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Inactivation of *Toxoplasma gondii* in dry sausage and processed pork, and quantification of the pathogen in pig tissues prior to production

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ABSTRACT

Toxoplasma gondii is an important zoonotic foodborne parasite. 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 pigs orally inoculated with 1000 oocysts ($n = 3$) or tissue cysts ($n = 3$) and naturally infected pigs ($n = 2$), 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 eight pigs, including in 41.7% (10/24) of muscle samples (shoulder, breast and ham) 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 (arithmetic mean (M) = 1, standard deviation (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 and processed pork, 94.4% (51/54) were positive for *T. gondii* by MC-qPCR or qPCR, with the mean *T. gondii* burden

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estimate equivalent to 31 parasites per gram (SD = 93). Only the untreated processed pork sample collected on the day of production was positive by mouse bioassay.

The results suggest an uneven distribution of *T. gondii* in the tissues examined, and possibly an absence or a concentration below the detection limit in some of them. Moreover, the processing of dry sausages and processed pork with NaCl, nitrates, and nitrites has an impact on the viability of *T. gondii* from the first day of production. Results are valuable input for future risk assessments aiming to estimate the relative contribution of different sources of *T. gondii* human infections.

1. 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 amongst 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 (Cook, 2000), 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 t, were dry sausages in 2019 (FICT, 2021). Dry sausages and salamis are widely consumed in France at almost 77,000 t in 2021, rising from <72,000 t 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 were explored in the present study.

The first objective of this study was to investigate the distribution of *T. gondii* in a selection of tissues (shoulder, breast, ham, heart) of naturally and experimentally infected pigs inoculated with two different infective stages (oocysts and tissue cysts) of a same strain, in order to rank the tissues, 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.

2. Materials and methods

A flow diagram visualization of the methodology can be found in Supplementary Fig. 1.

2.1. Toxoplasma gondii parasites

T. gondii oocysts (ME49 strain, type II, aged six months) isolated as described by Dubey (Dubey, 2010) were obtained as a courtesy of Dr. JP Dubey (USDA, ARS, Beltsville, USA) and stored in a 2% H_2SO_4 solution, at 4 °C until use.

T. gondii tissue cysts (ME49 strain, type II) used in the successful (third) oral inoculation of tissue-cyst-infected pigs were obtained from mice brains, collected four weeks after oral inoculation of approximately 500 *T. gondii* oocysts per mouse.

The experimental infection of mice was approved by the local ethical committee of University Champagne Ardennes under the agreement number B 514544, and projects APAFIS 10509-2017062614316905 and 14363-2018032908554996v3.

2.2. Pigs

Seven 97-day-old female pigs of the Large White breed were obtained from the Experimental Unit of the Animal Physiology Department of Inrae Nouzilly France, based on a negative in-house modified agglutination test (MAT) result (see 2.7.1) and transported to the Biomedical Research Centre of EnvA for an experimental infection with *T. gondii*.

Additionally, two naturally infected adult female pigs of the Large White breed were purchased from a slaughterhouse in the

Auvergne-Rhône-Alpes region, France, based on a positive enzyme-linked immunoassay (ELISA) result (see 2.7.2). The carcasses of these animals were transported to EnvA for the collection of tissue samples.

2.3. Experimental infection

The seven pigs were divided in three groups. Three pigs (O1, O2, O3) were orally 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, and kept together in a separate pen. Another three pigs (T1, T2, T3) were similarly 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. All pigs were kept under regular veterinary supervision and visited twice a day by local technicians and members of the research unit.

Infectivity of both the oocysts and tissue cysts was confirmed by mouse bioassay at the Biomedical Research Centre of EnvA. All experimental infections in animals were approved by the local Ethical Committee for animal experiments of Anses/EnvA/UPEC (APAFIS N° 14,363-2018032908554996v3) (see 2.10.5).

2.4. Sample collection for the dry sausage and processed pork production

The three pigs of the oocyst-infected group were euthanised on D58 p.i. Blood and all internal organs were removed and collected, before storing halved carcasses at +4 °C for 48 h.

The three pigs of the tissue-cysts group were euthanised on D68 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 for the processed pork production.

