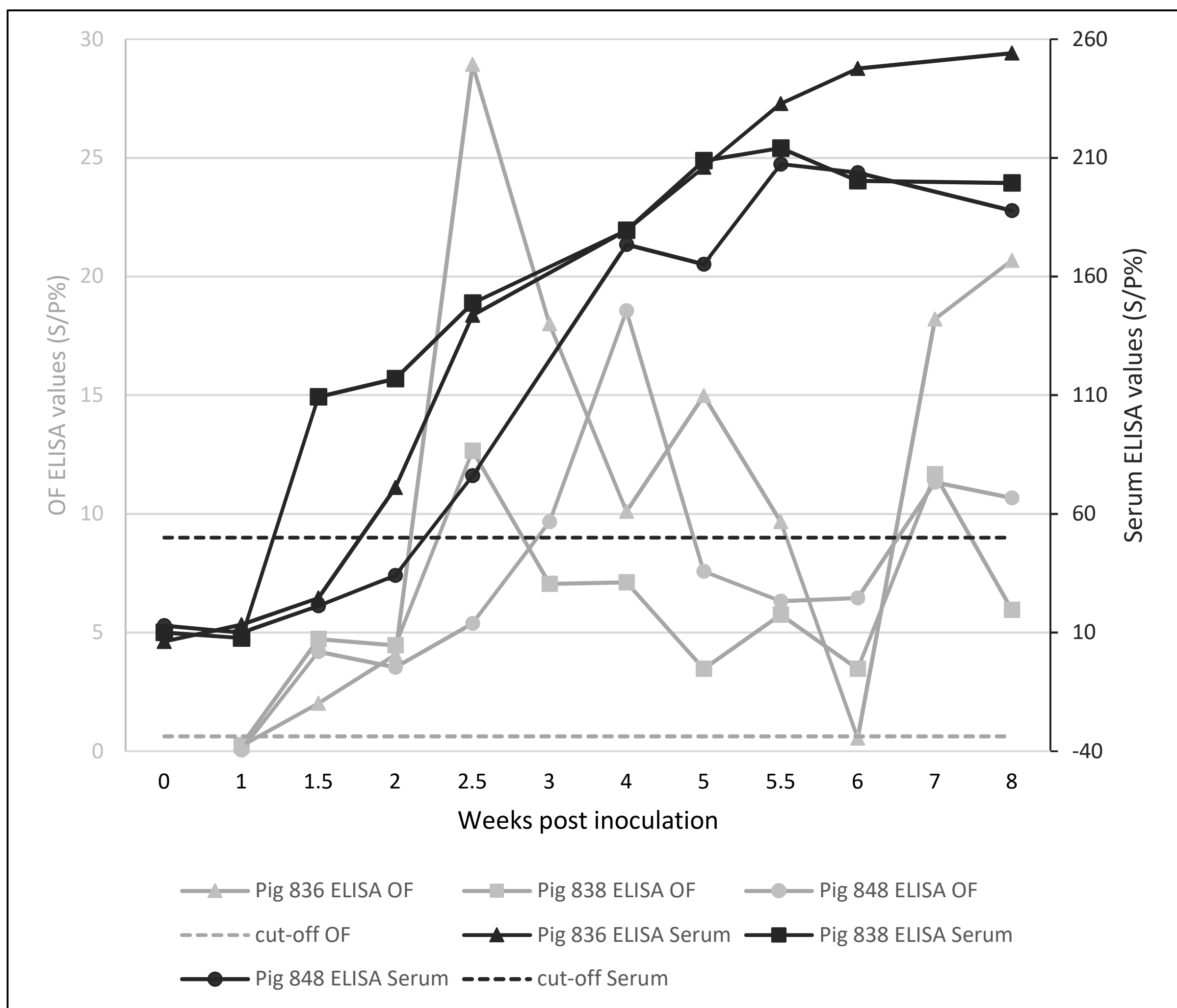

Figure 5 Anti-*Toxoplasma gondii* response in fattening pigs (n = 3) experimentally inoculated with 1,400 *T. gondii* oocysts (ME49 strain, clonal type II) in oral fluid- and serum- ELISA (ID Screen® Toxoplasmosis Indirect Multi-Species Test) over time. OF, oral fluid; S/P%, sample:positive control ratio.

The three experimentally inoculated fatteners (Pigs 836, 838, 848) started yielding positive results between 1.5 and 2.5 weeks pi and remained seropositive until the end of the experiment (8 weeks pi). The highest serum antibody levels were reached between 5.5 and 8 weeks pi (Fig. 5). All non-inoculated pigs used as negative controls (n = 6) tested negative. The sample set of nine potentially exposed sows included one seropositive (serum-ELISA S/P% 144.98), and eight seronegative sows.

OF-IB

OF-IB results from the eight experimentally inoculated sows were previously described by Campero et al. (2020), and were included in Table 1 of this paper. Briefly, positive OF-IB results for IgA and IgG were first detected at 1.5 weeks pi in eight and seven of the inoculated sows, respectively, and the frequency of detection of both antibody isotypes decreased over time. At 30 weeks pi only one out of three sampled sows yielded positive results in IgA-IB and none of them in IgG-IB. In a total of 62 samplings performed between detection of the first positive results and the end of the experiment at 30 weeks pi, 42 (67.7%) and 22 (35.5%) positive IB reactions for IgA and IgG, respectively, were recorded. Some of the sows showed inconsistent IB results when consecutive daily samplings during weeks 13 and 30 pi were per- formed (Table 1).

Immunoblot results from the three experimentally inoculated fatteners showed the same trend as in the sows: first positive IB results were detected between 1.5 and 2 weeks pi, with a decreasing frequency of detection of both Ig isotypes over time. None of the three pigs remained positive at all sampling times. In contrast to the sows, the frequency of IB detection of IgA and IgG was comparable (i.e., 15 IB reactions positive for IgA versus 16 reactions positive for IgG antibodies from a total of 25 samplings were registered after the first positive results) (Table 1). All non-inoculated pigs used as negative controls (n = 6) tested negative by IB.

The only seropositive sow within the group of potentially exposed sows yielded also positive results by OF-IB for IgA. All seronegative sows yielded negative IB results for both IgA and IgG.

OF-ELISA

A total of 147 OF samples from 26 individual animals (i.e., eight experimentally inoculated sows, three experimentally inoculated fatteners, three non-inoculated negative sows, three non-inoculated negative fatteners and nine potentially exposed sows) were tested for antibodies against *T. gondii* in OF by ELISA (out of these, 137/147 samples were also tested by IB). For the evaluation of the ELISA results, a cut-off for individual animals was calculated as described above (cut-off = 0.63 S/P%). The OF-ELISA results from the experimentally inoculated animals are shown in Table 1. In the sows, the first positive results were observed between 1.5 and 3 weeks pi. After that, positive OF-ELISA reactions were observed in a total of 37/57 (64.9%) samples. Two of the sows (Sows 1827 and 1890) yielded consistently positive results from the first detection until the end of the study (at 8 and 13 weeks pi, respectively). Five out of six sows sampled daily at 13 or 30 weeks pi for up to four consecutive days showed positive results at one or more of those time points.

The three experimentally inoculated fatteners showed the first positive results by OF-ELISA at 1.5 weeks pi. They continued yielding positive results in all samplings until the end of the experimental period (8 weeks pi), with the exception of Pig 836 which tested negative once, at 6 weeks pi (29/30 samplings; 96.7% positive). The highest antibody levels in OF were observed between 2.5 and 4 weeks pi but these values seemed to show a greater variation over time than the values in serum (Fig. 5).

Table 1
Results of immunoblot for IgA (IgA-IB) and IgG (IgG-IB) against *Toxoplasma gondii* (*Tg*)SAG1 antigen and ELISA (ID Screen® Toxoplasmosis Indirect Multi-Species Test, ID.vet, Grabels, France) (cut-off: 0.63 S/P%) in oral fluid samples (ELISA) from animals experimentally inoculated with *Toxoplasma gondii* oocysts.

	Diagnostic method	Weeks after inoculation (days after inoculation)																				
		0	1	1.5	2	2.5	3	4	5	5.5	6	7	8	12	13 (88)	13 (89)	13 (90)	13 (91)	30 (208)	30 (209)	30 (210)	30 (211)
Sow 1888	**IgA-IB**	-	-	+	++		+	+					+		+/-	+/-	++					
	IgG-IB	-	-	+	++		+	-					-		-	-	++					
	ELISA (S/P%)	0.08	0.02	0.04	0.62		**1.31**	0.62					**1.31**		0.38	0.5	**1.13**					
Sow 1874	**IgA-IB**	-	-	+	+		+	+/-					-		+/-	+/-	+	-				
	IgG-IB	-	-	+	+		+	+/-					-		-	-	+/-	-				
	ELISA (S/P%)	0.29	0.14	**0.74**	**1.37**		**1.1**	**3.44**					0.62		**0.98**	**1.1**	**1.58**	0.41				
Sow 1890	**IgA-IB**		-	+/-	+		+	-					+/-				+	+/-				
	IgG-IB		-	-	+		+/-	-					-				-	-				
	ELISA (S/P%)			0.53	**1.4**		**1.4**	**1.64**					**0.77**				**9.22**					
Sow 1818	**IgA-IB**			+	+		+/-	+					+/-	+						+	+/-	-
	IgG-IB			+	+		-	+					-	-						-	-	-
	ELISA (S/P%)			**1.61**	**1.67**		0.53	**4.44**					**1.07**	**1.4**							**1.22**	0.29
Sow 1798	**IgA-IB**			+/-	+/-		-	+/-					-	-					-	-	-	-
	IgG-IB			+/-	-		-	+/-					-	-					-	-	-	-
	ELISA (S/P%)			**0.98**	**0.98**		0.62	**3.53**					**0.98**	0.59					0.59	**0.98**	**1.01**	0.41
Sow 1829	**IgA-IB**			++	-		-						-	+/-					-	-	-	-
	IgG-IB			++	-		-						-	+/-					-	-	-	-
	ELISA (S/P%)			**2.09**	0.23		0.17						**0.86**						0.35	0.38	0.38	0.41
Sow 1806	**IgA-IB**			+	+		+	+					-	-								
	IgG-IB			+/-	-		-	-					-	-								
	ELISA (S/P%)			**1.64**	0.56		**0.89**	**1.28**					**0.92**	**0.89**								
Sow 1827	**IgA-IB**			+	+/-		+	+														
	IgG-IB			+	-		+	+														
	ELISA (S/P%)			**3.62**	**1.85**		**3.65**	**9.43**					**8.77**									
Pig 836	**IgA-IB**		-					++	++	-	-		++									
	IgG-IB		-					+/-	+	-	-		+									
	ELISA (S/P%)		0.22	**2.01**	**4.06**	**28.94**	**18**	**10.1**	**14.98**	**9.67**	0.55	**18.2**	**20.68**									
Pig 838	**IgA-IB**		-	-	-	+/-	+	+	+/-	-	-	+/-	-									
	IgG-IB		-	-	+/-	+	+	+	+/-	+/-	+/-	+	-									
	ELISA (S/P%)		0.25	**4.73**	**4.46**	**12.66**	**7.05**	**7.12**	**3.47**	**5.76**	**3.47**	**11.66**	**5.95**									
Pig 848	**IgA-IB**		-	+	++	+	+	++	+/-	+/-	-	-	-									
	IgG-IB		-	+	++	+	+	+/-	-	-	-	-	-									
	ELISA (S/P%)		0.05	**4.2**	**3.53**	**5.39**	**9.67**	**18.56**	**7.58**	**6.32**	**6.45**	**11.33**	**10.67**									

+/-, weak positive reaction; +, positive reaction; ++, strong positive reaction; -, negative reaction; blank: sample not available/not tested; S/P%: sample/positive control-ratio (SP% = (O.D. sample – O.D. negative control)/ (O.D. positive control – O.D. negative control) * 100); O.D., optical density at 450 nm; ELISA values in bold font, positive values which are above the cut-off.

The three serially tested non-inoculated control sows and the three non-inoculated fatteners yielded negative OF-ELISA and -IB results at all time points (results not displayed in Table 1 for better readability).

The potentially-exposed seropositive sow also presented an ELISA value above the cut-off in OF (S/P% OF-ELISA 1.16). All remaining sows were seronegative and had negative values by OF-ELISA.

Using the reference standard described above (either OF IB IgA-, OF IB IgG- or serum ELISA-positive), a total of 80 OF samples were classified as positive and 46 samples as negative by definition and could therefore be included for further calculations. Hence a relative specificity of 97.83% and a relative sensitivity of 78.75% could be calculated for OF ELISA, when used for testing of samples from individual animals. The negative predictive value (NPV) of this test was 72.58%, and the positive predictive value (PPV) 98.44%.

Analysis of samples from group-housed animals

Toxoplasma gondii *ELISA in serum and OF*

A total of 195 pooled OF samples from 195 groups of fattening pigs were tested for antibodies against *T. gondii* by OF-ELISA. The serum of each animal in these groups (n = 2,248) was tested individually for antibodies against *T. gondii*, revealing, that 167 of the groups contained 100% seronegative animals (n = 1,755). In the remaining 28 groups tested, at least one pig per pen was positive for antibodies against *T. gondii* by serum-ELISA. The percentage of seropositive pigs per pen ranged from 2.5% up to 92% (Table 2).

A cut-off value for OF-ELISA from pooled samples was calculated considering the OF-ELISA results of the seronegative animals as explained above (cut off: S/P% = 1.11). All pooled samples from pens with a seropositivity of at least 25% (n = 5) yielded positive results by OF ELISA (Table 2) with the exception of one group of four animals which contained one seropositive pig with a very low value by serum-ELISA (S/P% 24.46), which was not detected by OF-ELISA (S/P% 0.67). Also, when tested by OF ELISA, one out of 167 (0.67%) samples from pens with exclusively seronegative pigs yielded a value above the cut-off (1.17 S/P%), accounting for a false-positive result.

Table 2 Oral fluid (OF) ELISA (ID Screen® Toxoplasmosis Indirect Multi-Species Test, ID.vet, Grabels, France) and OF-TgSAG1 (P30)-immunoblot (IB) results of pens (n = 28, including 493 animals) containing different percentages of seropositive fattening pigs from Swiss pig farms.

Farm No.	Pen No.	Serum-ELISA *n* positive pigs /*n* pigs in the pen	Serum-ELISA% positive pigs in the pen (95% CI)	OF-IB IgA	OF-IB IgG	OF-ELISA (S/P%)
1	2	1/12	8.3 (0–23.9)	-	-	- (0.08)
3	4	1/11	9.0 (0–6.9)	-	-	- (-0.11)
	5	1/11	9.0 (0–6.9)	-	-	- (-0.31)
4	7	1/18	5.5 (0–16)	-	-	- (-0.11)
	9	8/18	44.4 (21.5–67.4)	-	-	+ (2.07)
	10	2/19	10.5 (0–24.3)	-	-	- (0.66)
5	1	3/23	13.0 (0–26.7)	-	-	- (0.02)
6	2	1/19	5.3 (0–15.3)	+/-	-	- (0.50)
7	1	3/24	12.5 (0–25.7)	-	-	- (0.21)
	2	3/23	13.0 (0–26.7)	-	-	- (0.24)
	3	22/24	92.0 (81.1–100)	++	+	+ (23.63)
	4	21/23	91.0 (79.3–100)	++	+	+ (9.75)
8	1	1/29	3.4 (0–10)	-	-	- (-0.02)
	2	1/13	7.7 (0–22.2)	-	-	- (0.47)
9	1	1/10	10.0 (0–28.6)	-	-	- (-0.05)
10	1	1/20	5.0 (0–14.6)	-	-	- (0.08)
11	1	1/40	2.5 (0–7.3)	-	-	- (0.45)
12	1	3/30	10.0 (0–20.7)	-	-	- (0.20)
	4	1/6	16.7 (0–46.5)	-	-	- (0.10)
13	1	8/36	22.2 (8.6–35.8)	-	-	- (0.00)
14	2	1/17	5.9 (0–17.1)	n.a.	n.a.	- (0.15)
	5	4/19	21.1 (2.7–39.4)	n.a.	n.a.	- (0.20)
15	1	1/4	25.0 (0–67.4)	-	-	- (0.67)
	6	1/5	20.0 (0–55.1)	-	-	- (0.39)
	25	1/4	25.0 (0–67.4)	+/-	-	+ (1.56)
	26	1/4	25.0 (0–67.4)	-	-	+ (2.11)
16	1	1/16	6.3 (0–18.1)	+	-	- (0.56)
	2	1/15	6.7 (0–19.3)	+	-	- (0.56)

*CI, confidence interval; S/P%: sample/positive control-ratio (SP% = (O.D. sample – O.D. negative control)/ (O.D. positive control – O.D. negative control) * 100); Positive, seropositive; OF-IB, oral fluid Toxoplasma gondii- TgSAG1 (P30)-immunoblot; +, positive sample; ++, strong positive sample; -, negative sample; n.a., not analysed. Results from pens containing exclusively seronegative pigs (n = 167 pens, including 1,755 pigs; 1–40 pigs/pen), which yielded negative OF-ELISA results, are not included.*

Toxoplasma gondii *antibodies in OF-IB*

A total of 67 pooled OF samples were additionally tested by IgA and IgG IB (i.e., 26/28 samples from pig groups containing at least one seropositive animal and 41/167 samples from groups containing 100% seronegative pigs).

All 41 OF samples from groups containing exclusively seronegative pigs yielded negative IB results for IgG, but five (12.2%) samples yielded (‘‘false”) positive results for IgA. The

seropositive groups with the highest rates of seropositive pigs (91% and 92%) showed positive results for IgG and IgA (Campero et al., 2020). The remaining 26 groups with lower percentages of seropositive pigs yielded negative IgG IB results; however, samples derived from five groups with 5.3%, 6.3%, 6.7%, 7.7% and 25% of seropositive pigs yielded positive results for IgA (Table 2).

DISCUSSION

The objective of this study was to test whether a commercial indirect ELISA kit, evaluated for its use in serum and meat juice, could represent a suitable tool for the detection of antibodies against *T. gondii* in OF samples from pigs. Standard serology as well as OF-IB were used as standards for comparison. Adequate monitoring methods at the primary production level are particularly being looked for in order to detect high-risk herds and to implement control measures against this highly important zoonotic food-borne parasite (Campero et al., 2020), and an OF-based anti- body detection method would offer many advantages in comparison to standard serology. Collection of OF represents a non-invasive, animal welfare friendly method with less discomfort or stress for the animals (Henao-Diaz et al., 2020). Furthermore, it is easy to perform and requires fewer personnel (Pol et al., 2017). The natural exploratory behaviour of these curious animals makes OF sampling using cotton ropes possible. When cotton ropes are presented to pigs as new environmental enrichment, the typical porcine response consists of chewing on them, with the consequent deposition of OF, which can then be easily collected from the ropes (Campero et al., 2020; Kittawornrat and Zimmerman, 2011).

For a broad evaluation of the adapted OF-ELISA, both serial individual samples from experimentally inoculated animals, as well as a large number of field samples from potentially exposed pigs, were examined. Samples from experimentally infected animals represent valuable reference material because in these cases, the exact time point of infection is known and an evolution of antibody levels over time can be evaluated. Therefore, these samples were included in the study, even though testing of individual animals was not the primary objective for future use. Since the prevalence of *T. gondii* in Swiss conventional pig farms is low (Berger-

Schoch et al., 2011; Kelbert et al., 2021) only 28 OF pooled samples from groups containing seropositive pigs were available. However, this sample set had the advantage that the OF samples were very well characterised, as every single pig in the groups had been serologically tested.

To our knowledge, there is not yet any commercial ELISA kit validated for the detection of antibodies against *T. gondii* in OF. Several studies showed that ELISA kits could be effectively adapted to detect antibodies against different swine pathogens in OF by changing the testing conditions (e.g., temperature and time of incubation with the antigen and dilution of the sample), which were optimal for a serum matrix (Bjustrom-Kraft et al., 2016; Kittawornrat et al., 2012; Olsen et al., 2013; Panyasing et al., 2018; Schott et al., 2021). Therefore, different testing conditions were evaluated in this study too (Supplementary data). The most promising results were achieved when the small sample set was tested using the protocol indicated in the Section 2 (protocol 2 in Supplementary data), which was then used for all OF-ELISA runs in the study.

Due to potential differences between the field pen-based samples from potentially exposed pigs and the individual samples from mostly experimentally inoculated pigs, two different OF-ELISA cut-offs were a priori calculated for both sample groups (i.e., 0.63% S/P for individual OF samples and 1.11% S/P for pooled OF samples).

Seroconversion occurred between 1.5 and 2.5 weeks after experimental inoculation with *T. gondii* oocysts, as reported in the present study in sera of fattening pigs. This is in agreement with observations from previous reports (Basso et al., 2013, 2017). Similarly, the first positive results in OF-ELISA were observed at 1.5 weeks pi, in agreement with the results from Campero et al. (2020) using IB. In some animals, positive ELISA results in OF were observed shortly before seroconversion (i.e., Pig 836, Pig 848, Sow 1874, Sow 1818 and Sow 1806). A possible explanation for these findings could be that locally produced antibodies appear earlier because local production is faster than passive diffusion and/or transportation from the circulatory and/or lymphatic system. As the mouth is part of the mucosal immune system, the local immune response with antibody production in the salivary glands can be stimulated not only through pathogen contact in the oral cavity but through pathogen contact in other

mucosal linings at distant sites such as the gut (Challacombe et al., 2015). Local antibody production in the oral cavity might be generally faster than systemic antibody production. Therefore, this fact was considered when defining the positive reference standard; i. e., in addition to positive serum- ELISA results, positive OF- IB (IgA or IgG) results were used. This definition led to a reduced number of false-positive results by OF ELISA and in combination with the relatively low cut-offs, to a high relative specificity of 97.8% for OF ELISA in individual animals. A high specificity, or a low rate of false-positive results, is particularly important when used as a surveillance method (Henao-Diaz et al., 2020). However, this high diagnostic specificity goes together with a relatively low diagnostic sensitivity (78.8%). When calculating sensitivity and specificity as well as the predictive values, it must be considered that the experimentally inoculated animals were tested multiple times, so that several samples from the same animal were used for the calculations. Thus, relative diagnostic sensitivity and specificity as reported in this study are probably biased to some extent (Trevethan, 2017). Furthermore, predictive values (NPV: 72.6%; PPV: 98.4%) are influenced by the prevalence of a disease (Trevethan, 2017). In this experimental setting, prevalence is artificially increased, leading to an overestimation of PPV and an underestimation of NPV. To define the statistical safety conclusively, a larger group of naturally exposed, individual animals would be needed.

Comparing ELISA and IB results from 84 OF samples from individual, experimentally inoculated animals collected from 1.5 weeks pi, when positive results may be first expected, OF-ELISA detected a higher number of positive results (62/84; 73.8%) than IgA-IB (54/84; 64.3%) and IgG-IB (37/84; 44.0%) (Table 1). Considering the time and effort in the laboratory, the possibility of standardization and the availability, ELISA has clear practical advantages over IB techniques.

Pens with animals potentially exposed to *T. gondii* generally contained only a very few seropositive animals (i.e., 22 pens with seropositivity between 1–24%, four pens with seropositivity between 25–90%, two pens with seropositivity over 90%). OF-ELISA seemed to reliably detect antibodies against *T. gondii* in pens with a proportion of seropositive pigs of at least 25%; however, the number of pooled samples with a potentially detectable proportion of seropositive animals was small, and further studies to confirm these detection limits would

be desirable. In contrast, a high number of negative pooled OF samples from potentially exposed animals (n = 167) was tested, and only one false-positive result was obtained. OF-ELISA offers a number of advantages over traditional serology as a screening method such as cost-effectiveness (given that pooled and not individual samples are tested), ease of implementation, safety for the personnel and improved animal welfare (Henao-Diaz et al., 2020; Olsen et al., 2013; Pol et al., 2017; Ramirez et al., 2012; Rotolo et al., 2017). A further advantage of OF over serum, which is particularly important when used for pathogens with a low prevalence, would be that OF samples are directly pooled in the pens when several animals chew on the same rope, reducing the impact of day-to-day variability in individual animals. However, this method may also introduce variability via the unpredictable chewing frequency of individual animals in the group.

OF ELISA values were generally very low compared with those from sera. Even with the adjustment of testing conditions as discussed above, values were approximately 100 times lower. One possible explanation is that IgA and IgG antibody concentrations in OF from pigs are 10 and 800 times lower than in serum, respectively (Olsen et al., 2013). OF samples from the three experimentally infected fattening pigs clearly yielded higher S/P% values in ELISA than did samples from the eight experimentally infected sows during the same sampling period (mean from reference standard-positive, inoculated fattening pigs: 8.4 S/P%; mean from reference standard-positive sows: 1.6 S/P%). Sample collection and testing of the samples were performed in the same way. Putative factors that might account for the higher ELISA values in OF from fattening pigs are the difference in age, a lower volume of saliva production in the fatteners, which could lead to a higher anti- body concentration in OF, the *T. gondii* strain used for the infection (fattening pigs: ME49 strain, type II; sows: CZ isolate-clone H3, type II) and individual factors of each animal.

In conclusion, this study showed that antibodies to *T. gondii* can be detected in OF with an ad hoc modified commercial ELISA, and that the diagnostic sensitivity of the method seems to be higher than that previously observed using either IgG- or IgA-IB techniques (Campero et al., 2020). Nevertheless, we observed that it may fail to consistently detect individual infected pigs over time, probably due to timely variations in the OF antibody concentration, as was

previously reported using IB techniques (Campero et al., 2020). In pooled OF samples from group-housed fatteners, antibodies against *T. gondii* could be reliably detected by ELISA in groups in which at least 25% of the animals tested positive in sera. These results suggest that this ad hoc adapted OF-ELISA may represent a promising non-invasive screening tool to detect groups of pigs with a high exposure to *T. gondii* at the farm level; however, it may fail to detect individual seropositive animals in large groups of seronegative pigs.

ACKNOWLEDGEMENTS

We would like to thank the team of the Veterinary Diagnostics Unit of the Institute of Parasitology, University of Bern, Switzerland for their kind help and support with the laboratory work and the staff of the Division of Pig Medicine of the Vetsuisse Faculty, University of Zurich, Switzerland for help with the sampling. In addition, we would like to thank ID.vet France (Loic Comtet, Kristine Klewer and Laura Olagnon) for generously providing the ELISA kits and advice. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. ID.vet, France provided the ELISA kits used in the study but was not involved in the study design, collection, analysis and interpretation of data, or in the decision to submit the article for publication.

Chapter 3

Systematic Review and Modelling of Age-Dependent Prevalence of *Toxoplasma gondii* in Livestock, Wildlife and Felids in Europe

Filip Dámek [1,†], Arno Swart [2,†], HelgaWaap [3], Pikka Jokelainen [4], Delphine Le Roux [1], Gunita Deksne [5], Huifang Deng [2], Gereon Schares [6], Anna Lundén [7], Gema Álvarez-García [8], Martha Betson [9], Rebecca K. Davidson [10], Adriana Györke [11], Daniela Antolová [12], Zuzana Hurníková [12], Henk J. Wisselink [13], Jacek Sroka [14], Joke W. B. van der Giessen [2], Radu Blaga [1,‡] and Marieke Opsteegh [2,*,‡]

[1] Anses, INRAE, Ecole Nationale Vétérinaire d'Alfort, Laboratoire de Santé Animale, BIPAR, F-94700 Maisons-Alfort, France
[2] Centre for Infectious Disease Control—Zoonoses and Environmental Microbiology, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, P.O. Box 1, 3720 BA Bilthoven, The Netherlands
[3] Laboratório de Parasitologia, Instituto Nacional de Investigação Agrária e Veterinária, 2780-157 Oeiras, Portugal
[4] Infectious Disease Preparedness, Statens Serum Institut, 2300 Copenhagen, Denmark
[5] Institute of Food Safety, Animal Health and Environment BIOR, LV-1076 Riga, Latvia
[6] Institute of Epidemiology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, 17493 Greifswald, Germany
[7] Department of Microbiology, National Veterinary Institute, 75189 Uppsala, Sweden
[8] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, 28040 Madrid, Spain
[9] School of Veterinary Medicine, University of Surrey, Guildford GU2 7AL, UK
[10] Food Safety and Animal Health, Norwegian Veterinary Institute, 9016 Tromsø, Norway
[11] Department of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 400372 Cluj-Napoca, Romania
[12] Institute of Parasitology, Slovak Academy of Sciences, 040 01 Košice, Slovakia
[13] Wageningen Bioveterinary Research, Wageningen University & Research, P.O. Box 65, 8200 AB Lelystad, The Netherlands
[14] Department of Parasitology and Invasive Diseases, National Veterinary Research Institute, 24-100 Pulawy, Poland

[*] Correspondence: marieke.opsteegh@rivm.nl; Tel.: +31-6-29651388
[†] These authors contributed equally to this work.
[‡] These authors contributed equally to this work.

Pathogens 2023, 12, 97. Article: https://doi.org/10.3390/pathogens12010097, supplementary data to this article can be found online at https: //www.mdpi.com/article/10.3390/pathogens12010097/s1.

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Toxoplasma gondii is a zoonotic parasite of importance to both human and animal health. The parasite has various transmission routes, and the meat of infected animals appears to be a major source of human infections in Europe. We aimed to estimate *T. gondii* prevalence in a selection of animal host species. A systematic literature review resulting in 226 eligible publications was carried out, and serological data were analyzed using an age-dependent Bayesian hierarchical model to obtain estimates for the regional *T. gondii* seroprevalence in livestock, wildlife, and felids. Prevalence estimates varied between species, regions, indoor/outdoor rearing, and types of detection methods applied. The lowest estimated seroprevalence was observed for indoor-kept lagomorphs at 4.8% (95%CI: 1.8–7.5%) and the highest for outdoor-kept sheep at 63.3% (95% CI: 53.0–79.3%). Overall, *T. gondii* seroprevalence estimates were highest within Eastern Europe, whilst being lowest in Northern Europe. Prevalence data based on direct detection methods were scarce and
were not modelled but rather directly summarized by species. The outcomes of the meta-analysis can be used to extrapolate data to areas with a lack of data and provide valuable inputs for future source attribution approaches aiming to estimate the relative contribution of different sources of *T. gondii* human infection.

INTRODUCTION

Toxoplasma gondii is an important zoonotic protozoan parasite capable of infecting potentially all warm-blooded vertebrates (Dubey, 2008). Felids are the only known definitive hosts of *T. gondii* and a source of environmental oocyst contamination through shedding in feces (Dubey, 1998; Frenkel et al., 1969; Hutchison et al., 1969). Upon infection, *T. gondii* rapidly multiplies, enters the host's tissues, and forms tis-sue cysts. There it remains infective, enabling *T. gondii* to reach a new host through the carnivory of its former host (Sullivan and Jeffers, 2012). Infection is considered to be lifelong, and unspecific clinical signs may be observed. Most of the infections are, however, subclinical. Toxoplasma gondii can also be transmitted to the fetus, causing congenital toxoplasmosis and potentially resulting in a miscarriage or stillbirth (McAuley, 2014). The potential consequences of congenital toxoplasmosis in particular have contributed to the high ranking of *T. gondii* compared to other major food-borne parasites (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2018). The global annual incidence of congenital toxoplasmosis was estimated to be 190,100 cases (95% CI: 179,300–206,300). This was equivalent to a burden of 1.20 million (95% CI: 0.76–1.90) disability-adjusted life years (DALYs) per annum (Torgerson and Mastroiacovo, 2013). Particularly high burdens were observed in South America and some Middle Eastern and low-income countries (Torgerson and Mastroiacovo, 2013).

There are two main routes of acquired infection in humans: An environmental route through the ingestion of *T. gondii* oocysts present in contaminated water, soil, or fresh produce, and a meat-borne route through the consumption of tissue cysts that may be present in the meat of infected animals. The consumption of raw or undercooked meat of infected animals appears to be the main source of transmission of the parasite for humans in Europe (Cook, 2000). The prevalence of *T. gondii* infections in the definitive hosts, felids, can give an indication of the environmental contamination with oocysts, and the surveillance of *T. gondii* prevalence within animal populations intended for human consumption helps to assess the risk of human infection from various meat products. Most livestock species have a herbivorous diet, therefore these animals mainly acquire the infection through ingesting sporulated *T. gondii* oocysts (Faull et al., 1986; Plant et al., 1974; Skjerve et al., 1998). Wildlife may become

infected by ingesting the meat of infected animals in addition to oocysts shed by felids. Stray cats and wild felids, in addition to domestic cats, contribute to soil contamination, possibly followed by runoff to surface waters (Shapiro et al., 2019; Stelzer et al., 2019).

Due to environmental contamination, the risk of acquiring *T. gondii* infection is higher in animals kept outdoors, thus leading to the difference in *T. gondii* prevalence between animals kept indoors or outdoors, as has been well-documented in various species (Djokic et al., 2016a; Stelzer et al., 2019; Tenter et al., 2000). In addition, prevalence is known to increase with age (Stelzer et al., 2019), in line with the assumption that the infection persists for the lifetime of the host.

Two types of methods are used to demonstrate *T. gondii* infection in animals. Direct methods such as a bioassay or PCR can be used to demonstrate the presence of the para-site or its DNA; however, indirect methods that demonstrate the presence of antibodies against *T. gondii* are more frequently used (Montoya, 2002; Robert-Gangneux and Dardé, 2012; Ybañez et al., 2020). There appears to be a good correlation between the detection of antibodies and the presence of parasites in most animal species, thus seroprevalence provides an indication of the proportion of animals that presents a risk for human infection if consumed (Opsteegh et al., 2016a, 2016b). Moreover, since *T. gondii* parasites are clustered in sparsely distributed tissue cysts, and in case the sample used for direct detection methods is small, tissue cysts may remain undetected with direct detection methods. Therefore, the detection of anti-*T. gondii* specific immunoglobulin G (IgG) antibodies is considered the most sensitive indicator of *T. gondii* infection in most animal species. However, in cattle, buffaloes, and equids, the seroprevalence is known not to correspond well with the presence of infective cysts in the tissue of these animals (Opsteegh et al., 2016a, 2016b, 2019). For this reason, direct detection methods such as PCR, using sufficient volumes of tissues as a matrix, might be optimal to obtain an indication of the proportion of animals presenting a risk for human infection in these species.

The aim of this study was to obtain age-dependent prevalence estimates in selected animal species. To this end, data on both indirect and direct detection methods were extracted from

the literature, and a meta-analysis was carried out using a hierarchical Bayesian age-dependent model including relevant covariates.

MATERIALS AND METHODS

Data

The literature review was conducted, and the results were reported following the PRISMA guidelines (Page et al., 2021). A structured literature search was carried out on 16 June 2020, covering the literature published up to this date using the Embase literature database. A search string (see Supplementary data) was developed based on previous work (Opsteegh et al., 2019) using Emtree terms. The search was designed to cover farm animal and wildlife species intended for human consumption, as well as feline species, sampled within Europe. Forty-one countries, including the 27 European Union member states, were included in the search strategy. The selection of included animal species was based on the FoodEx2 database (The EFSA Comprehensive European Food Consumption Database - Data Europa EU, n.d.) by including all animal species with consumption data from national food consumption surveys from the years 1997–2018.

The eligibility of retrieved publications was assessed by a team of 20 scientists from 13 countries across Europe using Cadima (Kohl et al., 2018), an open-access online tool for conducting systematic reviews. The screening of publications was based on a set of predefined criteria. Only peer-reviewed articles featuring original data on the detection of *T. gondii* (e.g., prevalence studies, epidemiological surveys) using both direct and indirect methods in selected host species in Europe, published in English, with at least a part of the data collected from the year 2000 onwards, were considered eligible. Experimental infection studies, case-control studies, literature reviews, meta-analyses, books, conference proceedings, grey literature, and publications with incomplete data necessary for the modelling (unreported sampling period, animal species, number of total and positive animals, or country), or cases where the full-text could not be obtained, were excluded. Each publication was screened by two randomly chosen scientists from the group, first at the level of the title and abstract, and

after a consensus on inclusion between the two scientists was reached, full-text screening was performed on the remaining publications.

Publications that met the inclusion criteria after full-text screening proceeded to data extraction, which was conducted by a smaller group of nine scientists. For each publication, the data were extracted by one of the nine scientists, and inputs were checked by an-other scientist. Inconsistencies and disagreements were discussed until an agreement was reached. A data extraction template file (see Supplementary Files—Data extraction tem-plate.xlsx) was created in a spreadsheet (Microsoft Excel) to record the required data. For each study, data describing the study design, sampling, and testing methods were collected as follows: Country or the region covered in the epidemiological screening, animal species, total number of farms or herds sampled, the total number of animals sampled, animal age or age group estimates, first and last years of the sampling period, and the sample type used. Data on sample testing encompassed the type of diagnostic assay, the commercial test name (when applicable), and cut-off values. Extracted data were harmonized and categorized for modelling. Firstly, animal species were sorted into thirteen categories (Table 3) based on their common physiological traits and phenotype.

Table 3 Animal categories and applied testing methods. Animal categories with associated animal species (animal species as defined by ITIS, Integrated Taxonomic Information System) and the types of test methods considered relevant to obtain prevalence estimates (direct methods demonstrate the presence of the parasite, and indirect methods demonstrate the presence of antibodies).

Group	Animal Species Included	Testing Methods
Buffalo	*Bubalus bubalis*	Direct
Cattle	*Bos taurus*	Direct
Duck/Goose	*Anas platyrhynchos, Anser anser, Anser cygnoides*	Indirect
Equids	*Equus caballus, Equus asinus* and their cross-breeds	Direct
Felids	*Felis catus, Felis silvestris, Lynx lynx, Lynx pardinus*	Direct and Indirect
Goat	*Capra hircus*	Direct and Indirect
Lagomorphs	*Oryctolagus cuniculus, Lepus europaeus, Lepus granatensis, Lepus timidus*	Direct and Indirect
Poultry	*Galus galus, Meleagris gallopavo*	Indirect
Pig	*Sus scrofa*	Direct and Indirect
Sheep	*Ovis aries*	Direct and Indirect

Wild birds	*Anas crecca, Aythya ferina, Anas penelope, Anas strepera, Anas platyrhynchos* (feral), *Anas acuta, Anas clypeata, Phasianus colchicus, Columbidae* (family), *Anas platyrhynchos* (feral)	Direct and Indirect
Wild boar	*Sus scrofa* (feral)	Direct and Indirect
Wild ruminants	*Rupicapra rupicapra, Cervidae* (family), *Dama dama, Alces alces, Ovis aries musimon, Ovis gmelini musimon, Ovis musimon, Ovis orientalis musimon, Ovis aries, Ovis ammon, Cervus elaphus, Rangifer tarandus, Rangifer tarandus platyrhynchus, Capreolus capreolus, Cervus nippon, Capra pyrenaica hispanica, Capra pyrenaica victoriae, Capra pyrenaica, Odocoileus virginianus*	Direct and Indirect

As grouping on a coarser level is more suitable for hierarchical modelling, the countries present in the dataset were assigned to one of five European regions—Western, Northern, Eastern, Southeastern, and Southwestern (Figure 6, see Supplementary data), as described previously (Bouwknegt et al., 2018).

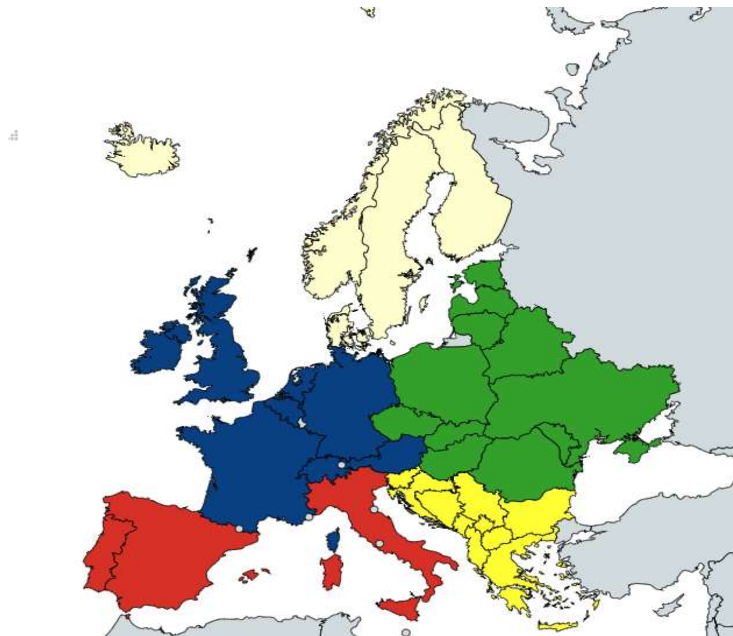

Figure 6 Categorization of Europe into regions. European countries are categorized into five European regions: Western (blue), Northern (white), Eastern (green), Southeastern (yellow), and Southwestern (red). Generated from: https://www.mapchart.net/index.html, ac-cessed on 18 August 2022.

The detection methods described in the publications were classified as direct or indirect, depending on whether the parasite (or its DNA) or the antibody response is detected.

The matrices tested were grouped into three categories—“blood”, “meat juice”, and “other”. The blood category comprised mostly serum samples but also included whole blood and

plasma samples. The meat juice category consisted of liquid samples obtained by freezing and thawing muscle tissues. The “other” category incorporated mainly matrices used for direct detection (e.g., organ and muscle tissues, milk, and, in the case of felids, feces) but also pleural fluids and cardiac fluids used for indirect detection (see Supplementary data).

Some publications presented more than one set of results on *T. gondii* detection for a group of animals (e.g., results based on multiple indirect methods, different matrices, or with multiple cut-off values), resulting in several rows of data for the same population in the spreadsheet. In order to avoid counting the same animal population multiple times, a population identifier was introduced. A population was defined as a set of animals of the same species, age group, and from the same study. For each population identifier, a weighting was derived as one over the number of entries. This weighting was used in the model to scale the influence of the entry. Weighting is similarly introduced in case two direct methods are used; however, the number of entries for indirect and direct are considered separately.

Indoor animals were defined as those kept strictly in an enclosed environment (e.g., pigs kept under controlled housing) and outdoor-held animals as animals commonly kept in the open, with potential contact with the outside environment and free-roaming felids. Indoor and outdoor access were based on details from the publication or the typical holding and living conditions (e.g., strictly outdoor access for wildlife). For buffalo, cattle, equids, sheep, and game species (wild boar, wild birds, and wild ruminants), outdoor access was assumed by default. For publications that included data on animals with and without outdoor access, these data were extracted on separate rows and given a different population identifier.

Since the ages of individual animals at the time of sampling were not given, we de-fine an uncertainty distribution based on estimates of the minimum, maximum, and most probable age at sampling. The most probable age at sampling often coincides with the slaughter age. Age ranges, with the minimum, maximum, and most probable age at sampling, were extracted from publications whenever available. In case only the age range was indicated in the publication, for livestock, the arithmetic mean of the provided age range was used as a default value for the most probable age at sampling. For wildlife, in cases where age information was missing in the publication, an estimate for the mini-mum, maximum, and most probable age

of sampling was applied based on the maxi-mum recorded age for the animal species while taking into account the most common hunting age. Details can be found in the Supplementary Materials (see Supplementary data).

Analysis of direct detection data

The amount of data from direct tests was insufficient to parameterize the full Bayesian model. Instead, the prevalence was calculated per species by dividing the number of positive animals by the total number of tested animals and multiplied by the population weighting factor (for details see Supplementary data). Confidence intervals were based on the binomial distribution.

Analysis of indirect detection data

Age-structured model

Indirect tests do not provide a good indication of the presence of infective *T. gondii* in cattle, buffalo, and equids, therefore these species were excluded from the age-dependent seroprevalence modelling. Two different compartmental infection models were considered to fit the age-dependent prevalence data based on indirect tests: (1) The susceptible-infected (SI) model where animals move from susceptible (i.e., seronegative) to infected (i.e., seropositive), and (2) the susceptible-infected-susceptible (SIS) model, where we also allow for a reversion to susceptible (i.e., loss of detectable antibody response).

In both of these models, animals are born susceptible and can move into the 'infected' compartment based on a constant force of infection λ (incidence rate, measured in new infections per year per animal). The SIS model also allows for reversion to seronegative with a rate of γ.

The variables S and I are fractions of the total population, dependent on age a, adding up to one for each age. For the SIS model, the equation for the prevalence is

$$I_{SIS}(a) = \frac{\lambda}{\lambda + \gamma}[1 - \exp(1 - (\gamma + \lambda)a)] \quad (1)$$

The SI model was recovered by setting $\gamma = 0$.

Bayesian Hierarchical Model

A Bayesian model was built to estimate the prevalence in different animal populations and regions to quantify uncertainty. Moreover, a hierarchical model was built to be able to estimate the prevalence in countries with no data by "borrowing" estimates through partial pooling, granting us the possibility to overcome the data gaps. The Bayesian model was built based on the variables as shown in Table 4 (for model details, see Supplementary data).

Table 4 Data used in the Bayesian hierarchical model. Variables included in the model with corresponding values.

Variable	Values
species[i]	Buffalo; Felids; Cattle; Duck, Goose; Goat; Equids; Pig; Chicken, Hen, Turkey [a]; Lagomorphs; Sheep; Wild Birds; Wild Boar; Wild Ruminants
region[i]	East, North, Southeast, Southwest, West
pop[i]	A unique identifier for a population
test[i]	Direct, Indirect
outdoors[i]	Outdoor, Indoor, Unknown
sample_type[i]	Blood, Meat juice, Other [b]
ntot[i]	Total number of animals tested
npos[i]	Total number of animals found positive
age[i]	Best estimate of average age range
agemin[i]	Lower bound of the age range
agemax[i]	Upper threshold of the age range
agemean[i]	The most probable age at sampling

[a] referred to as poultry in the following, [b] organ- and muscle tissues, milk, pleural fluids, cardiac fluids and feces.

The basis of the Bayesian model was to describe the number of positive animals as a realization of a random process where each individual of the population of size $ntot[i]$ had a probability $I(a)$ to be found infected, resulting in $npos[i]$ positives:

$$npos[i] \sim Binomial(ntot[i], I(a[i])) \tag{2}$$

The function $I(a)$ is from either the SI or SIS model described above. The age-distribution $a[pop[i]]$ of each population $pop[i]$ was a beta distribution, scaled to lie between $agemin[pop[i]]$ and $agemax[pop[i]]$,

$$a * [pop[i]] \sim Beta(\alpha[i], \beta[i]) \quad (3)$$

$$a[pop[i]] = \frac{a * [pop[i]] - agemin[pop[i]]}{agemax[pop[i]] - agemin[pop[i]]}. \quad (4)$$

That way, the age-distribution set for each population will be updated to facilitate the model fit in the posterior age distribution. As a prior the mean was set using $\mu[i] = \alpha[i]/(\alpha[i] + \beta[i])$, giving the best estimate of the age $age[i]$, and the precision $\phi[i] = \alpha[i] + \beta[i]$ to a low value of 10.

Differences between species, outdoor access, sample type, and regions were taken into account using a hierarchical model. These differences were modelled as linear contributions to the logarithmic force of infection:

$$\log(\lambda_i) = \lambda_0 + \lambda_{species[i]} + \lambda_{outdoor[i]} + \lambda_{sampetype[i]} + \lambda_{region[i]} \quad (5)$$

$$\lambda_{species[i]} \sim N\left(0, \sigma_{\lambda_{species}}\right), \quad \lambda_{outdoor[i]} \sim N\left(0, \sigma_{\lambda_{housing}}\right),$$

$$\lambda_{sampletype[i]} \sim N\left(0, \sigma_{\lambda_{sampletype}}\right), \quad \lambda_{region[i]} \sim N\left(0, \sigma_{\lambda_{region}}\right).$$

All hierarchically modelled forces of infection are soft-centered at zero to render the model identifiable. The parameter λ_0 can then be regarded as a baseline force of infection, which was given a vague prior λ_0 ~N(1,2). All standard deviations are supplied with vague priors. We assume that the loss of detectable antibodies is a physiological process, which only depends on species, and modelled γ analogously to λ,

$$\log(\gamma_i) = \gamma_0 + \gamma_{species[i]}, \quad \gamma_{species[i]} \sim N\left(0, \sigma_{\gamma_{species}}\right). \tag{6}$$

To prevent multiple counting of populations and resulting artificial inflation of precision, we weighted contributions to the likelihood using the weighting factor defined before.

Model fitting was performed using Stan (Stan, n.d.) interfaced from R v4.1.3. (Team, 2009). Trace plots of the Markov chains were visually assessed to confirm the convergence of the model (see Supplementary data).

RESULTS

Data collection

A total of 1985 publications were retrieved, with more than 50 animal species included (Table 3). Twenty-four articles were excluded as duplicates, 1599 publications did not meet the inclusion criteria during the title and abstract screening, and a further 86 were excluded during the full-text screening (see Supplementary data). Following the screening process, 276 publications were considered eligible for data extraction, out of which 226 publications with a complete set of data were included. Relevant data on at least one of the animal species of interest were recovered from 29 out of the 41 countries included in the search string. The number of articles on each of the individual species, including the number of animals and the type of detection method used, is summarized in the supplementary data (see Supplementary data). Direct testing methods included various molecular methods (PCR, quantitative PCR, nested PCR, and magnetic capture PCR) and other *T. gondii* parasite detection methods (bioassay, direct immunofluorescence test, direct Western Blot, flotation, and sedimentation followed by microscopy). The indirect methods used to detect specific anti-*T. gondii* IgG antibodies included the modified agglutination test, the direct agglutination test, the latex agglutination test, ELISA, the indirect fluorescence test, the Sabin–Feldman dye test, and the indirect Western Blot.

Animal prevalence results of direct methods

The highest prevalence based on direct detection data was 68.4% (95% CI: 54.8–80.1%) observed in the brains of lagomorphs in the Western region (other tissues, the United Kingdom) (Figure 7, see Supplementary data). The lowest *T. gondii* prevalence was observed in the hearts of equids from the Eastern region (*n* = 82, other tissues, Romania) at 0.0% (95% CI: 0.0–0.4%) (Figure 7, see Supplementary data). A low prevalence, equal to 0.0% when rounded up to a single decimal space, was also recorded in buffaloes from the Eastern region (*n* = 74, 95% CI: 0.0–4.9%), lagomorphs from the Southeastern region (*n* = 52, 95% CI: 0.0–6.9%), pigs from the Southwestern region (*n* = 44, 95% CI: 0.0–8.0%), and wild birds from the Southwestern region (*n* = 5, 95% CI: 0.0–52.2%). A consistently low *T. gondii* prevalence was observed in fecal samples of felids, especially in the Northern region at 0.2% (*n* = 598, 95% CI: 0.0–0.9%) and the Western region at 0.2% (*n* = 104309, 95% CI: 0.1–0.2%). Average weighted prevalence estimates for included animal species reported separately for different sample matrices can be found in supplementary files (see Supplementary data). No eligible direct detection data were available for ducks and geese and poultry.

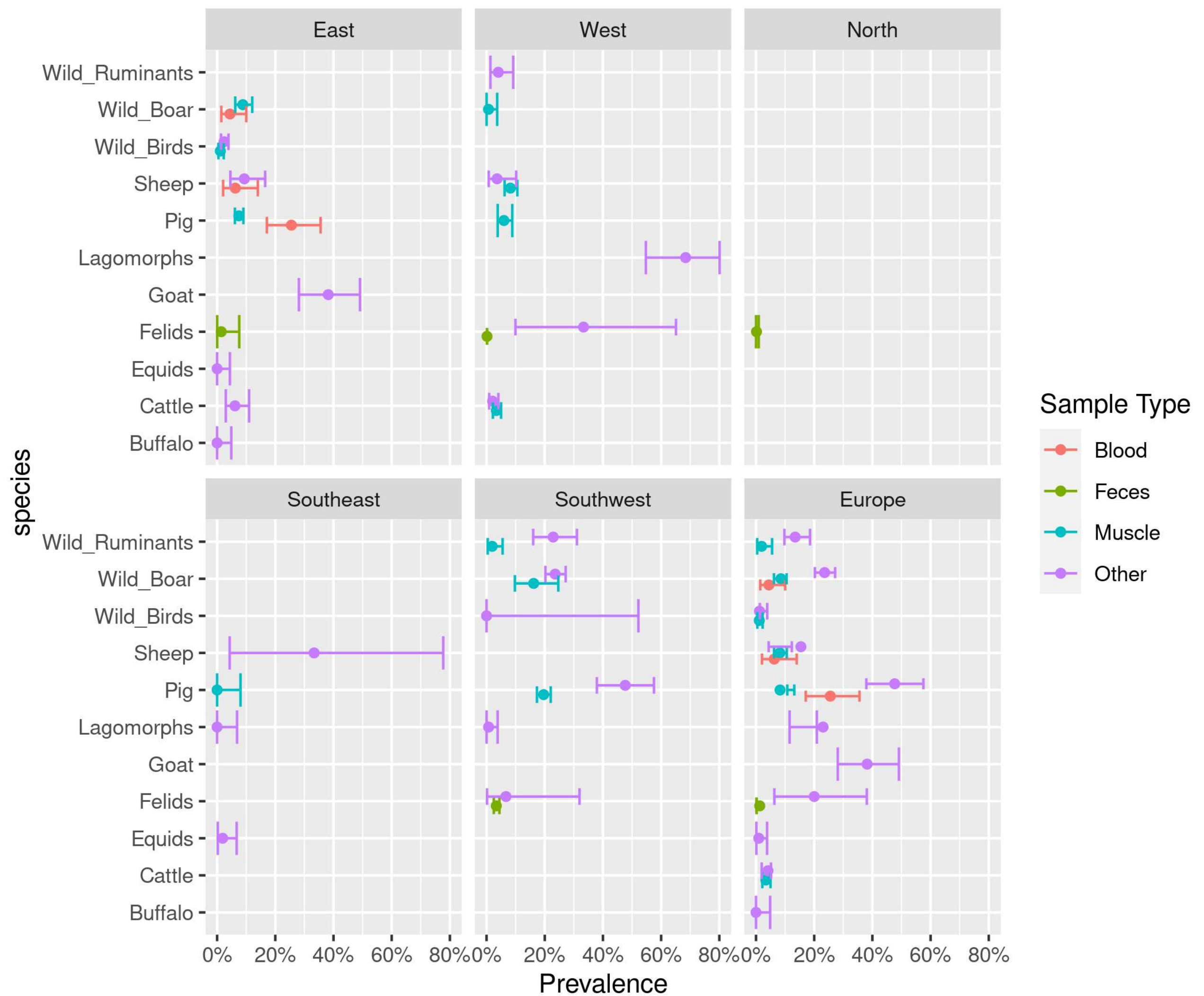

Figure 7 Direct detection estimates. Average weighted prevalence estimates with 95% CI per region based on direct detection and separated by sample types of blood, feces, muscle, and other types (muscle and organ tissues, milk, pleural fluids, cardiac fluids, and, in the case of felids, feces). Only animal species that had data available from at least one of the five regions are included.

Age-dependent animal seroprevalence results using the Bayesian hierarchical model

Trace plots and comparison of between and within chain estimates indicated good convergence of the model (see Supplementary data).

The effects of region, species, sample type, and outdoor access on the exponentiated force of infection and reversion rate are visualized in Figure 8. The baseline force of infection ($\exp(\lambda_0) = 1.92$, 95% CI: 1.15–2.93) and the reversion rate ($\exp(\lambda_0) = 0.25$, 95% CI: 0.07,

0.53) express the average with regards to all other components. The effect of these posteriors is multiplicative, with the mean value set to 1. The variation over regions was the lowest ($\sigma_{\lambda_{region}}$ = 0.36, 95% CI: 0.16–0.84), with the difference in the force of infection over the sample type ($\sigma_{\lambda_{sampletype}}$ = 0.51, 95% CI: 0.16–1.41) and outdoor access ($\sigma_{\lambda_{outdoor}}$ = 0.65, 95% CI: 0.23–1.66) being slightly higher. The largest variation was found for an interspecies force of infection ($\sigma_{\lambda_{species}}$ = 0.85, 95% CI: 0.54–1.40) and reversion rates ($\sigma_{\gamma_{species}}$ = 1.92, 95% CI: 1.15–2.93) (Figure 8). Given the baseline force of infection of $exp(\lambda_0) = 1.92$ and the reversion rate $exp(\gamma_0) = 0.25$, these are all considerable contributions, as can also be visually assessed from Figure 8.

For the force of infection or reversion, one over the posterior coefficient can be interpreted as an average waiting time in years until the event. For the force of infection, this yields a waiting time of $1/_{1.92}$ ≈ 0.5 years until *T. gondii* infection and a waiting time of $1/_{0.25}$ ≈ 4 years on average to become susceptible (seronegative) again.

The model results show clear differences in the force of infection between the animal species (Figure 8). In four of the ten groups (pigs, poultry, lagomorphs, and wild birds), the force of infection was below the overall average (lowest in lagomorphs $\exp(\lambda_{species}) = 0.24$, 4 years waiting time). Six animal groups (ducks and geese, felids, goats, sheep, wild boars, and wild ruminants) had a higher force of infection than average (highest for felids $\exp(\lambda_{species}) =$ 3.53 corresponding to a waiting interval of just over three months). In some animal species, especially those with a higher force of infection, a high uncertainty was observed due to a lack of data. Note that these estimates do not include the effects of the other parameters and do not take into account, for example, if species are held indoors or outdoors. To incorporate this aspect, the result must be multiplied by $\exp(\lambda_{outdoor})$ or $\exp(\lambda_{indoor})$.

Regarding reversion, four animal groups (chickens and hens, lagomorphs, pigs, and wild ruminants) scored a reversion rate lower than the European average of approximately four years to become susceptible again. The shortest reversion rate on average was calculated for pigs at six months ($\exp(\gamma_{species})$ = 0.1). Six animal groups (ducks and geese, felids, goats, sheep, wild boars, and wild ruminants) had a reversion rate above the calculated average,

with the longest interval of approximately seven years, observed in felids ($exp(\gamma_{species}) =$ 3.7) (Figure 8).

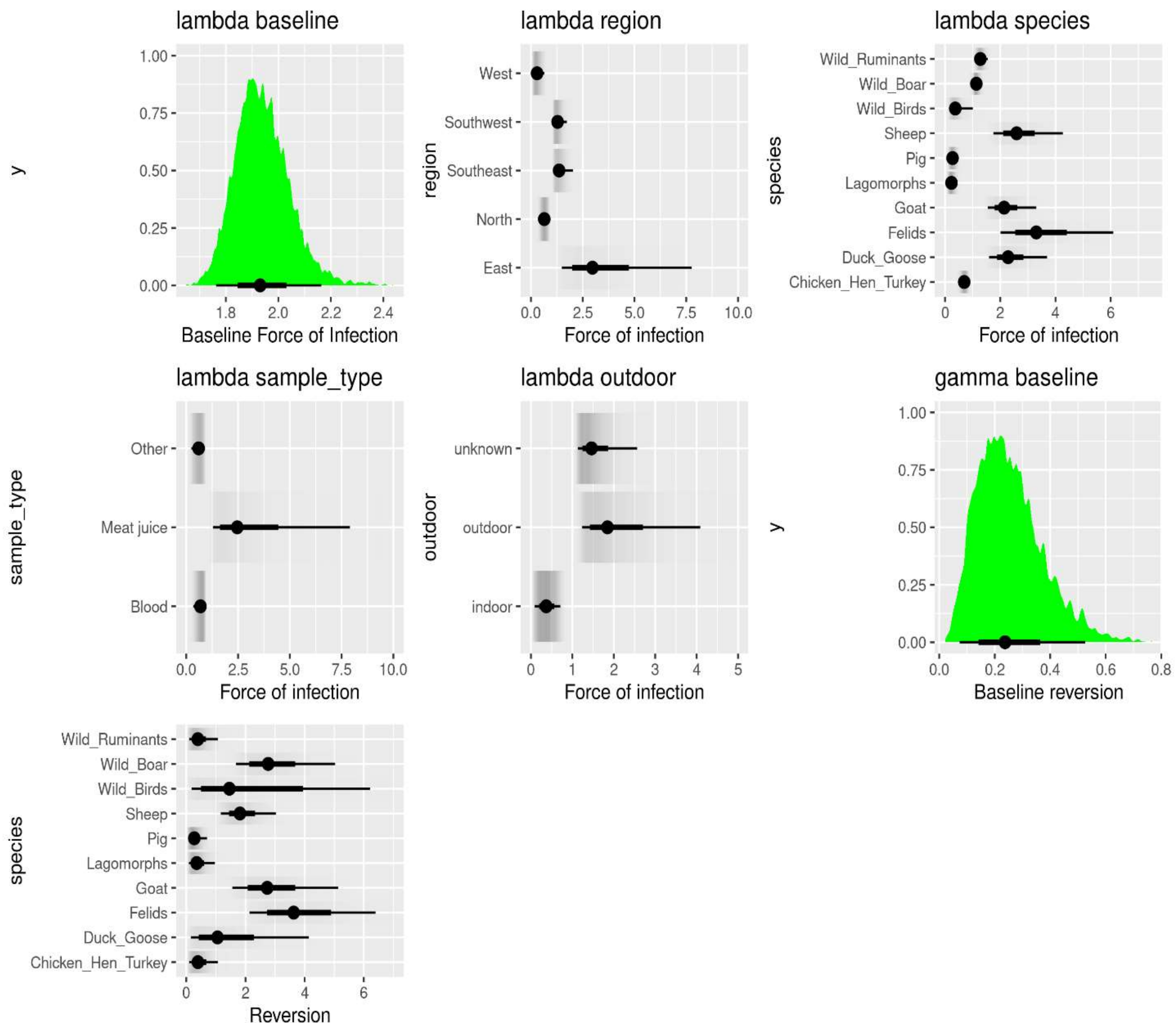

Figure 8 Posterior probabilities of the model parameters. Values for the force of infection in regions, in species, by sample type and outdoor access are all exponentiated, which means that a value of one indicates the absence of an effect on the baseline probability (see Supplementary Data—Figure S4). To reconstruct the total force of infection, the exponentiated contributions must be multiplied. Gray area represents the uncertainty distribution. In all panels, the thin and thick black lines indicate 95% and 50% Bayesian credible intervals, respectively, with dots indicating the mean of exponentiated forces of infection dependent on region, species, sample type (other = pleural or cardiac fluids), and holding status as multiplicative corrections to the baseline (see Equation (6)).

The lowest overall force of infection across the species' spectrum within the European regions was observed in the Western region ($exp(\lambda_{region}) = 0.3$), followed closely by the Northern region ($exp(\lambda_{region}) = 0.6$). The Southwestern ($exp(\lambda_{region}) = 1.3$) and Southeastern regions ($exp(\lambda_{region}) = 1.4$) showed a similar force of infection, slightly above the European

average. The highest force of infection was observed in the Eastern region ($exp(\lambda_{region}) = 3.5$) (Figure 8). It is estimated that the time until encountering *T. gondii* infection in animals from the Eastern region averages approximately three months, which would be more than eight times shorter than in the Western region (approximately 3 years).

The force of infection was almost five times higher in outdoor-held animals ($exp(\lambda_{outdoor}) = 2.1$) than in those kept strictly indoors ($exp(\lambda_{indoor}) = 0.4$) (Figure 8). The time until *T. gondii* infection was, on average, 6 months for outdoor-kept animals and 2.6 years for indoor-kept animals. Animals with unknown holding backgrounds are similar to those with outdoor access ($exp(\lambda_{outdoor}) = 2.1$).

Model results indicate that meat juice samples were almost five times as likely to be positive for *T. gondii* antibodies than blood samples (Figure 8). The force of infection of other matrices ($exp(\lambda_{sampletype}) = 0.6$) yielded results closer to the blood sample type ($exp(\lambda_{sampletype}) = 0.7$) than the meat juice sample type ($exp(\lambda_{sampletype}) = 3.4$).

An example of the age-dependent seroprevalence curves for pigs is shown in Figure 9. Similar curves are available for all species (see Supplementary data) and are used to estimate the seroprevalences at the age most relevant for human infection, split by outdoor access of the animal as shown in Table 5. The highest seroprevalence was estimated for sheep with outdoor access in the Eastern region at 78.5% (95% CI: 77.0–79.8%; 3.7 years of age) (Table 3). In contrast to the result of direct methods, the lowest seroprevalence was estimated for indoor-kept lagomorphs in the Eastern region at 2.0% (95% CI: 1.7–2.4%; 0.2 years of age). *Toxoplasma gondii* seroprevalence estimates were the highest in the Eastern region, followed by the Southeastern and Southwestern and the lowest in the Northern and Western regions.

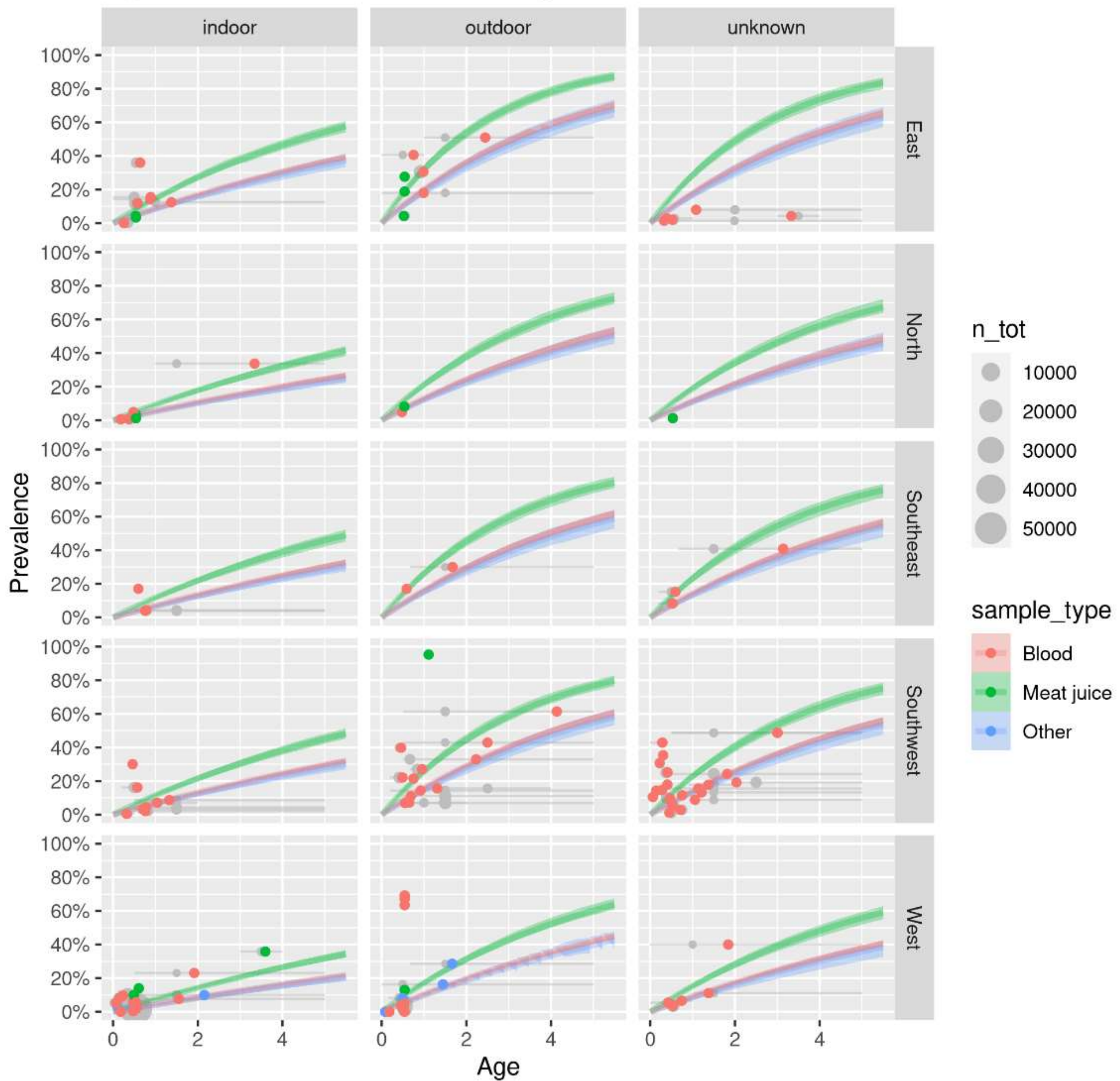

Figure 9 SIS model fit for age-dependent seroprevalence of *T. gondii* in pigs by outdoor access in five regions of Europe. The lines indicate the fitted seroprevalence by age, based on detection in the blood (red), muscle fluid (green), or other matrices (blue). The grey dots represent seroprevalence data points at the best estimate of age in the data for the studied populations. The size of the dots indicates number of animals, in categories from 10,000 to 50,000. The dots are shifted horizontally along the grey line extending from the minimum to maximum possible age, to the best fitting age (red dots) (see Supplementary Files—Figure S15).

Table 5. Modelled regional seroprevalence estimates. Regional seroprevalence estimates (sample type = "blood") were modelled for selected animal species groups at their most probable age of sampling, reported separately for indoor and outdoor holding conditions. Numbers in square brackets are the ages most relevant for human infection. The asterisk indicates the age of the animal species that have been estimated from the demographics of the other countries within the region.

Species	Holding conditions	East	North	Southeast	Southwest	West	Europe
Chicken_Hen_Turkey	indoor	10.3% (9.2%, 11.6%) [0.6]	10.4% (9.2%, 11.8%) [0.9 *]	13.0% (11.5%, 14.7%) [0.9 *]	1.6% (1.4%, 1.8%) [0.1]	3.4% (3.1%, 3.9%) [0.4]	7.8% (1.5%, 14.0%)
	outdoor	23.8% (21.5%, 26.3%) [0.6]	23.5% (21.1%, 26.2%) [0.9 *]	28.8% (25.9%, 32.0%) [0.9 *]	6.3% (5.6%, 7.0%) [0.2]	27.4% (25.1%, 30.0%) [1.4]	22.0% (5.9%, 30.7%)
Duck_Goose	indoor	10.2% (8.8%, 11.8%) [0.2]	11.4% (9.7%, 13.4%) [0.4 *]	14.3% (12.1%, 16.7%) [0.4 *]	14.0% (11.9%, 16.2%) [0.4 *]	5.1% (4.4%, 5.9%) [0.2]	11.0% (4.7%, 15.9%)
	outdoor	25.6% (22.3%, 29.3%) [0.2]	25.5% (21.9%, 29.6%) [0.4 *]	31.2% (26.9%, 35.9%) [0.4 *]	30.7% (26.7%, 35.1%) [0.4 *]	31.0% (26.2%, 35.7%) [0.6]	28.8% (22.8%, 35.1%)
Felids	indoor	48.0% (45.2%, 50.9%) [7.3]	27.2% (24.7%, 29.6%) [4.6 *]	32.9% (30.1%, 35.7%) [4.6 *]	24.7% (22.3%, 27.1%) [3.0]	26.5% (24.8%, 28.2%) [6.2]	31.9% (23.3%, 49.6%)
	outdoor	73.8% (70.9%, 76.6%) [7.5]	41.2% (37.6%, 44.7%) [2.9]	49.9% (46.1%, 53.6%) [3.0]	54.0% (50.9%, 56.9%) [3.7]	51.5% (49.3%, 53.8%) [6.6]	54.1% (39.1%, 75.5%)
Goat	indoor	26.5% (24.1%, 28.9%) [2.8*]	17.4% (15.7%, 19.1%) [2.8 *]	21.6% (19.7%, 23.5%) [2.8 *]	21.2% (19.2%, 23.1%) [2.8 *]	16.0% (14.5%, 17.5%) [3.3]	20.5% (15.1%, 27.9%)
	outdoor	47.5% (44.2%, 50.6%) [2.4]	46.0% (42.9%, 49.1%) [4.0]	44.0% (41.1%, 46.6%) [2.8]	48.2% (45.2%, 51.0%) [3.3]	30.6% (28.2%, 32.9%) [2.8 *]	43.3% (29.2%, 50.2%)
Lagomorphs	indoor	2.0% (1.7%, 2.4%) [0.2]	5.4% (4.6%, 6.3%) [1.1 *]	6.8% (5.7%, 8.0%) [1.1 *]	5.4% (4.6%, 6.3%) [0.9]	4.3% (3.7%, 5.0%) [1.1 *]	4.8% (1.8%, 7.5%)
	outdoor	18.8% (16.3%, 21.6%) [1.1]	16.8% (14.4%, 19.4%) [1.5]	20.8% (17.9%, 24.0%) [1.5]	16.0% (13.9%, 18.3%) [1.1]	12.1% (10.5%, 13.9%) [1.3]	16.9% (11.1%, 22.7%)
Pig	indoor	5.2% (4.9%, 5.4%) [0.6]	3.6% (3.3%, 3.8%) [0.6]	8.3% (7.7%, 8.9%) [1.2]	6.6% (6.3%, 6.9%) [1.0]	2.4% (2.4%, 2.5%) [0.5]	5.2% (2.4%, 8.7%)
	outdoor	22.0% (21.1%, 23.0%) [1.1]	6.5% (6.0%, 7.0%) [0.5]	16.9% (15.8%, 18.1%) [1.0]	18.5% (17.7%, 19.3%) [1.2]	5.5% (5.3%, 5.7%) [0.5]	13.9% (5.4%, 22.6%)
Sheep	indoor	43.9% (41.8%, 46.1%) [3.2 *]	30.3% (28.4%, 32.3%) [3.2 *]	36.7% (34.5%, 38.9%) [3.2 *]	36.1% (34.5%, 37.7%) [3.2 *]	24.8% (23.7%, 26.0%) [3.2 *]	34.4% (24.1%, 45.1%)
	outdoor	78.5% (77.0%, 79.8%) [3.7]	61.0% (58.7%, 63.3%) [3.5]	63.2% (61.0%, 65.4%) [2.9]	60.0% (58.9%, 61.1%) [2.7]	53.8% (52.5%, 55.1%) [3.6]	63.3% (53.0%, 79.3%)
Wild_Birds	indoor	5.4% (3.2%, 9.0%) [3.8 *]	3.4% (2.0%, 5.7%) [3.8 *]	4.3% (2.5%, 7.1%) [3.8 *]	4.2% (2.5%, 7.0%) [3.8 *]	2.7% (1.6%, 4.5%) [3.8 *]	4.0% (1.9%, 7.4%)
	outdoor	12.5% (7.6%, 20.3%) [3.8 *]	8.0% (4.7%, 13.3%) [3.8 *]	9.5% (5.7%, 15.6%) [3.5]	9.8% (5.9%, 16.1%) [3.8]	6.7% (4.0%, 11.1%) [4.0]	9.3% (4.7%, 17.0%)
Wild_Boar	indoor	19.1% (17.6%, 20.7%) [2.3 *]	12.4% (11.3%, 13.5%) [2.3 *]	15.4% (14.0%, 16.9%) [2.3 *]	15.1% (14.0%, 16.3%) [2.3 *]	9.9% (9.2%, 10.6%) [2.3 *]	14.4% (9.5%, 20.0%)
	outdoor	44.3% (42.1%, 46.7%) [2.8]	28.7% (26.7%, 30.6%) [2.5]	33.2% (30.8%, 35.6%) [2.3 *]	33.5% (31.8%, 35.3%) [2.4]	19.9% (18.7%, 21.1%) [2.0]	31.9% (19.2%, 45.7%)
Wild_Ruminants	indoor	22.0% (20.3%, 23.8%) [4.8 *]	14.3% (13.1%, 15.5%) [4.8 *]	17.7% (16.2%, 19.5%) [4.8 *]	17.4% (16.2%, 18.8%) [4.8 *]	11.4% (10.6%, 12.3%) [4.8 *]	16.6% (10.9%, 23.0%)
	outdoor	51.6% (49.0%, 54.3%) [5.8]	27.7% (25.9%, 29.5%) [4.2]	37.9% (35.3%, 40.6%) [4.8 *]	39.1% (37.1%, 41.1%) [5.1]	24.6% (23.2%, 26.1%) [4.6]	36.2% (23.7%, 53.2%)

DISCUSSION

An extensive literature review on the prevalence of *T. gondii* infection in animal species that can be relevant as sources of human infection was carried out. Although the prevalence of *T. gondii* in animals does not give a direct indication of the risk of human infection, the prevalence in combination with exposure data is important to estimate the relative contribution of different sources of *T. gondii* human infection by quantitative risk assessment. Performing the literature search within the Embase database allowed us to utilize its advanced guided mapping of keywords to Emtree, conveniently covering a broad range of animals and countries in the literature search. In the present study, the animal prevalence was modelled using a Bayesian approach, which is increasingly being used in epidemiological studies because of the ability to quantify uncertainty. Moreover, hierarchical modelling allows handling cases with little or no data by "borrowing" estimates through partial pooling, granting us the possibility to overcome the data gaps (Gelman et al., 1995).

In this review, the modelled outcomes based on serological data show the lowest overall seroprevalence of *T. gondii* in indoor-kept lagomorphs at 4.8% (95% CI: 1.8–7.5) and the highest in outdoor-kept sheep at 63.3% (95% CI: 53.0–79.3). Previous attempts at estimating *T. gondii* seroprevalence in animals using meta-analyses were mostly bound to a single country outside of Europe (Deng et al., 2018; Guo et al., 2016) or focused on a limited number of animal species (Foroutan et al., 2019; Montazeri et al., 2020; Rostami et al., 2017). The only recently published meta-analysis overlooking the European livestock and poultry seroprevalence data within a comparable time period provides only a single combined seroprevalence (43.5%, 95% CI: 32.1–55.6%) for all included species (Hajimohammadi et al., 2022). It is not justified or possible to validate the outcomes of the present model by comparison to original seroprevalence studies in animals in Europe because these studies were the input for our model.

Data based on direct testing methods in Europe is relatively scarce and insufficient for inclusion in the Bayesian hierarchical model. Therefore, the results of studies using direct detection methods are summarized separately with no age-modelling applied to them (Figure 7, Supplementary data). A comparable study performed on a global scale and using data from

direct methods showed the lowest pooled prevalence in cattle, followed by pigs, and the highest in sheep for Europe as a whole (Belluco et al., 2016). Similarly, a low prevalence of *T. gondii* in cattle was determined in the current study; however, the prevalence in pigs and sheep varied greatly between the regions and matrices included (see Supplementary data).

Seroprevalence results may be a more representative indicator than direct methods for *T. gondii* infection as tissue cysts are not homogenously distributed within infected animals and may go undetected (Opsteegh et al., 2019). Nonetheless, sheep, which have the highest prevalence by direct detection according to Belluco et al. (2016), also have the highest seroprevalence estimates modelled for outdoor-kept sheep in Europe.

The present model is methodologically the closest to the published model of Deng et al. (2018) who introduced two of the covariates presented in the current model—animal species and geographical region. Additionally, the current model incorporates the effect of the sample type used and the outdoor access of the animal. The regional variation, introduced previously by Deng et al. (2018), was modified to fit the needs of the present study. Even with the variation over regions being the lowest among all the factors included ($\sigma_{\lambda_{\text{region}}} = 0.36$, 95% CI: 0.16–0.84), the differences between regions were considerable, justifying the reporting of the seroprevalences per region. The Bayesian hierarchical modelling made it possible to fill the data gaps present for some combinations of species and regions. Future model extensions for incorporating regional variation could involve the use of spatially explicit modelling as presented by Gotteland et al. (2014), or the use of automated clustering to be performed directly by the model (Teng et al., 2020).

For the majority of animal species, the seroprevalence of *T. gondii* is known to increase with age (Olsen et al., 2019; Opsteegh et al., 2016a; Stelzer et al., 2019). Therefore, the most important addition to the current model is the introduction of a dynamic transmission (SIS) model to allow the inclusion of data reported for heterogeneous age ranges in one model. This approach, using a compartmental infection model, was applied previously in age-dependent modelling for estimating *T. gondii* prevalence in animals (Opsteegh et al., 2012, 2011b). It should be noted that the “I” in our epidemiological model stands for “Infected” or seropositive rather than “Infectious”, as the majority of *T. gondii* infections in animals occur

due to an external source of infection (e.g., oocysts or infected prey) instead of via transmission between animals within one animal population (Stelzer et al., 2019; Tenter et al., 2000). The dogma of a lifelong persistence of *T. gondii* antibodies (Dubey and Beattie, 1988), where susceptible animals move to the infected compartment and the seroprevalence always reaches 100% if animals live long enough, corresponds to an SI epidemiological approach (Restif and Koella, 2004; Roy Malcolm Anderson and Robert Mccredie May, 1981). However, the SIS approach with a reversion rate was better able to fit the plateau observed in the seroprevalence data at high age. The reversion rate did not revert to zero in any of the species and was lowest in pigs ($\exp(\gamma_{species}) = 0.1$). Alternatively, an SI model could also reach a plateau of less than 100% when a subpopulation of animals is not exposed or does not develop a detectable antibody response. To settle these hypotheses, animals would have to be sampled and monitored multiple times in life.

Another feature of the model is the estimation of the effect of outdoor access on *T. gondii* seroprevalence. Our results show an almost five times higher force of infection in outdoor-kept animals compared to those kept strictly indoors. These findings agree with the outdoor access of animals being one of the most commonly identified risk factors for acquiring *T. gondii* infection (Djokic et al., 2016a; Stelzer et al., 2019; Tenter et al., 2000). This outcome can be explained by a higher infectious pressure caused mostly by environmental contamination with oocysts and the presence of potentially infected rodents and birds that could be preyed on (Dubey et al., 1995; Hutchinson et al., 1980; Shapiro et al., 2019). As the presence of cats on farms is also a well-known risk factor for *T. gondii* infection in animals (Shapiro et al., 2019; Stelzer et al., 2019), ideally, this should be taken into consideration in the modelling, but this information is only rarely reported. For the final results, with prevalence by region at the most relevant age, it was, unfortunately, not possible to combine the prevalence into a weighted prevalence with regards to indoor/outdoor access due to insufficient data on the proportions of indoor to outdoor-kept animals in the different regions.

Variability in the force of infection over sample type (σ_(λ_sampletype)= 0.51, 95% CI: 0.16–1.41) was observed. Surprisingly, the results show that the serological testing of meat juice samples was almost five times more likely to result in *T. gondii* antibody detection than when

using blood or other matrices (milk, pleural fluid, and cardiac fluid) (Figure 8), suggesting a higher sensitivity of meat juice matrix. Thirteen studies provided data on *T. gondii* detection using meat/muscle juice; however, sera of the same animals were never tested in parallel. Even though meat juice can be considered an alternative matrix to serum for antibody detection (Viana et al., 2020), performing indirect tests using serum samples appears to provide more reliable results than other matrices such as meat juice, where the concentration of the specific antibodies is less homogenous and depends on the muscle the meat juice has been extracted from (Wallander et al., 2015). Due to the lack of parallel testing of the two matrices, we cannot exclude the possibility of selection bias (i.e., meat juice has been used more often in populations with a higher risk of *T. gondii* infections) and consider blood serum as a matrix of choice for the specific anti-*T. gondii* antibody detection, despite the modelled outcome. More data from a parallel indirect screening of samples from the same animal is needed to establish diagnostic test sensitivity and specificity of indirect tests when using different matrices. Adding these data as prior information could be a useful addition to the current model.

In the study by Deng et al. (2018), separate literature reviews were carried out for the different serological tests used in order to take into account test characteristics in the seroprevalence modelling and provide corrected true prevalence estimates. A similar approach was considered not feasible for animal studies, as there is more diversity in serological tests applied and a lack of harmonized studies on performance characteristics in comparison to the assays used in human diagnostic laboratories. Moreover, in order to include as many data as possible, we did not exclude studies based on the test or cut-off value used and included the results as presented by the authors. The uncertainty in the Bayesian framework consists of model uncertainty and parameter uncertainty. Model uncertainty refers to the fact that the exact processes governing reality are unknown. Most pertinent in the current case is the choice between an SI model and an SIS model in light of the observed plateau in seroprevalence by age, as explained above. To deal with parameter uncertainty, the parameters of the model are supplied with (prior) uncertainty distributions. After running the model with the data, output (posterior) uncertainty distributions are obtained. The uncertainty present due to the lack of data for the covariates is reflected in the posterior

probability intervals from the Bayesian hierarchical model. For the regions or species with insufficient data, the Bayesian hierarchical model allowed us to obtain seroprevalence estimates based on the data from the remaining regions or species but with larger uncertainty than those with data. Furthermore, the uncertainty was affected by the coarse granularity and missing metadata of reported data. Our suggested way of dealing with this is by following a single data reporting template, which includes all the necessary (meta)data. We would like to stress the importance of publishing a full set of raw data and adhering to a set of fixed guidelines for reporting. Preferably, data should also be published on a per-animal basis, for example in an online supplement. A simple spreadsheet template for epidemiological data reporting that could act as such a guideline was developed (see Supplementary data). Individual rows should be used to report data on individual animals rather than populations. The template itself is divided into three parts (sampling, processing, and reporting) and facilitates data reporting and the future use of the data for reviews and meta-analyses, thus increasing the visibility and reach of the data and corresponding articles. Instead of only mentioning results based on a fixed cut-off value, it is advised to provide OD values or titers so a custom cut-off can be applied by the user. Ultimately, the creation of a single publicly accessible online data repository incorporating homogenously structured data from original research studies would be an invaluable resource for future studies.

The current study describes a literature review and meta-analysis using a complex Bayesian hierarchical model including a novel dynamic transmission model, based on data obtained from an extensive meta-analysis. Age as well as all other covariates, such as indoor/outdoor status and region, can be extrapolated to obtain prevalence estimates for the actual composition of the animal population (with regards to animal species, outdoor access, etc.) in any European country despite data gaps. Prevalence estimates presented in this study, as well as those obtained from the model by users, could be used as a valuable tool for various purposes such as calculating adequate sample sizes for serological screening based on the seroprevalence in the region, a comparison tool, and could even provide a powerful instrument for the policymakers, e.g., to evaluate consequences of slaughtering age on the risk of *T. gondii* infection. Moreover, the modelled seroprevalence estimates, together with consumption data of the meat products of these animals, are a starting point for future risk

assessments and are being implemented as such in a multi-country quantitative microbial risk assessment for *T. gondii* source attribution in humans.

ACKNOWLEDGEMENTS

We would like to express our appreciation to Rob van Spronsen for his expertise and guidance with the literature search, Siv Klevar for her much-appreciated assistance with the literature screening process, and members of the TOX-OSOURCES consortium for their continuous support and ideas.

This work was performed within the One Health European Joint Project TOXOSOURCES and ToxSauQMRA and received funding from the European Union's Horizon 2020 Research and Innovation Programme, under grant agreement No 773830: One Health European Joint Programme.

Chapter 4

Effects of experimental toxoplasmosis in pigs inoculated with different parasite stages and genotypes of *Toxoplasma gondii*

Filip Dámek [1,*], Walter Basso [2], Maike Joeres [3], Sandra Thoumire [1], Arno Swart [4], Anaëlle da Silva [1], Inés Gassama [1], Miša Škorič [5], Jiří Smola [5,6], Gereon Schares [3], Radu Blaga [1,†], Břetislav Koudela [5,6,†]

[1] Anses, INRAE, Ecole Nationale Vétérinaire d'Alfort, Laboratoire de Santé Animale, BIPAR, Maisons-Alfort, France

[2] Institute of Parasitology, Vetsuisse-Faculty, University of Bern, Länggassstrasse 122, CH-3012 Bern, Switzerland

[3] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Epidemiology, Südufer 10, 17493 Greifswald - Insel Riems, Germany

[4] Centre for Infectious Disease Control—Zoonoses and Environmental Microbiology, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, P.O. Box 1, 3720 BA Bilthoven, The Netherlands

[5] Faculty of Veterinary Medicine, University of Veterinary Sciences, Brno, Czech Republic

[6] Central European Institute of Technology (CEITEC), University of Veterinary Sciences, Brno, Czech Republic

* Correspondence: Filip Dámek, filip.damek@vet-alfort.fr

† These authors contributed equally to this work.

Pre-submission manuscript

Toxoplasma gondii is a food-borne zoonotic parasite widespread in a variety of hosts, including humans. With a majority of infections in Europe estimated to be meat-borne, pork, as one of the most consumed meats worldwide represents a potential risk for consumers. Therefore, we aimed to investigate the progress of *T. gondii* infection and distribution of the parasites within tissues of experimentally infected pigs, using different *T. gondii* isolates and infectious stages, i.e. tissue cysts or oocysts.

Twenty-four pigs were divided into four groups of six, with each group inoculated orally with an estimated low dose of either 400 oocysts or 10 tissue cysts of two European *T. gondii* isolates, a type II and a type III isolate, mimicking the dose of natural infections.

The majority of pigs seroconverted two weeks post-inoculation. Pigs infected with the type III isolate had significantly higher levels of anti-*T. gondii* antibodies compared to those infected with the type II isolate. Unfortunatelly, two pigs inoculated with type III oocysts died 12 days post-inoculation (dpi). Histopathological exams revealed reactive hyperplasia of the lymphatic tissue of all pigs. Additionally, a selected set of nine tissues was collected during necropsy at 50 dpi from each of the remaining 22 pigs for the analysis of *T. gondii* DNA presence by quantitative real time PCR. A positive result was obtained in 29.8% (59/139) of tested tissues. Brain was identified as the most frequently positive tissue in 63.6% (14/22) of the animals. In contrast, liver samples tested negative in all animals. The highest mean parasite load, calculated by interpolating the average Cq values on the standard curve made of ten-fold serial dilutions of the genomic DNA, corresponding to 10^0 to 10^4 tachyzoites/µL, was observed in shoulder musculature with an estimated concentration of 84.4 [0.0 - 442.5] parasites per gram of tissue.

The results showed an individual variability in the concentration of parasites in tissues of the tested animals. Within the tested isolates, statistically significant effects of parasite stages, i.e. tissue cysts or oocysts, and strain on the proportion of *T. gondii*-positive tissues and parasite load were demonstrated.

INTRODUCTION

Toxoplasma gondii is a zoonotic apicomplexan parasite capable of infecting essentially all warm-blooded animal species, including humans. Felids are the only known definitive hosts of the parasite, responsible for shedding oocysts into the environment with the faeces. The two main routes of infection for susceptible animals are via accidental ingestion of sporulated oocysts from the environment (e.g., contaminated water, feed, or by soil intake) and via consumption of tissues of infected hosts containing viable *T. gondii* bradyzoites. Despite the existence of other transmission routes such as vertical transmission from mother to foetus, through an organ transplant or via blood transfusion, consumption of raw or undercooked meat of infected animals appears to be the main source of human *T. gondii* infections in Europe (Cook, 2000). Intermediate hosts may develop tissue cysts in their muscles and organs upon infection.

Although *T. gondii* infections are often subclinical or manifest with non-specific symptoms, an infection during pregnancy may cause abortion, stillbirth or congenital development disorders (Jones et al., 2001). Moreover, the infection may lead to serious cases of encephalitis and retinochoroiditis in particular (Luft and Remington, 1992; Maenz et al., 2014).

With the relatively high prevalence observed in various animal species across the world (Dubey, 2022) and one third of the global human population estimated to be infected (Montoya and Liesenfeld, 2004), *T. gondii* is of a great importance to animal and human health. The major impact of this parasite on human health is reflected in the high ranking of *T. gondii* among the top-priority food-borne in multicriteria-based ranking for risk management of food-borne parasites at both the global (4th out of 24 food-borne parasites) (Boireau et al., 2014) and European levels (2nd out of 25 food-borne parasites) (Bouwknegt et al., 2018).

The majority of human *T. gondii* infections are estimated to be meat-borne (Cook, 2000) and pork, as one of the most consumed meats in the world and the most consumed meat in Europe (OECD and Food and Agriculture Organization of the United Nations, 2022), plays an important role in public health safety. The European Food Safety Authority (EFSA) Panel on Biological

Hazards (BIOHAZ) identified *T. gondii* as one of the most relevant biological hazards in the context of meat inspection of swine (Scientific Opinion on the public health hazards to be covered by inspection of meat (swine), n.d.).

Besides, pigs may represent an experimental animal model for the study of different aspects of human toxoplasmosis (Garcia et al., 2017a; Nau et al., 2017). Which factors are responsible for the development of clinical toxoplasmosis in swine are poorly understood, but factors such as host age and immune status, coinfections with viral agents, parasite stage, inoculum and genotype seem to play an important role (Basso et al., 2017; Fernández-Escobar et al., 2020; Stelzer et al., 2019).

In the last years, more attention is being paid to the genetic background of *T. gondii* isolates and their association with different virulence phenotypes. It seems that some genotypes may be more prone to cause clinical disease in some animal species than others. A variety of *T. gondii* strains has been identified but the majority in Europe and North America falls into three distinct clonal lineages (types I, II, and III) (Saeij et al., 2005). Isolates of type I tends to be lethal to mice regardless the infective dose and is overall more aggressive than type II and type III (Dardé et al., 1992; Howe et al., 1996; Howe and Sibley, 1995; Sibley and Boothroyd, 1992). Virulence is more variable for type II and type III strains, however, some type III isolates were proven to induce overall higher morbidity and mortality rates in animals compared to type II strains (Fernández-Escobar et al., 2021, 2020; Largo-de la Torre et al., 2022). Effect of experimentally-induced *T. gondii* infection with type III isolates, arguably the least examined of the three lineages, and the comparison with type II isolates that dominate in Europe (Fernández-Escobar et al., 2022; Khan et al., 2007; Lorenzi et al., 2016; Shwab et al., 2014) helps to fill the knowledge gap in the development of a valid animal model for human *T. gondii* infections based on the effects of experimentally induced toxoplasmosis in pigs.

To shed some light on the factors that influence the clinical outcome in swine toxoplasmosis we evaluated the course of infection in pigs experimentally inoculated with a low dose of oocysts and tissue cysts of a type II and a type III *T. gondii* isolate, mimicking the natural infection, and assessing the clinical signs, histopathological changes, and the evolution of the specific immunoglobulin G (IgG) humoral response in the different inoculation groups.

Furthermore, we aimed to evaluate the anatomical distribution of *T. gondii* within the tested tissues of these experimentally infected pigs, according to the two infectious stages and isolates used, and rank the tissues based on the estimated parasite load per gram of tissue.

MATERIALS AND METHODS

Toxoplasma gondii parasites

For experimental infection of pigs, two archetypal *T. gondii* isolates were used for comparison. The first isolate ("CZ-Tiger") was obtained from the feces of a Siberian tiger (*Panthera tigris altaica*), kept at the Dvůr Králové Zoo in the Czech Republic in February 2005 (Juránková et al., 2013a). The oocysts of CZ-Tiger used in this study were obtained from the fourth cat passage, two months prior to the inoculation of the pigs.

The second isolate ("CZ-Šimková") derived from the faeces of a domestic cat (*Felis catus*) imported from the Balkan region in May 2015 to Czech Republic. The oocysts of CZ-Šimková used in this study were obtained from the second cat passage, five months prior to inoculation into pigs.

Both isolates were and maintained by subsequent passages in outbred ICR (CD-1) mice and domestic cats prior to this experiment. Sporulated oocysts of both above mentioned isolates were purified from cat feces as described by Wainwright et al. (2007), microscopically examined, and stored in PBS solution at 4°C until further use. PCR-RFLP and microsatellite genotyping of the two *T. gondii* strains used for experimental infection in this study revealed or confirmed that both belonged to different clonal lineages, i.e. *T. gondii* Type II and III (details in Results – Characterization of isolates).

Oocyst viability was confirmed by bioassay in outbred ICR mice six weeks before the inoculation of the pigs (see Materials and methods - Mouse bioassay).

For production of *T. gondii* tissue cysts of both isolates, two groups of two mice each were orally inoculated with approximately 100 oocysts in 0.5 mL of water/mouse by gavage. After six weeks post-inoculation (wpi), the mice were euthanized, their brains were collected, gently

homogenised in PBS solution supplemented with gentamycin using a mortar and pestle and used for the inoculation of pigs within 24 hours. The number of tissue cysts was determined by counting five 20 µL aliquots of the suspension in a cavity well microscope slide using a Nomarski interference contrast microscope (Olympus BX30, Japan). Obtained tissue cysts were diluted to 10 tissue cysts in 10 mL of PBS and used for the inoculation of pigs within 24 hours.

The experimental infection of mice was approved by the local ethical committee of the University of Veterinary Sciences Brno (UVS) under agreement number 19/2017.

Genotyping typing *T. gondii* strains

For genotyping *T. gondii*, both PCR-RFLP and microsatellites typing methods were used. Genotyping of *T. gondii* by PCR-RFLP was performed using eight chromosomal genetic markers (nSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1) as previously reported and an Apicoplast marker (Herrmann et al., 2012) by amplifying DNA extracted from cell culture-derived tachyzoites using internal primers as previously reported (Su et al., 2006).

For microsatellite typing, DNAs extracted from in-vitro isolated tachyzoites were genotyped after multiplex PCR, using 15 markers as described (Ajzenberg et al., 2010). These markers included 8 typing markers (TUB2, W35, TgM-A, B18, B17, M33, IV.1, XI.1) and 7 fingerprinting markers (M48, M102, N83, N82, AA, N61, N60) that display a high level of polymorphism within clonal lineages type I, type II or type III (Ajzenberg et al., 2010). The only divergence from the original methods was that in case of M102, AA and N60 the fluorophore Atto550 was used instead of NED to label amplicons during multiplex PCR.

Experimental inoculation of pigs with *T. gondii*

Twenty-seven weaned Danhybrid-LY gilts, weighing 35-40 kg, which tested seronegative to *T. gondii* by an in-house immunofluorescence test (data not shown), were purchased from an intensive pig production unit. The pigs were divided into four groups of six animals each (Groups 1 to 4), and one group of three animals (Group 5), with each group housed in a separate pen with concrete floor and with water and feed accessible *ad libitum.*

One pig (Pig No. 10) had to be euthanized before the start of the experiment, and was replaced by pig No. 25 from Group 5.

The pigs of Group 1 (Pigs No. 1-6) and Group 3 (Pigs No. 14-18, and 25) were orally inoculated using a gastric tube with a suspension containing approximately 400 *T. gondii* oocysts of the isolates, respectively, in 10 mL of PBS solution. Subsequently, pigs of Group 2 (Pigs No. 7-9 and 11-13) and Group 4 (Pigs No. 19-24) were orally inoculated using a gastric tube with a suspension containing 10 *T. gondii* tissue cysts of the same isolates, respectively, in 10 mL of PBS solution (Table 6). The two remaining pigs of Group 5 (Pigs No. 26 and 27) received 10 ml of PBS and served as negative, non-infected controls. Pig groups with corresponding administered inoculation doses are summarized in Table 6.

Table 6 Division of pigs into groups. Four experimentally inoculated groups were based on the different combinations of parasite stages and isolates, and one group comprised negative control pigs orally inoculated with PBS solution. N/A = not applicable.

Group	Pig numbers	Isolate	Stage	Dose
Group 1	1, 2, 3, 4, 5, 6	CZ-Tiger	oocysts	400
Group 2	7, 8, 9, 11, 12, 13	CZ-Tiger	tissue cysts	10
Group 3	14, 15, 16, 17, 18, 19	CZ-Šimková	oocysts	400
Group 4	20, 21, 22, 23, 24, 25	CZ-Šimková	tissue cysts	10
Group 5	26, 27	N/A	N/A	0

All pigs were clinically monitored twice a day and their rectal temperatures measured daily.

One week after inoculation, all bedding material was removed, and the boxes were thoroughly cleaned and disinfected with 3 % Neopredisan 135-1® (Menno Chemie, Germany) in order to eliminate the potential oocysts from the inoculum that might have passed non-excysted within the faeces.

Experimental inoculation of pigs with *T. gondii* parasites for the purpose of this study was authorized by the Ministry of Education, Youth and Sports, Czech Republic (permission No. 55/2016) and were performed under the control of the Regional Veterinary Administration.

Mouse bioassay

The inoculation of mice was performed at the animal facility of the Parasitology Laboratory UVS Brno and was approved by the local ethical committee of UVS Brno under agreement number 19/2017.

The viability of *T. gondii* oocysts used in the current study was tested by mouse bioassay. For each of the tested *T. gondii* isolates, two groups of six mice each were orally inoculated by gavage, with dilutions containing 10 and 100 oocysts in 0.5 ml water, respectively. All mice (n = 24) developed unspecific clinical signs (apathy, rough and shaggy hair) between 7 and 13 days after inoculation and were euthanized. Necropsies were performed and *T. gondii* tachyzoites were detected microscopically in peritoneal exudates and also visualized in touch imprint slides from lung tissues stained with Giemsa, confirming the viability of the parasites.

Blood sampling and serological analyses of specific anti-*T. gondii* antibodies

Blood samples from all pigs were collected from the right jugular vein into sterile collection tubes for serological analyses on a weekly basis at 0, 1, 2, 3, 4, 5, and 6 wpi.

Collected blood samples from pigs were left to coagulate for four hours at room temperature and centrifuged at 1500 g for 10 minutes to separate sera and clots. Subsequently, blood sera were collected into sterile collection tubes and stored frozen at -20°C until further use.

All pig sera samples were tested for specific anti-*T. gondii* IgGs using a commercial ELISA kit (PrioCHECK *Toxoplasma* Ab porcine, Prionics, Schlieren, Switzerland). The sera samples were tested at a dilution of 1:50 and results were expressed as the percentage of positivity (PP) relative to the reaction of the positive control

$(PP\ sample = \frac{(OD\ sample - OD\ negative\ control)}{(OD\ positive\ control - O\quad negative\ control)} * 100)$.

The seroconversion in pigs was assessed as a statistically significant difference in PP values compared to the negative control PP values.

Histopathological examination of pig tissues

Necropsy was performed immediately following the euthanasia of all pigs. Tissue samples (brain, spleen, thymus, lymph nodes, tonsils, heart, liver, kidneys, stomach, liver, and lungs) were collected from each pig and fixed in buffered 10% neutral formalin, and processed for a routine histopathological examination. Formalin-fixed tissues were embedded in paraffin, cut on a microtome 4µm thick, mounted on glass slides, stained with haematoxylin and eosin

(H&E), and screened for the presence of *T. gondii* stages and pathological changes under a microscope (Olympus BX30, Japan).

Tissue sampling for the tropism study

The pigs were euthanised 50 days after the inoculation and tissue samples were collected at the slaughterhouse of the Faculty of Veterinary Hygiene and Ecology of the UVS Brno by professional butchers under veterinary supervision. Nine tissues composed of three meat cuts ("shoulder" = *musculus triceps brachii, musculus supraspinatus, musculus infraspinatus*; "loin" = *musculus longissimus dorsi pars lumbalis*; "ham" = *musculus semitendinosus, musculus semitendinosus, musculus gluteus medialis*) and six organs (heart, brain, lungs, liver, spleen, and kidneys) were collected from each pig. A minimum of 200 grams of each tissue, or the whole tissue in case of a smaller size of the tissue, were stored in labelled sterile plastic bags at -20°C until further use.

Trypsin artificial digestion

Two hundred grams of each of the nine tissues (shoulder, loin, ham, heart, brain, lungs, liver, spleen, and kidneys) from each of the twenty-two pigs inoculated with *T. gondii* (n = 198 samples) were blended and incubated (90 minutes at 37°C, 200 RPM on a shaking plate) in trypsin solution (Trypsin (1:250), powder, Gibco™, Scotland, final concentration of 4 g/L). The mixture was filtered through a double layer of a gauze, transferred to 50 mL sterile centrifuge tubes, and centrifuged at 1800 g for 10 minutes. The formed pellet was washed twice of leftover trypsin using a saline solution (0,9% NaCl, Sigma-Aldrich, US). Weight of the final pellet was recorded and samples were stored at - 20°C until DNA extraction and subsequent qPCR analysis.

Molecular analyses of *T. gondii* DNA

DNA was extracted from 80mg of digested pellet (see Materials and mthods – Trypsin digestion), using NucleoSpin DNA RapidLyse, Mini kit for rapid DNA purification (Macherey-Nagel, France), according to the manufacturer's instructions. Extracted DNA was cleaned of PCR inhibitors and concentrated using ethanol precipitation protocol (Zeuigin and Hartley, 1985) and resuspended in 30µL of Milli-Q water. The detection and quantification of *T. gondii*

DNA in each sample was performed in duplicate by amplification of a sequence within the 529 bp repetitive element, according to Opsteegh et al. (2010) with minor modifications (25 µL total reaction mixture volume, 2X Premix Ex Taq™ (TakaraBio, Japan) and 5 µL of DNA as template) and competitive internal amplification control (CIAC) probe modification by Deng et al. (2021b), using LightCycler® 480 System 96-plate thermocycler (Roche, Germany).

Genomic DNA from a suspension of 10^6 cultured RH-strain (Type I, ToxoDB Genotype #1) (ToxoDB, n.d.) was extracted using NucleoSpin DNA RapidLyse, Mini kit (Macherey-Nagel, France), according to the manufacturer's instructions. A standard curve was prepared with ten-fold serial dilutions of the genomic DNA, corresponding to 10^0 to 10^4 tachyzoites/µL (Thomas et al., 2022). Positive and negative controls, distilled H_2O sample, and a standard curve were included in each qPCR run.

Samples with smooth amplification curve and a Cq value ≤ 40 in at least one of the duplicate reactions were considered positive.

Absolute quantification of parasites

One µL of each serial dilution of the standard curve were included on each qPCR plate. The qPCR results were analysed using LightCycler® 480 System software, Version 1.2.9.11 (Roche, Switzerland). The starting quantity of parasites per qPCR reaction was estimated from the mean Cq values, obtained for duplicates of each sample, using a regression line from the ten-fold dilutions of standard curve. Negative reactions were not considered in the calculation of the Cq mean. For the calculation of the final parasite load, the original weight of the sample, the final weight of the digested pellet, the proportion of the pellet used for DNA extraction, and the proportion of the DNA extract volume used in the subsequent qPCR reaction were taken into account. The final result was expressed as a number of parasites per gram of tissue (ppg).

Statistical analysis

For the statistical analysis of the serological data, a mixed linear model was applied to the data before ANOVA analysis of data to provide a more complete and accurate representation of

the data by accounting for both fixed and random effects, thereby improving the accuracy and reliability of the ANOVA results.

The mixed linear model was fitted to the ELISA percentage positivity (PP)-values with the fixed effect of pig group (Groups 1-4) and blood sampling times in wpi (0-6 wpi) multiplied, and an individual animal kept as a random effect to test the differences in the data. The estimates from this analysis were used in a two-way repeated-measures ANOVA and post-hoc pairwise comparisons of all combinations of pig groups and blood sampling times, using the Tukey's method for comparing a family of estimates. Outcomes with p-value of less than 0.05 were considered statistically significant.

The analyses of the parasite tropism and parasite load in the tissues were done in two steps. A generalized linear model with binomial distribution was fitted to estimate the frequency of *T. gondii*-positive tissues by qPCR. Parasite stage (oocyst or tissue cyst), isolate of the parasite ((CZ-Tiger (type II) and CZ-Šimková (type III)) and tissue were set as fixed effects, with the pig kept as a random effect in the model. The predicted effects of the isolate, type and tissue itself on the frequency of positive tissues in pigs was expressed as odd ratio (OR) with 95% confidential interval (CI).
Subsequently, a linear mixed model was used to estimate the effect of the stage and isolate of the parasite as well as tissue on the parasite load in positive tissues which were previously estimated in the analysis frequency of positive tissues. The relationship between the infection route and parasite burden in the tissues was expressed as a log10-transformed coefficient estimate with the standard error, with the random effect excluded from the prediction.
Results with p-value of less than 0.05 were considered statistically significant. Statistical analyses were performed using RStudio (Team, 2009), R version 4.2.2 (2022-10-31 ucrt), using the following packages: ggplot2 (Wickham, 2016), tidyverse (Wickham et al., 2019), lmerTest (Kuznetsova et al., 2017), readxl (Wickham and Bryan, 2022), MuMIn (Barton, 2022), emmeans (Russell V. Lenth, 2023), scales (Wickham and Seidel, 2022), and lme4 (Bates et al., n.d.).

RESULTS

Characterization of isolates

Genotyping by PCR-RFLP revealed Type II variant (ToxoDB #3) in case of CZ-Tiger confirming previous reports (Fabian et al., 2020; Geuthner et al., 2019; Juránková et al., 2013a; Koethe et al., 2015; Ramakrishnan et al., 2019). PCR-RFLP analysis typed CZ-Šimková as Type III (ToxoDB #2). Microsatellite typing confirmed lineage typing and provides a detailed genotypic characterization by fingerprinting markers (Table 7).

Table 7 Genotyping data. Microsatellite typing of the two strains used in the current study.

Isolate	Origin	Marker (Chromosome)															MS type
		Typing								Fingerprinting							
		TUB2 (IX)	W35 (II)	TgM-A (X)	B18 (VIIa)	B17 (XII)	M33 (IV)	IV.1 (IV)	XI.1 (XI)	M48 (Ia)	M102 (VIIa)	N60 (Ib)	N82 (XII)	AA (VIII)	N61 (VIIb)	N83 (X)	
RH	Reference	291	248	209	160	342	169	274	358	209	170	150	119	268	87	306	I
ME49	Reference	289	242	207	158	336	169	274	356	215	176	148	111	268	91	310	II
NED	Reference	289	242	205	160	336	165	278	356	209	192	152	111	270	91	312	III
CZ-Tiger	Tiger	289	242	207	158	336	169	274	356	213	180	147	117	279	91	312	II
CZ-Šimková	Cat	289	242	205	160	336	165	278	356	211	194	155	111	273	91	312	III

Clinical observation of experimentally infected pigs

All inoculated pigs manifested with an intermittent fever of > 40.0°C during 1 to 2 wpi (data not shown), accompanied by inappetence and letargy with occurrence of mild diarrhoea in some animals. These clinical signs persisted in all oocyst-infected pigs and four pigs infected with tissue cysts of the type III (CZ-Šimková) isolate during the second wpi.

Two pigs from Group 3 (Pigs No. 18 and 25) deceased due to serious health issues at 12 days post-inoculation. Tissues from the negative control pigs of Group 5 (Pigs No. 26 and 27) and the two prematurely deceased pigs of Group 3 (Pigs No. 18 and 25) and were not included in the sampling for the subsequent tropism study.

Serological response

The fitting of the linear mixed model to the serological data showed significant effects of time (wpi) and time in combination with pig groups on the level of the specific antibodies based on ELISA PP-values. Overall, no significant difference in ELISA PP-values was found in Groups 2 (type II tissue cysts), 3 (Type III oocysts) and 4 (type III tissue cysts) compared to Group 1 (type II oocysts). In all data combined, a statistically significant rise of antibodies based on the ELISA PP-values was observed starting from 2 wpi (< 0.001) when compared to the values before the inoculation (0 wpi). For the combined effect of pig group and time, statistically significant differences in comparison to Group 1 were observed for Group 2 at 3, 4, and 5 wpi ($p < 0.05$, < 0.01, and < 0.01, respectively), for Group 3 at 2, 3, 4, 5, and 6 wpi ($p < 0.001$ for all), and for Group 4 at 2, 3, 4, 5, and 6 wpi ($p < 0.001$ for all).

The following statistical analyses of the effects of time (wpi), pig groups and their combinations on anti-*T. gondii* antibodies development using ANOVA showed an overall significant effect all three variables ($p < 0.001$ for all). The subsequent Tukey's pairwise comparisons of the combinations of the effects of time, group, and their combination are summarized in the pairwise comparison charts.

Overall, statistically significant rise in the production of *T. gondii*-specific antibodies was observed starting from 2 wpi (Table 8). The differences in the serological response among the tested pigs groups were significant between Groups 1 and 2 ($p < 0.05$), Groups 1 and 3 ($p < 0.001$), Groups 1 and 4 ($p < 0.001$), Groups 2 and 3 ($p < 0.05$), and Groups 2 and 4 ($p < 0.001$) (Table 9). No difference was observed in the serological response between Groups 3 and 4 ($p = 0.975$). The comparisons of the serological response between the pig groups in time showed significant differences between some of the pig groups starting from 2 wpi, as detailed in Table 9.

No specific anti-*T. gondii* IgGs were detected by ELISA in sera samples of the two negative control pigs during the monitored period. The evolution of specific IgG anti-*T. gondii* antibodies over time in the different inoculated pig groups is displayed in Fig. 10.

wpi	Contrast	Estimate	SE	df	t-ratio
0	Group 1 - Group 2	-2.24	15.5	65.6	-0.144
	Group 1 - Group 3	-4.67	17.4	65.6	-0.269
	Group 1 - Group 4	-4.68	15.5	65.6	-0.301
	Group 2 - Group 3	-2.43	17.4	65.6	-0.140
	Group 2 - Group 4	-2.43	15.5	65.6	-0.157
	Group 3 - Group 4	-0.01	17.4	65.6	0.000
1	Group 1 - Group 2	-2.42	15.5	65.6	-0.156
	Group 1 - Group 3	-3.39	17.4	65.6	-0.195
	Group 1 - Group 4	-10.90	15.5	65.6	-0.702
	Group 2 - Group 3	-0.97	17.4	65.6	-0.056
	Group 2 - Group 4	-8.48	15.5	65.6	-0.546
	Group 3 - Group 4	-7.51	17.4	65.6	-0.433
2	Group 1 - Group 2	-33.79	15.5	65.6	-2.177
	Group 1 - Group 3	-80.04	17.4	65.6	-4.612
	Group 1 - Group 4	-87.46	15.5	65.6	-5.634
	Group 2 - Group 3	-46.25	17.4	65.6	-2.65
	Group 2 - Group 4	-53.66	15.5	65.6	-3.457
	Group 3 - Group 4	-7.41	17.4	65.6	-0.427
3	Group 1 - Group 2	-41.07	15.5	65.6	-2.646
	Group 1 - Group 3	-95.63	17.4	65.6	-5.510
	Group 1 - Group 4	-95.40	15.5	65.6	-6.145
	Group 2 - Group 3	-54.56	17.4	65.6	-3.144
	Group 2 - Group 4	-54.33	15.5	65.6	-3.500
	Group 3 - Group 4	-5.11	17.4	65.6	0.013
4	Group 1 - Group 2	-49.19	15.5	65.6	-3.517
	Group 1 - Group 3	-81.07	17.4	65.6	-5.907
	Group 1 - Group 4	-92.26	15.5	65.6	-6.933
	Group 2 - Group 3	-31.89	17.4	65.6	-2.761
	Group 2 - Group 4	-43.08	15.5	65.6	-3.417
	Group 3 - Group 4	-11.19	17.4	65.6	-0.295
5	Group 1 - Group 2	-34.88	15.5	65.6	-3.168
	Group 1 - Group 3	-88.59	17.4	65.6	-4.671
	Group 1 - Group 4	-92.43	15.5	65.6	-5.943
	Group 2 - Group 3	-53.71	17.4	65.6	-1.037
	Group 2 - Group 4	-57.55	15.5	65.6	-2.775
	Group 3 - Group 4	-3.83	17.4	65.6	-0.645
6	Group 1 - Group 2	-34.88	15.5	65.6	-2.247
	Group 1 - Group 3	-88.59	17.4	65.6	-5.104
	Group 1 - Group 4	-92.43	15.5	65.6	-5.954
	Group 2 - Group 3	-53.71	17.4	65.6	-3.095
	Group 2 - Group 4	-57.55	15.5	65.6	-3.707
	Group 3 - Group 4	-3.84	17.4	65.6	-0.221

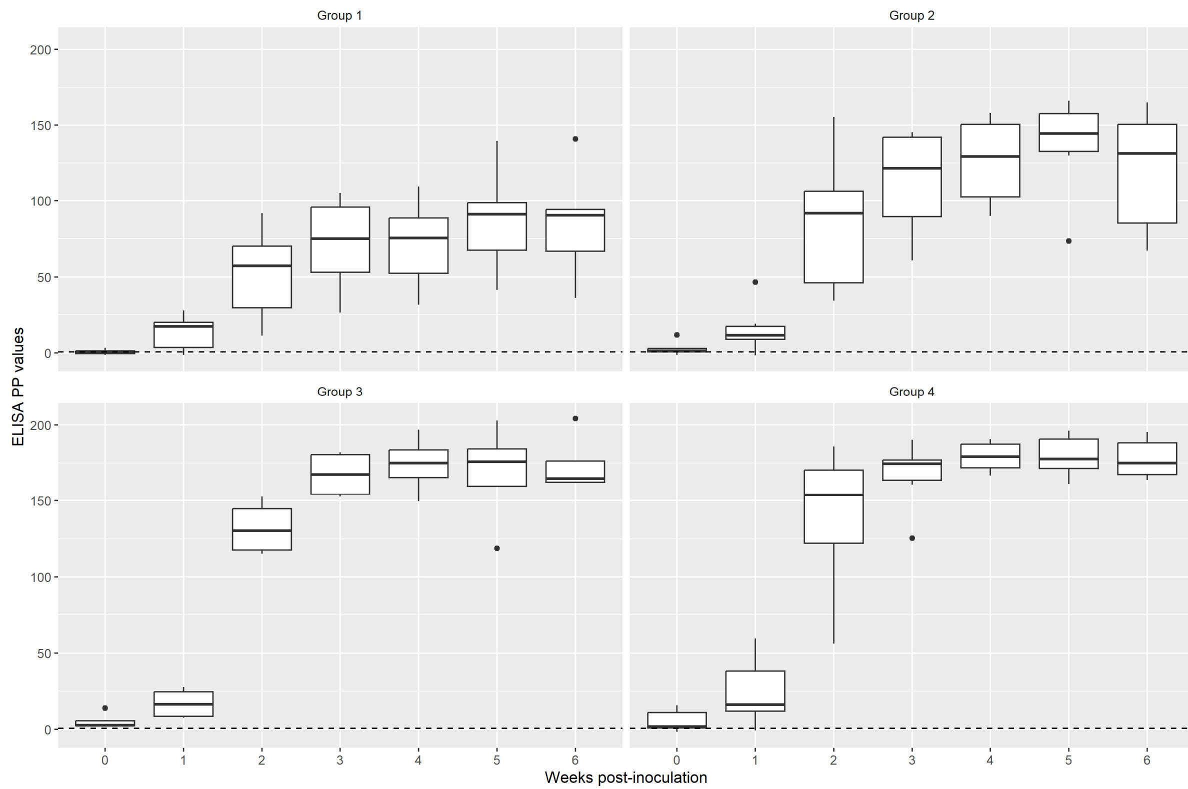

Figure 10 Vizualization of the seroconversion of pigs. The time of sera collection is shown on the x-axis and observed ELISA PP values are shown on the y-axis. The results are visualised per groups of pigs - Group 1 (type II oocysts), Group 2 (type II tissue cysts), Group 3 (type III oocysts), and Group 4 (type III tissue cysts). Dashed horizontal line signalizes mean ELISA negative control PP value.

wpi								
	0	1	2	3	4	5	6	Total
0	-	-14.2	-98.9	-126.3	-134.4	-139.5	-135.9	3.5 (-8.2-15.1)
1	-14.2	-	-84.7	-112.1	-120.2	-125.3	-121.7	17.7 (6.03-29.3)
2	-98.9	-84.7	-	-27.4	-35.4	-40.5	-37.0	102.4 (90.8-114.0)
3	-126.3	-112.1	-27.4	-	-8.0	-13.1	-9.6	129.8 (118.2-141.4)
4	-134.4	-120.2	-35.4	-8.0	-	-5.1	-1.6	137.8 (126.19-149.4)
5	-139.5	-125.3	-40.5	-13.1	-5.1	-	3.55	142.9 (131.3-154.5)
6	-135.9	-121.7	-37.0	-9.6	-1.6	3.55	-	139.36 (127.74-151.0)

Table 8 Pairwise comparison chart with contrasts of coefficient estimates of ELISA PP-values for combinations of groups (Groups 1-4), and mean with minimum and maximum values in round brackets for each of the wpi. Level of significance are as follows: $p < 0.001$ (red), $p < 0.01$ (yellow), $p < 0.05$ (green) and $p > 0.05$ (blue).

Group	1	2	3	4	Mean (Standard Error)
1	-	-31.17 (10.7)	-65.13 (12.0)	-70.11 (10.7)	54.6 (7.6)
2	-31.17 (10.7)	-	-33.96 (12.0)	-38.94 (10.7)	85.8 (7.6)
3	-65.13 (12.0)	-33.96 (12.0)	-	-4.98 (12.0)	119.7 (9.3)
4	-70.11 (10.7)	-38.94 (10.7)	-4.98 (12.0)	-	124.7 (7.6)

Table 9 Pairwise comparison chart with contrasts of coefficient estimates of ELISA PP-values for combinations of combination of weeks post-inoculation (wpi) and groups (Groups 1-4) in all their combinations. Level of significance are as follows: $p < 0.001$ (red), $p < 0.01$ (yellow), $p < 0.05$ (green) and $p > 0.05$ (blue). SE = standard error, df = degree of freedom.

wpi	Contrast	Estimate	SE	df	t-ratio	*p*-value
0	Group 1 - Group 2	-2.24	15.5	65.6	-0.144	0.9989
	Group 1 - Group 3	-4.67	17.4	65.6	-0.269	0.9931
	Group 1 - Group 4	-4.68	15.5	65.6	-0.301	0.9904
	Group 2 - Group 3	-2.43	17.4	65.6	-0.140	0.9990
	Group 2 - Group 4	-2.43	15.5	65.6	-0.157	0.9986
	Group 3 - Group 4	-0.01	17.4	65.6	0.000	1.0000
1	Group 1 - Group 2	-2.42	15.5	65.6	-0.156	0.9986
	Group 1 - Group 3	-3.39	17.4	65.6	-0.195	0.9973
	Group 1 - Group 4	-10.90	15.5	65.6	-0.702	0.8958
	Group 2 - Group 3	-0.97	17.4	65.6	-0.056	0.9999
	Group 2 - Group 4	-8.48	15.5	65.6	-0.546	0.9472
	Group 3 - Group 4	-7.51	17.4	65.6	-0.433	0.9726
2	Group 1 - Group 2	-33.79	15.5	65.6	-2.177	0.1405
	Group 1 - Group 3	-80.04	17.4	65.6	-4.612	< 0.001
	Group 1 - Group 4	-87.46	15.5	65.6	-5.634	< 0.001
	Group 2 - Group 3	-46.25	17.4	65.6	-2.65	0.0466
	Group 2 - Group 4	-53.66	15.5	65.6	-3.457	0.0052
	Group 3 - Group 4	-7.41	17.4	65.6	-0.427	0.9736
3	Group 1 - Group 2	-41.07	15.5	65.6	-2.646	0.0489
	Group 1 - Group 3	-95.63	17.4	65.6	-5.510	< 0.001
	Group 1 - Group 4	-95.40	15.5	65.6	-6.145	< 0.001
	Group 2 - Group 3	-54.56	17.4	65.6	-3.144	0.0131
	Group 2 - Group 4	-54.33	15.5	65.6	-3.500	0.0046
	Group 3 - Group 4	-5.11	17.4	65.6	0.013	1.0000
4	Group 1 - Group 2	-49.19	15.5	65.6	-3.517	0.0043
	Group 1 - Group 3	-81.07	17.4	65.6	-5.907	< 0.001
	Group 1 - Group 4	-92.26	15.5	65.6	-6.933	< 0.001
	Group 2 - Group 3	-31.89	17.4	65.6	-2.761	0.0366
	Group 2 - Group 4	-43.08	15.5	65.6	-3.417	0.0059
	Group 3 - Group 4	-11.19	17.4	65.6	-0.295	0.9910
5	Group 1 - Group 2	-34.88	15.5	65.6	-3.168	0.0122
	Group 1 - Group 3	-88.59	17.4	65.6	-4.671	< 0.001
	Group 1 - Group 4	-92.43	15.5	65.6	-5.943	< 0.001
	Group 2 - Group 3	-53.71	17.4	65.6	-1.037	0.2654
	Group 2 - Group 4	-57.55	15.5	65.6	-2.775	0.0353
	Group 3 - Group 4	-3.83	17.4	65.6	-0.645	0.9170
6	Group 1 - Group 2	-34.88	15.5	65.6	-2.247	0.1216
	Group 1 - Group 3	-88.59	17.4	65.6	-5.104	< 0.001
	Group 1 - Group 4	-92.43	15.5	65.6	-5.954	< 0.001
	Group 2 - Group 3	-53.71	17.4	65.6	-3.095	0.0150
	Group 2 - Group 4	-57.55	15.5	65.6	-3.707	0.0024
	Group 3 - Group 4	-3.84	17.4	65.6	-0.221	0.9961

Histopathological examination

Reactive hyperplasia of lymphatic tissue (lymph nodes, spleen and thymus) (Fig. 11A, B) and minor lesions of chronic interstitial pneumonia (Fig. 11C, D), predominantly mild, were observed in all pigs. These symptoms were occasionally accompanied by hyperaemia in tissues of some individuals.

At necropsy, both prematurely deceased pigs (Pigs No. 18 and 25) manifested gross lesions represented by anaemia and focal cyanosis of mucosal surfaces of conjunctiva, oral and nasal cavities, accompanied by atrophy of nasal conchae and purulent tonsillitis. Moreover, both pigs showed evident anaemia of skeletal muscles and subcutaneous tissue. Focal hyperaemia was observed in certain parts of the brain tissue and brain vessels, and in pig No. 18 were these symptoms joined by mild perivascular lymphoplasmatic inflammatory infiltrate while no inflammatory reaction was found in the brain of pig No. 25. In both pigs, a vast majority of examined lymph nodes (submandibular, cervical, mediastinal, inguinal, mesenteric, splenic lymph nodes) were enlarged and hyperaemic. Histopathologically, all examined lymph nodes exhibited prominent reactive hyperplasia of lymphatic follicles, with minor focal haemorrhages and neutrophilic inflammatory infiltrate in some lymph nodes. Similarly, reactive hyperplasia of lymphatic follicles and tonsils was observed in these pigs, paired with marked neutrophilic inflammatory infiltrate with presence of bacterial colonies in the tonsillar tissue.

Necropsy of pig No. 18, revealed splenomegaly with multiple haemorrhages and haematomas of various sizes in the parenchyma. Some of the spleen haematomas were ruptured and the abdominal cavity was filled with approximately 1.5 litres of partially coagulated blood. Other organs were hyperaemic or without visible pathological changes. Closer examination of spleen showed multiple areas of haemorrhages with blood-filled cavities and focal coagulation necrosis in the parenchyma. Tissues of the digestive system, kidneys, liver and lungs exhibited no macroscopic changes. Histopathology revealed a multifocal, mostly neutrophilic, inflammatory reaction and a small volume of lymphoplasmacytic infiltrate containing macrophages in the spleen parenchyma.

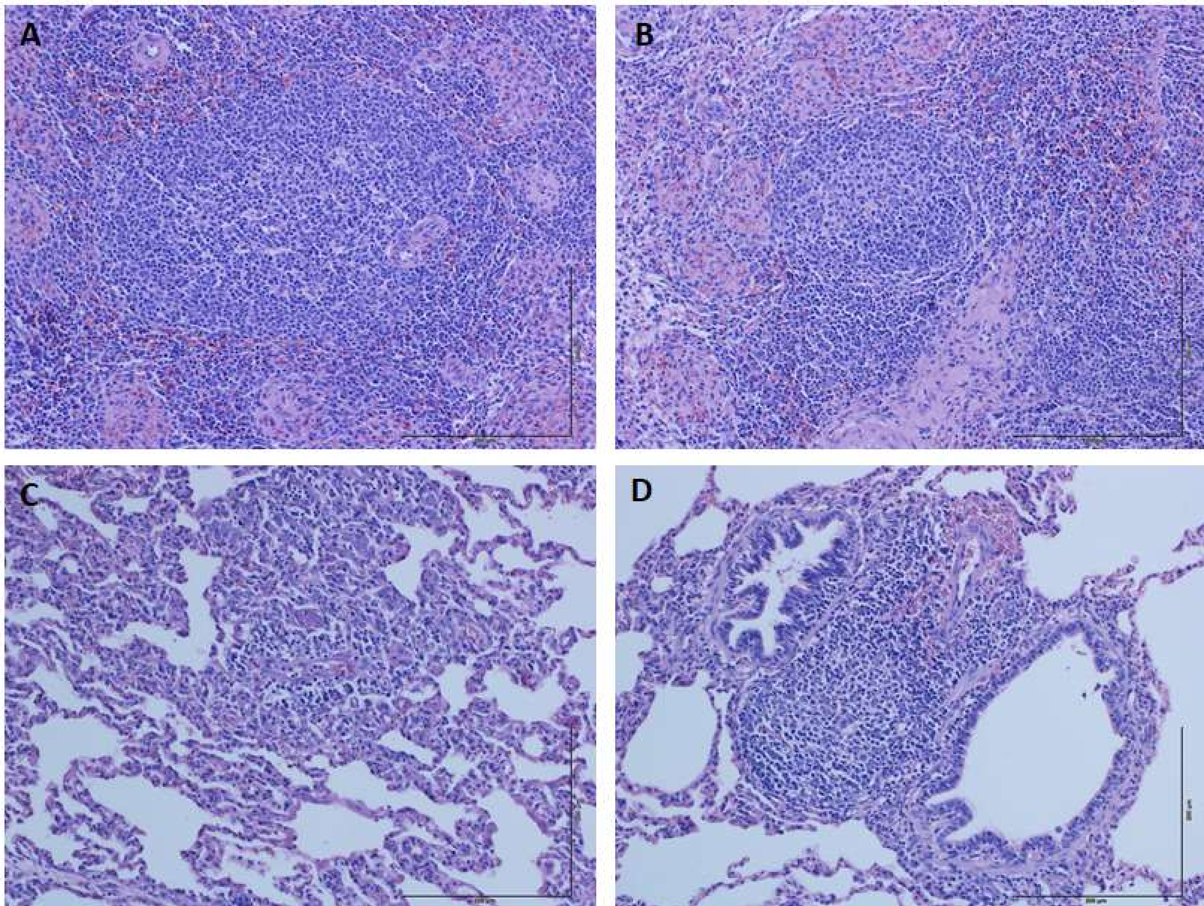

Figure 11 Histological observations in tissues of experimentally infected pigs. A: hyperplastic lymphadenitis of mesenterial lymph nodes in pig No. 17 (oocysts, type III), B: hyperplastic lymphadenitis of mesenterial lymph nodes in pig No. 8 (tissue cysts, type II), C: Intersticial pneumonia in pig No. 21 (tissue cysts, type III), D: Intersticial pneumonia in pig. No. 4 (oocysts, type II). Scale in the rigt bottom corner = 200µm.

On the contrary, spleen of pig No. 25 was of normal size and shape. Anaemia was observed in the parenchymal organs of abdominal cavity, kidneys and liver. Interestingly, partially digested blood was found in the lumen of the stomach, with a haematoma formation observed in the site of large mucosal ulceration (approximately 10 cm in diameter) in the cardiac part of the stomach. Similarly, the large intestine contained melena. In thoracic cavity, a faintly clouded effusion containing precipitated fibrinous filaments attached to the surface of pleura was observed. In the cranial lung lobes, dark-red-coloured atelectatic foci of parenchyma were discovered, together with smaller foci of necrosis and haemorrhages, hyperaemia, and oedema. Tracheobronchial and mediastinal lymph nodes were considerably hyperplasic and hyperaemic. Histopathological examination of lung showed alveolar oedema, foci of marked

neutrophilic and fibrinous inflammation, foci of parenchymal coagulation necrosis, and a presence of bacteria in the exudate. Lymphatic tissue of spleen and lymph nodes exhibited reactive hyperplasia, with neutrophilic inflammatory infiltrate in some of the lymph nodes.

Detection of *T. gondii* DNA in tissues

A total of 198 tissue samples (9 tissues x 22 pigs) were analysed by qPCR, resulting in 29.8 % (59/198) positive tissues. The tissues of pigs of Group 4 (type III tissue cysts) were the most frequently positive in 57.4% (31/54) of cases, with at least four positive tissues each. All pigs of Groups 2 (type II tissue cysts), 3 (type III oocysts) and 4 were positive in at least one tissue, with the exception of pig No. 11 of Group 2. The pigs of Groups 2 and 3 were positive in 24.1% (13/54) and 33.3% (12/36) tissues per pig, respectively. On the other side, tissues of pigs of Group 1 (type II oocysts) were the least frequently positive with only 0.06% (3/54) positive tissues overall, all three in pig No. 5 (see Supplementary data).

With all tissues, except for liver and spleen, positive for *T. gondii* DNA by qPCR, pigs No. 21 and 22 were found the most frequently infected. At the same time, no parasite DNA was detected by qPCR in any of the tested tissues of pigs No. 1-4, 6, and 11. Overall, the most frequently positive tissue was brain at 63.6% (14/22) while no liver samples were found to be positive (see Supplementary data).

Interestingly, all sampled tissues from pigs of Group 1 tested negative for the presence of *T. gondii* DNA by qPCR, except for three tissues (ham, heart and brain) in pig number 5. The tissues from pigs of Group 4 on the other hand were the most frequently positive (Fig. 12A).

Tissue samples of pigs infected with type II isolate (Groups 1 and 2) were positive in 14.8% (16/108) of cases, whereas tissues of pigs infected with type III strain (Groups 3 and 4) were positive in 47.8% (43/90) of cases (Fig. 12B, see Supplementary data). Moreover, the pigs infected with *T. gondii* oocysts (Groups 1 and 3) and tissue cysts (Groups 2 and 4), resulted in 16.7% (15/90) and 40.7% (44/108) positive tissues respectively (Fig. 12C, see Supplementary data).

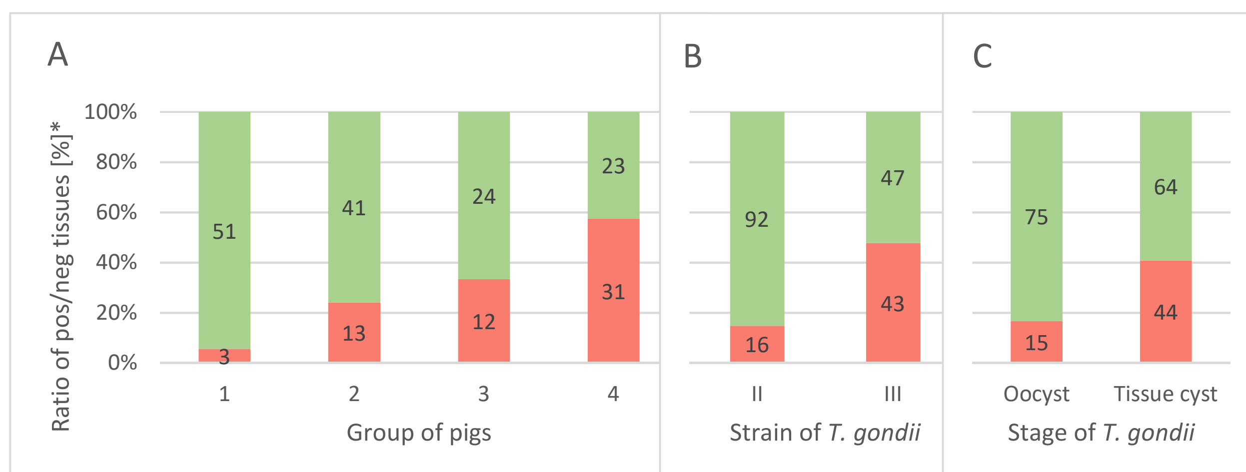

Figure 12 Proportion of positive tissues per Group (A), per strain (B), and per stage (C) of the parasite. The number of tissues positive and negative for *T. gondii* DNA by qPCR is provided for the group, strain/isolate and stage of the parasite and their proportions are visualised in red for positive and green for negative tissue samples. *the results are scaled proportionally to each other

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Multivariate binomial regression model showed statistically significant effects of the parasite stage, isolate and tissue (Table 10). Infection with *T. gondii* tissue cysts in the pigs showed significantly higher frequency of positive tissues by qPCR (OR 17.41, 95% CI: 3.03 - 99.88) compared to the oocyst infection (Table 10). Similarly, type III strain CZ-Šimková caused significantly higher frequency of positive tissues (OR 45.20, 95% CI: 7.07 – 288.80) in comparison with type II strain CZ-Tiger (Table 10). The tissues of heart, kidney, lung and spleen contained *T. gondii* significantly less frequently than brains of the tested pigs, as detailed in Table 10. The outcomes of the statictical analysis comparing the predicted values and the actual frequencies of positive samples per parariste stage, isolate and tissue are visualized in Fig. 13.

Table 10 Odds ratios for the parasite stage, strain of the isolate and tissue itself as a risk factor for presence/absence of *T. gondii*-positive tissues. Binomial regression model outcome are presented as odds ratios with 95% confidential intervals (CI), with effects in bold being significant at this level.

Variable	Value	Odds ratio (95% CI)	*p*-value
	intercept	**0.12 (0.02 - 0.78)**	**0.026***
Parasite stage	oocyst (reference)		
	tissue cyst	**17.41 (3.03 - 99.88)**	**0.001****
Isolate (strain)	CZ-Tiger (type II, reference)		
	CZ-Šimková (type III)	**45.20 (7.07 – 288.80)**	**< 0.001*****
Tissue	brain (reference)		
	ham	0.46 (0.08 – 2.76)	0.395
	heart	**0.04 (0.00 - 0.31)**	**0.002****
	kidneys	**0.00 (0.00 – 0.06)**	**< 0.001*****

	liver	0.00 (0.00 – 0.00)	0.729
	loin	0.46 (0.08 – 1.95^{e}+29)	0.395
	lungs	**0.01 (0.00 – 0.09)**	**< 0.001*** **
	shoulder	0.31 (0.05 – 1.88)	0.202
	spleen	**0.00 (0.00 – 0.03)**	**< 0.001*** **

significance codes : < 0.001***, < 0.01**, < 0.05*

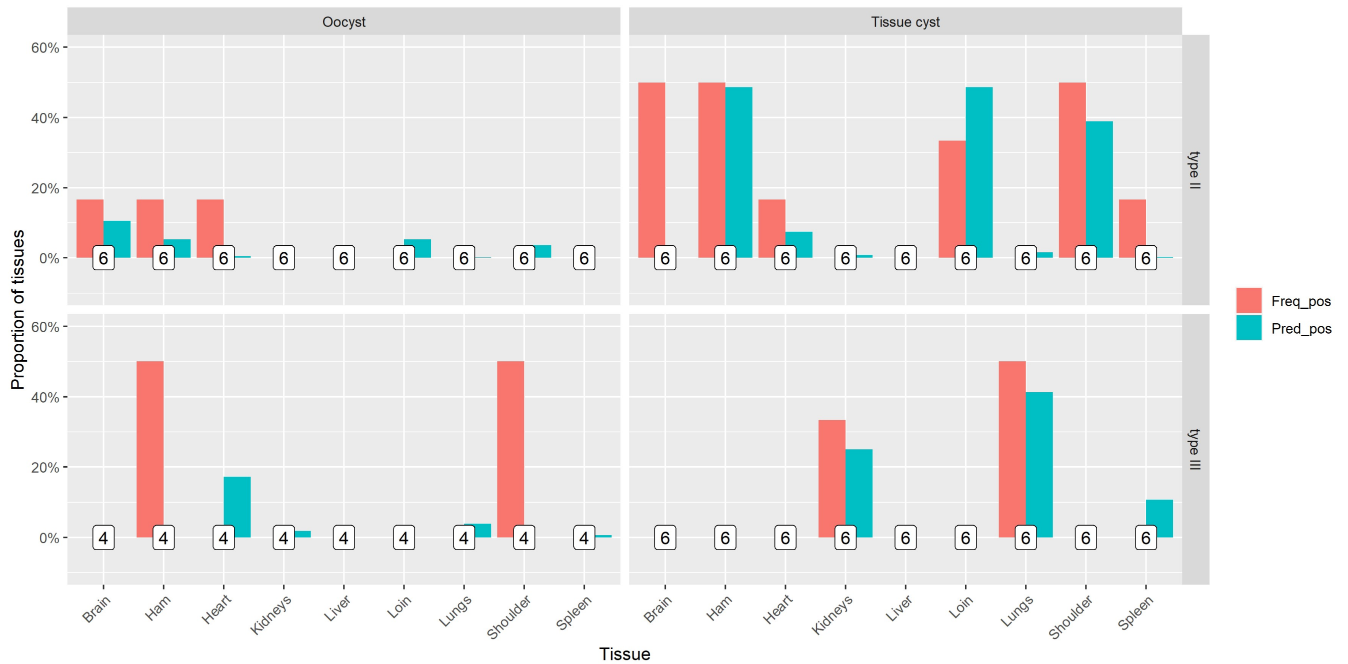

Figure 13 Proportions of positive tissues (red columns) were compared to the predited values (blue columns). Numbers at the bottom of each column stand for the number of samples tested within the corresponding parasite stage, isolate/strain and tissue. The pig groups are represented as combinations of parasite stage (oocyst or tissue cyst) and parasite strain (type II or type III) separate boxes, with individual tissues on the x-axis. The proportion of postitve findings out of the total number of samples is visualized on y-axis in percentage.

Parasite distribution in tissues

Detectable *T. gondii* DNA was found in 59 sampled tissues, ranging from 0.1 to 697.5 ppg. The highest mean parasitic burden was found in pigs of Group 4 and the lowest in pigs of Group 1.

The highest parasite burden of all tested tissues was observed in shoulder at 84.4 (0.0 - 442.5) ppg, closely followed by the other tested meat cuts, loin and ham, at 82.2 (0.0 - 697.5) and 61.0 (0.0 - 675.4) ppg. No parasites were detected in liver of sampled pigs (Table 11).

Tissue samples from pigs of Group 1 carried the lowest parasite load, in contrast to the highest load observed in pigs of Group 4. Means of 5.1 ppg and 60.7 ppg were found in pigs infected with type II and type III isolates respectively. The tested samples of oocyst-infected pigs (Groups 1 and 3) contained the mean parasite load of 6.4 ppg, compared to a mean of 50.3 ppg in tissue-cyst-infected pigs (Group 2 and 4) (Table 11, see Supplementary data).

Table 11 Parasite burden estimates. Detectable parasite burden (arithmetic mean of *T. gondii* in nine tissues composing of three meat cuts ("shoulder" = *musculus triceps brachii, musculus supraspinatus, musculus infraspinatus*; "loin" = *musculus longissimus dorsi pars lumbalis*; and "ham" = *musculus semitendinosus, musculus semitendinosus, musculus gluteus medialis*) and six organs (heart, brain, lungs, liver, spleen, and kidneys). The results are presented in parasites per gram of tissue and ranked from high to low. Arithmetic mean with the range of observed minimal and maximal mean of the corresponding group is provided for individual pigs (column on the right) and for single tissue (line at the bottom). ND = not detected in the sample.

Strain	**Stage**	**Group**	**Pig number**	**Shoulder**	**Loin**	**Ham**	**Brain**	**Heart**	**Lungs**	**Kidneys**	**Spleen**	**Liver**	**Aritm. mean [min - max]**
III	tissue cyst	4	22	442.5	697.5	675.4	20.8	58.8	26.6	10.7	ND	ND	214.7 [0.0 - 675.4]
III	tissue cyst	4	19	336.4	310.1	223.1	295.5	23.7	ND	ND	ND	ND	132.1 [0.0 - 336.4]
III	tissue cyst	4	24	356.3	479.6	137.3	116.3	ND	33.0	ND	ND	ND	124.7 [0.0 - 479.6]
III	oocyst	3	14	295.5	17.3	24.1	66.2	ND	ND	ND	ND	ND	44.8 [0.0 - 295.5]
II	tissue cyst	2	13	58.7	109.5	54.3	46.7	ND	ND	ND	ND	ND	29.9 [0.0 - 109.5]
III	tissue cyst	4	23	47.6	72.9	71.9	56.3	0.1	ND	ND	ND	ND	27.6 [0.0 - 72.9]
III	tissue cyst	4	21	70.9	34.1	39.1	23.7	12.3	39.1	23.9	ND	ND	27.0 [0.0 - 70.9]
III	tissue cyst	4	20	99.2	26.6	63.0	6.2	ND	ND	ND	ND	ND	21.7 [0.0 - 99.2]
II	tissue cyst	2	7	57.3	18.3	23.0	ND	14.7	ND	ND	ND	ND	12.6 [0.0 - 57.3]
III	oocyst	3	16	37.2	19.1	ND	8.6	ND	ND	ND	ND	ND	7.2 [0.0 - 37.2]
II	tissue cyst	2	8	55.69	ND	ND	ND	ND	ND	ND	ND	ND	6.2 [0.0 - 55.7]
II	tissue cyst	2	9	ND	ND	1.9	46.7	ND	ND	ND	1.3	ND	5.5 [0.0 - 46.7]
II	oocyst	1	5	ND	ND	27.1	0.9	13.5	ND	ND	ND	ND	4.6 [0.0 - 27.1]
III	oocyst	3	17	ND	11.6	0.8	22.2	ND	ND	ND	ND	ND	3.8 [0.0 - 22.2]
III	oocyst	3	15	ND	11.5	ND	17.6	ND	ND	ND	ND	ND	3.2 [0.0 - 17.6]
II	tissue cyst	2	12	ND	ND	ND	18.2	ND	ND	ND	ND	ND	2.0 [0.0 - 18.2]
II	tissue cyst	2	11	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0 [0.0 - 0.0]
II	oocyst	1	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0 [0.0 - 0.0]
II	oocyst	1	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0 [0.0 - 0.0]
II	oocyst	1	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0 [0.0 - 0.0]
II	oocyst	1	4	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0 [0.0 - 0.0]
II	oocyst	1	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0 [0.0 - 0.0]
Aritm. mean				84.4	82.2	61.0	33.9	5.6	4.5	1.6	0.1	0.0	

The statistical analysis of the parasite load in tissues showed significant effect of the parasite stage and tissue, however, none of the isolate/strain of *T. gondii.* In animals infected with tissue cysts, the tissues contained on average approximately 1.8 times (0.59 log10-units) more parasite load than the tissues of the oocyst-infected pigs (Table 12). Although not statistically significant, the tissues of pigs infected with type III isolate contained approximately 1.4 times (0.35 log10-units) more parasites than the tissues of the type II-infected pigs (Table 13). Compared to the parasite burden found in brains of the pigs, a significantly higher parasite burden was observed in shoulder muscles with approximately 1.65 (0.51 log10-units) times more parasite load. This is in contrast with the significantly lower parasite burden found in hearts of the pigs, approximately 1.8 (0.58 log10-units) times lower compared to the brains (Table 12). The comparisons of the parasite load of the remaining tissues can be found in Table 12. The log10-transformed parasite burden per gram of tissue and split by parasite stage inoculated is summarized in Fig. 14.

Table 12 Coefficient estimates with standard errors for each of the infection routes (natural or experimental, via ingestion of oocysts or tissue cysts) in log-transformed parasite load in the *T. gondii*-positive tissues. Bold are significant effects at the 0.05 level. Significance codes : < 0.001***, < 0.01**, < 0.05*

Variable	Fixed effects	Estimate (log10-transformed)	CI 95%	Standard error	*p*-value
	intercept	**0.77**	**0.27-1.27**	**0.28**	**0.011***
Parasite stage	oocyst (reference)				
	tissue cyst	**0.59**	**0.17-1.00**	**0.22**	**0.020***
Isolate (strain)	CZ-Tiger (type II, reference)				
	CZ-Šimková (type III)	0.35	-0.05-0.77	0.22	0.131
Tissue	brain (reference)				
	ham	0.09	-0.29-0.48	0.21	0.657
	heart	**-0.58**	**-1.07- -0.09**	**0.27**	**0.034***
	kidneys	-0.58	-1.34-0.20	0.42	0.173
	liver	ND	ND	ND	ND
	loin	0.26	-0.12-0.65	0.21	0.217
	lungs	-0.32	-0.96-0.35	0.35	0.369
	shoulder	**0.51**	**0.11-0.92**	**0.22**	**0.024***
	spleen	-1.11	-2.23- -0.03	0.59	0.068

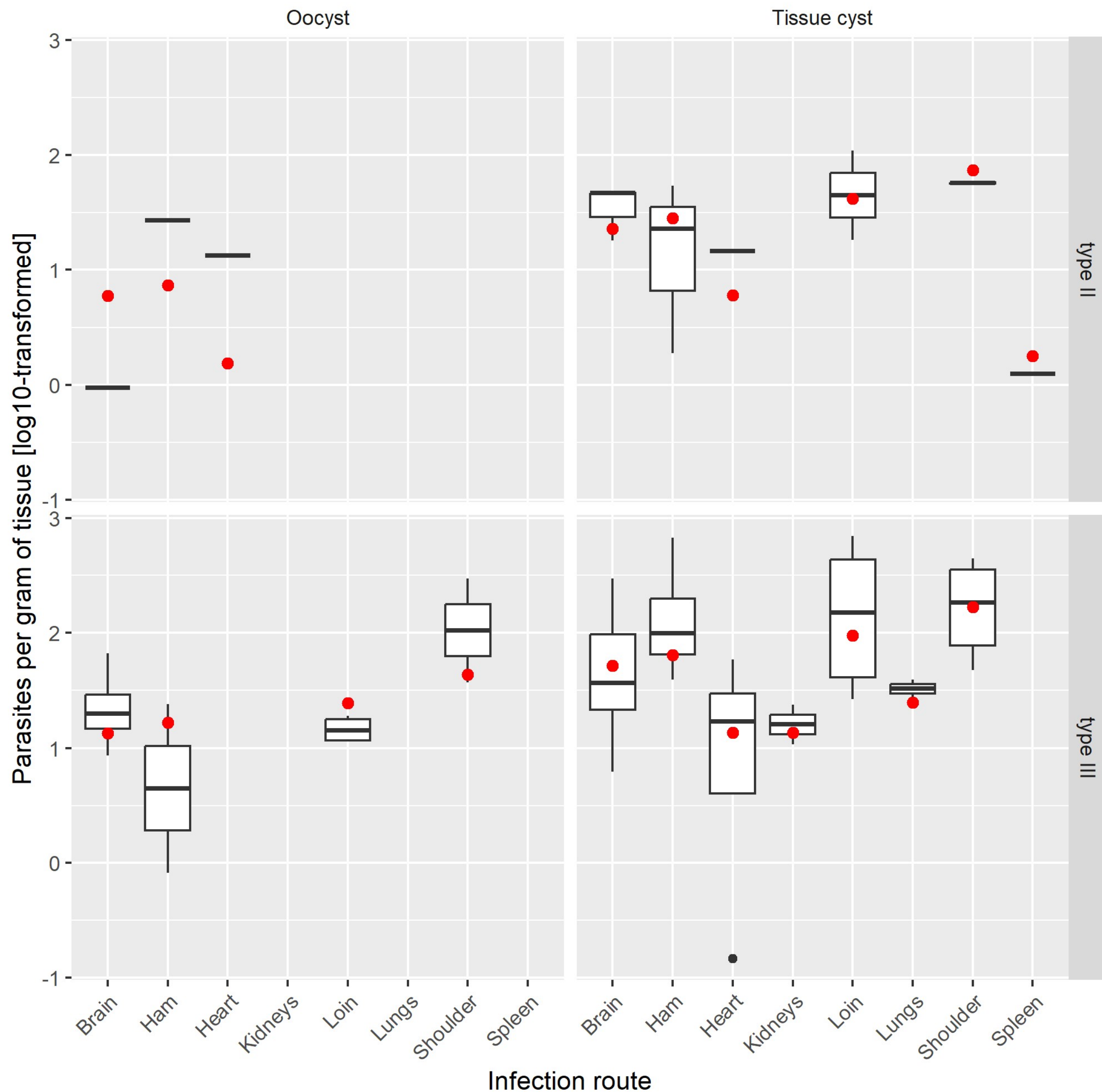

Figure 14 Log10-transformed parasite loads in *T. gondii*-positive tissues of the pigs, presented in boxes by pig groups (Group 1-4, combinations of parasite stage and strain) with Group 1 represented in the upper left section, Group 2 in the upper right, Group 3 in the lower left, and Group 4 in the lower right. Individual tissues are showed on x-axis and the parasite load is presented on y-axis as log10-transformed number of *T. gondii* parasites per gram of tissue. No *T. gondii* DNA was detected in livers of the tested pigs. Boxes represent the main body of the boxplot showing the quartiles and the median's confidence intervals, with a median value represented by a horizontal line, vertival lines ("whiskers") represent spread of sample parasite burdens within the interquartile range (IQR = third quartile – first quartile). Dots represent the predicted outcomes.

DISCUSSION

In order to exclude differences in the outcome of the infection due to the number of parasites inoculated, pigs were inoculated an equal dose of oocysts (~ 400) of both isolates. Storage of the oocysts used in this experiment of two and five months for the type II and III isolates respectively, should not have affected the infectivity of the oocysts which are capable of surviving for months even under harsh environmental conditions (J. P. Dubey et al., 2011; Shapiro et al., 2019). Accordingly, we aimed to inoculate the same number of bradyzoites in tissue cysts as sporozoites contained in oocysts, considering that 400 oocysts should contain 3200 sporozoites in case of perfect sporulation rate and assuming that 10 tissue cysts harbour approximately 3500 bradyzoites (Watts et al., 2015). Since the infective dose of bradyzoites used in the current study was based on an average number of bradyzoites per cyst instead of counting the content of each of these tissue cysts, a potential deviation of the true numbers from the expected numbers is possible. Additionally, potential inter-strain differences in the number of bradyzoites per tissue cyst could not taken into account, considering there is currently no data supporting this hypothesis and unexpected heterogeneity amongst bradyzoites and tissue cysts (Watts et al., 2015) might outweigh potential inter-strain variability. In contrast to most studies using thousands of oocysts or tissue cysts for experimental infection, an intentionally lower dose was chosen in this experiment in order to mimic natural *T. gondii* infection. Infective doses as low as a single oocyst were proved to cause infections in pigs (Dubey et al., 1996). Moreover, a lower inoculation dose does not necessarily result in a lower parasite load in tissues (Jennes et al., 2017), suggesting a space for further investigation of the dose effect on the progress of *T. gondii* infections in intermediate hosts.

The seroconversion of pigs in this study, manifested by a significant rise of specific anti-*T. gondii* antibodies, occurred between 1 and 2 wpi. The lack of significant difference in the immunological response observed between pigs of Groups 3 and 4 may suggest a similar immunogenicity of the CZ-Šimková type III isolate regardless of the parasite stage inoculated. The level of the measured antibody response in pigs of Group 1 was overall lower compared to the rest of the pigs. Combined with the overall lower levels of measured antibodies in

Groups 1 and 2 compared to the levels in Groups 3 and 4 (Fig. 10), this result may suggest a lower immunogenicity of CZ-Tiger type II strain in pigs compared to the CZ-Šimková type III strain and oocysts infections compared to those induced by tissue cysts. Similar strain effect to the one described in the current study was observed in a previous comparison of type II and III strains from Spain in pigs (Largo-de la Torre et al., 2022). Interpretation of the effect of parasite stage might be confined only to the isolates used in this study, CZ-Tiger and CZ-Šimková, as the overall effect of parasite stage on immunogenicity is impossible to assess due to lack of studies comparing infections induced by oocysts and tissue cysts of the same strain and dose. Regarding the time until seroconversion, the results observed in the remaining Groups 2-4 are in line with most experimental studies in pigs, reporting seroconversion between two and four wpi (Garcia et al., 2017a; Genchi et al., 2017; Jennes et al., 2017; Juránková et al., 2014a; Verhelst et al., 2011).

Lungs, spleen, liver and muscular tissue are often macroscopically altered due to the pathogenic effect of the parasite. The results of the necropsy and subsequent histopathological exam showed signs of lymphadenitis due to reactive interstitial hyperplasia of the lymphatic tissue in all pigs of this study. This effect is primarily due to an elevated flow of lymphocytes into the node from the blood caused by the parasitaemia by-products, exceeding the rate of outflow from the node (Mohseni et al., 2014). Additionally, lesions of interstitial pneumonia were found in lungs of all pigs in the present study. Almost identical findings of multifocal lesions in skeletal muscles, lymphadenomegaly, hepatosplenomegaly, and non-collapsed lungs as well as histological findings of hepatitis and splenitis, necrotizing myositis, and lymphoplasmacytic interstitial pneumonia were observed in pigs infected with atypical *T. gondii* genotype (Piva et al., 2022). Likewise, lesions of chronic interstitial pneumonia were also linked with toxoplasmosis in piglets (Thiptara et al., 2006). Affinity of *T. gondii* towards lung tissue, resulting in high concentration of the parasite within, was observed in previous experimental infections of pigs (Algaba et al., 2018; Juránková et al., 2014a). Pathology of toxoplasmosis manifesting with toxoplasmic pneumonitis (Mariuz et al., 1997; Oksenhendler et al., 1990) and lymphadenitis (Miettinen, 1981; Saxena et al., 2018) are well-described even in humans.

The observations during necropsy and results of histopathological examination of tissues of the two prematurely deceased pigs revealed different probable causes of their grave condition, both potentially linked to *T. gondii.* The most severe finding in pig No. 18 was purulent necrotic splenitis which might have caused the rupture of the spleen and subsequent internal bleeding in the peritoneal cavity. Cases of purulent necrotic splenitis are predominantly of bacterial origin, although *T. gondii* cannot be excluded as the potential cause as splenitis is linked with *T. gondii* infections in animals (Campbell et al., 2022; Oz, 2014; Rodrigues Oliveira et al., 2022). Moreover, the same pig suffered from purulent tonsillitis where apart of the possible bacterial ethology, *T. gondii* plays a role as an infectious agent as well. The other deceased pig, No. 25, suffered from chronic ulcerative gastritis. This condition is in pigs often caused by excessive stress factors (Norton et al., 1972), especially those with lower social hierarchical ranking, but toxoplasmosis cannot be ruled out as a potential cause due to documented pathogenicity in the gastrointestinal tract of certain animal species, including humans (Carossino et al., 2021; Glover et al., 2017).

Since the pathogenicity of *T. gondii* strains in mice varies from other animal species, and humans as an extension, the mice model was contested as the most suitable animal model prevising the pathogenicity in human toxoplasmosis (Calero-Bernal et al., 2022; Miranda et al., 2015), possibly in the prospect of pigs becoming a promising replacement due to anatomical, physiological, immunological and dietary similarity to humans (Mukhopadhyay et al., 2020; Perleberg et al., 2018). Anatomical similarity of pigs and humans enabled us to observe reactive hyperplasia in tonsil tissue which would be otherwise completely missed in mice due to lack of this tissue (Liebler-Tenorio and Pabst, 2006). Similarly, minor lesions of chronic interstitial pneumonia observed in all pigs could be undetected in mice because of known differences in lung disease development (Rogers et al., 2008). Larger size of the pigs allowed for an adequate description of the infection-related macroscopical changes during necropsy and might be beneficial during sample collection, especially in need of precise anatomical collection of individual small muscles and tissues. In this study the obvious advantage of the pig model was observed in the possibility of repeated weekly blood collection for the seroconversion monitoring which would not be otherwise possible in mice

models. Pig model is also deemed to be a more appropriate model for human immunological studies compared to mice (Dawson, 2011). Moreover, due to the longevity of pigs compared to mice, the possibility to study long-time effects of toxoplasmosis arises.

The genetic variation of *T. gondii* isolates collected from screening of toxoplasmosis in animals reflects the infection background and hereby indicates the potential composition of strains present in the human population. Type II strains were frequently identified as dominating in the Northern hemisphere (Fernández-Escobar et al., 2022; Shwab et al., 2014), hence the effects of this lineage have been relatively well-described through experimental pig infections with type I and II isolates (Algaba et al., 2018; Dubey et al., 1996). The hereby presented comparison of a type II and a type III strain but using isolates different to ours was partially explored in pigs only recently (Fernández-Escobar et al., 2021; Largo-de la Torre et al., 2022). A significant variability in virulence of the different *T. gondii* strains was shown across animal species (Calero-Bernal et al., 2022; Dubey et al., 2012). The present study thus presents an opportunity to fill the knowledge gap in the more complete view of the strain effect on the course of toxoplasmosis in animals and humans.

Previous studies exploring inter-strain differences in the disease outcome between type I and II isolates in swine reported earlier antibody production in infections with type II isolate (Jennes et al., 2017) and a higher parasite load in tissues (Algaba et al., 2018). This study showed a significantly higher probability of a tissue becoming positive with the tested type III isolate. An infection with the CZ-Šimková type III isolate in this study resulted significantly more positive tissues by qPCR when compared to an infection with the CZ-Tiger type II isolate (Table 10). However, no significant difference between the isolates was observed regarding the parasite load in tissues, despite the overall higher parasite load in pigs infected with the type III strain compared to the type II strain. This is in agreement with the previously observed higher parasite loads in tissues of animals infected with type III strains (Fernández-Escobar et al., 2021; Largo-de la Torre et al., 2022).

Among other factors, *T. gondii* infections can be influenced by the infective stage of the parasite (Dubey et al., 2020). By involving all four possible combinations of an infection with

type II (CZ-Tiger) or type III (CZ-Šimková) isolates and the two infective stages, oocysts and tissue cysts, we present a complete picture of the effects of these variables on *T. gondii* experimental infection in pigs while successfully demonstrating that pigs can be successfully infected with either *T. gondii* stage. An infection with tissue cysts in this study resulted in more than twice more positive tissues by qPCR when compared to an infection with oocysts (Table 10). The fact that the parasite burden in tissues of the oocyst-infected pigs in the current study was observed to be lower than in the tissue-cyst-infected counterparts suggest that clinical symptoms in pigs are independent on the number of tissue cysts developed in the tissues of the pig. However, the severity of clinical signs in the infection with type III oocysts in this study, resulting in premature euthanasia of two pigs, showed that clinical symptoms are parasite-stage-dependent. This observation is in agreement with the previously published results where oocyst-induced infections in animals were more often associated with more severe symptoms (Dubey and Beattie, 1988).

Comparison of the most frequently infected pig tissues and parasite burdens within is complicated by the variability of reported doses, isolates of different strains, and stages of *T. gondii*, which all have an effect on the outcome. Parasites could be recovered from a variety of tissues and the most frequently infected groups of pigs in this study contained the highest observable parasite load at the same time. Brain and heart were repeatedly mentioned as tissues with higher *T. gondii* parasite load (Algaba et al., 2018; Opsteegh et al., 2010; Verhelst et al., 2011), with lungs carrying higher loads of the parasite especially in the early stages of the infection (Algaba et al., 2018; Juránková et al., 2014a; Rahman et al., 2020). An interesting distribution pattern was observed in the tested organs of pigs included in this study which often tested negative for the presence of *T. gondii* DNA by qPCR, with the exception of brain. A similar outcome, was presented by Juránková et al. (2014a) who identified brain as a predilection site by MC-qPCR using the same type II (CZ-Tiger) isolate, however we did not observe high parasite loads in lungs, perhaps due to the lower inoculation dose we used. Alternatively, this might have been a result of the method used, as MC-qPCR was previously described as a more sensitive alternative to qPCR (Gisbert Algaba et al., 2017; Juránková et al., 2013a; Opsteegh et al., 2010). In contrast to the results of this study, high numbers of

parasites were described also in hearts of pigs experimentally infected with different type II and type III isolates (Largo-de la Torre et al., 2022) as well as with isolates of other lineages (Algaba et al., 2018; Jennes et al., 2017; Verhelst et al., 2011; Xia et al., 2020). Low positivity rates reported for liver and spleen are in concordance with the result presented in the current study. However, the relatively low prevalence and parasite load found in hearts of tested pigs does not correlate with the 100% positivity rates reported for the same isolate of type II (CZ-Tiger) (Juránková et al., 2014a). Moreover, liver and spleen were reported to carry less parasites which might be due to their unique structure rather than a shortcoming of the method, considering the two different methodological approaches applied. Overall, the tissues of the pigs included in this study that were infected with tissue cysts were significantly more frequently positive and contained on average significantly more parasites than tissues of animals infected with *T. gondii* oocysts. A different outcome observed by Algaba et al. (2018) in pigs, may have been caused by the different *T. gondii* isolate used for the inoculation or by the notably higher number of tissue cysts compared to oocysts. The result of this study correlates with the previously observed higher parasite burdens in tissue-cyst-infected definitive hosts (Dubey, 2022).

The current study presents an animal model highlighting the differences in the severity of clinical signs and distribution of the parasite within tissues of pigs experimentally infected with *T. gondii*, according to the combination of stages and strains of the parasite inoculated. An experimental infection with the type III isolate, and oocysts of this isolate in particular, caused a stronger antibody response in the pigs while leading to a higher parasite burden in the tissues, especially in pigs infected with tissue cysts. The observed variability in the virulence of *T. gondii* strains in pigs, a model species for human infections, and the significant effect of the type III (CZ-Šimková) isolate in this study, suggest the need for further investigation of type III isolates to better understand the potential risks to humans.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Marieke Opsteegh from the Dutch National Institute for Public Health and the Environment (RIVM) for the critical review of the article and her constructive remarks, and to the personnel of the Ruminant and Swine Clinic of the UVS Brno who were involved in the monitoring of the pigs within the present study.

FUNDING

This work is a part of TOXOSOURCES and ToxSauQMRA projects, funded by the European Union's Horizon 2020 Research and Innovation programme under grant agreement No. 773830: One Health European Joint Programme.

Chapter 5

Detailed anatomical distribution of *Toxoplasma gondii* in tissues of experimentally infected pigs: effects of infection sources

Filip Dámek [1], Sandra Thoumire [1], Arno Swart [2], Delphine Le Roux [1], Maxime Delsart [3], Sandra Vuillermet [1], Dominique Aubert [4], Isabelle Villena [4], Benoît Durand [5], Radu Blaga [1]

[1] Anses, INRAE, Ecole Nationale Vétérinaire d'Alfort, Laboratoire de Santé Animale, BIPAR, Maisons-Alfort, France

[2] Centre for Infectious Disease Control—Zoonoses and Environmental Microbiology, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, P.O. Box 1, 3720 BA Bilthoven, The Netherlands

[3] EPIMAI, USC Anses, Ecole Nationale Vétérinaire d'Alfort, Maisons-Alfort, France

[4] National Reference Center on Toxoplasmosis, Toxoplasma Biological Resources Center, CHU Reims and EA7510, SFR CAP-Santé, University of Reims Champagne-Ardenne, USC EpiToxo ANSES, France

[5] Université Paris-Est, Anses, Laboratoire de santé animale, Unité d'épidémiologie, 94700 Maisons-Alfort, France

Pre-submission manuscript

Consumption of raw or undercooked meat containing *Toxoplasma gondii* tissue cysts is a significant risk factor for acquiring toxoplasmosis. With the majority of human infections in Europe estimated to be meat-borne, pork as the most widely consumed meats globally plays an important role in potential transmission. Therefore, we aimed to investigate and evaluate the anatomical distribution of *T. gondii* within tissues of experimentally and naturally infected pigs, and to identify the predilection sites according to the different parasite stages.

A set of 36 muscles and 14 organs was collected from pigs experimentally infected with approximately 1000 oocysts (n=3) or 1000 tissue cysts (n=3), both belonging to the ME49 strain. Also, 31 muscles were obtained from naturally infected sows (n=2). The anatomical distribution of the parasite was investigated in the three groups of pigs by means of qPCR and the parasite load was then quantified.

A total of 38.2 % (138/361) tested positive for *T. gondii* DNA with the most frequently infected muscles found among head muscles, the most frequently infected organs were eyes (66.7 %, 4/6). Surprisingly, none of the brain samples tested positive. Overall, a non-homogenous parasite burden was observed across the tested tissues with the highest mean estimates among muscles in musculus longissimus dorsi at 1,678.1 parasites per gram (ppg) and in uterus at 3,976.1 ppg among organs.

The results showed an individual variability of the tissue positivity and *T. gondii* parasite counts in the tested tissues. Infection with oocysts resulted in significantly lower amount of positive tissues compared to naturally infected pigs, revealing an interesting distribution pattern according to the route of infection used and reflecting biological variability of different sources of infection.

INTRODUCTION

Toxoplasma gondii is a globally present foodborne parasite of importance to both human and animal health (Dubey, 2010). Practically all warm-blooded animals may act as intermediate hosts and get infected by various infection routes, developing subsequently tissue cysts in their tissues (Tenter et al., 2000). Among the various horizontal and vertical transmission pathways, two are considered to be the most prevalent. The host may get infected by ingesting sporulated *T. gondii* oocysts, shed in the environment by the definite hosts, felids, or through ingestion of meat of raw or undercooked tissues of infected animals containing viable parasites (Tenter et al., 2000).

The meat-borne infection route appears to be the most common for humans in Europe (Cook, 2000). Correspondingly, pork was recognized as an important source of predicted *T. gondii* infections in humans (Belluco et al., 2018; Deng et al., 2020; Opsteegh et al., 2011a).

Pork is one of the most consumed meats worldwide, and was leading the meat consumption preferences in Europe and Asia between the years 2019 and 2021 (OECD and Food and Agriculture Organization of the United Nations, 2022). With the 34.5 percent of the global meat production share, the pork meat output was estimated to reach 122.5 million tonnes in 2021, an increase of 11.5 percent in a single year (FAO, 2022). Since pork and pork products worldwide were reported to harbour *T. gondii* parasites (Dubey et al., 2020), the European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ) identified *T. gondii* as one of the most relevant biological hazards in the context of meat inspection of swine (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2018).

Despite the majority of *T. gondii* infections in animals and humans alike being subclinical, swollen lymph nodes, fever-like symptoms, and muscle aches may be observed. In some cases, toxoplasmosis can manifest with severe cases of retinochoriditis, abortion or stillbirth, abnormalities in the brain development in foetuses, and was even reported to lead to death, especially in immunocompromised people (Gilbert et al., 1999; Hampton, 2015; Luft and Remington, 1992). The major impact of this parasite on human health is reflected in the consistent high ranking of *T. gondii* among the top-priority food-borne in multicriteria-based ranking for risk management of food-borne parasites at both the global (4th out of 24 food-

borne parasites) (Boireau et al., 2014) and European levels (2nd out of 25 food-borne parasites) (Bouwknegt et al., 2018).

The rich genetic background of *T. gondii* comprises numerous variants of the parasite, however a majority of the isolates in the Northern hemisphere belong to three distinct clonal lineages, Type I, II, and III (Saeij et al., 2005). Various levels of pathogenicity are associated with different lineages and even isolate within, varying also by the host species (Fernández-Escobar et al., 2020; Saeij et al., 2005; Sánchez-Sánchez et al., 2018). Type II strains are reported to dominate in European populations (Shwab et al., 2014).

With the aim of filling the knowledge gap regarding the tropism of *T. gondii* in pig tissues, we explored the detailed anatomical distribution of *T. gondii* parasites in tissues of pigs infected naturally and experimentally, with tissue cysts and oocysts. The comparison of the detectable parasite burden in tissues of tested animals contributed to the deeper understanding of the biology behind the distribution patterns of this *T. gondii* isolate according to the different sources of infection.

MATERIALS AND METHODS

The methodology and materials used in current study are detailed in Chapter 7, with a brief summary provided below.

Toxoplasma gondii parasites

T. gondii oocysts of ME49 strain (Type II, ToxoDB #3) were provided by Dr. JP Dubey (USDA, ARS, Beltsville, USA) and were six months old at the time of inoculation of pigs. Oocysts were counted under microscope and were stored in a 2% H2SO4 solution, at 4 °C until use.

T. gondii tissue cysts of ME49 strain (Type II, ToxoDB #3) were obtained from brain tissues of mice, infected with the previously mentioned *T. gondii* oocysts. Mice brains were pooled, gently homogenized and diluted in PBS before being counted under microscope.

The infectivity of both the oocysts and tissue cysts was confirmed by mouse bioassay and subsequent serological screening.

Pigs and experimental infection

Seven female pigs of Large White breed, aged 97 days, tested negative for the presence *T. gondii* antibodies by modified agglutination test (MAT) and were transported to the Biomedical Research Center of Ecole Nationale Vétérianire d'Alfort, Maisons-Alfort, France (EnvA) for an experimental infection with *T. gondii*. The seven pigs were divided into three groups and kept in separate pens. Three pigs (O1, O2, O3) were orally inoculated with a dose of ~10^3 *T. gondii* sporulated oocysts per animal in a bite-size bolus. Another three pigs (T1, T2, T3) were orally inoculated with a dose of 10^3 *T. gondii* tissue cysts per animal using a single bolus. Finally, one pig (C1) received a non-infected bolus. All animals were kept under regular veterinary supervision with daily measurements of their rectal temperatures. The seroconversion of the experimentally inoculated pigs was monitored twice a week for the first 2 weeks post-inoculation and on a weekly basis afterwards using an in-house modified agglutination test (data not shown).

Additionally, two adult sows of the same Large White breed were purchased from a slaughterhouse based on a positive result for *T. gondii*-specific antibodies by ELISA ID Screen Toxoplasmosis Indirect Multi-Species (ID Vet, France), according to the manufacturer's instructions, and their carcasses without heads and organs were transported to EnvA for the collection of remaining tissue samples.

All animal experimental infections were approved by the local Ethical Committee for animal experiments of Anses/EnvA/UPEC (APAFIS N° 14363-2018032908554996v3).

Sample collection

Fourteen organs (brain, eyes, tongue, oesophagus, stomach, small intestine, large intestine, liver, spleen, heart, lungs, kidneys, ovaries, and uterus) and thirty-six muscles from head, body, thoracic limb and pelvic limb (Table 13) were sampled 58 days post-inoculation (p.i.) from three oocyst-inoculated pigs (O1, O2, O3) aged 155 days, and 68 days p.i. from three pigs experimentally inoculated with *T. gondii* tissue cysts (T1, T2, T3) aged 278 days (see the age difference explanation in Chapter 7). In addition, thirty-one muscles from body, thoracic limb and pelvic limb (Table 13) were sampled from the two naturally infected pigs (N1, N2). All the tissues were stored at -20°C until artificial digestion.

Tissue	Tissue group	Tested infection routes
musculus sternocephalicus	body	TC + OOC + NI
musculus brachiocephalicus	body	TC + OOC + NI
musculus trapezius	body	TC + OOC + NI
musculus latissimus dorsi	body	TC + OOC + NI
musculus longissimus	body	TC + OOC + NI
musculus obliquus externus abdominis	body	TC + OOC + NI
musculus pectoralis profundus	body	TC + OOC + NI
musculus serratus ventralis thoracis	body	TC + OOC + NI
diaphragm	body	TC + OOC + NI
brain	brain	TC + OOC
oesophagus	digestive system	TC + OOC
stomach	digestive system	TC + OOC
small intestine	digestive system	TC + OOC
large intestine	digestive system	TC + OOC
liver	digestive system	TC + OOC
eyes	eyes	TC + OOC
musculus digastricus	head	TC + OOC
musculus buccinator	head	TC + OOC
musculus temporalis	head	TC + OOC
musculus masseter	head	TC + OOC
musculus pterygoideus medialis	head	TC + OOC
heart	heart	TC + OOC
kidney	kidneys	TC + OOC
lungs	lungs	TC + OOC
musculus gracilis	pelvic limb	TC + OOC + NI
musculus semimebranosus	pelvic limb	TC + OOC + NI
musculus flexor digitorum superficialis	pelvic limb	TC + OOC + NI
musculus gastrocnemius caput lateralis	pelvic limb	TC + OOC + NI
musculus gluteus medius	pelvic limb	TC + OOC + NI
musculus semitendinosus	pelvic limb	TC + OOC + NI
musculus pectineus	pelvic limb	TC + OOC + NI
musculus adductor	pelvic limb	TC + OOC + NI
musculus sartorius	pelvic limb	TC + OOC + NI
musculus tensor fasciae latae	pelvic limb	TC + OOC + NI
musculus extenson digitorum brevis	pelvic limb	TC + OOC + NI
musculus psoas mayor	pelvic limb	TC + OOC + NI
musculus biceps femoris	pelvic limb	TC + OOC + NI
musculus quadriceps femoris	pelvic limb	TC + OOC + NI
musculus obturatorius externus	pelvic limb	TC + OOC + NI
ovaries	reproductive system	TC + OOC
uterus	reproductive system	TC + OOC
spleen	spleen	TC + OOC
musculus flexor digitorum superficialis	thoracic limb	TC + OOC + NI
musculus subscapularis	thoracic limb	TC + OOC + NI
musculus infraspinatus	thoracic limb	TC + OOC + NI
musculus biceps brachii	thoracic limb	TC + OOC + NI
musculus triceps brachii caput lateralis	thoracic limb	TC + OOC + NI
musculus supraspinatus	thoracic limb	TC + OOC + NI
musculus extensor digitorum lateralis	thoracic limb	TC + OOC + NI
tongue	tongue	TC + OOC

Artificial trypsin digestion

Two hundred grams of collected tissues, or the whole tissue in case of a smaller sample, were collected from the six experimentally infected pigs and two naturally infected pigs. Tissue samples were each blended and incubated in trypsin solution (Trypsin (1:250), powder, Gibco™, Scotland, final concentration of 4g/L) for 90 minutes at 37°C on a shaking plate. Following the artificial digestion, the mixture was filtered through a double layer of gauze, transferred to 50 mL centrifuge tubes and centrifuged at 1800 g for 10 minutes. The obtained pellet was washed twice of leftover trypsin using a saline solution (0,9% NaCl, Sigma-Aldrich) and stored at - 20°C until DNA extraction.

DNA extraction and ethanol precipitation

Eighty milligrams of the pellet suspension were used to extract genomic DNA using NucleoSpin DNA RapidLyse kit (Macherey-Nagel, France), according to the manufacturer's instructions. The extracted DNA was desalted and concentrated using ethanol precipitation (Zeugin and Hartley, 1985) and resuspended in 30 µL of Milli-Q water prior to the qPCR analysis.

Molecular detection of *T. gondii* DNA

The detection and quantification of *T. gondii* DNA was performed by amplification of a sequence within the 529bp repetitive element, according to Opsteegh et al. 2010 with minor modifications described in the protocol in Chapter 7, using LightCycler® 480 System 96-plate thermocycler (Roche, Germany).

Absolute quantification of parasites

Five µL of each serial dilution of the standard curve were included on each qPCR plate. The qPCR results were analysed using LightCycler® 480 System software, Version 1.2.9.11 (Roche, Switzerland). The starting quantity of parasites per qPCR reaction (n) was estimated from the mean Cq values, obtained from duplicates of each sample, using a regression line from the ten-fold dilutions of a standard curve as shown in Eq. 1. Negative reactions were not considered in the calculation of the Cq mean.

$$n = \frac{Cq\ mean - interc}{slope} \quad (1)$$

For the calculation of the final parasite load (N), the original weight of the sample (m_{sample}), the final weight of the digested pellet (m_{pellet}), the proportion of the pellet used for DNA

extraction, and the proportion of the DNA extract volume used in the subsequent qPCR reaction were taken into account. The final result (*N*) based on Eq. 2 was expressed as a number of parasites per gram of tissue (ppg).

$$N = \frac{n * \frac{30}{5} * \left(\frac{m\ pellet}{0.08}\right) * \left(\frac{m\ sample}{m\ pellet}\right)}{m\ sample} \quad (2)$$

Statistical analysis

Two statistical analyses of the data were performed. In the first analysis, a binomial regression was fitted to predict the presence or absence of *T. gondii* DNA in tissues of each tissue groups (for division of tissues into tissue groups see Table 13), with infection route as a fixed effect, and individual pig and tissue group as random effects. The predicted effect of infection route on the frequency of positive tissues was expressed as odd ratio (OR) with 95% confidential interval (CI). Negative binomial distribution was selected in order to account for over-dispersion.

In the second part of the analysis, a mixed linear model was used to estimate the effect of the infection route on parasite load in the previously predicted positive tissues (parasite load > 0) in tissue groups. The relationship between the infection route and parasite burden in the tissues was expressed as a log10-transformed coefficient estimate with the standard error. The random effects were not included in the prediction and the effect of a variable was considered significant if the p-value was less than 0.05.

Statistical analyses were performed using RStudio (Team, 2009), R version 4.2.2 (2022-10-31 ucrt), using the following packages: ggplot2 (Wickham, 2016), tidyverse (Wickham et al., 2019), lmerTest (Kuznetsova et al., 2017), readxl (Wickham and Bryan, 2022), scales (Wickham and Seidel, 2022), and lme4 (Bates et al., n.d.).

RESULTS

Clinical observations

All pigs experimentally inoculated seroconverted between the first and second week p.i. Additionally, some of the animals, especially the pigs infected with *T. gondii* tissue cysts, showed signs of inappetence, fatigue and intermittent fever during this period.

Real time qPCR

Despite *T. gondii* DNA presence in tissues of all sampled pig, none of the pigs was positive in all its tested tissues. Specifically, a total of 38.2 % (138/361) of tissues tested positive in the molecular analysis. The most frequently affected muscles were two head muscles at 83.3 % (5/6) musculus (m.) pterygoideus medialis and m. temporalis, both belonging to the group of head muscles. Among organs tested, the most frequently positive were eyes at 66.7 % [4/6]. In contrast, no positive sample was found in either m. semitendinosus, kidneys, brain, small intestine, or stomach (see Supplementary data).

Multivariable analysis of all data showed no significant effect of infection route on the frequency of positive tissues in pigs (Table 14, Figure 15). Although not significant at 0.05 level of confidence, experimental oocysts infection in pigs resulted in lower frequency of positive tissues (OR 0.40, 95% CI: 0.12-1.37) compared to the natural infection. Conversely, experimental tissue cyst infection in pigs showed higher frequency of positive tissues (OR 1.65, 95% CI: 0.49-5.57) than natural infection.

Table 13 Odds ratios for infection route (natural or experimental, via ingestion of oocysts or tissue cysts) as a risk factor for presence/absence of *T. gondii*-positive tissues in tissue groups (see Table S1 for the definition of tissue groups). Binomial regression model outcome are presented as odds ratios with 95% confidential intervals (CI).

Variable	Value	Odds ratio (95% CI)	*p*-value
	intercept	0.50 (0.16 - 1.59)	0.242
Infection route	natural (reference)		
	oocyst	0.40 (0.12 - 1.37)	0.146
	tissue cyst	1.65 (0.49 - 5.57)	0.422

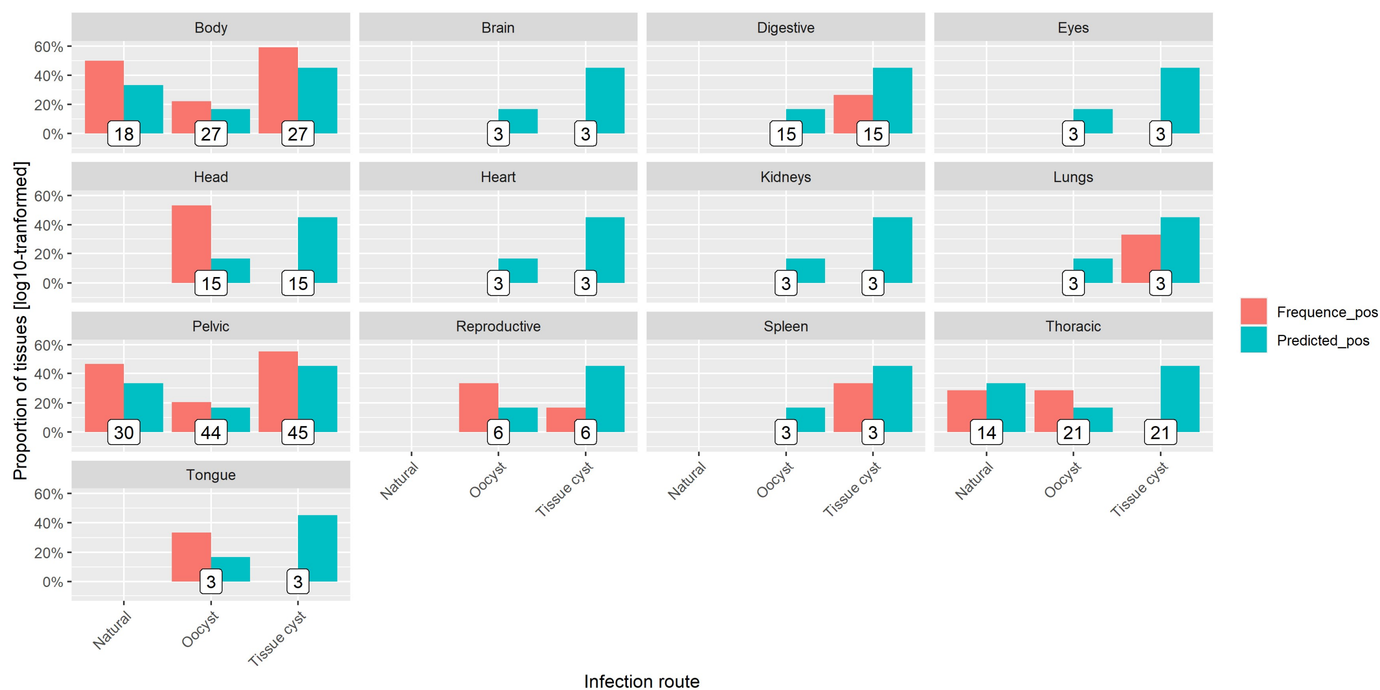

Figure 15 Proportions of positive tissues in each tissue group (red columns) were compared to the predited values (blue columns). Numbers at the bottom of each column stand for the number of samples tested within the corresponding tissue group and infection route. Tissues are represented in separate boxes, with infection route on the x-axis. The proportion of postitve findings to the total number of samples is visualized on y-axis in percentage. Freq_pos = frequency of positive samples, Pred_pos = predicted frequency of positive outcomes.

Parasite burden estimates

No significant effect of infection route on *T. gondii* parasite load in the tissues was observed (Table 15). Alltogether, oocyst infection in the experimentally infected pigs averaged in approximately 0.6 (0.49 log10-units) times the parasite load found in the tissues of naturally infected pigs. Tissues of the tissue cyst-infected pigs, on the other hand, contained on average moderately higher parasite load, 1.65 log10-units (approximately 5.2) times the burden of tissues of naturally infected pigs (Table 15). The observed *T. gondii* parasite burden estimates, grouped by infection routes, were visualized for each of the tissue groups, as shown in Fig. 16.

Table 14 Coefficient estimates with standard errors for each of the infection routes (natural or experimental, via ingestion of oocysts or tissue cysts) in log-transformed parasite load in *T. gondii*-positive tissues. Bold are significant effects at the confidential interval (CI).

Variable	Fixed effects	Estimate (log10-transformed)	CI 95%	Standard error	*p*-value
	intercept	**2.66**	**2.20 – 3.13**	**0.25**	**< 0.001**
Infection route	natural (reference)				
	oocyst	-0.49	-0.95 - -0.02	0.25	0.090
	tissue cyst	0.20	-0.23 – 0.63	0.23	0.432

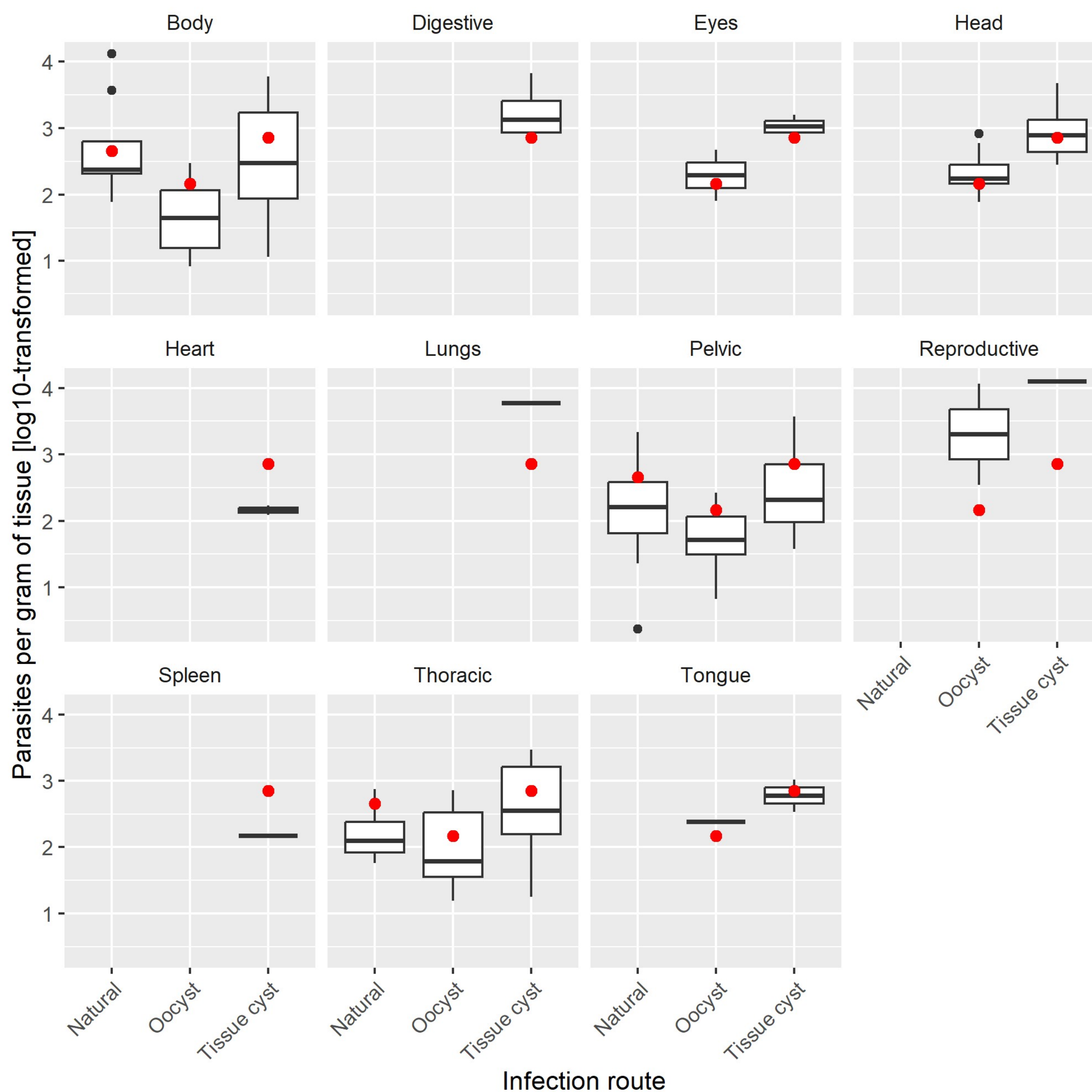

Figure 16 Log10-transformed parasite loads in positive tissues, presented by tissue groups. Infection route is showed on x-axis (infection routes: natural and experimental through oocysts or tissue cysts). Parasite load is presented on y-axis as log10-transformed number of *T. gondii* parasites per gram of tissue. No *T. gondii* DNA was detected in brain and kidneys. Boxes represent the main body of the boxplot showing the quartiles and the median's confidence intervals, with a median value represented by a horizontal line, vertival lines ("whiskers") represent spread of sample parasite burdens within the interquartile range (IQR = third quartile – first quartile). Dots represent predicted modelled outcomes.

The overall observed mean parasite load in tested organs varied from 0 to 12,391 ppg, with the highest mean parasite burden estimated for uterus. Since the qPCR of brain, stomach, small intestine, and kidneys did not reveal any *T. gondii* DNA, the lowest average detectable parasite load was measured in the spleen, with a mean value of 24.4 ppg. The highest *T. gondii*

parasite count in an individual sample was observed in heart and liver of pig T1, both equal to an equivalent of 8.54 ppg (Table 16).

Table 15 Ranking of the tested organ tissues based on the observed parasite load. The results are represented as a number of *T. gondii* parasites per gram of tissue (ppg). ND = not detected, NA = not available.

	Oocyst infection			Tissue cyst infection			
Organ	**O1**	**O2**	**O3**	**T1**	**T2**	**T3**	**Arithmetic mean [min-max]**
uterus	11,465.7	ND	ND	ND	12,391.0	ND	3,976.1 (0.0-1,239.1)
large intestine	ND	ND	ND	ND	6,768.3	1,876.4	1440.8 (0.0-6,768.3)
lungs	ND	ND	ND	5,847.0	ND	ND	974.5 (0.0-5,847.0)
eyes	ND	81.1	471.8	701.9	ND	1,591.9	474.5 (0.0-1,591.9)
tongue	ND	ND	242.2	1,045.5	ND	346.0	272.3 (0.0-1,045.5)
liver	ND	ND	ND	944.9	ND	ND	157.5 (0.0-944.9)
oesophagus	ND	ND	ND	ND	638.9	ND	106.5 (0.0-638.9)
ovaries	ND	351.5	ND	ND	ND	ND	58.6 (0.0-351.5)
heart	ND	ND	ND	122.7	ND	171.8	49.1 (0.0-171.8)
spleen	ND	ND	ND	146.3	ND	ND	24.4 (0.0-146.3)
brain	ND	ND	ND	ND	ND	ND	0.0 (0.0-0.0)
stomach	ND	ND	ND	ND	ND	ND	0.0 (0.0-0.0)
small intestine	ND	ND	ND	ND	ND	ND	0.0 (0.0-0.0)
kidney	ND	ND	ND	ND	ND	ND	0.0 (0.0-0.0)
Arithmetic mean [min-max]	818.9 (0.0-11,465.7)	30.9 (0.0-351.5)	51.0 (0.0-471.8)	629.2 (0.0-5,847.0)	1,414.2 (0.0-12,391.0)	284.7 (0.0-1,876.4)	538.2 (0.0-12,391.0)

Accordingly, we assessed the overall mean parasite load within muscles, resulting in a range of 0 to 13,119.2 ppg. The highest *T. gondii* parasite count in an individual sample was observed in m. longissimus of pig N2, this muscle contained at the same time the highest average mean parasite burden among muscles. The only muscle tissue without a detectable presence of *T. gondii* DNA was m. semitendinosus (Table 17).

Table 16 Ranking of the tested muscle tissues based on the observed parasite load. The results are represented as a number of T. gondii parasites per gram of tissue (ppg). ND = not detected, NA = not available, m. = musculus.

	Oocyst infection			Tissue cyst infection			Natural infection		
Muscle	O1	O2	O3	T1	T2	T3	N1	N2	Arithmetic mean
m. longissimus	55.1	8.4	ND	11.6	ND	ND	230.6	13,119.2	1,678.1
m. masseter	824.1	ND	162.1	912.5	ND	4,729.2	NA	NA	1104.6
diaphragm	ND	ND	149.0	1,638.8	322.8	2,630.7	ND	3724.9	1058.3
m. temporalis	ND	78.1	159.4	3,155.9	1419.2	390.4	NA	NA	867.2
m. pectoralis profundus	ND	ND	ND	609.4	ND	5,945.3	ND	238.5	849.2
m. infraspinatus	730.3	ND	ND	21.0	1178.7	2,969.3	ND	ND	612.4
m. quadriceps femoris	ND	ND	16.2	106.8	608.0	3,684.3	456.4	2.3	609.3
m. sternocephalicus	ND	ND	ND	89.2	ND	3,683.8	ND	116.5	486.2
m. pectineus	ND	ND	ND	2,069.3	ND	ND	58.36	1,083.8	401.4
m. triceps brachii cpt. lat.	ND	ND	ND	2,364.9	113.3	ND	ND	164.3	330.3
m. serratus ventr. thorac.	11.8	ND	ND	1,940.2	ND	51.1	ND	626.3	328.7
m. psoas mayor	ND	ND	ND	89.8	38.4	207.2	22.6	2,178.5	317.1
m. pterygoideus med.	ND	111.5	221.5	608.5	298.7	656.9	NA	NA	316.2
m. subscapularis	ND	51.9	ND	155.6	353.8	1,913.8	ND	ND	309.4
m. gluteus med.	ND	ND	51.9	65.4	ND	1,521.2	91.9	403.5	266.7
m. adductor	ND	ND	ND	918.6	ND	710.2	191.7	ND	227.6
m. biceps brachii	71.1	ND	31.1	17.6	ND	1,645.9	ND	ND	220.7
m. buccinator	ND	ND	184.1	1,119.0	ND	ND	NA	NA	217.2
m. tensor fasciae latae	ND	ND	ND	ND	ND	1,547.8	ND	ND	193.5
m. obturator extern.	ND	ND	268.0	109.0	ND	1,084.4	ND	ND	182.7
m. supraspinatus	ND	ND	15.5	ND	ND	1,349.8	ND	57.1	177.8
m. biceps femoris	ND	ND	ND	581.1	ND	710.2	ND	110.9	175.3
m. ext. digit. lat.	561.2	ND	ND	ND	ND	ND	ND	745.4	164.4
m. digastricus	594.8	ND	ND	279.9	ND	ND	NA	NA	145.8
m. brachiocephalicus	300.1	ND	ND	276.1	ND	183.2	ND	208.7	121.0
m. trapezius	ND	ND	ND	940.9	ND	ND	ND	ND	117.6
m. flexor dig. superf.	ND	ND	ND	96.3	ND	582.5	217.8	ND	112.1
m. flexor dig. brevis	ND	ND	ND	307.3	ND	263.2	ND	93.7	83.0
m. latissimus dorsi	ND	35.6	ND	178.7	ND	64.5	ND	350.5	78.7
m. ext. digit. brevis	ND	ND	216.9	262.6	61.9	ND	ND	34.3	71.9
m. sartorius	ND	ND	ND	ND	ND	125.5	ND	332.8	57.3
m. gastrocnemius cpt. lat.	30.8	84.6	117.2	180.4	37.1	ND	ND	ND	56.3
m. gracilis	6.7	NA	ND	42.9	132.9	ND	ND	ND	26.1
m. semimebranosus	ND	ND	43.3	ND	ND	ND	137.8	ND	22.6
m. obliquus ext. abdom.	ND	ND	ND	ND	83.8	ND	ND	76.9	20.1
m. semitendinosus	ND	ND	ND	ND	ND	ND	ND	ND	ND
Arithmetic mean	88.5	10.6	45.4	531.9	129.1	1,018.1	45.4	763.8	327.5

DISCUSSION

T. gondii infections are common in pigs (Dubey, 2022). With pork being undoubtedly one of the most consumed meats in the world, toxoplasmosis in this animal species plays an important role in the risk of human *T. gondii* infections. Traditionally, some pork products such as certain types of dry hams, sausages, and meat spreads are made from raw pork meat. Considering the meat-borne transmission of *T. gondii*, the undercooked meat and organs of infected pigs may pose a health risk to the consumers, as was acknowledged in multiple studies focusing on the risk from pork (Belluco et al., 2018; Condoleo et al., 2018; Crotta et al., 2017; Guo et al., 2016).

In agreement with the previous pig infection models (reviewed by Dubey, 2022), we confirmed that pigs can be infected with both *T. gondii* oocysts and tissue cysts, and that the parasite can be subsequently detected in various tissues of the pig body. The results of this study suggest an individual variability of *T. gondii* distribution in tested pigs with non-homogenous parasite load across the tested tissues.

The potential effect of the clonal lineage of *T. gondii* was eliminated by design of this experiment including the inoculation of pigs with the same isolate (ME49, Type II, ToxoDB#3). The effect of the dose on the other hand might have played some role as the inoculation dose was uneven for the two groups of pigs – approximately 8,000 sporozoites compared to a minimum of 194,000 bradyzoites contained in the target amount of 1,000 *T. gondii* oocysts and tissue cysts respectively, based on the estimates from a mouse model (Watts et al., 2015). Probability of a successful *T. gondii* infection generally increases with the dose, as was shown for animals (Bonačić Marinović et al., 2020), although, the exact effect of dose is not yet fully explored in pigs as lower doses may result in higher final parasite load in tissues (Jennes et al., 2017). Past studies suggested an effect of the infection route as the oocyst-induced infections were more often associated with more serious clinical symptoms in animals (Dubey and Beattie, 1988). Contrary to this statement, it was the tissue-cyst-induced infection in the present study that led to a more severe response of the pigs, manifesting with higher antibody production and rectal higher temperatures during the acute parasitemia (see Chapter 7). This effect however might have been influenced also by the mentioned parasite count disproportion in inoculate. Similarly, the oocyst infection of the pigs led to a significantly lower

number of positive tissues, although no significant effect of infection route was observed regarding the parasite load in pig tissues. More data, exploring the effect of the infection route on the parasite load in tissues, is needed in order to prove the hypothesis as Algaba et al. (2018) reported a higher parasite load in oocyst-infected pigs.

It is generally accepted that *T. gondii* has an affinity towards the neural tissues and skeletal muscles (Dubey, 1998). Accordingly, brain and heart have been repeatedly mentioned as a predilection site for *T. gondii* (Algaba et al., 2018; Jennes et al., 2017; Opsteegh et al., 2010; Verhelst et al., 2011). In this study, we did not observe these distribution patterns as brain remained one of the few tissues without a detectable presence of *T. gondii*. On the other hand, eyes, essentially a neural tissue, were identified as the most frequently positive organ. Eyes are rarely sampled and tested for the presence of *T. gondii*, yet the parasite was previously found in eyes of pigs (Garcia et al., 2017a), and is currently considered the most common cause of retinal infection in humans (Holland, 2003). The high frequency of the positive findings in the current experiment can be traced to some organs, especially uterus. Generally, organs contained high parasite loads yet were not consistently positive in more tested animals. An exception in this regard could are the eyes, where the presence of the parasite can be linked to the affinity of *T. gondii* towards retina and other neural tissues (Dubey, 2022). Surprisingly, another neural tissue, and a prevised predilection site of *T.* gondii, brain, did not harbour detectable parasites. Relatively high concentration of *T. gondii* tissue cysts in muscles of head and body can be explained by the affinity of *T. gondii* towards skeletal muscles or possibly the frequent use of these muscles during mastication and breathing, and related increased blood flow. The variable presence of *T. gondii* DNA within pig organs was not surprising as tissue cysts were not often retrieved from organs of pigs, with an exception of brain and heart, and occasionally lungs during an acute phase of the infection (Algaba et al., 2018; Juránková et al., 2014a). Although not proven, the lower prevalence of *T. gondii* DNA observed in organs might be due to a potentially poorer performance of the trypsin digestion of these tissues, compared to proteinase-K (Dau et al., 2020). Moreover, stomach and intestines contain higher contents of connective tissue compared to muscle or neural tissue, and might therefore be more resistant to the enzymatic digestion with trypsin. Works of other authors proved also a higher sensitivity of detection methods like magnetic capture qPCR (MC-

qPCR) (Algaba et al., 2018; Juránková et al., 2014a; Opsteegh et al., 2010). Possibly a similar effect of a DNA extraction efficiency was noted in hearts of the pigs of the current study with only two out of six positive, compared to five of the same hearts being positive for *T. gondii* DNA when tested by MC-qPCR in parallel (Chapter 7). Interestingly, the average parasite load in positive samples obtained through both of these methods was very similar, mean of 198.6 parasites for MC-qPCR and 147.25 parasites in this experiment. For the improved and more specific capture of *T. gondii* DNA, we propose MC-qPCR as a more effective alternative, especially for the detection of *T. gondii* in organs. Statistical analyses did not reveal a significant effect of infection route on frequency of positive tissues or parasite load in tissues. Nevertheless, the experimental infection of pigs with oocysts in this study resulted in lower frequency of positive tissues and lower parasite burden compared to both the experimental infection with tissue cysts and natural infection. In case we consider an effect of inoculation dose on the final parasite load in tissues, this outcome could be explained by the higher inoculation dose in tissue cyst-infected pigs compared to their oocyst-infected counterparts. Consequently, we could argue that the infection dose in the two naturally infected pigs included in this study was caused by ingestion of a similar dose to that of tissue cyst-infected pigs.

In conclusion, this study describes the to this date largest and most detailed overview of *T. gondii* distribution and parasite burden quantification in pig tissues. By using the different parasite stages originating from the same *T. gondii* isolate, we aimed to offer a unique comparison of the effect of these routes on the distribution patterns in pig tissues. While filling one of the knowledge gaps in the newly proposed pig model for human *T. gondii* infections, we would like to stress the importance of further quantitative measurements of the parasite burden in pig tissues. Quantifying *T. gondii* in pork helps to determine the potential risk of meat-borne illness caused by the parasite. Knowing the levels of *T. gondii* in pork products allows for the implementation of effective food safety measures to minimize the risk of infection and ensure that infected meat and meat products do not reach consumers. This information also helps to monitor the efficacy of control measures, such as those used in animal husbandry and meat processing, to reduce *T. gondii* contamination in pork.

Acknowledgements

The authors are thankful to all personnel of the Biomedical Research Centre of Maisons-Alfort, EnvA, who were involved in monitoring the pigs within the present study, and Dr. JP Dubey (USDA, ARS, Beltsville, USA) for providing *T. gondii* oocysts used in the experimental infection.

Funding

This work was supported by funding from the France-Agri-Mer: contract number 0917003490 and the European Union's Horizon 2020 Research and Innovation programme under grant agreement No 773830: One Health European Joint Programme (projects ToxSauQMRA and TOXOSOURCES).

Chapter 6

Detection of *Toxoplasma gondii* in retail meat samples in Scotland

Jacqueline Plaza [a,1], Filip Dámek [a], Isabelle Villena [b], Elisabeth. A. Innes [a], Frank Katzer [a] and Clare M. Hamilton [a,*]

[a] Moredun Research Institute, Pentlands Science Park, Midlothian EH26 0PZ, UK.
[b] Laboratory of Parasitology, EA 7510, University of Reims Champagne-Ardenne and National Reference Centre on Toxoplasmosis, Hospital University Centre of Reims, France.
[*] Corresponding Author

[1] Current address: Instituto de Parasitología, Facultad de Medicina, Universidad Austral de Chile, Isla Teja S/N, Valdivia. Región de los Ríos, Código postal 5090000, Chile.

Short communication published in Food and Waterborne Parasitology

Volume 20, September 2020, e00086, https://doi.org/10.1016/j.fawpar.2020.e00086

Supplementary data can be found at https://ars.els-cdn.com/content/image/1-s2.0-S2405676620300159-mmc1.docx

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Toxoplasma gondii is a globally important zoonotic parasite ranked as one of the most significant causes of disease burden amongst the major foodborne pathogens. Consumption of undercooked meat is a well-known risk factor for infection so the aim of this study was to investigate the presence of *T. gondii* in meat samples from retail outlets in Scotland. In Sampling Period 1, 300 meat samples (39 beef, 21 chicken, 87 lamb, 71 pork and 82 venison) were purchased from butchers', farmers' markets, farm shops and supermarkets, and in Sampling Period 2, 67 pure venison samples only were purchased from farmers' markets, farm shops and supermarkets. DNA was extracted and screened for *T. gondii* using a quantitative PCR targeting the 529bp repeat element, and any positive samples were genotyped using PCR-RFLP targeting 10 markers. Meat juice was screened for *T. gondii* antibodies using a commercial ELISA or modified agglutination assay. *Toxoplasma gondii* DNA was detected in 0/39 (0%) beef samples, 1/21 (4.8%) chicken samples, 6/87 (6.9%) lamb samples, 3/71 (4.2%) pork samples and 29/82 (35.4%; Sampling Period 1) and 19/67 (28.4%; Sampling Period 2) venison samples. Partial PCR-RFLP genotyping revealed both clonal and non-clonal genotypes. Antibodies to *T. gondii* were detected in the meat juice of 2/38 (5.3%) beef samples, 3/21 (14.3%) chicken samples, 14/85 (16.5%) lamb samples, 2/68 (2.9%) pork samples and 11/78 (14.1%; Sampling Period 1) and 8/50 (16%; Sampling Period 2) venison samples. This is the first study to report the presence of *T. gondii* in retail meat products in Scotland and has highlighted venison as a potentially high risk meat. Further work is required to determine viability of parasites in this particular meat product.

INTRODUCTION

Toxoplasma gondii is a zoonotic parasite of global importance. Humans can become infected with the parasite via ingestion of oocysts (shed in cat faeces) directly from the soil or in contaminated food or water; via ingestion of tissue cysts in undercooked/raw infected meat; or vertically from mother to baby during a primary infection in pregnancy. Symptoms of toxoplasmosis in immune competent people are mostly mild and self-limiting; however, immune compromised people can suffer severe or life-threatening disease and acute infection during pregnancy may result in miscarriage or devastating congenital defects (Dubey, 2010).

In the UK, an average of 365 cases of toxoplasmosis are clinically diagnosed in England and Wales each year and an average of 33 cases are clinically diagnosed annually in Scotland (Halsby et al., 2014; HPS, 2019). However, given the lack of pathognomonic signs in the majority of infections and the fact that the disease is not notifiable, these figures are likely to be a vast underestimate of the true incidence of toxoplasmosis. In a recent study of over 1,400 blood donors in Scotland, 13.2% had antibodies to *T. gondii* (Burrells et al., 2016). In the same study, *T. gondii* DNA was also detected in 17.9% of brain tissue deposited at the Medical Research Council Sudden Death Brain Bank. In both study groups, prevalence of *T. gondii* increased with age suggesting an important role for acquired infection.

Foodborne transmission of *T. gondii* is an important route of infection and consumption of undercooked/raw meat is known to be a significant risk factor (Belluco et al., 2018). Due to the severe sequealae of infection which may persist for the lifetime of the host, the disease burden of toxoplasmosis can be high (Scallan et al., 2015). Recent studies in the USA and the Netherlands ranked *T. gondii* as the second and third highest cause of disease burden among the major foodborne pathogens, respectively (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2018; Scallan et al., 2015). Despite bodies such as the Food Standards Agency and European Food Safety Authority highlighting the need for identifying the role of different meat products in foodborne toxoplasmosis, there have been few studies examining the presence of *T. gondii* in commercial meat products (Dubey et al., 2005; Warnekulasuriya, 1998).

The aim of the present work, therefore, was to investigate the presence of *T. gondii* in different meat products available for human consumption from retail outlets in Scotland.

MATERIALS AND METHODS

Sample collection

Convenience meat samples were collected over two periods. Initially, 300 meat samples (39 beef, 21 chicken, 87 lamb, 71 pork and 82 venison) were purchased from different retail outlets between April and November 2017 (Sampling Period 1; Table 18). Of the 300 samples, 31 were purchased from butchers' shops, 163 were purchased from farmers' markets or farm shops, and 106 were purchased from supermarkets. All samples were purchased fresh (not frozen) and were pre-packaged except those purchased at butcher shops which were packaged upon purchase. Different cuts of meat were purchased for each meat type on a convenience basis (Table 18).

Table 17 Detection of *T. gondii* in different cuts of meat sampled.

Meat Type	Cut of meat	No. purchased
Sampling Period 1:		
Beef	Ground meat[a] (sausages, minced, burgers, meatballs)	24
	Steak (shoulder, rump, fillet)	10
	Stewing meat	5
Chicken	Breast	19
	Drumstick	1
	Offal (liver)	1
Lamb	Steaks (fillet, leg)	29
	Ground meat[b] (sausages, grillsteaks, minced, burgers)	15
	Meat on the bone (chops, shank)	13
	Stewing meat	6
	Offal (heart, kidney, liver)	24
Pork	Ground meat[c] (sausages, burgers, minced)	35
	Steak (fillet, loin, leg, shoulder)	21
	Stewing meat	7
	Bacon and loin medallions	4
	Chops	2
	Offal (heart, liver)	2
Venison	Ground meat[d] (sausages, grillsteaks, burgers, meatballs, mince)	58
	Stewing meat	14
	Steak (haunch, striploin)	5
	Offal (liver, kidney)	3
	Other (frying meat)	2
Sampling Period 2:		
Venison	Steak (haunch, sirloin)	28
	Ground meat[e] (burgers and mince)	21
	Stewing meat	12
	Other (meat for frying)	6

[a]Samples contained ground beef only; [b]Samples contained ground lamb only; [c]Samples contained ground pork only; [d]43 out of 58 samples also contained ground pork; [e]Samples contained ground venison only.

The rearing conditions of the animals was not always available but where it was: 22 out of 39 beef samples were from pasture-reared animals, 10 out of 21 chicken samples were from outdoor-reared animals, 20 out of 71 pork samples were from outdoor-reared animals and all lamb samples were from animals reared in the UK so were assumed to be outdoor-reared (see Supplementary data). Of the 82 venison samples, 46 were known to be from wild deer, 23 were from farmed deer, 2 were from a mix of wild and farmed deer and for 11 samples the origin was not specified (see Supplementary data). As some of the venison products in Sampling Period 1 also contained pork, a second collection (Sampling Period 2) was carried out in which 67 samples of pure venison only were purchased (fresh, not frozen) between June and August 2018 (Table 18). Of these, 50 were purchased at farmers' markets or farm shops and 17 were purchased from supermarkets. Out of 67 samples, 28 were from wild deer and 39 were from farmed deer (see Supplementary data).

Although samples were purchased over an 8-month period (Sampling Period 1) and a 3-month period (Sampling Period 2) from a range of different retail outlets, it is possible that some of the samples originated from the same animal. However, the purpose of the study was not to determine the prevalence of *T. gondii* in individual animals, it was to determine the incidence in meat products for sale to the general public.

Sample processing and testing

Fifty grams per meat sample were digested with acid-pepsin and DNA was extracted from 2 ml homogenised pellet, as previously described (Hamilton et al., 2015). Meat juice (fluid within the meat packaging) was collected from fresh samples where possible as previously described (Hamilton et al., 2015). Where none was available, juice was collected following freezing and thawing of the remaining meat sample once 50g had been taken for DNA extraction.

DNA from each sample was screened for *T. gondii* DNA using a quantitative PCR (qPCR) targeting the 529 bp repeat element, as previously described (Hamilton et al., 2015). Any samples which were positive by qPCR were electrophoresed on a 3% agarose gel incorporating Biotium GelRed™ (Cambridge Bioscience Ltd, U.K) to confirm the size of amplicons. Genotyping was attempted on qPCR-positive samples using a multiplex nested PCR-RFLP targeting 10 genetic markers, including SAG1, SAG2 (5'-3' SAG2 and alt.SAG2), SAG3, BTUB,

GRA6, c22-8, c29-2, L358, PK1 and Apico. PCR-RFLP conditions for all markers were carried out as previously described (Hamilton et al., 2015).

Meat juice collected from beef, lamb, pork and venison were screened for antibodies to *T. gondii* using an indirect ELISA (ID Screen® Toxoplasmosis Indirect Multi-species, IDvet, Montpellier, France) according to the manufacturer's instructions. Meat juice collected from chicken samples were sent to the Toxoplasma National Reference Centre (Reims, France) to be screened using a modified agglutination test as previously described (Halos et al., 2010). As the MAT has previously been shown to have lower diagnostic specificity for fluids collected from muscle tissue such as chicken breast (Schares et al., 2018), we used a higher cut-off of 1:20 to reduce the number of false positive results.

Statistical analysis

Statistical analysis was carried out using Minitab Statistical Software (version 17). Since two detection methods (serological and molecular) were used to test the meat samples for the presence of *T. gondii*, the level of agreement between the tests was investigated using Cohen's kappa coefficient (Landis and Koch, 1977).

RESULTS

Of the 300 meat samples purchased for testing in Sampling Period 1, 39 (13.0%) were found to be positive for *T. gondii* DNA (Table 19). Specifically, *T. gondii* DNA was detected in 1 out of 21 (4.8%) chicken samples, 6 out of 87 (6.9%) lamb samples, 3 out of 71 (4.2%) pork samples and 29 out of 82 (35.4%) venison samples (Table 19). None of the 39 beef samples were positive by qPCR. Of the 67 venison samples purchased in Sampling Period 2, 19 (28.4%) were positive for *T. gondii* DNA (Table 19). The number of positive samples for the different rearing conditions and by the different cuts of meat are reported in Supplementary data. Partial genotyping was obtained for only 3 out of the 58 qPCR-positive meat samples (from both sampling periods) due to lack of amplification at the single-copy markers. Of the 3 samples genotyped, all of which were from venison, amplification could only be achieved at 3 markers for one sample (venison sample 1) and 4 markers for two samples (venison samples 2 and 3). Venison sample 1 had Type I alleles at markers SAG2 (3' and 5'), L358 and Apico. Venison sample 2 had Type II alleles at markers SAG2 (3' and 5'), Alt SAG2, SAG3 and BTUB. Venison sample 3 had a Type I allele at GRA6, Type II alleles at C22-8 and L358, and a Type III allele at SAG3 suggesting a non-clonal genotype (atypical).

Table 18 Detection of *T. gondii* in meat samples from animals raised under different rearing conditions.

Meat product	No. tested by qPCR	No. pos by qPCR (%)	No. tested by serology[a]	No. positive by serology (%)	No. positive by PCR and/or serology (%)
Sampling Period 1					
Beef	39	0 (0%)	38	2 (5.3%)	2 (5.1%)
Chicken	21	1 (4.8%)	21	3 (14.3%)	4 (19.0%)
Lamb	87	6 (6.9%)	85	14 (16.5%)	17 (19.5%)
Pork	71	3 (4.2%)	68	2 (2.9%)	4 (5.6%)
Venison	82	29 (35.4%)	78	11 (14.1%)	30 (36.6%)
TOTAL	300	39 (13.0%)	290	32 (11.1%)	57 (19.0%)
Sampling Period 2					
Venison	67	19 (28.4%)	50	8 (16.0%)	20 (29.9%)

[a] Beef, Lamb, Pork and Venison meat juices were all tested by ELISA whereas chicken meat juice was tested by MAT.

Meat juice could not be collected from all meat samples (Table 19). In Sampling Period 1, fresh meat juice could only be collected from 193 samples and juice from frozen and thawed samples was collected from 97 samples (no juice could be collected from 10 samples despite numerous freeze/thaw cycles). Antibodies to *T. gondii* were detected in 32 out of 290 (11.0%) meat juice samples tested in Sampling Period 1 (Table 19). Specifically, antibodies were detected in 2 out of 38 (5.3%) beef juice samples, 3 out of 21 (14.3%) chicken juice samples, 14 out of 85 (16.5%) lamb juice samples, 2 out of 68 (2.9%) pork juice samples and 11 out of 78 (14.1%) venison juice samples. In Sampling Period 2, fresh meat juice could only be collected from 28 samples and juice from frozen and thawed samples was collected from 22 samples (no juice could be collected from 17 samples). *T. gondii* antibodies were detected in 8 out of 50 (16%) venison juice samples tested in Sampling Period 2 (Table 19).

Overall, 21 samples tested positive by serology (ELISA or MAT) but were negative by qPCR, and 34 samples tested positive by qPCR but were negative by serology. Cohen's kappa coefficient demonstrated only a fair agreement between the tests ($\kappa = 0.348$).

DISCUSSION

The consumption of undercooked or raw meat is a significant risk factor for infection with *T. gondii*. Despite this, few data are currently available on the risk for consumers from retail meat in the UK. In the present study, *T. gondii* DNA was detected in chicken, lamb, pork and venison products purchased for human consumption from different retail outlets in Scotland. Of particular note was the incidence of *T. gondii* in venison which was much higher than any of the other meat types tested. Although some of the venison products in the first sampling period also contained pork meat, the prevalence of *T. gondii* in pure venison samples in the second sampling period was similar indicating that the source of *T. gondii* was most likely the venison, particularly given the low prevalence in pork products overall.

Consumption of undercooked or raw game meat has been identified as the source of *T. gondii* infection in a number of cases of toxoplasmosis (England et al., 2019; McDonald et al., 1990; Ross et al., 2001; Sacks et al., 1983) and very recently there was an outbreak of acute toxoplasmosis in a group of Canadian deer hunters who had consumed undercooked venison steak on a hunting trip to the USA (Gaulin et al., 2020). Venison is seen as a healthy meat

choice and in the UK as a whole the venison market is worth an estimated £100 million (Scotland Food and Drink, 2018). As part of Scotland Food and Drink Ambition 2030, there is a significant planned expansion of the farmed venison sector with an emphasis on making venison the main meat of choice for consumers. As it is common (and sometimes recommended on packaging) to consume venison undercooked, this meat could present a potentially significant source of foodborne toxoplasmosis.

The incidence of *T. gondii* detected in meat products in the present study is lower than those reported in a worldwide systemic review and meta-regression analysis on studies reporting the direct detection of *T. gondii* in meat products (Belluco et al., 2016). In this, they reported pooled prevalences of 14.7% in sheep products, 12.3% in pig products and 2.6% in cattle products. These higher prevalences may be a reflection of the type of samples tested as the majority of samples in the analysis were liver, muscle, brain, heart or diaphragm which are known to be predilection sites of the parasite. The lack of detection of *T. gondii* DNA in beef products in the present study is similar to results of other studies (Opsteegh et al., 2011c). Cattle were thought to be able to clear *T. gondii* infections (Burrells et al., 2018) and as such have not been seen as an important source of foodborne toxoplasmosis. However, recent studies have highlighted beef as a high risk meat due it being commonly consumed undercooked or raw (e.g. steak tartare) and therefore the risk from this meat should not be ignored (Belluco et al., 2018; Opsteegh et al., 2011a). In a recent study in Australia, 68% of lamb mincemeat samples purchased at the supermarket were positive for *T. gondii* by PCR highlighting lamb as a potentially high risk meat (Dawson et al., 2020).

It should be noted that the incidence of *T. gondii* detected in meat products in the present study does not reflect the prevalence of *T. gondii* in these food animals. It is likely that some of the products originated from the same animal or were a combination of animals (e.g. ground meat). However, the aim of this study was not to determine the prevalence of *T. gondii* in food animals but instead to determine the incidence in meat products and thus the potential risk to consumers.

The seroprevalence of *T. gondii* in livestock varies widely depending on geographical region, serological test, age of animal and farm management system (Dubey, 2010). Few serological studies have been conducted in the UK. In the present study, antibodies to *T. gondii* were

detected in the meat juice of lamb, venison, chicken, beef and pork. Although these results do not directly represent the seroprevalence in these food animals, the results are similar to previous studies in the UK which have reported seroprevalences of 37.3% in 1-year old sheep (Katzer et al., 2011), 32.5% in red deer (Williamson et al., 1980), 11.8% in cattle (Opsteegh et al., 2019) and 3.6% in pigs (Limon et al., 2017). The lack of concordance between serology and direct detection of parasites has been reported previously (Halos et al., 2010; Opsteegh et al., 2016b). In the present study, there were samples of all meat types which were positive by serology yet negative by qPCR. This may reflect the size of meat sample processed, the portion of meat tested (an edible portion rather than a predilection site) or the inhomogeneous distribution of tissue cysts. Surprisingly, 18 venison samples which were positive by qPCR were negative by serology. The meat juice samples in this study were screened using a commercial ELISA which, although is suitable for ruminants, has not been specifically validated for deer. The discrepancy may also indicate that the venison originated from deer harbouring a chronic infection and thus although they had cysts in their tissues, their antibody response may have waned (Williamson et al., 1980).

It is of note that out of three DNA isolates, which were partially genotyped in this study, only one had Type II alleles at all the amplified markers and the others had Type I or a mix of alleles. Strains containing Type I or atypical alleles are more pathogenic or more likely to cause severe disease than other isolates (Xiao and Yolken, 2015) and have been associated with cases of clinical human toxoplasmosis in England and Wales (Aspinall et al., 2003) and a case of re-infection of an immune competent patient with devastating consequences (Elbez-Rubinstein et al., 2009). The presence of *T. gondii* with Type I alleles in a meat product which is commonly consumed undercooked, such as venison, could pose a potentially significant public health problem. Type I alleles have also been reported in *T. gondii* DNA isolates from wildlife in Scotland (Burrells et al., 2013) suggesting that other clonal or atypical strains may dominate in the environment.

In conclusion, this is the first study to report the presence of *T. gondii* in retail meat products in Scotland and has highlighted venison as a potentially high risk meat. This data could be used to inform quantitative microbial risk assessments of foodborne toxoplasmosis in Scotland. Further work is underway with additional fresh samples of venison to determine the viability

of *T. gondii* in these meat products to truly assess the foodborne risk. Given the difficulty in controlling transmission in food animals, thorough cooking or freezing of meat is currently the best option for reducing human infection (Dubey, 2010). With an ever increasing demand for organic or outdoor-reared meat, there comes an increased risk of infection with *T. gondii* as animals raised in this way are more likely to be seropositive and, therefore, potentially harbouring tissue cysts (Stelzer et al., 2019). Experimental vaccination of pigs (Burrells et al., 2015) and lambs (Katzer et al., 2014) with the S48 strain of *T. gondii* demonstrated a reduction in the number of viable tissue cysts following oocyst challenge resulting in safer meat for consumption; however, there is currently no incentive for farmers to vaccinate their animals purely for food safety. Also, animals would need to be vaccinated shortly after birth to avoid natural infection with *T. gondii* from the environment.

ACKNOWLEDGEMENTS

This work was funded by the University of Edinburgh and the Scottish Government (Rural and Environment Science and Analytical Services Division). This work was funded by the Scottish Government Rural Affairs, Food and the Environment (Strategic Research Programme 2016-2021). JP was funded through a Becas-Chile scholarship from the National Commission for Scientific and Technological Research (CONICYT), Chile.

Chapter 7

Effect of NaCl, nitrates and nitrites on viability of *Toxoplasma gondii* in French dry sausage and processed pork and quantification of the parasite burden in pig tissues used for their production and heart

Filip Dámek [1*], Bastien Fremaux [2], Dominique Aubert [3], Sandra Thoumire [1], Maxime Delsart [4], Jean-Luc Martin [2], Sandra Vuillermet [1], Marieke Opsteegh [5], Pikka Jokelainen [6], Delphine Le Roux [1], Pascal Boireau [1], Isabelle Villena [3], Radu Blaga [1]

[1] Anses, INRAE, Ecole Nationale Vétérinaire d'Alfort, Laboratoire de Santé Animale, BIPAR, Maisons-Alfort, France

[2] IFIP - Institut du Porc, Ecole Nationale Vétérinaire d'Alfort, Maisons-Alfort, France

[3] National Reference Centre on Toxoplasmosis, Toxoplasma Biological Resources Centre, CHU Reims and EA7510, SFR CAP-Santé, University of Reims Champagne-Ardenne, USC EpiToxo Anses, France

[4] Anses, Ecole nationale vétérinaire d'Alfort, Laboratoire de Santé Animale USC EPIMAI, Maisons-Alfort, France

[5] Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands

[6] Statens Serum Institut, Infectious Disease Preparedness, Copenhagen, Denmark

[*] Corresponding author: Filip Dámek, UMR BIPAR. INRA-Anses-EnvA, 22 rue Pierre et Marie Curie, 94701 Maisons-Alfort Cedex, Maisons-Alfort, France, filip.damek@vet-alfort.fr

Submitted manuscript

Toxoplasma gondii is an important zoonotic foodborne parasite. Amid the possible transmission routes, meat of infected animals appears to be a major source of infection in Europe. Pork is the most consumed meat in France, with dry sausages well represented. The risk of transmission via consumption of processed pork products is largely unknown, mainly since processing will affect viability but may not entirely inactivate all *T. gondii* parasites.

We investigated the presence and concentration of *T. gondii* DNA in the shoulder, breast, ham, and heart of six pigs orally inoculated with 1,000 oocysts or tissue cysts and two naturally infected pigs, by means of magnetic capture qPCR (MC-qPCR). Muscle tissues of experimentally infected pigs were further used to evaluate the impact of manufacturing processes of dry sausages, including different concentrations of nitrates (0, 60, 120, 200 ppm), nitrites (0, 60, 120 ppm), and NaCl (0, 20, 26 g/kg), ripening (2 days at 16 – 24°C) and drying (up to 30 days at 13°C), by a combination of mouse bioassay, qPCR and MC-qPCR.

DNA of *T. gondii* was detected in all pigs, including in 41.7 % (10/24) of muscle samples and 87.5 % (7/8) of hearts by MC-qPCR. The number of parasites per gram of tissue was estimated to be the lowest in the hams (M = 1, SD = 2) and the highest in the hearts (M = 147, SD = 233). However, the *T. gondii* burden estimates varied on the individual animal level, the tissue tested and the parasitic stage used for the experimental infection (oocysts or tissue cysts). Of dry sausages, 94.4 % (51/54) were positive for *T. gondii* by MC-qPCR or qPCR, with the mean *T. gondii* burden estimate equivalent to 31 parasites per gram (SD = 93), and the untreated dry sausage sample collected on the day of production was positive by mouse bioassay.

The results suggest an uneven distribution of *T. gondii* in the samples examined, and possibly an absence or a concentration below the detection limit in some of them. Moreover, the results indicate an effect of NaCl, nitrates, and nitrites used in dry sausages on the viability of *T. gondii* as soon as the first day of the production process. Results are valuable input for future risk assessments aiming to estimate the relative contribution of different sources of *T. gondii* human infections.

INTRODUCTION

Toxoplasma gondii is an important zoonotic coccidian parasite and one of the most successful parasites worldwide, widely spread amongst various warm-blooded animal species, including humans (Dubey, 2022). Sexual reproduction resulting in shedding oocysts occurs only in its definitive hosts, felids, but virtually all warm-blooded animals can carry tissue cysts and may act as intermediate hosts (Dubey, 1998). Humans may acquire *T. gondii* infection via ingestion of sporulated oocysts from the environment (soil, water, unwashed vegetables or fruits) or via ingestion of bradyzoites by consumption of raw or undercooked meat of infected animals containing tissue cysts (Hill and Dubey, 2002). If a seronegative woman acquires a *T. gondii* infection during pregnancy, the parasite is transmitted to the fetus in approximately 30 % of the cases (SYROCOT (Systematic Review on Congenital Toxoplasmosis) study group et al., 2007). This may result in abortion or abnormalities in the central nervous system, retinochoriditis, and other symptoms in the child (Hampton, 2015). Postnatal *T. gondii* infection is recognized as an important cause of retinochoriditis (Gilbert et al., 1999). Moreover, toxoplasmosis was a major cause of death in AIDS patients before the introduction of highly active retroviral therapy (Luft and Remington, 1992). The overall impact of toxoplasmosis on human health was highlighted in multicriteria-based ranking for risk management of food-borne parasites where *T. gondii* ranked among the top priority food-borne parasites at both global (4th out of 24 food-borne parasites) (Boireau et al., 2014) and European levels (2nd out of 25 food-borne parasites) (Bouwknegt et al., 2018).

In a European multi-centre case control study (Gaulin et al., 2020), 30 to 63 % of *T. gondii* infections in pregnant women were attributed to meat of infected animals, whereas 6 to 17 % were most likely soil-borne. With an average annual per capita consumption of 31.7 kg in 2021 (FranceAgriMer, 2022), pork is the most consumed meat in France. Approximately three-quarters of the pork produced in France is used in charcuterie (meat specialities mainly made from pork) out of which 10 %, corresponding to nearly 113,000 tonnes, were dry sausages in 2019 (FICT, 2020). Dry sausages and salamis are widely consumed in France at almost 77,000 tonnes in 2021, rising from less than 72,000 tonnes in 2019 (FranceAgriMer, 2022). The microbiological safety of these products is therefore of major importance. The decrease in *T.*

gondii prevalence amongst pregnant women in France has been substantial, from 83 % in 1965 (Desmonts et al., 1965) to 31.3 % in 2016 (Blondel et al., 2017), indicating improved infection prevention, but also underlining that the infection remains common. One of the ways to further progress with the prevention of human *T. gondii* infections could be an improvement in the safety of meat products. This can be performed in various key points from farm measures (rodent-control plans, avoiding cats in the farms, etc.) to interventions in the production process of meat products such as dry sausage, which was explored in the present study.

The first objective of this study was to investigate the tissue tropism of *T. gondii* in a selection of tissues (shoulder, breast, ham, heart) of naturally and experimentally infected pigs inoculated with various infective stages (oocysts and tissue cysts) in order to rank the tissues, which can be used in the production of dry sausages, according to their parasite burden. Secondly, we aimed to evaluate the impact of NaCl, nitrates, and nitrites as well as processing steps (ripening, drying and storage) on the viability of *T. gondii* in dry sausages and sausage batter not encased in natural pig intestines (referred to as “processed pork” in the following text), according to an industrial process.

MATERIALS AND METHODS

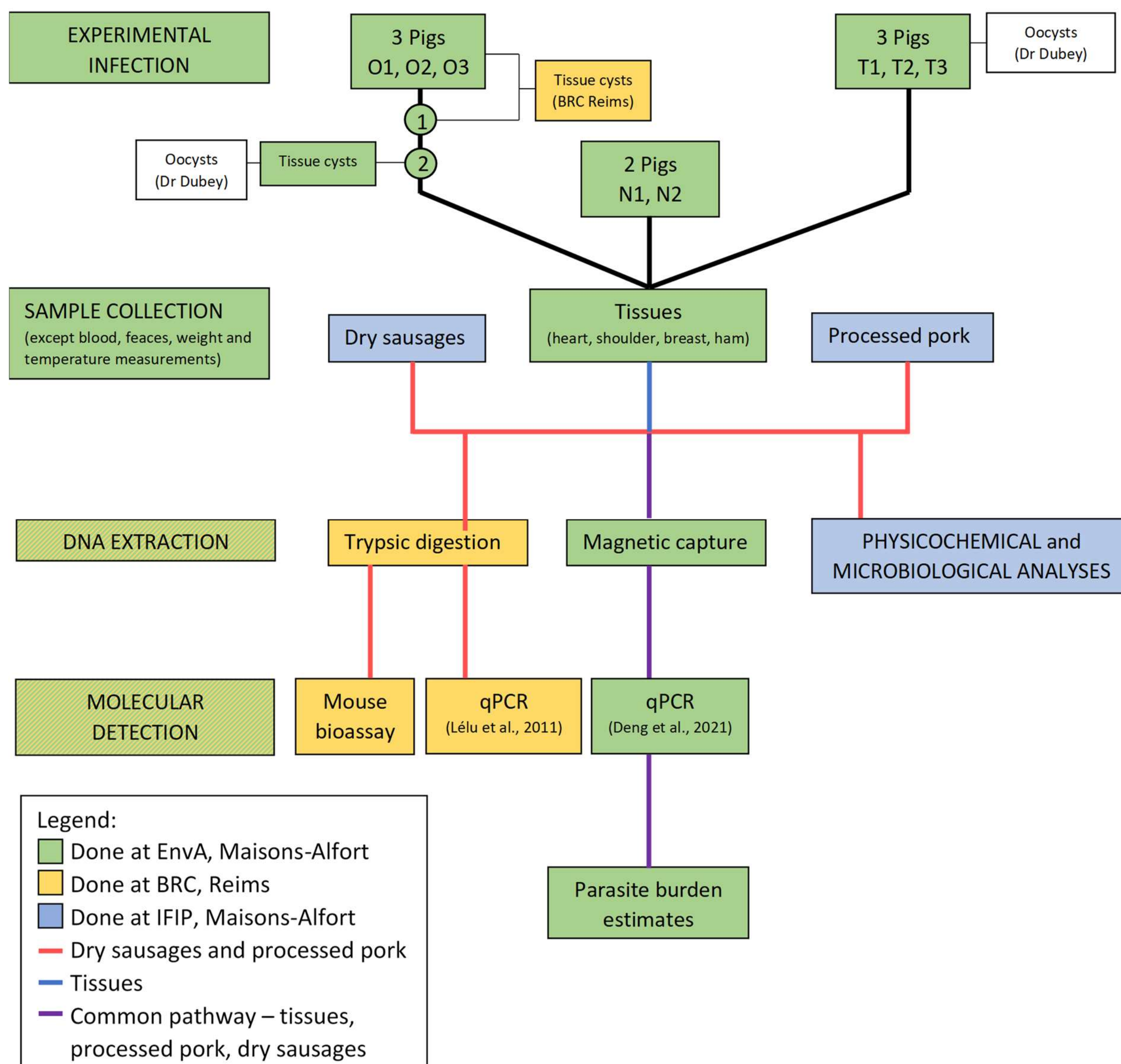

Figure 17 Flowchart representing the work process and distribution. Green-filled fields represent work done at EnvA, Maisons-Alfort; yellow-filled fields represent work or materials coming from BRC, Reims, blue-filled fields represent work done at IFIP, Maisons-Alfort. Red lines represent common workflow pathways for the dry sausages and processed pork, blue line represent a workflow for the tissues, purple represents a common workflow pathway for the tissues, dry sausages and processed pork.

Toxoplasma gondii parasites

T. gondii oocysts (ME49 strain, genotype II, aged six months) isolated as described by Dubey (2010) were obtained as a courtesy of Dr. JP Dubey (USDA, ARS, Beltsville, USA) and stored in a 2% H2SO4 solution, at 4 °C until use.

T. gondii tissue cysts (ME49 strain, genotype II, age of mice two months) isolated from mice brains as described by Villena et al. (Villena et al., 2012) were obtained from the Biological Resources Centre of Toxoplasma, Reims, France (BRC) and used in Ecole Nationale Vétérinaire d'Alfort (EnvA) facilities within 24 hours upon their reception. The experimental infection of mice was approved by the local ethical committee of University Champagne Ardennes under agreement no° B 514544, project APAFIS 10509-2017062614316905. *T. gondii* tissue cysts used in the third oral inoculation of tissue-cyst-infected pigs (see Discussion) were obtained from brain tissues of 14 Swiss albino female mice, collected four weeks after oral inoculation with an average of 500 *T. gondii* oocysts of ME49 strain per mouse.

Pigs

Twenty pigs of the Large White breed, belonging to the Experimental Unit of the Animal Physiology Department of Inrae Nouzilly, were tested by in-house modified agglutination test (MAT) (cut-off: a positive result at dilution 1:6) for *T. gondii* antibodies detection (see Modified agglutination test (MAT)). Seven of these pigs (females, 97 days old) were selected based on a negative MAT result and transported to the Biomedical Research Centre of EnvA (Maisons-Alfort, France) for experimental infection with *T. gondii*.

Additionally, fourteen adult female pigs of the Large White breed were tested using ELISA ID Screen Toxoplasmosis Indirect Multi-Species (ID Vet, France), according to the manufacturer's instructions, at a slaughterhouse in the Auvergne-Rhône-Alpes region of France. Two animals that tested positive for *T. gondii* antibodies, indicating natural *T. gondii* infection, were selected and the carcasses of these animals were transported to EnvA for the collection of tissue samples.

Experimental infection

The experimental infections were approved by the local Ethical Committee for animal experiments of Anses/EnvA/UPEC (APAFIS N° 14363-2018032908554996v3) and carried out at the Biomedical Research Centre of EnvA (Maisons-Alfort, France). The seven pigs were first submitted to a seven-day-long acclimatization period upon arrival. Later on, three pigs (O1, O2, O3) were inoculated by hand-feeding a dose of 10^3 *T. gondii* sporulated oocysts per animal

on bite-size portions of pain au lait, a French pastry to which the pigs were previously accustomed to as a reward, and kept together in a separate pen. Similarly, another three pigs (T1, T2, T3) were inoculated with a dose of 10^3 *T. gondii* tissue cysts per animal and kept together in a separate pen. Finally, one pig (C1) was kept as a negative control, most of the time in a separate pen. Infectivity of both the oocysts and tissue cysts was confirmed by mouse bioassay in the Biomedical Research Centre of EnvA (Maisons-Alfort, France) according to the approval of the local Ethical Committee for animal experiments of Anses/EnvA/UPEC (APAFIS N° 14363-2018032908554996v3) (see Mouse bioassay).

Blood samples from pigs were collected from the jugular vein into both EDTA-covered collection tubes and serum separator tubes three times a week for the first two weeks post-infection (p.i.) and on a weekly basis afterwards until the euthanasia of the animals. Specifically, the sample collection was scheduled on days (D) 2, 5, 9, 12, and 14 p.i. from the oocyst-infected group, D2, D5, D9, and D12 p.i. from the tissue-cyst-infected group, and all of the mentioned dates from the negative control pig.

Faecal samples were collected daily for the first week following the experimental infection in order to monitor potential cross-contamination. Specifically, the faecal samples were collected on D1, D2, D3, D5, and D6 from the oocyst-infected group, D1, D2, D3, D4, D5, and D7 from the tissue-cyst-infected group, and all of the mentioned dates for the negative control pig. Unless processed immediately, all the blood and faecal samples were stored at –20°C until further processing.

Rectal temperature measurements were taken on a daily basis for the first two weeks following the experimental infection and three times a week afterwards until the euthanasia of the animals. Specifically, the measurements were taken on D1, D2, D3, D5, D6, D8, D9, D12, D13, D14, D19, D27, D36, D37, D38, D39, D40, D44, D47, D48, D50, D51, and D54 from the oocyst-infected group, D0, D1, D2, D3, D4, D5, D7, D8, D9, D12, D13, D15, D16, D19, and D21 from the tissue-cyst-infected group, and all of the mentioned dates except D21 from the negative control pig.

Weight of the pigs was measured on D2, D14, D19, D26, D34, D40, D47, D54, and D57 p.i. for the oocyst-infected group, and D1, D5, D12, D19, and D22 p.i. for the tissue-cyst-infected

group. The negative control pig was weighted on all of the mentioned dates except D22. The optimal weight objective at the end of the measurement (D57 p.i.) was estimated using the mean weight of the oocyst-infected group at the first measurement (D2 p.i.) and adding the average daily growth gain of 815 grams (Hiboost, 2016) per day until D57 p.i. Subsequently, the weight objective was set to 97.2kg at day 57 p.i. and 156 days of age.

All pigs were handled in strict accordance with good clinical practices, kept under regular veterinary supervision, and visited twice a day by local technicians and members of the research unit.

Sample collection

Muscle samples were collected for the production of French dry sausages “saucissons secs” and processed pork, as well as for the study of tropism within tissues.

Sample collection for the dry sausage and processed pork production

The three pigs of the oocyst-infected group were euthanised 58 days p.i. Blood and all internal organs were removed and collected, before storing halved carcasses at + 4°C for 48 hours. All available muscle tissues, including shoulder, breast, and ham, were collected from the three pigs, totalling 72.8kg of muscles for the dry sausage production.

The three pigs of the tissue-cysts group were euthanised 68 days p.i. and their carcasses were processed similarly to those of the oocyst-infected group. Twelve kilograms of muscles from the shoulder, breast, and ham in total were collected from the three pigs for the processed pork production.

Sample collection for T. gondii tropism study

Three hundred grams from the breast, shoulder, ham, and heart were collected from each of the experimentally and naturally infected animals (n = 8) to compare the distribution of the parasites between the groups and various tissues.

Dry sausage and processed pork production

The manufacture of dry sausages and processed pork was carried out at the Institut du Porc (IFIP), including seven recipes (F1 to F6, FT) for dry sausages and four recipes (F2, F4, FT, VH) for processed pork, according to the traditional industrial production protocol. Dry sausages were produced using the muscles of animals infected with *T. gondii* oocysts (Table 20). Processed pork was manufactured using the muscles of animals infected with *T. gondii* tissue cysts.

Table 19 Dry sausages and processed pork recipes. Additives and ingredients, used in the production of the traditional French dry sausage, were added to the minced pork batter according to the corresponding recipes (FT, F1 to F6) for the dry sausages, and for the processed pork (VH, FT, F2, F4).

	Dry sausage recipes							Processed pork recipes			
Additives and ingredients	**FT**	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**VH**	**FT**	**F2**	**F4**
NaNO2 [mg/kg]	0	0	0	60	60	120	120	0	0	0	60
KNO3 [mg/kg]	0	200	200	60	60	120	120	0	0	200	60
NaCl [g/kg]	26	20	26	20	26	20	26	0	26	26	26
Lactose [g/kg]	10	10	10	10	10	10	10	0	10	10	10
Dextrose [g/kg]	5	5	5	5	5	5	5	0	5	5	5
Pepper [g/kg]	1,5	1,5	1,5	1,5	1,5	1,5	1,5	0	1,5	1,5	1,5

Three dry sausages were produced for each of the corresponding recipes (F1 to F6, FT) and each of the collection days (D1, D6, D13, D20, D30, D50), while two bags of processed pork were manufactured for each of the four different corresponding recipes (F2, F4, FT, VH) and collection days (D0, D1, D6). Dry sausages were encased in natural intestine casings, while the processed pork was bagged into sterile plastic bags. Samples were taken for the detection of DNA, quantification, and viability testing of *T. gondii* (qPCR, MC-qPCR, and mouse bioassay), as well as for the microbiological and physicochemical analysis (CFU, pH, aw, nitrates/nitrites, and NaCl) on each of the sampling days (D0, D1, D6, D13, D20, D30, and D50).

The muscle tissues (n = 72.8kg) intended for the dry sausage production, retrieved from oocysts-infected pigs was pre-cut, and divided equally into seven parts to ensure homogenous distribution of tissues between the seven different dry sausage recipes (control recipe = FT and recipes F1 to F6) as described in Table 20. Pig back fat was added to respect the 80/20 ratio for muscle/fat content. All tissues were stiffened at 1°C overnight before being homogenized using a PSV DRC98 chopper (PSV, France). After the homogenization, each of the seven parts was mixed with appropriate amounts of dry ingredients and additives as described in Table 20. BactoFlavor® Flora Italia (Chr. Hansen, Denmark) starting culture (a mix of Lactobacillus sakei and Staphylococcus carnosus strains) was added at a rate of 0.5%, according to the supplier's recommendations. Each recipe part was then homogenized manually by two operators for 10 minutes and filled into a natural casing (pork small intestine, previously desalted in spring water for 24 hours, rinsed and drained) using a tabletop filler PSV VILLA ST13 (PSV, France). Encased products of a minimum of 300g per piece (+/- 10g) were secured using a clipper and soaked in a suspension of Penicillium nalgiovense (Mold 600 – Chr. Hansen) spores. Subsequently, dry sausages were suspended in a ripening chamber with a specified controlled environment for the production of traditional French dry sausages with parameters specified in Supplementary data. Following the ripening and drying step, a period of conservation at 18°C for 20 days was simulated. Relative humidity and temperature in the chamber were monitored and adjusted by an HMP110 probe (Vaisala, Finland) connected to the Labguard 3D™ system (bioMérieux, France).

The muscle tissues (n = 12 kg) intended for the processed pork production, retrieved from tissue-cysts infected pigs, were pre-cut and divided equally into four parts to ensure homogenous distribution of tissues between the corresponding four processed pork recipes (control recipe = FT, minced meat = VH, recipes F2 and F4) while respecting the 80/20 ratio for muscle/fat content. Muscles and fat were stored overnight at 1°C at the IFIP laboratories and processed pork samples were then produced in the same way as the dry sausages, except for the filling step. Different concentrations of salt and nitrites (potassium nitrite = saltpetre) were added to processed pork samples corresponding to the different recipes, as described in Table 20. Starter fermentation flora BactoFlavor® Flora Italia (Chr. Hansen, Denmark) was added to recipes F2, F4, and FT, following the producer's recommendations. Processed pork

samples were transferred into sterile plastic bags and left open during a six-day-long incubation process, including 24 hours at 24°C (ripening simulation) followed by five days at 13°C (shortened drying simulation).

Flotation and sedimentation of faecal samples

Five grams of faeces per pig, collected at D1, D2, D3, D5, and D6 p.i., were processed without storage at -20°C by the magnesium sulphate flotation technique described by Quinn et al. (1980). Similarly, five grams of faeces per pig were processed without storage at -20°C by formalin-ethyl acetate sedimentation technique as described by Young et al. (1979). Samples were then observed on microscopic slides under an optic microscope (Nikon Eclipse E100, 10 × magnification) to detect potential *T. gondii* oocyst passage and eggs of other parasites.

Modified agglutination test (MAT)

Sera of pigs and mice were obtained by centrifugation of clotted blood, at 1500g for 10 minutes and analysed by the Modified Agglutination Test (MAT) for the detection of *T. gondii*-specific immunoglobulin G (IgG) according to Dubey and Desmonts (Dubey and Desmonts, 1987). Briefly, whole formalin-fixed tachyzoites of RH strain, provided by the National Reference Centre for Toxoplasmosis in Reims, France (Villena et al., 2012), were used as an antigen. The sera were diluted two-fold, starting at a 1:6 dilution up to a 1:192 dilution. The threshold for serum dilution to consider a sample to be positive was 1:6.

Enzyme-linked immunosorbent assay (ELISA)

Sera of pigs and mice were obtained as described in MAT and analysed by ID Screen® Toxoplasmosis Indirect Multi-species (IDVet, France) for the detection of anti-*T. gondii* antibodies according to the manufacturer's instructions.

Microbiological and physicochemical analysis of the dry sausages and processed pork

For the six recipes (F1 to F6) of dry sausages, pH was monitored during the whole ripening process using a LoT406-M6-DXK-S7/25 penetration probe (Mettler-Toledo, Switzerland), accompanied by a temperature probe Pt 1000/3M, both placed in the centre of a sausage.

Data were recorded using a Mettler Transmitter M200 easy with the help of the ALMEMO 5690-1M acquisition system (Ahlborn, Germany). The weight loss of three sausages per recipe was regularly monitored on laboratory scales (precision platform scales KERN DS). Measurements of pH in the processed pork samples were taken with a LE427-IP67 penetration probe (Mettler-Toledo, Switzerland) connected to a pH-meter Mettler-Toledo FiveGo (norm NF V04-408). In addition to the pH measurements, water activity levels (aw) were measured in one dry sausage and processed pork bag per recipe on each of the sample collection days: D0, D6, D13, D20, D30, D50 and D0, D1, D6, respectively. Water activity measurements were performed with the help of an Aw-meter Aqualab Series 4TEV (norm ISO 21807). Doses of nitrites, nitrates, and sodium (NaCl equivalent) were measured on D0 and D50 in each of the three sausages of all seven recipes (FT, F1 – F6). Traces of nitrites, nitrates (Flow Injection Analysis=FIA), and sodium (Atomic absorption spectroscopy=AAS, MOPC 075) were measured at Actalia laboratory (Villers Bocage, France). At each analysis date, the lactic acid bacteria from one sausage per recipe were cultivated on an MRS medium (bioMérieux, France), according to the norm NF ISO 15214.

For the four recipes of processed pork, microbiological and physicochemical analyses were carried out on D0, D1, and D6. Moreover, on the same samples, the lactic acid bacteria were counted according to standard NF ISO 15214. Another two samples, collected on these dates, were used to determine pH and aw values, as well as levels of nitrites, nitrates, and NaCl, using the same methods as described previously.

Molecular detection and viability of *T. gondii*

Whole blood samples and faecal samples were used for the detection and quantification of *T. gondii* DNA by qPCR. Dry sausages and processed pork were also processed for *T. gondii* detection by qPCR (see qPCR below).

Three dry sausages were collected for each of the seven recipes (FT, F1 to F6) and collection dates (D0, D6, D13, D20, D30, D50). Each dry sausage was divided into three equal parts. Two parts of each of the three sausages were pooled to form approximately a two-hundred-gram sample and blended before undergoing trypsin artificial digestion (see Trypsin artificial

digestion). The obtained pellet was used for qPCR analysis and mouse bioassay. The remaining parts of each of the three sausages were pooled to form a single one-hundred-gram sample, were blended, digested by proteinase K solution, and used for magnetic capture qPCR (MC-qPCR) according to the protocol (Opsteegh et al., 2010).

Processed pork samples were collected for each of the four recipes (FT, VH, F2, F4) and collection dates (D0, D1, D6). Two hundred grams were taken directly from the sterile plastic bags, processed by trypsin digestion, and were used for qPCR and mouse bioassay, while one hundred grams were digested by proteinase K solution and used for MC-qPCR analysis.

For tissue samples, a pool of 300g of tissue per sample (shoulder, breast, ham, heart) from each of the eight animals were homogenised and divided subsequently: 200g were digested by trypsin solution and intended for *T. gondii* DNA quantification by qPCR and 100g of the tissues were digested by proteinase K and intended for *T. gondii* DNA quantification by MC-qPCR.

Samples tested by both the MC-qPCR and qPCR (dry sausages and processed pork samples) were considered positive if at least one of the results was considered positive.

MC-qPCR and parasitic burden estimates

One hundred grams of each of the following muscles: shoulder, breast, ham, and heart from the six experimentally infected pigs and two naturally infected pigs (n = 32 samples), as well as the dry sausages and processed pork produced corresponding to the eleven recipes (n = 54 samples), were digested using a lysis buffer containing proteinase K (Merck, Germany), followed by MC-qPCR, according to the protocol (Opsteegh et al., 2010), using Streptavidin Sepharose® High-Performance beads (Merck, Germany), Streptavidin coated paramagnetic beads (Solulink, USA) and Stomacher 400C Sterile Strainer/Filter Bags (Seward, UK). The detection and quantification of *T. gondii* DNA in each sample was performed in duplicate by amplification of a sequence within the 529bp repetitive element, according to Opsteegh et al. (2010) with minor modifications (25µL total reaction mixture volume instead of 20µL, 2X Premix Ex Taq™ (Takara Bio) instead of 5 × concentrated Taqman master mix (Roche) and 5µL instead of 10µL of DNA as template) and CIAC probe modification by Deng et al. (2021b), using

LightCycler® 480 System 96-plate thermocycler (Roche, Germany). Parasitic burden estimates in tissues, dry sausages, and processed pork samples were based on mean quantification cycle (Cq) values obtained by MC-qPCR, compared to a standard curve. The standard curve was obtained as follows: 100 grams of negative meat (no *T. gondii* DNA detected in the tissue by MC-qPCR and no *T. gondii*-specific antibodies detected by ELISA and MAT in meat juice, data not shown) was spiked with a known number of parasites ranging from 1 to 10,000 tachyzoites in tenfold dilutions and quantified by MC-qPCR. The negative reactions were not considered in the calculation of the final mean Cq value and the subsequent parasite burden estimates in samples. The curve fitted to MC-qPCR Cq-values by parasite number for the spiked samples was used to estimate parasite burden in samples.

Trypsin artificial digestion

Two hundred grams of each of the following muscles: shoulder, breast, ham, and heart from the six experimentally infected pigs, the two naturally infected pigs, and each of the dry sausage and processed pork samples produced corresponding to the eleven recipes (n = 54 samples) were blended and incubated (90 minutes at 37°C, 200 RPM on a shaking plate) in trypsin solution (Trypsine (1:250), powder, ThermoFisher Scientific, final concentration of 4 g/L). The mixture was filtered through a double layer of gauze, transferred to 50mL centrifuge tubes, and centrifuged at 1800g for 10 minutes. The formed pellet was washed twice of leftover trypsin using a saline solution (0,9% NaCl, Sigma-Aldrich). Three hundred µL of the final suspension was stored at - 20°C until DNA extraction and subsequent qPCR analysis. The leftover pellet was used for mouse bioassay.

qPCR

DNA was extracted from 80 mg of whole blood or faeces, using QIAamp DNA Blood Mini Kit (Qiagen, Germany) and NucleoSpin DNA Stool kit (Macherey-Nagel, France) respectively, according to the manufacturer’s instructions. Extracted DNA was desalted and concentrated using ethanol precipitation (Zeuigin and Hartley, 1985) and resuspended in 30µL of Milli-Q water. The detection and quantification of *T. gondii* DNA in each sample was performed in duplicate by amplification of a sequence within the 529bp repetitive element, according to Opsteegh et al. (2010) with minor modifications (25µL total reaction mixture volume, 2X

Premix Ex Taq™ (TakaraBio, Japan) and 5µL of DNA as template) and CIAC probe modification by Deng et al. (2021a), using LightCycler® 480 System 96-plate thermocycler (Roche, Germany).

For dry sausages and processed pork, DNA was extracted from a total of 300µL of the trypsin artificial digestion pellet using QIAamp DNA minikit (Qiagen, France), and qPCR analysis for each sample was performed in six-plicate for dry sausages and in quadruplicate for processed pork with the use of QuantStudio™ 3 System thermocycler (Fisher Scientific, France). The detection and quantification of *T. gondii* DNA were achieved by amplification of a sequence within the 529bp repetitive element (Lélu et al., 2011). The negative qPCR reactions were not considered in the calculation of the Cq mean.

None of the tissue samples of shoulder, breast, ham, and heart from each of the eight pigs were processed by trypsin digestion and the following testing by qPCR, and no mice bioassays were performed with these samples.

Mouse bioassay

Following the trypsin digestion of the dry sausages and processed pork (see Trypsin artificial digestion), Penicillin-streptomycin (Fisher Scientific, France) was added to the pellet homogenate as described in Opsteegh et al. (2016b). Five hundred µL of the pellet homogenate was inoculated intraperitoneally to three female Swiss albino mice, for each of the seven dry sausage recipes and six collection days. Four female Swiss albino mice were inoculated for the unsupplemented processed pork sample (VH) and three collection days, and six female Swiss albino mice for each of the three processed pork samples containing additives (F2, F4, FT) tested on three collection days, totaling at 66 mice. The serum of the mice was collected four weeks p.i. and tested for the presence of *T. gondii*-specific immunoglobulin (IgG) antibodies by MAT (see chapter MAT). Mouse deceased on D2 p.i. was tested for the presence of *T. gondii* tissue cysts in the brain using microscopy. From D7 p.i. on, deceased mice were tested also for specific antibodies. Positive controls, fed *T. gondii* oocysts used in the infection of pigs (see chapter Experimental infection), were included in all mouse bioassays. The inoculation of mice was performed at the animal facility of the Parasitology

Laboratory, CHU Reims under the agreement B 514544 as a part of the project APAFIS 10509-2017062614316905.

Note that none of the tissue samples of the shoulder, breast, ham, and heart of the eight pigs were processed by trypsin digestion and not tested by mouse bioassay.

Statistical analysis

Statistical analyses were performed using RStudio (Team, 2009), R version 4.2.2 (2022-10-31 ucrt), using the following packages: ggplot2 (Wickham, 2016) and lme4 (Bates et al., n.d.).

Arithmetic mean and standard deviation were calculated for numerical data. The normality of the data distribution was assessed by visualization in a QQ plot. Wilcoxon Signed-Rank test was subsequently used to assess the differences between samples concerning the parasite burden estimates and the three pig groups (oocyst, tissue cyst, and naturally infected group). Since non-normality was observed in the data distribution, the non-parametric Kruskal-Wallis test was used to assess the significance of the within-group parasite burden estimates. The level of significance was set at 0.05 for all the tests performed.

RESULTS

Clinical observation of experimentally infected pigs

To assess infection success and clinical signs in experimentally infected pigs, the rectal temperature of the pigs was recorded on specific dates, as described in detail in chapter Experimental infection. The rectal temperature of experimentally infected pigs measured during the course of the experiment did not exceed the range of 38 – 40.9°C (Fig. 18a,b, see Supplementary data).

Concerning the oocyst-infected group, the temperature values stayed within the range of 38 - 39.5°C, corresponding to the normal body temperature values for an adult pig, except for three days for O1 (D6, D8, and D9 p.i.), one day for O2 (D1 p.i.) and one day for O3 (D9 p.i.).

The maximum temperature value recorded was 40.1°C in O1 at 8 days post-infection (Fig. 18a, see Supplementary data).

Concerning the tissue cysts-infected group, all three pigs showed a febrile response, defined as a rectal temperature of 39.5°C or higher, as soon as D5 p.i., with a maximum temperature of 40.9°C for T1 at D6 p.i., 40.8°C for T2 at D7 p.i. and 40.7°C for T3 at D6 p.i. The rectal temperature values returned to normal for all three pigs on D8 p.i. (Fig. 18b, see Supplemenetary data). The febrile response in the tissue-cyst infected group was represented by a steep rise of body temperature on D5, falling shortly after on D7 p.i.

The rectal temperature of the negative control pig (C1) stayed within the range of 38 - 39.5°C, except for D37 and D44 when it reached a value of 40.0°C (Fig. 18a, see Supplementary data).

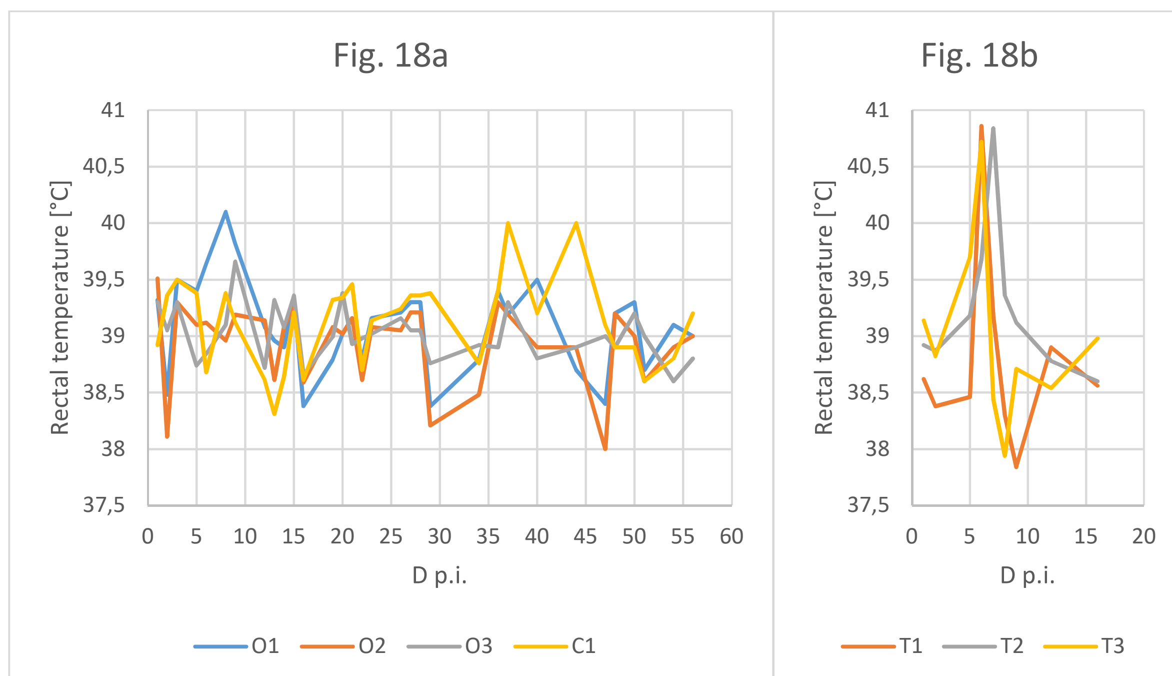

Figure 18 Figure 1 Rectal temperature measurements. Rectal temperature measurements in °C (y-axis) and days post-infection (x-axis) were taken for the pigs experimentally infected with *T. gondii* oocysts and the negative control pig (Fig. 18a), and the pigs experimentally infected with *T. gondii* tissue cysts (Fig. 18b). Individual animals are represented by the curves of different colours.

The weight and weight gains of the pigs were measured to monitor the health status of the pigs and the success of the experimental *T. gondii* infection. At the beginning of the experiment, at the age of 99 days (D2 p.i.), the pigs weighed 58, 49.5, and 49.5 kg (M = 52.3 kg, SD = 4.9 kg) for O1, O2, and O3 respectively, and 52, 54, and 47 kg (M = 51 kg, SD = 3.6 kg)

for T1, T2 and T3 respectively. The weight of pig C1 was not recorded at this age. The oocyst-infected pigs (O1, O2, O3) were euthanised on D58 p.i. at the age of 155 days, weighing 108, 93.5, and 88.5 kg respectively at D57 p.i. (see Supplementary data). The mean individual daily weight gains in the oocyst-infected pigs were 909 grams in pig O1, 800 grams in pig O2, and 709 grams in pig O3. The weight gain in the negative control pig (C1) was 953 grams per day within the observed period (D14 - D57). Due to a technical problem, the inoculation of the second group of pigs had to be repeated three times before a successful infection was confirmed. Therefore, these animals (T1, T2, T3) were 210 days old at the time of the third infection and weighed 128, 132, and 132.5 kg respectively. These pigs were euthanised on D68 p.i. and they weighed approximately 170 kg at the age of 278 days (data not shown).

Serological response and qPCR on blood, and flotation, sedimentation, and qPCR on faeces

Following the oral inoculation with 10^3 sporulated oocysts of *T. gondii*, specific seroconversion was detected by MAT in all three pigs on D12 p.i. The MAT titres on D12 p.i. were 12 for pig O3 and 24 for pigs O1 and O2. These titres consistently increased in the following days to reach 192, the highest titre tested, on D19 p.i. for all three pigs and persisted at this titre until the end of the experiment (Fig. 19a, see Supplementary data). Similar seroconversion was detected by ELISA on D12 p.i. in pig O2, D14 p.i. in pig O1, and D19 p.i. for pig O3 (Fig. 19b, see Supplementary data).

The oral infection with 10^3 tissue cysts/animal had to be performed three times since no specific seroconversion was detected either by MAT or by ELISA in this group of pigs. Following the third oral infection with 10^3 tissue cysts, a specific seroconversion was detected by MAT on D9 p.i. for all three animals. The MAT titres on D9 p.i. were 24 for pig T2, 48 for pig T1, and 192 for pig T3. Titres reached 192 in all three tissue cyst-infested animals as early as D16 p.i. and stayed at this level until D68 p.i., corresponding to the end of the experiment (Fig. 19c, see Supplementary data). Seroconversion by ELISA was detected on D16 p.i. for all three pigs (T1, T2, T3) (Fig. 19d, see Supplementary data).

No *T. gondii* DNA was detected by qPCR in the 40 samples of whole blood and in the 40 faecal samples collected from the oocyst and tissue-infected pigs, and the negative control pig.

Following the analysis of faeces by the flotation and sedimentation methods, no gastro-intestinal parasitic infection was detected.

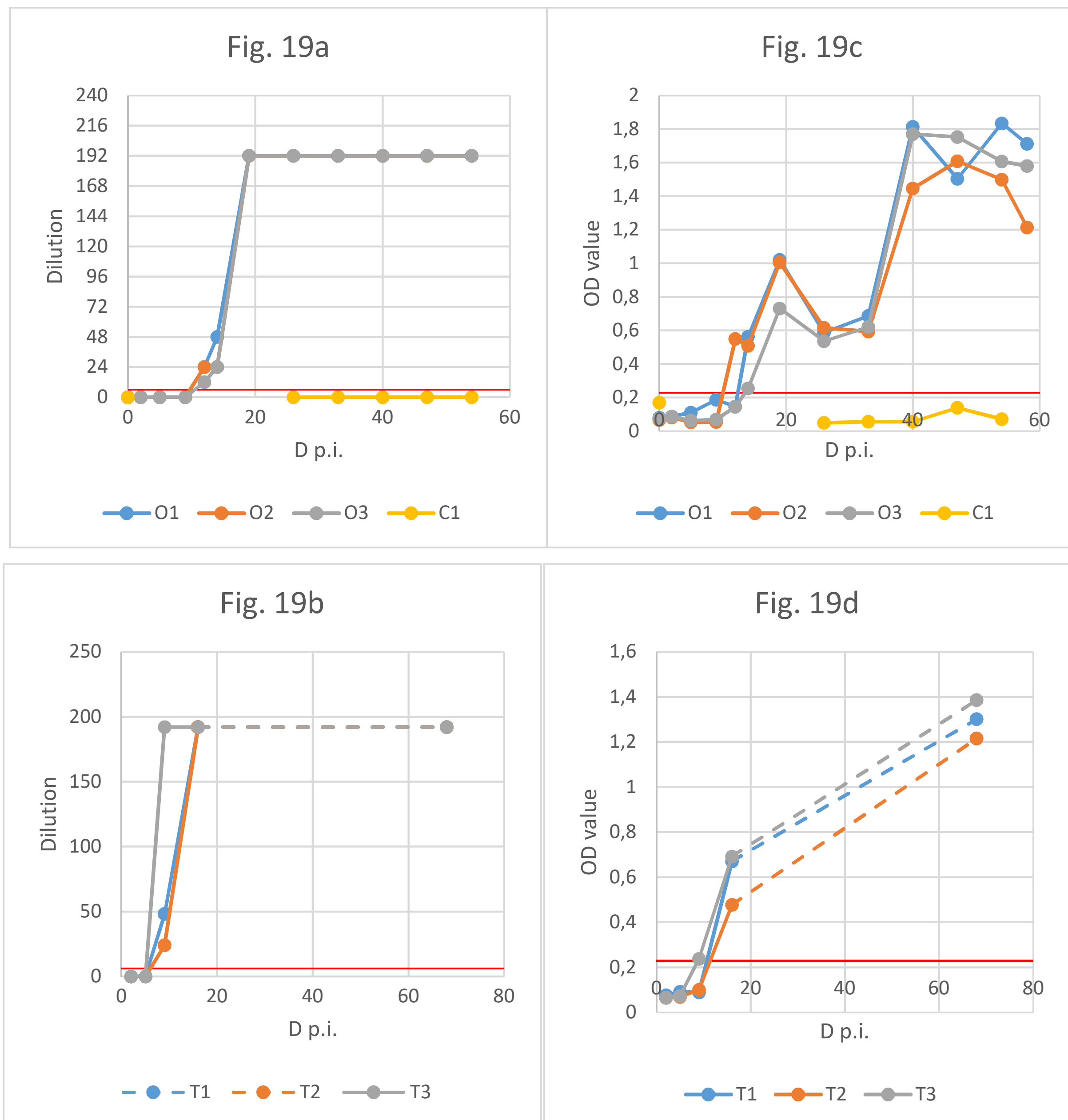

Figure 19 MAT and ELISA results. Anti-T. gondii immunoglobulins development visualized for the pigs experimentally infected with T. gondii oocysts with the use of MAT (Fig. 19a) and ELISA (Fig. 19b), and for the pigs experimentally infected with tissue cysts with the use of MAT (Fig. 19c) and ELISA (Fig. 19d). Collection points in Fig. 19b and 19d are visualized as points to highlight the missing collection days between D16 and D68 p.i. ELISA OD values and MAT dilutions respectively are represented on the y-axis, days post-infection are represented on the x-axis. The horizontal red line represents the cut-off values applied.

Dry sausage and processed pork production

Overall, a total of 126 dry sausages (3 dry sausages × 7 recipes × 6 collection days) and 24 processed pork bags (2 batches of processed pork × 4 recipes × 3 collection days) were produced.

Physicochemical and microbiological properties of dry sausages and processed pork

pH levels

For all eleven recipes tested (F1 to F6 and FT of the dry sausages, and F2, F4, FT, VH of the processed pork), acidification was observed during the ripening phase (D0 - D6) with an average pH drop of 0.82 and 0.72 units for the sausages and processed pork respectively (see Supplementary data). The pH then increased across the recipe spectrum of all eleven recipes, with values for the seven dry sausage recipes between 5.32 and 6.02 units at the end of drying process of sausages (D30). The only recipe for which the pH remained stable during the measured period (D1 – D6) was the control recipe (VH) (see Supplementary data). Continuous pH monitoring in one of the sausages from each recipe during the ripening process is shown in the supplementary files.

Lactic acid microbiota

Initial levels of the lactic acid bacteria in the dry sausages developed from 6.66 ± 0.189 Log10 CFU/g at D0 to 9 ± 0.156 Log10 CFU/g at D13 (see Supplementary data), with no remarkable difference between recipes. In processed pork samples, the first incubation of 24 hours at 24°C allowed a notable development of the lactic acid bacteria, with an increase in populations of about 2.5 Log10 CFU/g. As early as D1, the F2, F4, and FT recipes, supplemented with bacterial starter cultures had populations of 8.85, 8.81, and 8.89 Log10 CFU/g, respectively, compared to 5.75 Log10 CFU/g for the unsupplemented control recipe (VH) (see Supplementary data). For the latter, the lactic acid bacteria continued to develop during the incubation phase at 13°C to reach a population of 8.3 Log10 CFU/g at D6.

Water activity (a_w)

Loss of water, equivalent to an approximate 40% weight loss at the end of the drying process, was measured regardless of the recipe, leading to a decrease in mean a_w values from 0.969 (SD = 0.0043) at D6 to 0.907 (SD = 0.0136) at D30 (see Supplementary data). The a_w of the processed pork samples remained constant during the six days of bagging, with a mean value of 0.975 (SD = 0.0088) across all four recipes (see Supplementary data).

NaCl, nitrates, and nitrites

Nitrates (NaNO3), nitrites (NaNO2), and salt (NaCl) levels measured at D0 and at the end of ripening (D50) are presented in supplementary files. The lower limit of quantification of nitrites for the applied method is 2mg/kg. For this reason, residual nitrite doses in dry sausages ranged between 2 - 15mg/kg and were observed at D0 for recipes F1 to F6. The salt concentration in sausages was significantly higher ($p < 0.001$) at D50 (M = 5.75, SD = 0.73), compared to the salt levels at D0 (M = 2.38, SD = 0.25), regardless of the recipe tested (see Supplementary data). In processed pork, NaCl levels remained stable (M = 2.53, SD = 0.06) during the six days of ripening, while nitrate levels decreased in recipes F2 and F4 (the only recipes containing added potassium nitrate), with the highest reduction in recipe F2 from 148.5mg/kg to 15mg/kg. The only noteworthy residual nitrite dose was measured in recipe F4, the only recipe containing added nitrite salt, at 6.5 mg/kg (see Supplementary data).

T. gondii molecular detection, parasite burden estimates, and viability analysis in dry-sausages, processed pork, and tissue samples

Dry-sausages

Seven different recipes, collected over six different collection days (D1 to D50), were analysed by both qPCR and MC-qPCR, for a total of 42 sausages.

Twenty-eight out of forty-two dry sausages (28/42; 66,7 %) were positive for the presence of *T. gondii* DNA by MC-qPCR and thirty out of forty-two dry sausages (30/42; 71,4 %) were positive by qPCR. A total of nineteen dry sausages (19/42; 45.2 %) were positive by both methods, eleven samples (11/42; 26.1 %) were positive by only qPCR, additional nine samples

(9/42; 21.4 %) were positive only by MC-qPCR, and three samples (3/42; 7.1 %) were negative by both methods applied. The parasite burden estimates varied from 0 to 625 parasites per gram (ppg) (M = 25, SD = 99), as described in supplementary files. Statistical analysis showed no significant effect of the tested recipes (F1 to F6, FT) or days of sample collection (D1, D6, D13, D20, D30, D50) on parasite burden. The parasite burden estimates were below ten parasites per gram in all sausage samples, except for five of them: recipe F3, collected on D6 (40 ppg), recipe F6, collected on D6 (122 ppg), recipe F2, collected on D20 (111 ppg), recipe F6, collected on D20 (75 ppg) and recipe FT, collected on D30 (625 ppg).

Three mice were used for the mouse bioassay for each of the six recipes (F1 to F6) and one control recipe (FT) produced and five collection days (D1, D6, D13, D20, D30,). In total, 105 mice were included in the viability analysis. No specific *T. gondii* antibodies (IgG) were detected in any of the 105 mice sera, four weeks p.i. All the included positive controls, fed *T. gondii* oocyst, were positive by MAT.

Processed pork

Twelve processed pork samples corresponding to four different recipes (F2, F4, FT, VH), collected over three different collection days (D0, D1, D6), were analysed by both qPCR and MC-qPCR.

Twelve out of twelve (12/12; 100 %) samples were positive for the presence of *T. gondii* DNA by both the MC-qPCR and by qPCR, with parasite burden estimates varying from 1 to 241 ppg (M = 50, SD = 71), as described in supplementary files. The highest parasite burden was estimated for the recipe FT, collected on D6 (241 ppg).

Four mice were used for the mouse bioassay of the unsupplemented processed pork sample (control recipe = VH) at each of the three different collection days (D0, D1, D6) as well as six mice per collection day for the three recipes produced (F2, F4, FT). A total of 66 mice were included in the processed pork mouse bioassay study. Out of four mice used for testing the unsupplemented processed pork sample recipe (VH), with a parasite burden of 8 parasites per gram, one mouse died within the first 2D p.i. without detection of *T. gondii* in the brain by microscopy, one mouse developed specific anti-*T. gondii* antibodies detectable by MAT four

weeks p.i, and two mice were negative for specific antibodies by MAT. All the included positive controls, fed *T. gondii* oocyst, were positive by MAT. All other mice were negative for *T. gondii*-specific antibodies by MAT.

Tissue samples

Four different tissues (shoulder, breast, ham, and heart), collected from eight different animals (O1 - O3, T1 - T3, N1, N2), were analysed by MC-qPCR, adding up to a total of 32 samples (Table 21).

Seventeen out of thirty-two tissues (53.1 %) tested positive for the presence of *T. gondii* DNA by MC-qPCR (Table 21). Similar parasite burden estimates per gram of tested tissue were found in hearts (M = 147, SD = 233), breasts (M = 143, SD = 256), and shoulders (M = 117, SD = 225), with hams being the tissue with the lower parasite per gram estimates (M = 1, SD = 2) (Table 22).

Statistical analysis by the Wilcoxon test showed a non-normal distribution of samples with no difference between the pig groups infected with different stages of the parasite in regards to the parasite burden within the tissues ($p > 0.05$). Following Kruskal-Wallis test showed no significant difference ($p > 0.05$) between the muscle region tested (shoulder, breast, ham, heart) or the parasite stage (oocyst, tissue cyst) on the parasite burden estimate.

Table 20 *T. gondii* DNA detection and parasite burden estimates in tested tissues. The mean quantification cycle (Cq) values (based on up to two replicates) and parasite burden estimates per gram by tissue for the individual pigs. Negative MC-qPCR results were not taken into account in the calculation of the Cq mean and the subsequent calculation of the parasite burden. The number of positive reactions in each duplicate qPCR run is presented in a separate column. ND = no T. gondii DNA detection recorded.

Pig	Sample	MC-qPCR Cq mean	Positive replicates MC-qPCR	Estimated number of parasites per gram of sample
O1	shoulder	ND	0/2	0
	breast	32.86	2/2	22
	ham	ND	0/2	0
	heart	ND	0/2	0
O2	shoulder	ND	0/2	0
	breast	ND	0/2	0
	ham	ND	0/2	0
	heart	34.57	2/2	6

O3	shoulder	ND	0/2	0
	breast	28.56	2/2	642
	ham	ND	0/2	0
	heart	30.96	2/2	98
T1	shoulder	30.86	2/2	106
	breast	32.75	2/2	24
	ham	ND	0/2	0
	heart	28.45	2/2	700
T2	shoulder	ND	0/2	0
	breast	ND	0/2	0
	ham	ND	0/2	0
	heart	31.61	2/2	59
T3	shoulder	32.39	2/2	32
	breast	28.99	2/2	459
	ham	35.19	1/2	4
	heart	30.60	2/2	130
N1	shoulder	30.47	2/2	144
	breast	ND	0/2	0
	ham	34.99	2/2	4
	heart	30.20	2/2	177
N2	shoulder	28.53	2/2	657
	breast	ND	0/2	0
	ham	ND	0/2	0
	heart	34.55	2/2	6

Table 21 Mean *T. gondii* parasite burden estimates by individual tissues, individual pigs, and a group of pigs based on the type of infection/stage of *T. gondii* parasite. Standard deviation values are provided in round brackets.

	Pig group			
Tissue	**Oocyst-infected**	**Tissue-cyst-infected**	**Naturally infected**	**Total - groups**
shoulder	ND	46 (54)	401 (363)	117 (225)
breast	221 (364)	161 (258)	ND	143 (256)
ham	ND	1 (2)	2 (3)	1 (2)
heart	35 (55)	296 (351)	92 (121)	147 (233)
Total - tissues	64 (105)	126 (100)	124 (60)	

DISCUSSION

Pork is the most consumed meat in France, and approximately three-quarters of the consumed pork is eaten in the form of charcuterie (FranceAgriMer, 2022). For this reason, the present study aimed to investigate the tissue tropism of *T. gondii* in naturally and experimentally infected pigs and to evaluate the impact of key ingredients and additives as well as processing steps on the viability of *T. gondii* in meat products.

Pigs included in the current study were divided into three groups based on the selected stage of parasite used for the experimental infection: the oocyst-infected group, the tissue-cyst-infected group, and the negative control. Animal welfare and the 3R principle (Russell and Burch, 1992) were taken into account in the study design. For example, the negative control pig (C1) was housed with the oocysts-infected group beginning with D2 p.i., but separated in an individual pen two weeks later, due to the aggressive behaviour of the other animals towards it. Pigs are animals with a complex social hierarchy system within a group, thus such behaviour was not surprising (Tong et al., 2019).

Inoculation doses used in previous experimental infection studies in pigs were reviewed in order to select the appropriate dose. Ideally, such dose should be high enough to induce a successful experimental infection in pigs, described as antibody detection and/or PCR confirmation, while limiting the potential negative effects (fever, discomfort, etc.) at the same time. Oocyst inoculation doses described in literature varied from a single oocyst (Dubey et al., 1996) to 800,000 oocysts (Algaba et al., 2018) per animal. The number of *T. gondii* tissue cysts used for experimental infections ranged from 700 (Jennes et al., 2017) to 6,000 tissue cysts (Algaba et al., 2018). For the present study, we used 1,000 oocysts per animal for one group, and 1,000 tissue cysts per animal for the other group, corresponding to the most frequently used inoculation doses. The morphological structure of *T. gondii* oocyst, containing four sporozoites within each of the two sporocysts, is well-described (Dubey et al., 1970a) while, the average number of bradyzoites per single tissue cyst in mouse brain varies from 194 bradyzoites at three weeks p.i. to 344 bradyzoites at four weeks p.i., and 427 bradyzoites at five weeks p.i., after which the bradyzoite count seems to stabilize around this mean (Watts et al., 2015). Therefore, even when taking into account the lowest bradyzoites account per

cyst, it is obvious that the effective dose was not equal, with 8,000 sporozoites per animal for the oocysts-infected group and an estimated 194,000 bradyzoites per animal for the tissue-cysts infected group. Using an unbalanced number of parasites we were mimicking a natural infection of a pig, given the uncertain dose ingested during natural infection with *T. gondii* parasites either by food (or water) contaminated with faeces of infected, shedding felines or by ingestion of tissues of infected mammals or birds. Pigs were fed the corresponding infectious dose on bite-size portions of pain au lait, mainly for the stress reduction linked with an oral infection, representing thus a natural way of infection. An alternative to this method would have been the anaesthesia of the pigs and the use of a gastro-oesophageal tube. By using pain au lait, a highly appetizing French pastry to which the pigs were previously accustomed as a reward, we were certain of the total uptake of *T. gondii* parasites by each individual animal.

Concerning the experimental infection of the pigs with *T. gondii* tissue cysts, the infection had to be repeated twice because no specific anti-*T. gondii* antibodies were detected during the 30-day follow-up. To our knowledge, this is the first report of repeated experimental infection in pigs, an uncommon event since Dubey et al. (1996) had demonstrated the sensitivity of pigs to doses as low as 1 oocyst (VEG strain) or 700 tissue cysts (Jennes et al., 2017).

This rather rare event may be explained by the fact that the mice brains containing *T. gondii* tissue cysts were kept for three months in liquid nitrogen at the BRC (Reims, France) before the pig infection, inducing most likely a decreased viability, and a short delay of less than 24-hour-long transport allowed us to accept the latest hypothesis as the most likely one, especially when the successful experimental infection using locally produced *T. gondii* tissue cysts proved the sensitivity of the pigs to the infection. Therefore, one should keep in mind the disadvantages of frozen storage of tissue cysts as well as the benefits of performing laborious and complex animal experimental infections within a single lab, from infective stages up to tissue collection, eliminating thus the transportation, prolonged storage, and temperature fluctuations.

Symptoms of acute parasitemia following *T. gondii* infection in pigs depend on several factors such as the breed and age of the pigs, the parasitic stage, the infection route, and the infective

dose of the parasites (Jungersen et al., 2001). In this case, a febrile response was present in all pigs. Concerning the pigs infected with *T. gondii* tissue cysts, a sharp rise in body temperature measured between D5 and D7 p.i. was notable. Whereas, in the oocyst-infected pigs, individual points of higher temperature were observed. This might be explained by the higher number of *T. gondii* parasites in the infection dose administered (194,000 bradyzoites versus 8,000 sporozoites). Moreover, the clear peak in temperature measurements of the tissue-cyst-infected pigs in this study could have been possibly caused by a higher virulence of bradyzoites when compared to sporozoites, manifesting the effect of the form of the parasite used, as was suggested previously by Garcia et al. (Garcia et al., 2017a). However, it should be noted that oocyst-induced infections in animals were more often associated with severer symptoms (Dubey and Beattie, 1988), thus suggesting that the observation was possibly a result of the higher dose administered. The rise in the body temperature of an animal infected with *T. gondii* is commonly observed and described in studies with experimentally infected pigs (Algaba et al., 2018; Genchi et al., 2017; He et al., 2020; Hou et al., 2019; Juránková et al., 2014a), adult sheep, goats, and lambs (Juránková et al., 2013a; Thomas et al., 2022; Verhelst et al., 2014). Concerning the negative control and the fluctuations in the body temperature, those were not related to *T. gondii* infection, as anti-*T.gondii* antibodies were never detected by serology during the whole testing period. The body temperature of a pig may fluctuate during the day based solely on the rhythms of respiration and blood pressure of the pig or, most likely in our case, the increased movement during handling, enhanced by the fearful nature of this animal (Baldwin and Ingram, 1968). At the same time, the temperature measurements were not taken at the very same time of the day and thus increasing the chance to observe a fluctuation.

The weight measurements in the oocyst-infected pigs showed that on the individual level, only pig O1 reached the set weight objective with a surplus of 10.8 kg, and pigs O2 and O3 lagged behind the set goal with a deficit of 3.7 kg and 8.7 kg respectively. Similarly, the average daily weight gains were lower than the estimated goal in pigs O2 and O3 while higher than average for pig O1. It is important to note that both the pig O2 and pig O3 started the experiment with a weight of 2.8 kg below the group's average and this may have influenced their lower daily weight gain considering it is the weaning weight that can predict their lifetime performance

as measured by growth potential (Douglas et al., 2014). Both the weight gain (953 grams per day) and the final weight (100kg) in the negative control pig (C1) were above the set objectives. Lower final weight and weight gains in oocyst-infected pigs may also be caused by the loss of appetite commonly identified in pigs between days 2 and 12 after *T. gondii* infection (Dubey, 2010). Additionally, as a result of the immune system stimulation by pathogens, pro-inflammatory cytokines which have a negative effect on growth and feed efficiency, are produced (Johnson, 1997), hence negatively influencing weight gains. The weight of the tissue-cyst-infected pigs was measured only at the beginning of the infection and could not be followed throughout the experiment due to a general locked down within the COVID-19 pandemic measures. At the same time, the size of these pigs at the end of the experiment did not allow for fitting them on a scale and therefore, the final weight was based on the expert veterinary opinion at around 170 kg at 278 days of age. Such weight is comparable to the weight of a minimum nine month old pigs destined for the production of Italian dry ham (160 ± 16 kg) (Gallo et al., 2017; di Parma, 1992).

Specific immune response was observed in sera of all pigs in both experimental groups, starting as early as D9 p.i. in tissue-cyst-infected pigs and D12 p.i. in the oocyst-infected pigs, while peaking at D16 p.i. and D19 p.i. respectively and lasting at this level until the slaughter. Animals can develop long-lasting high levels of specific IgG antibodies following the infection with *T. gondii* (Dubey, 2022; Dubey and Beattie, 1988). The first measurable levels of anti-*T. gondii* antibody production in pigs can be commonly observed between one and three weeks p.i. (Alves et al., 2019; Basso et al., 2017; Bokken et al., 2012; Garcia et al., 2017a; Juránková et al., 2014a; Lind et al., 1997; Verhelst et al., 2011). Similar seroconversion periods were observed also in other farmed animal species such as lambs and sheep (Dubey, 2022; Esteban-Redondo and Innes, 1998; Lundén et al., 1994; Thomas et al., 2022; Trees et al., 1989). In the present study, the specific antibody production and consequently the peaking of the antibody titres in the oocyst-infected pigs developed later than *T. gondii* antibody levels in tissue-cyst-infected pigs. The cause of this delay could be explained by the difference in the parasitic stage or infective dose used. It was previously reported that higher infective doses induce higher antibody titres (Esteban-Redondo and Innes, 1998; McColgan et al., 1988; Opsteegh et al., 2016b) which might be the case with 8,000 sporozoites and 194,000 bradyzoites used in this

study. At the same time, tissue-cyst-infected animals tend to achieve their peak antibody levels earlier, despite the similarity in the kinetics of the immune response (Garcia et al., 2017b). The sample collection from the tissue-cyst-infected pigs was largely impacted by a lockdown during the COVID-19 pandemic, causing a 52-day-long break in blood collection, compared to a regular blood sample collection twice to three times a week from the oocyst-infected group. Therefore, even though we were able to observe the seroconversion in these pigs, the specific antibody development was not documented in detail. For the same reasons (COVID-19 pandemic restrictions) the tissue-cyst-infected pigs were culled with a ten-day delay (D68 p.i.) compared to the oocysts-infected pigs. The delay in the culling leading to the overall increased size of pigs in the tissue cyst group may have impacted the raw number of parasites per gram of tissue but it should not have influenced the distribution of the parasite in tissues between the two experimentally infected groups as the average tissue cyst burden remains stable over time, only the cysts may increase in size and generally contain more bradyzoites (Watts et al., 2015).

Our results show that no *T. gondii* DNA was found in the hams of pigs infected with oocysts, further underlined by the overall low *T. gondii* prevalence and a low parasite burden in the hams of the naturally and tissue-cyst-infected pigs. Similarly, no *T. gondii* DNA was found in the shoulder muscles of oocyst-infected pigs which were in stark contrast with the relatively high prevalence and parasite burden found the in shoulder muscles of pigs in the tissue cyst group, and naturally infected pigs in particular. The low parasite burden in hams is compatible with results reported in pigs naturally infected with *T. gondii*, despite the frequent parasite occurrence in hams of these seropositive pigs (Herrero et al., 2017). Comparable results with relatively low parasite burden along a high prevalence of *T. gondii* DNA by MC-qPCR in both hams and shoulders were previously observed also in experimentally infected pigs (Gomez-Samblas et al., 2016). The higher frequency of the positive *T. gondii* DNA findings in hams reported by Herrero et al. (2017) and Gomez-Samblas et al. (2016) may be explained by the high number of ham tissues tested compared to the present study. The high frequency of the parasite and relatively high parasite burden in the shoulders of naturally infected pigs in the present study may have been caused by the potentially higher dose of *T. gondii* parasites ingested as the distribution is known to be dose-dependent (Djurković-Djaković et al., 2012).

However, as mentioned previously, a limited sampling size in the present study (n = 2) did not provide the power to find the differences statistically significant. Pigs experimentally infected with tissue cysts were previously confirmed to harbour *T. gondii* tissue cysts in shoulder muscles (Rani et al., 2019) thus offering another potential difference in relation to the parasitic stage used for infection. Generally, individual variation and variation between tissues are observed in studies providing a detailed comparison of *T. gondii* tropism in pig tissues. Unfortunately, the variability of results between animals in the current study and in comparison to the limited number of other studies is insufficient to identify whether part of the variation depends on the infectious dose and the parasite stage.

High parasite counts and relatively frequent *T. gondii* DNA findings were observed in the breasts of both experimentally infected pig groups but surprisingly no *T. gondii* was found in the same tissue of naturally infected pigs. Considering the higher number of *T. gondii* parasites found in the breasts of the naturally infected pigs, the lack of parasites in the breasts examined may be explained by an uneven distribution of tissue cysts within the tissues of infected pigs (Rani et al., 2019). The majority of the pigs (n = 7/8) included in the current study tested positive for *T. gondii* DNA in hearts by qPCR, thus confirming one of the predilection sites for the parasite (Dubey, 1986; Opsteegh et al., 2016b). The high prevalence of the parasite in the heart tissues was accompanied by a relatively high parasite burden. Similarly to our findings, a high burden of *T. gondii* was found in the hearts of experimentally infected pigs regardless of the phase of the infection (Algaba et al., 2018; Juránková et al., 2014a) or animal species (Esteban-Redondo et al., 1999; Juránková et al., 2015; Thomas et al., 2022).

Common predilection sites of *T. gondii* tissue cysts, brain, and heart, were identified for a range of intermediate hosts: pig, sheep, and goat (Opsteegh et al., 2016b). Tissue cysts may also be found in skeletal muscles and to a lesser extent in visceral organs, such as the lungs, liver, and kidneys (Dubey et al., 1998). However, the interspecies comparison should be applied with caution as differences in the distribution of *T. gondii* between various intermediate hosts are well-documented. In cattle, a low presence of viable *T. gondii* was observed without an obvious predilection site (Burrells et al., 2018; Dubey and Thulliez, 1993; Esteban-Redondo et al., 1999; Opsteegh et al., 2016b).

Parasite distribution varied on the anatomical and individual level, suggesting a random tissue cyst distribution amongst organs and muscles with the tongue and heart being more heavily infected than other tissues. Similar observations were done by Dubey and Beattie (Dubey, 1986; Dubey and Beattie, 1988). The parasite burden seems to follow the previously described patterns with the highest burden of the tested tissues in the hearts (Algaba et al., 2018; Juránková et al., 2014a). The variability in the parasitic load might be also a result of the uneven distribution of the tissue cysts in an organism (Rani et al., 2019). The MC-qPCR method was used for the detection and quantification of *T. gondii* DNA in tissue samples as well as the parasite distribution study within different tissues (shoulder, breast, ham, heart) of infected pigs because of the improved sensitivity over commercial DNA isolation followed by PCR (Juránková et al., 2014a; Opsteegh et al., 2010). The absence of *T. gondii* in a selection of tissues may suggest a concentration below the detection limit of the method. However, given the detection limit of the MC-qPCR method as low as a single parasite in 100 grams of a sample (Gomez-Samblas et al., 2016, 2015; Juránková et al., 2014a, 2013b; Opsteegh et al., 2010), this option seems unlikely. The specific magnetic capture protocol (Opsteegh et al., 2010) with the adjusted CIAC design (Deng et al., 2021b) was selected as we failed to reproduce the UV-based DNA elution step described in the protocol by Gisbert Algaba et al. (Gisbert Algaba et al., 2017). The selected protocol was slightly modified in terms of consumables used due to the limited availability of some products on the market. The design and concentration of the MC-qPCR primers and probes remained unchanged.

The infected pork is a source of *T. gondii* infection for humans in many countries (Dubey, 2009b). Among the many food animals, pigs are considered the most important for *T. gondii* transmission in the USA and China (Dubey et al., 2020). Even though quantitative risk assessment models from Italy (Belluco et al., 2018) and the Netherlands (Deng et al., 2021b; Opsteegh et al., 2011a) identified beef as the most important source of predicted meat-borne *T. gondii* infections in humans, the consumption habits in France fully justify the focus on pork meat products. For this reason, the second part of the experiment aimed to estimate the effect of commonly used additives (nitrates and nitrites) and salt, on the viability of *T. gondii* parasites within dry fermented sausage and processed pork. The seven sausage recipes, focusing on the impact of salt and the additives on the infectivity of the parasite, were

produced using tissues of pigs infected with *T. gondii* oocysts and were encased in natural intestine casings. Although, given the relatively low weight of the pigs and the seven different sausage recipes to be produced (the control recipe FT and recipes F1 to F6), it was necessary to collect additional muscle tissues from these animals, especially the loins, compared to the traditional recipe including only shoulder, breast and/or ham. No influence of this dilution by non-traditionally used muscles was expected as it was proved by our results that the distribution of *T. gondii* varies more on an individual level than within muscle tissues of these pigs, supported by a random distribution of tissue cysts amongst muscles described previously (Dubey, 1986; Dubey and Beattie, 1988). Moreover, the purpose of the tissue collection was to produce meat products containing measurable *T. gondii* DNA levels in each dry sausage and processed pork sample, regardless of the variation in the parasite DNA concentration. Arguably, the goal of the production of homogenous sausages was not completely successful since 14 of 42 sausages were negative for *T. gondii* DNA. Possible explanations are the observed individual parasite burden variation in the tissues in the current study, the previously discussed uneven tissue cyst distribution in pig muscles (Rani et al., 2019), and a suggested low number of tissue cysts produced in some experimental infections of pigs (Abdulmawjood et al., 2014). It should be stressed that ideally, all sausages included in the testing of the effect of NaCl, nitrates, and nitrites would contain a similar and high parasite and high parasite burdens, a feat that was not achieved.

To further investigate the impact of different concentrations of nitrites (0 or 60 mg/kg), nitrates (0, 60, or 200 g/kg) and salt (0, 60, or 200 g/kg) on *T. gondii* viability, a supplementary experiment with processed pork was performed. All processed pork samples contained DNA of *T. gondii*, suggesting a homogenous batter of the processed pork. The negative control recipe (VH) without salt, nitrate, and nitrite content was the only recipe causing *T. gondii* infection in mouse bioassay. The fact that not every mouse included in this control recipe bioassay was positive for *T. gondii* antibodies can be explained by the premature death of two of the four mice used. Toxoplasmosis could not be proven as a cause of death as tissue cysts in the brain need more time to form, and only mice older than seven days are being routinely screened for the presence of anti-*T. gondii* antibodies. The remaining mouse bioassay using

the negative control recipe was unexpectedly negative, which may be explained by an uneven distribution of parasites in the digested pellet, part of which was fed to the mice.

Unlike the tissues, the dry sausages and processed pork were tested for the presence of *T. gondii* DNA with the use of both magnetic capture isolation combined with qPCR and DNA extraction with a commercially available kit combined with qPCR. All the processed pork samples were found positive for *T. gondii* DNA by both detection methods but eleven dry sausages tested solely positive by qPCR, and nine were only positive by MC-qPCR. The slightly higher number of qPCR positive findings compared to the magnetic capture method in the dry sausage testing can be explained by the input of 200g of sample in the trypsin digestion, followed by the commercial DNA extraction kit, and only half of the sample weight (100g) in the magnetic capture method, thus increasing the chance of encountering *T. gondii* tissue cysts. At the same time the qPCR was run in duplicate for MC-qPCR samples and in six-plicate for dry sausage samples isolated using a commercial kit, which increased the odds of successful identification of the parasite DNA in samples with a low parasite count but also a potential false positive result. A similar comparison of the two methods was done previously in a survey of *T. gondii* in wild house mice (Juránková et al., 2014b), however, with the magnetic capture method coming out as the more sensitive of the two.

The pH of pork meat decreases naturally down to pH 5.5 over a period of hours after the slaughter of the pig due to the formation of lactic acid in muscles (Lonergan, 2008). The decrease in the measured pH levels below this level in dry sausages and processed pork during the ripening phase (D0 – D6 post-production) was expected and was caused by the proliferation of lactic acid bacteria added to the recipe mix. Only in the recipe without any additives (VH), the pH levels stayed relatively stable at around pH 5.5 over the same period (see Supplementary data). Lower pH levels may limit the growth of certain bacteria but are expected to play only a limited role in *T. gondii* tissue cysts inactivation. The naturally occurring acidification alone is not sufficient in the inactivation of bradyzoites as tissue cysts are naturally resistant to low pH values and are capable of surviving at pH 5.0 for a prolonged period of time (Pott et al., 2013), still, a complete inactivation can be successfully achieved

with a combination of low pH (>4.6 and ≤5.2), NaCl content ≥1.3 % and >4 hours of fermentation (Fredericks et al., 2019).

The nitrate, nitrite, and salt (NaCl) levels measured were generally in line with those expected (see Supplementary data), and were consistent with the additive doses in corresponding recipes. Due to the loss of water from the sausages during the drying stage, the salt concentration was considerably higher at D50, regardless of the recipe tested. Residual nitrite doses are lower than those added (2 - 15 mg/kg at D0 for doses ranging from 0 to 120 mg/kg). Nitrates in dry sausages are unstable and are reduced to nitrites, mostly due to the nitrate-reductase activity of the bacterial starter flora, and further to nitric oxide which converts myoglobin to nitrosomyoglobin (Gül Karahan et al., 2005), contributing to the red colour of dry sausages. Nitrates and nitrites are frequently added to meat products in the form of salts to act as preservatives and antioxidants (Honikel, 2008). Christieans et al. showed that a concentration of nitrites in dry fermented sausages of ≥ 80 ppm represented an essential barrier in the growth of bacterial pathogens like Listeria monocytogenes and Salmonella spp. (Christieans et al., 2018), and therefore, a question was raised concerning the potential reduction of viability of protozoan parasites. Nitrites and nitrates have a negative effect on human health, with nitrates widely considered more toxic and linked with higher chances of developing colorectal cancer (Santarelli et al., 2008). Moreover, a recent opinion of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) confirms the existence of an association between the risk of developing colorectal cancer and exposure to nitrites and nitrates (Reducing dietary exposure to nitrites and nitrates, 2022). Overall, more than 66 % of Europeans consume processed meat products (Crowe et al., 2019), where nitrites and salts may be in surplus. Understandably, consumer preferences regarding the consumption of meat products are changing and products with lowered sodium content are viewed positively (Guàrdia et al., 2006), especially when considering the proven risk of cardiovascular problems caused by hypertension due to increased salt intake (Frisoli et al., 2012; He et al., 2012).

The absence of viable parasites in the dry sausages collected on D0 (i.e. a few hours after the contact of the *T. gondii*-positive meat with the dry ingredients/additives) was observed for all

the recipes, including the control recipe (FT) without added nitrite salt, containing only NaCl at a concentration of 26 g/kg. This result suggests a notable sensitivity of *T. gondii* to both NaCl used in the production and the processing applied. Similar to our findings, other authors proved the sensitivity of tissue cysts to increased salt concentrations (Dubey, 1997; D. E. Hill et al., 2006; Hill et al., 2004; Navarro et al., 1992). A recent study on *T. gondii* bradyzoites inactivation during the curing process of pork sausages showed that NaCl concentrations in ground pork at 1.3% or above are effective at inactivating bradyzoites within tissue cysts during the first six hours of the fermentation process (Hill et al., 2018). The enhanced protective effect of NaCl, when combined with nitrite salts, was well-described only recently (Fredericks et al., 2020, 2019; Gomez-Samblas et al., 2016; D. E. Hill et al., 2006).

According to our findings, the NaCl content of 2 % and above in traditional French dry sausage and processed pork alike appears sufficient to inactivate *T. gondii* parasites within these products as early as one day following the incorporation, especially when combined with nitrates and nitrites. Follow-up studies, focusing on processed pork products, are needed to build on the valuable information obtained during this study as the mice fed untreated processed pork were not consistently positive in all bioassays. This result may also suggest an uneven distribution of the parasite in individual dry sausages even when made of a homogenized meat batter. Higher parasite burden in the homogenized meat batter may have resulted in a presence of *T. gondii* in all dry sausage recipes and may have resulted in a positive mouse bioassay in the least effective concentrations of NaCl, nitrates, and nitrites. To confirm the exact potential of ingredients and additives used in the dry sausage recipes F1 to F6, an experiment with the exactly counted number of individual *T. gondii* parasites instead of tissue cysts of various sizes, while taking into account a potential reduction factor of the tissue cyst wall, would have to be designed. Additionally, a higher number of samples per tested recipe could be included to increase the statistical power of the experiment. More frequent sampling of meat products in the early collection time points, combined with lower concentrations of salt and additives used, would be advised to determine more precisely the conditions capable of *T. gondii* inactivation. Despite the optimistic results showcased in the present study, it is advised to adequately cook all meat and meat products, if possible, before consumption to ensure the inactivation of potential *T. gondii* parasites.

Our results suggest an uneven distribution of tissue cysts within the porcine tissues tested, regardless of the infectious stage of *T. gondii* used. An animal experiment with similar settings but the delayed slaughter of pigs could be performed to ensure sufficient time for the distribution of *T. gondii* in tissues and potentially increase the frequency of *T. gondii*-positive tissues. Alternatively, since no significant difference in parasite distribution with regards to the infectious stage of *T. gondii* was observed, a significantly higher number of naturally infected pig carcasses could be collected to add the statistical power to a future experiment aimed to assess the distribution patterns of *T. gondii* within pigs. The heart, of the tissues tested, was shown to be one of the predilection sites with a high frequency of *T. gondii* DNA detection and consistently high parasitic burden estimates.

ACKNOWLEDGEMENTS

The authors are thankful to all staff of the Biomedical Research Centre of Maisons-Alfort, EnvA, who were involved in monitoring the pigs within the present study. Furthermore, we thank Dr. JP Dubey (USDA, ARS, Beltsville, USA) for providing *T. gondii* oocysts used in the experimental infection, Dr. Joke van der Giessen (RIVM, Bilthoven, the Netherlands) for her expertise and much appreciated suggestions, and Dr. Benoît Durand (ANSES, Maisons-Alfort, France) for his valuable advice concerning the data analysis.

FUNDING

This work was supported by funding from the France-Agri-Mer: contract number 0917003490 and the European Union’s Horizon 2020 Research and Innovation programme under grant agreement No 773830: One Health European Joint Programme (projects ToxSauQMRA and TOXOSOURCES).

Chapter 8

General discussion

As a food-borne parasite present in tissues of various animal species across all continents, *T. gondii* poses a major risk to consumers. General prevention strategies and recommendations were put in place to reduce the burden on public health. Proper cooking and handling of meat, as well as avoiding raw or undercooked meat consumption, are important steps to minimize the risk of infection. However, there are more risk factors of toxoplasmosis in the play, factors such as the prevalence and concentration of the parasite in animal populations, the processing and preparation of the meat, and the inactivation methods associated with these processes all play a role in determining the overall risk of infection from consuming meat. Additionally, the frequency of consumption and portion size of the meat are also important considerations that can impact the risk of infection. By understanding the factors that contribute to the risk of *T. gondii* infection, public health officials and consumers can take steps to reduce the spread and health-related burden of this parasite.

The articles included in this work aimed to estimate the various factors that can influence whether a meat product is a potential source of *T. gondii* infection. Reliable detection of *T. gondii* in animal herds is crucial to successfully locate and eliminate potential sources of contamination at the farm level. A cost-effective strategy to reduce the risk of human infection through pork is to identify herds with a high prevalence of *T. gondii* and implement preventive measures. A commercial ELISA kit for detection of anti-*T. gondii* antibodies has been validated for use with oral fluid, offering a rapid, economical, and welfare-friendly alternative to traditional serological methods that does not require a presence of a veterinarian for blood collection (Chapter 2). Data from such screenings are valuable for providing an overall awareness of the epidemiological situation of *T. gondii* infection in animal populations. Data from the recent European animal prevalence studies focusing on the most commonly consumed animal species and felids were collected to create an updated database reflecting

the latest epidemiological situation. A novel age-dependent model utilizing Bayesian principles was developed in order to answer the need for a precise prevalence estimates in animal populations in the regions with little or no data (Chapter 3). The prevalence of *T. gondii* in livestock influences the risk of human infection, making it an important factor in estimating such risk. As one of the most widely farmed animals globally, pigs were selected as the ideal candidates to study the variable pathologies caused by different strains and stages of *T. gondii*. The findings showed a stage- and strain-dependent effect on the distribution patterns and parasite load within porcine tissues. Infection with tissue cysts and the type III CZ-Šimková strain led to a higher immunogenicity, increased rate of positive tissue samples and a higher parasite burden compared to infection with the type II CZ-Tiger strain and *T. gondii* oocysts (Chapter 4). This thesis aimed to provide a detailed picture of *T. gondii* tropism in pig tissues by examining experimentally infected pigs with oocysts and tissue cysts, and naturally infected pigs, with anatomical precision. The results showed a higher parasite burden and more frequent positive results in the muscles of the head and body compared to other parts of the pig's carcass. (Chapter 5). This is a favorable finding for the use of pork hind limbs in dry ham production. The results indicated that some pigs had surprisingly high parasite burdens in tissues of their reproductive tract, which may suggest a connection between *T. gondii* and congenital transmission of the infection. Despite the individual variability between pigs, the observed patterns showed lower presence and parasite burden in the oocyst-infected pigs compared to the infection with tissue cysts (Chapter 5). Available meat and meat products in retail, with special attention to the increasingly popular game meat, were screened for the presence of *T. gondii* DNA to provide a more precise estimate of the potential risk to consumer. The presence of *T. gondii* DNA was confirmed in meats of various species with the exception of beef, despite presence of the specific antibodies in meat juice. The results suggested that venison could be a high-risk meat for *T. gondii* infection given the popularity of the meat and preparation preferences (Chapter 6). Raising awareness regarding the consumption meat of wild animals especially in populations at risk (e.g. pregnant women) is therefore advisable. With regards to the tradition of meat products of pork origin in Europe, the effect of salting, additives and processing on meat products was evaluated for French dry sausage, representing similar meat products across Europe. Salt in concentrations of 2%

inactivated *T. gondii* in the meat products in less than a day regardless of supplementary additives (Chapter 7). These results suggest that salt in sufficient concentration may be a viable option for managing risks in processed pork products.

In the remainder, the results from the studies are analyzed in a comprehensive manner, were put into perspective with the help of the recent advances in related fields, and the limitations of the research conducted in the thesis are discussed.

Monitoring the epidemiological situation of *T. gondii* infection in animal herds in Europe

Keeping track of the progression of *T. gondii* infections among animal populations is crucial to minimize the risk of potential human exposure and ensure food safety. Serological screening is commonly used for tracking the prevalence of *T. gondii* in animal herds, with an objective of providing insight into the effectiveness of control measures and to help identify areas where additional interventions may be necessary to prevent the spread of the parasite. With an economical potential in animal husbandry, alternatives for sensitive screening of anti-*T. gondii* antibodies are in demand. Matrices such as milk and oral fluids were explored recently, showing promising, although variable, levels of sensitivity and specificity, often due to use of different laboratory-based methods (i.e. immunoblot and PCR) (Campero et al., 2020; Ranucci et al., 2020).

The presented method achieved a specificity of 97.83% and sensitivity of 78.75% compared to immunoblot. The lower sensitivity is likely due to the lower antibody levels present in oral fluids compared to the blood (McKie et al., 2002). While oral fluid may not represent the most reliable matrix for assessing *T. gondii* status in individual animals, our result indicate that the specific antibodies can be consistently detected on a herd level. This opens the potential for larger-scale applications on a farm level, considering also the fast and animal welfare-abiding sample collection. Despite the capability of such application, this method was not originally intended for individual screening of animals and occasional false negative results are therefore less important than false positive. The novel approach of detecting antibodies in oral fluids is

demonstrated by its limited use worldwide, with exceptions being the detection of SARS-CoV-2 antibodies (Hoschler et al., 2022; MacMullan et al., 2021). Until recently, oral fluids have primarily served as a matrix for detecting anti-viral antibodies in humans (Cruz et al., 2019) and pigs (Campero et al., 2020; Henao-Diaz et al., 2020). Campero et al. (2020) were the first to use oral fluids for indirect parasite detection, which served as the basis for our confirmatory immunoblot testing in this study.

To effectively reduce the risk of human *T. gondii* infection via meat, identifying herds with high prevalence of *T. gondii* and implementing preventive measures is crucial. This need for up-to-date prevalence estimates led to the largest literature review of *T. gondii* in European animals in the past two decades. Over 2,000 articles were screened, providing a comprehensive dataset for developing a Bayesian model, which can accurately reflect the reality in animal herds in regions with limited data. The species-specific and age-dependent estimates from this study are unique for the three matrices used. However, despite efforts to gather the latest *T. gondii* screening literature, none reported the use of oral fluids, emphasizing the pioneering nature of the work presented in Chapter 2. Unfortunately, the lack of recent literature on the use of oral fluids for *T. gondii* screening has precluded the opportunity to compare with modelled estimates from Chapter 3. Potential confirmation of the modelled outcomes by such screening data could have further strengthen the credibility of the model.

The majority of the data utilized in the model originated from serological screenings, resulting in a predominant utilization of a single matrix. Despite the predominance of a single matrix in the data collection process, standardizing the format of data obtained from various studies remained a challenge. One of the critical variables in determining *T. gondii* prevalence, age, was frequently reported imprecisely or was missing individual-level detail. Additionally, the absence of a clear definition of adult age for each animal species necessitated the use of literature-based age ranges and the implementation of modelled approaches for data analysis, instead of simple pooling. Standardizing the reporting process would greatly benefit future modelled applications. A reporting template was therefore proposed as a supplementary initiative.

The modelled seroprevalence of *T. gondii* in animals exhibits a clear increase towards the south, which suggests that temperature affects the environmental transmission of the parasite. This can be attributed to the inactivation of *T. gondii* oocysts during the winter, with the most notable gradient observed in Nordic countries where cold winters increase the probability of inactivation. However, global warming and the retraction of permafrost in these regions may lead to a potential increase in prevalence even in the northernmost parts of Europe. On the other hand, the eastward increase in animal *T. gondii* prevalence probably indicates the importance of maintaining proper hygienic and biosecurity standards in animal husbandry, particularly in the confined indoor breeding systems commonly found in western countries. This approach minimizes the risk of external infections and the choice of farming system also plays a role in the prevalence gradient. In eastern Europe, smaller farms are more prevalent compared to the intensive farming practices in western Europe. Smaller farms have been shown to be associated with a higher *T. gondii* prevalence, possibly due to the presence of cats or rodents (Stelzer et al., 2019). The results of seroprevalence estimates for *T. gondii* reveal a distinct pattern, with the highest values observed in outdoor-reared sheep in the eastern region of Europe and the lowest values reported in indoor-raised pigs in western Europe. This pattern aligns with the various factors and trends discussed previously, including the effects of climate, farming practices, and biosecurity standards.

Impact of parasite stage, strain, and dose on detection, distribution, and concentration

The dose-response relationship has important implications for the risk of infection from meat products. The concentration of parasites in meat is variable, with factors such as the stage of the parasite, the strain of the parasite, the mode of infection, and the infective dose in the source animal potentially playing a role. These factors will be explored in greater detail later in this chapter. Higher doses of parasites increase the likelihood of infection and therefore the risk associated with consuming contaminated meat products, as has been observed in animal studies (Bonačić Marinović et al., 2020). As specific meat products are made out of different

cuts of meat, differences in the parasite concentration will have impact on the risk posed by meat products. It is important to consider the origin of the meat and the specific cuts used when evaluating the risk associated with meat products.

In Chapters 4, 5, and 7, we analyzed the effects of various factors on the clinical signs of toxoplasmosis in pigs experimentally infected with *T. gondii*. Our investigation focused on the prevalence of positive tissue samples and the parasite burden within those samples. Despite some commonalities observed across the factors examined, individual animal variability must be taken into account when interpreting the results. Nevertheless, visible patterns emerged despite this variation.

The comparison of the effect of parasite stage (oocyst and tissue cyst) on *T. gondii* infection in pigs revealed an interesting outcome. Specifically, tissue cyst-induced infections resulted in a higher frequency of positive tissues and a greater overall parasite burden compared to oocyst-induced infections. However, the clinical signs observed in pigs infected with oocysts were more severe, as evidenced by the premature death of two oocyst-infected pigs in Chapter 4. Despite efforts to provide a consistent parasite dose to all pigs in this study (~10 tissue cysts or 400 oocysts), it is possible that differences in the dose may have influenced the results observed in Chapters 5 and 7, where approximately 1000 tissue cysts or oocysts were used. Overall, the impact of the dose on the success of *T. gondii* infection in animals remains unclear, with conflicting results in the literature regarding its effect on the final distribution and parasite burden in infected tissues. In order to establish the dose-response relationship more precisely, it is advised to report the inoculation dose applied during an experimental infection and to quantify the recovered parasite from the tested tissues. If the variability in initial inoculation dose is disregarded, the results of Chapters 5, and 7 present a consistent observation of the differences between oocyst and tissue cyst-induced *T. gondii* infections, independent of the parasite strain or isolate used, as was the case in Chapter 4.

When comparing the effect of different strains of *T. gondii*, represented by type II strains CZ-Tiger and ME49 and type III strain CZ-Šimková, it was observed that the type III isolate resulted in more severe clinical signs in infected pigs, as evidenced by premature death of the two pigs in Chapter 4. The infection caused by the CZ-Šimková isolate was also found to have higher

immunogenicity, with a higher number of positive tissues, and a higher parasite burden than the CZ-Tiger isolate. A comparison of within-strain differences, without taking into account the potential effect of dose and individual response of pigs, showed that the brain was a predilection site for infection with the CZ-Tiger strain, while all brains tested negative for *T. gondii* DNA with the ME49 isolate. This result is noteworthy as the same predilection site using the CZ-Tiger isolate was previously reported by Juránková et al. (2013b), but was not frequently reported by other authors using different type II strain isolates, suggesting an isolate-dependent effect in addition to the previously reported strain-related effect in various animal experiments. Similar results to those presented in this thesis regarding the effects of type III and type II strains of *T. gondii* in pigs, are shown in a comparison of TgShSp1 (type II, ToxoDB genotype #3) and TgShSp24 (type III, ToxoDB genotype #2) in piglets (Largo-de la Torre et al., 2022). However, it is likely that the same strain-related effects are not universal across all animal species, as reviewed by Calero-Bernal et al. (Calero-Bernal et al., 2022). Even with the genotype identification available, in case of a successful isolation of *T. gondii* parasite in sufficient numbers from a naturally infected animal, the effect of experimental and natural infections remains challenging to interpret. This is due to the unknown infectious dose in naturally infected animals, as well as the uncertainty of whether the source of infection in the intermediate host was an oocyst or tissue cyst.

Further data is required to evaluate the response of different strain isolates in various animal species, in order to determine the presence of a potential species-related pattern in addition to the observed strain- and stage-related effects. Controlled experimental infections are preferred over naturally infected animals to obtain quantitative data, as they provide a more structured and controlled environment. The data collected from experimental infections can then be utilized to develop models that can be applied to data obtained from naturally infected animals. Additionally, it is important to consider the impacts of parasite stage and strain on the prevalence of infected tissues and parasite load when determining the consumer risk associated with various meat products. This can be accomplished by adjusting the dose present in the tissues based on the parasite stage and strain, similar to how risk is currently assessed based on animal species.

Prevention and risk management in meat production

The manufacturing of pork products, which are often not heated, has a long tradition in Europe. Effective conservation strategies were necessary to prevent the growth of harmful bacteria that could lead to spoilage. Heating has been proven to be an effective method for inactivating *T. gondii* tissue cysts in meat and meat products, but this approach is not applicable to non-heated pork products. Several methods, relying mostly on lowering the water activity in products and restricting the access of water to pathogens, were developed to ensure the safety of meat products.

Drying itself or by effect of dry aging might be effective in inactivation of *T. gondii* tissue cysts in pork, as suggested by an experiment on dry aging in pork loins (Alves et al., 2020). However, the large storage facilities required to dry age all produced pork would not be economical and would pose additional challenges such as preventing bacterial contamination during prolonged storage and altering the texture and properties of the meat, while the maturation of pork will change the texture and properties of pork, making the production of some processed pork products impossible.

Freezing meat is a common method to prevent water access, and temperatures below the freezing point also impact the survival of *T. gondii* tissue cysts by disrupting the parasite's integrity through the formation of ice crystals within the parasite's cells. Although effective, freezing is not a preferred preventive measure for all meat products as it may alter their texture, flavour and natural physical barriers that slow the spread of potential bacterial contamination (Dang et al., 2021; Friedman, 2003; Reddy, 1981). Generally, pre-production freezing is commonly applied in the manufacture of meat spreads (e.g. filet americain), finely ground meat products (e.g. sausages), thinly sliced meat products (e.g. carpaccio) and meat products where visible fat particles are desirable (e.g. salamis) (Oyinloye and Yoon, 2020). On the contrary, the utilization of pre-frozen meat may pose challenges, particularly in the production of traditional dried hams (e.g. Parma ham, Serrano ham) (The Parma Ham Consortium, 2019). In these products, the extended drying process raises the risk of bacterial contamination and alternative preventive measures are recommended to inactivate potential *T. gondii* parasites.

The impact of salt on the viability of *T. gondii* tissue cysts has been well studied, but effective concentrations can vary based on other factors and seem to differ across studies. The method of salt application is one important factor influencing the effectiveness of salting, with different concentrations required for *T. gondii* inactivation depending on the accessibility of salt to tissue cysts. Salt accessibility appears to be better in solutions than in dry salting applications, making lower concentrations of salt during brining similarly effective as high salt concentrations in dry salting. Temperature during the salting is also influential for the salt penetration, with higher temperatures generally increasing the effectiveness of salt. In our experiments, temperature was not a focus of the study as it remained constant for all tested recipes during the production of dry sausage and processed pork. Further investigation into the effects of temperature in combination with salt concentrations could provide valuable data for developing a model predicting the effectiveness of salt in inactivating *T. gondii* in pork based on processing temperature and salt concentration. Additionally, more data quantifying the effect of salt concentration and salting method on parasite inactivation would aid in more precise modeling of the salting method's effect. The effect of salt can be amplified by the antimicrobial properties of nitrites and nitrates in curing salts, however these chemical compounds have been linked with carcinogenicity and their use should ideally be avoided.

Fermentation plays a role in preventing the multiplication of harmful bacterial flora in pork products, but its impact on the inactivation of *T. gondii* is limited to a decrease in pH. *T. gondii* tissue cysts are relatively stable at low pH, so relying solely on this factor may not be effective in inactivating the parasite. Results from the experiments conducted in Chapter 7 suggest that the change in pH of the meat batter in some of the control recipes may have contributed to the inactivation of *T. gondii*, but the effect was likely due to the presence of salt.

The impact of spices, such as paprika or pepper on the viability of *T. gondii* tissue cysts in meat was not established in the current study, as the inactivation effect observed in the tested recipes was more likely due to the effect of salt. Further research on the effect of various additives and ingredients known to have antimicrobial properties on the viability of *T. gondii* tissue cysts in meat could be an interesting area for future experiments.

Novel preventive measures for inactivating *T. gondii* in pork products can be drawn from other fields and specialties. Certain fungi are used for their antibacterial effects in various processed pork products, and there may be potential for similar effects of certain fungi on *T. gondii* infections [Nascimento 2022]. The application of other fungi species in the food industry for this purpose could be explored in the future.

The role of salting and processing in the inactivation of *T. gondii* in pork products is important, however, given the government initiated approach (ter Borg et al., 2022) on the lowering of salt concentration in food due to related health concerns, the successful implementation becomes complicated. In order to apply the lowest possible salt concentration still capable of inactivating *T. gondii* in the meat products, it is crucial to obtain more quantitative data from experiments, as the effective doses reported in the literature vary. Alternatively, prioritization of the prevention of meat contamination at the source could be pursued. Pre-harvest measures, such as limiting access of cats and rodents at farms, have been shown to reduce the spread of oocyst contamination and thereby reduce the risk of infection. The low *T. gondii* prevalence observed in Western Europe may be attributed to the prevalent intensive farming and related use of measures aimed at limiting the contamination of pigs (Djokic et al., 2016b; Eppink et al., 2022). The stable to table concept is defined by raising animals in a more natural and humane environment, which goes hand in hand with the One Health concept. This approach may also help to reduce the stress and susceptibility to infection in animals, thus possibly reducing the likelihood of the parasites ending in the meat on our shelves. The current trend towards welfare-oriented animal farming is commendable and leaning towards this direction, but care must be taken to ensure that the transition does not increase the risk of infection and *T. gondii* prevalence, as often seen in organic farms. As the outdoor access of animals is a known risk factor for acquiring *T. gondii* infections, meat from these animals should undergo sufficient freezing, heating or salt treatment either during production or be clearly labelled to do so before the consumption. Additionally, implementing measures such as vaccination (Burrells et al., 2015), separate storage of feed, restricted access to pig feed during feeding times, and the use of mechanical barriers like mesh to prevent bird access can help in reducing contamination and preserving the safety of the meat products.

The comparative exposure assessment is one of the generally recognized approaches to attributing foodborne disease (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2018). Previous source attribution studies, conducted in the Netherlands (Opsteegh et al. 2011, Deng et al., 2020) and Italy (Belluco, 2018; Condoleo et al., 2018), have investigated the meat-borne transmission of *T. gondii* in Europe, and have determined that beef products are the primary source of meat-related infections, with pork being a less frequent source. These findings, however, are influenced by regional differences in the epidemiology of *T. gondii* and consumer habits and should not be generalized to other countries in Europe.

Given the substantial data gaps in source attribution of *T. gondii* human infections across Europe, the comprehensive data from the presented studies will be utilized in the development of a comparative exposure assessment for the risk of infection from pork and individual pork products. This information, combined with supplementary data from other sources, will facilitate the establishment of source attribution for meat-borne transmissions of *T. gondii* and provide a more comprehensive understanding of the epidemiology of *T. gondii* infections, including environmental transmissions. Ultimately, this model may be updated with future data as new studies are conducted to provide up-to-date predictions.

Chapter 9

Summary and perspectives

Toxoplasma gondii is a highly prevalent foodborne parasite that is distributed across all continents. Initially described in 1908, it has been the subject of intensive research and has become a model organism for studying apicomplexan parasites. Felids serve as the definitive host for *T. gondii*, with other warm-blooded animals, including humans, acting as intermediate hosts. Toxoplasmosis, the disease caused by *T. gondii*, has significant impacts on human and animal health. The manifestation of toxoplasmosis is influenced by a number of factors, including the stage of *T. gondii* and the strain, and may result in a range of clinical symptoms, such as fever, swollen lymph nodes, retinal disease, abortion, or stillbirth. Environmental and meatborne transmission are the most prevalent routes of transmission for humans. Environmental transmission occurs through the accidental ingestion of sporulated oocysts from contaminated food, water, or the environment. Meatborne transmission, through the consumption of raw or undercooked tissue, is estimated to be the most common in Europe.

Pork is the most widely consumed meat globally and its consumption may pose a significant risk for acquiring *T. gondii* infections. Various risk factors, such as farm type, presence of cats, and outdoor access of animals, have been linked to *T. gondii* infections in pigs. To reduce the impact on public health, preventive measures have been implemented in pork production to address these issues. This thesis explores the various aspects of addressing these issues in pork production, following the concept of “stable to table”.

Chapter 2 detailed the validation of a commercial enzyme-linked immunosorbent assay (ELISA) kit for the detection of anti-*T. gondii* antibodies in pigs using oral fluid samples. This approach offers numerous advantages over the use of blood sera, including improved animal welfare, increased time efficiency, and economic benefits. The results demonstrated high

specificity, indicating successful validation of the method as an alternative option, particularly for large-scale screening of the specific antibodies in pig herds using bulk samples.

The comprehension of the epidemiological status of commonly consumed animal species and definitive hosts is crucial for identifying the risk factors associated with toxoplasmosis and reducing its spread at the farm level, as well as for the development of public health interventions. To this end, a comprehensive literature review of the most recent prevalence of *T. gondii* in selected European animal populations was conducted to create a database for the development of a Bayesian statistical model capable of estimating age-specific seroprevalence for individual animal species, considering the effects of region, outdoor access, and sample matrix. The implementation of this approach resulted in the successful estimation of the seroprevalence for the ten most common groups of animal species in Europe. In addition, a comprehensive prevalence review based on the direct detection of *T. gondii* in animal tissues was provided, which may prove useful, especially for animal species where the presence of specific antibodies does not reliably indicate the presence of the parasite in their tissues. The experiences gained during the conduct of the literature review and the development of the model led to the creation of guidelines to aid and standardize the conduct and reporting of future prevalence studies. The model and related resources, including data extraction and reporting templates, were made publicly available to maximize their potential impact and usefulness in informing risk assessment strategies. Furthermore, the generated seroprevalence estimates can provide valuable data for regions with limited data and, when updated with future data, can be used to produce up-to-date prevalence estimates or estimate seroprevalence in other animal species.

In Chapter 4, to enhance our understanding of the parasite, we investigated the potential effects of *T. gondii* genotype and stage on clinical response and tissue distribution in pigs. Pigs were inoculated with combinations of type II or type III and oocysts or tissue cysts, and the effect of dose was controlled by adjusting the number of parasites according to the use of oocysts or tissue cysts. *T. gondii* infection was characterized by common symptoms such as elevated rectal temperature and reactive hyperplasia of lymphatic tissue and interstitial pneumonia. Seroconversion was observed in pigs between the first and second week post-

inoculation. The highest prevalence of the parasite was observed in shoulder muscles, with the highest parasite load observed in the uterus. Significantly lower parasite load was on the other hand observed in hearts of the pigs. Although individual variability was observed, some strain- and stage-related patterns were noted. Despite not being significant, the specific antibody production was higher in pig groups infected with type III CZ-Šimková isolate and tissue cysts. The infection with the type III isolate and tissue cysts resulted in significantly more positive tissues and significantly higher parasite burden in tissue cyst-infected tissues. However, it is important to note the potentially higher virulence of CZ-Šimková (type III) isolate oocysts, which led to the premature death of two out of six inoculated pigs. These findings highlight the importance of considering infection routes and genotype in the development of preventive strategies against *T. gondii*.

In Chapter 5, we expanded upon the understanding of *T. gondii* tropism established in the previous chapter. To investigate the distribution of *T. gondii* in pig tissues in greater detail, we analyzed anatomically specific samples from 35 muscles and 14 organs using qPCR to detect *T. gondii* DNA. We also included naturally infected pigs in the comparison. The overall frequency of positive tissues was only slightly higher than observed in the previous chapter, and as previously noted, the frequency and parasite load in tissues were lower in pigs infected with oocysts compared to those infected with tissue cysts or naturally infected pigs. These findings provide additional support for the impact of parasite stage on distribution within pig tissues. The detailed data generated in this study can be utilized as an input for product-specific source attribution studies, particularly considering the sometimes specific tissue composition of pork products.

In Chapter 6, we evaluated the risk posed to consumers by analyzing commercially available meat and meat products sold in retail outlets. The results revealed a considerable prevalence of *T. gondii* in venison, which is a significant finding given the increasing popularity of this type of meat due to its nutritious value. Notably, a significant proportion of the screened venison samples were meat cuts or products that required only minimal heating during preparation, as frequently recommended on the product packaging. These results should be of particular interest to governments, and may prompt further investigation into the presence of *T. gondii*

in venison, potentially leading to the implementation of protective measures for products from this meat, such as pre-production freezing.

In Chapter 7, the efficacy of some of the preventive measures was evaluated by examining the viability of *T. gondii* in traditional French dry sausage and processed pork products containing different concentrations of salt, nitrites, and nitrates. The results showed that salt in sufficient concentrations, greater than 2%, in these processed pork products provided a protective effect against *T. gondii*, regardless of other used ingredients and additives, or processing steps applied. This finding offers valuable insights and provides a foundation for further research to determine the optimal level of salt that can inactivate *T. gondii* in pork products. Given the growing pressure from governments to reduce salt levels in food and the recent evidence linking some additives to carcinogenicity, these findings may be useful in both the meat industry and risk assessment studies.

The findings obtained in the thesis were discussed and contextualized in Chapter 8. This chapter delved into various important topics, including the significance of new screening tools for large-scale monitoring of *T. gondii* prevalence in pig herds and the advantages of epidemiological modeling and its applications. The impact of *T. gondii* stage, strain, and dose on the clinical response and distribution in pig tissues was emphasized for the reader. Furthermore, the known risks associated with the consumption of raw or undercooked meat and meat products were evaluated, considering the implementation of available preventive measures, with a focus on the post-harvest portion of the pork production. Suggestions for future research directions were also discussed.

In total, a substantial amount of information on the epidemiology, pathogenicity, distribution, and inactivation of *T. gondii* was gathered and analyzed, providing valuable insights into one of the most successful parasites in the world. The detailed understanding of the relative contributions of different sources of infection can aid in the implementation of targeted preventive measures. The thesis is part of the larger One Health European Joint Project Toxosources, with the ultimate goal of developing a comprehensive quantitative microbial risk assessment model aiming to estimate the relative contribution of different sources of *T. gondii* human infection, where many of the presented data will be used as input elements. The final

model includes components such as animal prevalence estimates, anatomical distribution of the parasite, industrial freezing, heating, and salting, consumer consumption frequencies, consumer freezing and heating, and a dose-response model for infection based on ingested dose. By retaining relevant properties along the model chain, such as animal, product, consumption preference, age, etc., it becomes possible to attribute the source of infection, which is useful for public health policy making.

In addition to their potential use in future risk assessment models, the findings presented in this thesis may serve as a stepping stone for further investigations of *T. gondii* inactivation in meat products. Future studies could focus on the effects of salt in low concentrations, gradually increasing the dose. Ideally, such studies would be conducted using a sausage-like meat product, with multiple collection points over time to determine the optimal salt concentration and duration of treatment. To assess the effects in other meat products, different salting techniques such as dry salting or brining could be applied. The effect of other commonly used meat processing ingredients and additives on the viability of *T. gondii* in not only pork products but also other meats should be tested. The primary objective should be to avoid subjecting animals to experimental infections for the purpose of testing these effects. Instead, it is recommended to use naturally infected meat from retail markets or explore other viable alternatives. The use of negative meat spiked with *T. gondii* tachyzoites is not recommended as it may not accurately represent the potential resistance due to the presence of tissue cyst wall in naturally infected tissues.

All of these proposed investigations, including further inactivation studies and improving the accuracy of the dose-response model, would be of great benefit to public health policy-making and implementation of targeted preventive measures.

ACKNOWLEDGEMENTS

The first place on this list has always been reserved for my Cher Chef, Radu. You trusted and valued my opinions since the very beginning, offering me everything that I needed and more while never expecting anything in return yourself. You taught me to see things from a perspective and your modesty, selflessness, and lightheartedness will always serve as an inspiration to me.

I would like to thank the members of the thesis jury for accepting the invitation and devoting their precious time to evaluate my work. It is an honour to have such well-esteemed experts among the jury members. Thank you very much Prof. Jean Dupouy-Camet, Prof. Arie Hendrik Havelaar, Prof. Caroline Frey and Prof. Břetislav Koudela.

I had the privilege of having amazing tutors who not only guided my progress, but also cared about me greatly beyond my lab work. I am grateful to each and every one of you for being there for me at every step, always happy to find a moment to help and share your wisdom. Three years have passed by so quickly, but there is definitely nothing to regret as I was lucky to have made a special bond with you all. I have deep appreciation and respect for each of you, and there are not enough words to express my gratitude. I have learned so much from you! Delphine, thank you for your unwavering support and kind encouragement. Your welcoming smile always brightens my day, and I am grateful for your assistance both in and outside of the lab. Marieke, thank you for teaching me that every problem has a solution if you ask the right questions. Your patient explanations and insightful remarks always pointed me in the right direction, showing me a completely different level of creativity. Arno, thank you for always going extra mile in ensuring that I have all I needed and for teaching me to see alternative solutions. No matter the obstacle, you always helped me see a way forward. I am almost certain that you use magic in your R code but your secret is safe with me, I only wish I have had learned more from you. My sincere thanks go to Monsieur Boireau for his kindness, unlimited support, and helpful advice. Despite your unbelievably busy schedule, you always kept your door open to me, and for that, I am grateful. Thank you for the opportunity to conduct this thesis in your lab.

I would also like to thank members of my PhD steering committee, Joke, Caroline, Clare, Dr. Dubey, Benoît, and Vito, for their time and commitment to navigating the course of my thesis and solving issues along the way. Joke, thank you for sharing so many fascinating conversations and sharing memorable moments both at work events and outside of them. Caroline, thank you for your kindness and support, as well as your expertise as a member of the steering committee and the thesis jury. Clare, I would like to thank you for trusting me with your projects and allowing me to express myself, teaching me so much in the process. Dr. Dubey, I am grateful to you for kindly sharing your immense knowledge in the field and for attending meetings despite the time differences. It was an honor to have the opportunity to discuss with you, Sir. Benoît, thank you for taking the time and willingness to teach me the basics of statistics in R to get me started. Vito, thank you for keeping an eye on me and taking care of me, following my work, and inviting me to spend time with your family. Special thanks belong to Pr. Koudela, my first mentor, who guided me through my first steps in the lab. I would have never imagined where knocking on your door would lead my future steps. I still reminisce about the long nights during the overnight experiments that couldn't wait until the morning. I would surely not be where I am now if it weren't for you!

There is so much to be thankful for, my dear GG… From the countless surprises and celebrations to the great adventures, games, harmless pranks, to even visiting me abroad. You have made each day so much brighter. Thank you Noah for keeping me sane and supporting me in the madness (I don't think I'll ever find all those stickers). For the time we spent locked together during the confinement and for our spontaneous trips exploring France, whether by TGV or road trip. I am so glad that I could share an office with you, thanks, mate! Camille and Lisa, thank you both for all the great running sessions, adventurous trips, wild camping, carefree moments during bumpy car rides, your attempts to make me speak French (c'était dur mais j'ai résisté) and countless more memories. The three years would be completely different without such a great group of friends, I will be always grateful for those moments.

Thank you, Sandra, for being my trusted and indispensable right hand in the lab. Your support in processing of countless samples and endless positive energy that kept me motivated has made all the difference.

There are more in Anses whom I would like to thank for making my time there a great experience, sharing not only thoughts and techniques but also great moments and memories. Many thanks to Isabelle for always having time to listen and help, to Najat for her everlasting positivity, to Marie for her advices and calming presence across the aisle, to Aurélie for setting the bar with her incredible productivity, to Laura Lucía for her true smile and positive nature, to Myriam for the life-saving gift of personal coffee machine, to Amandine for teaching me the ways of MAT, for to Léa for introducing me to the football breaks, to Anqi for being a kind and caring friend, and to Greg for lifting the mood with innapropriate jokes. Un grand merci à Dany et Madeleine pour s'être assuré que tout était prêt au laboratoire, à Océane pour tes délicieux muffins et à Alain pour tes commentaires hilarants. Merci beaucoup Sophie et Viviane de me faciliter la vie en s'occupant de toutes les commandes et ordres de missions.

I am grateful also to my friends and colleagues from Carré, to Sara for her unbelievable efficiency and speed in replying to emails, Laco for the great chats remiding me of home, Alejandro for his limitless friendship, Sabine for bringing happiness to my day even with a simple greeting, Lourdes for the daily dose of Spanish humour, Aurélie and Clotilde for their enthusiasm to help, Angélique, Élodie, Alejandra, Amalia, Stefania and Caina for cheerful encounters in the lab and after-work soirées, Clémence for setting an example in efficiency, and Apoline for being an unstoppable force of positivity.

A big thanks belongs also to Nathalie, Pascale and Catherine for the invitations to amazing lunch sessions and for helping me battle the sometimes overwhelming bureaucracy. My thanks go out to Adil, Anaëlle, Inès, and Sandra, our fantastic interns who provided invaluable help on our projects. I would also like to thank Lea, Fetta, and Baptiste for the great conversations and camaraderie over drinks after work. Thanks you, Maud, for engaging in discussions beyond statistics and to you, Luca, for your support in running endeavors.

I would like to thank to the colleagues at EnvA as well, to Bruno for his kindness and care, Nadia for never skipping a smile, Veronica and Momo for their empathy and support, Maxime

for his advices and immense help with the sampling of the pigs, Edouard for his enthusiasm, Jennifer for her help with the project and friendly encouragements. Merci beaucoup aussi à tous en BRC pour leur aide avec mes experiments.

I couldn’t forget to express my gratitutde to the colleagues at RIVM. Many thanks Tryntsje, Elisa, Jenny, Tal, Ingrid, Jurgen, Axel, Kees, and others for all the great moments spent with you and the many lessons taught. Thank you for accepting me warmly in your collective, it means a lot to me.

I would like to thank also everyone in my former parasitology lab in Brno for their kind support and advices, and to Krista in particular for her help in the uneasy task of delivering safely the samples on their journey across Europe.

My thanks belong also to the rest of the Moredun team: Lee, Frank, Alison, Beth, Tanja, Janice, Jackie, Renata, Claire, Sean, Paul and others. The three months in Moredun might not have been that long but made for a great summer full of experiences. Thank you for the opportunity to spend that time with you!

I want to thank to my European colleagues who made every scientific meeting and conference a friendly encounter. Thank you Pikka, Gunita and Helga for always saving a hug for me, Walter, Jakob and Frank for your amazing company and fascinating discussions, Gereon, Maike, Marco, Rafa, Gema and Furio for your kindness, care and willingness to help. My sincere thanks belong also to all the great colleagues in Toxosources, there are too many to name.

I am grateful to my family - my mom, dad, brother, my dear grandma, aunt, cousin and kids, for your unwaiverng support, encouragement and visits. None of my success would be possible without you, thank you!

This list would not be complete without expressing my deepest gratitude to my other half, Pavlína, for your kindness and heartwarming smiles that have the power to chase away all the clouds. I am especially grateful for your seemingly limitless patience during the final months of composing this manuscript. Your support and encouragement have been invaluable in helping me achieve this milestone, and I feel truly fortunate to have you by my side.

LIST OF PUBLICATIONS

Published

1. Systematic review and modelling of age-dependent prevalence of *Toxoplasma gondii* in livestock, wildlife and felids in Europe

   Dámek F, Swart A, Waap H, Jokelainen P, Le Roux D, Deksne G, Deng H, Schares G, Lundén A, Álvarez-García G, Betson M, Davidson RK, Györke A, Antolová D, Hurníková Z, Wisselink HJ, Sroka J, van der Giessen JWB, Blaga R, Opsteegh M. Pathogens. 2023; 12(1):97.

2. Detection of *Toxoplasma gondii*-specific antibodies in pigs using an oral fluid-based commercial ELISA: Advantages and limitations

   Kauter J, **Damek F**, Schares G, Blaga R, Schott F, Deplazes P, Sidler X, Basso W. International journal for parasitology. 2022; S0020-7519(22)00183-7.

3. Detection of *Toxoplasma gondii* in reail meat in Scotland

   Plaza J, **Dámek F**, Villena I, Innes EA, Katzer F. Food and Waterborne Parasitology. 2020; 20, pp.e00086.

Submitted

4. Effect of NaCl, nitrates and nitrites on viability of *Toxoplasma gondii* in French dry sausage and processed pork and quantification of the parasite burden in pig tissues used for their production

   Dámek F, Fremaux B, Aubert D, Thoumire S, Delsart M, Martin JL, Vuillermet S, Opsteegh M, Jokelainen P, Le Roux D, Boireau P, Villena I, Blaga R

Pre-submission

5. Effects of experimental toxoplasmosis in pigs inoculated with different stages and genotypes of *Toxoplasma gondii*

   Dámek F, Basso W, Joeres M, Thoumire S, Swart A, da Silva A, Gassama I, Škorič M, Smola J, Schares G, Blaga R, Koudela B

6. Detailed anatomical distribution of *Toxoplasma gondii* in tissues of experimentally infected pigs: effects of infection sources

   Dámek F, Thoumire S, Swart A, Le Roux D, Delsart M, Vuillermet S, Aubert D, Villena I, Durand B Blaga R

LIST OF FIRST-AUTHOR ORAL PRESENTATIONS AT INTERNATIONAL SCIENTIFIC CONGRESSES

1. **Dámek F**, Opsteegh M, Waap H, Jokelainen P, Le Roux D, Deksne G, Deng H, Schares G, Lundén A, Álvarez-García, Betson M, Davidson R, Györke A, Antolová D, Hurníková Z, Wisselink HJ, Sroka J, Klevar S, van Spronsen R, Blaga R, Swart A. Systematic review and modelling of age-dependent prevalence of *Toxoplasma gondii* in livestock, wildlife and felids in Europe. 6th international meeting on apicomplexan parasites in farm animals (ApiCOWplexa2022), Bern, Switzerland. 5-7th October 2022.

2. **Dámek F**, Opsteegh M, Waap H, Jokelainen P, Le Roux D, Deksne G, Deng H, Schares G, Lundén A, Álvarez-García, Betson M, Davidson R, Györke A, Antolová D, Hurníková Z, Wisselink HJ, Sroka J, Klevar S, van Spronsen R, Blaga R, Swart A. Systematic review and modelling of age-dependent prevalence of Toxoplasma gondii in livestock, wildlife and felids in Europe. 15th International Congress of Parasitology (ICOPA2022), Copenhagen, Denmark. 21-26th August.

3. **Dámek F**, Fremaux B, Aubert D, Opsteegh M, Thoumire S, Delsart M, Vuillermet S, Jokelainen P, van der Giessen J, Boireau P, Villena I, Blaga R. Tropism and persistence of *Toxoplasma gondii*: from pork carcass to dry sausage. 15th International Congress of Parasitology (ICOPA2022), Copenhagen, Denmark. 21-26th August.

4. **Dámek F**, Fremaux B, Aubert D, Thoumire S, Vuillermet S, Villena I, Blaga R. Detailed anatomical distribution of *Toxoplasma gondii* in tissues of experimentally infected pigs. 15th International Congress of Parasitology (ICOPA2022), Copenhagen, Denmark. 21-26th August.

5. **Dámek F**, Waap H, Opsteegh M, , Swart A, Jokelainen P, Le Roux D, Deksne G, Deng H, Schares G, Lundén A, Álvarez-García, Betson M, Davidson R, Györke A, Antolová D, Hurníková Z, Wisselink HJ, Sroka J, Klevar S, van Spronsen R, Blaga R. *Toxoplasma*

gondii seroprevalence in European wildlife: a systematic review. 13th European Multicolloquium of Parasitology (EMOP2021), Belgrade, Serbia. 12-16th October 2021.

6. **Dámek F**, Koudela B, Thoumire S., da Silva A, Kameník J, Blaga R. Tropism of *Toxoplasma gondii* in the tissues of experimentally infected pigs. 13th European Multicolloquium of Parasitology (EMOP2021), Belgrade, Serbia. 12-16th October 2021.

The work presented in this thesis was also shared through over 15 poster presentations presented at 7 national and international congresses.

ONE HEALTH EUROPEAN JOINT PROJECT PARTICIPATION

One Health European Joint Project ToxSauQMRA

All work done as a part of this project is presented in Chapters 5 and 7. The obtained results will be implemented in the QMRA model, produced in the One Health European Joint Project Toxosources. The project website is accessible at the following address: https://onehealthejp.eu/projects/foodborne-zoonoses/phd-toxsauqmra.

One Health European Joint Project Toxosources

The work presented in Chapters 3, 5, 6 and 7 are contributing to this project. The project website is accessible from: https://onehealthejp.eu/projects/foodborne-zoonoses/jrp-toxosources. The outcomes of this project are detailed in the following deliverables:

1. Opsteegh M, Swart A, Chardon J, Pires SM, Bier N, Mayer-Scholl A, Schares G, Jore S, Davidson R, Waap H, Calero Bernal R, Alvarez Garcia G, Blaga R, **Damek F**, LeRoux D, Stensvold CR, Sroka J, Rozycki M, Koudela B, Ottoson J, Lalle M, de Haas M, Benincà E, van der Giessen J, Jokelainen P. Deliverable D-JRP-TOXOSOURCES-WP2.2 Report on quantitative exposure data from survey of WP2. DOI: 10.5281/zenodo.5812060.

2. Blaga R, **Dámek F**, Waap H, Opsteegh M, Jokelainen P. Deliverable D-JRP-TOXOSOURCES-WP2.1 Report on prevalence of T. gondii infection in animals for human consumption and cats within Europe. DOI: 10.5281/zenodo.4730705.

3. Swart A, Ottoson J, **Dámek F**, Benincà E, Bonačić Marinović A, Chardon J, Pires SM, Opsteegh M, Jokelainen P. Deliverable D-JRP-TOXOSOURCES-WP2.4 Report on relative contribution of different sources and routes of exposure by country/region. DOI: 10.5281/zenodo.7384280.

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ANNEX

Personal contribution to the presented chapters

Chapters 1, 8 and 9

- Drafting of the content based on original ideas and available literature sources, application of supervisors' suggestions for improvements

Chapter 2

- Conducting experimental infection of pigs and collection of sera and oral fluids from these pigs
- Conducting of a trial of several oral fluid collection methods beforehand (not shown)
- Processing of the collected samples and confirmation of the seroconversion of pigs by ELISA and MAT (not shown), including data analysis of serological data
- Delivering samples and transitioning of the collected data for further processing and drafting of the manuscript

Chapter 3

- Active participation in detailing various aspects of the study prior to commencing
- Coordination of the literature review and screening of a portion of the publications at both levels - title & abstract and full text screening
- Conducting of data extraction and solving inconsistencies in the data interpretation
- Unification of the data format and adjustments for the application with R for the use with the model
- Co-creation of a customized data collection template based on previous studies and reporting template based on experiences during data extraction
- Participation in consultations and in-person meetings aimed at troubleshooting of data application during programming of the model
- Drafting of the manuscript and application of suggestions of co-authors

Chapter 4

- Dissection of the pig carcasses and collection of samples from pigs
- Processing of the samples (excluding sera and genotyping of the strains)
- Analysis of data and providing of a complete dataset including parasite burden estimates for statistical analysis
- Drafting of the manuscript and application of suggestions of co-authors

Chapter 5

- Care-taking of the pigs, conducting measurements and sampling since the beginning of the experiment
- Conducting anatomical dissection of pig carcasses and collection of samples
- Processing of the samples
- Analysis of data and providing of a complete dataset including parasite burden estimates for statistical analysis
- Drafting of the manuscript and application of suggestions of co-authors

Chapter 6

- Collection of venison samples from retail during the second collection period
- Conducting all steps of processing of these samples, including digestion of samples, DNA extraction, qPCR, and RFLP typing of the positive samples
- Provided a complete dataset for drafting of the manuscript
- Actively participated in revisions and suggestions

Chapter 7

- Active participation at all stages of the experiment, including taking care of the pigs, sampling and measurements, and dissection of pig carcasses
- Conducting serological analyses (ELISA and MAT)
- Processing of tissue and pork product samples by MC-qPCR
- Estimation of the parasite burden in samples
- Data analysis and statistical analysis
- Drafting of the manuscript and application of suggestions of co-authors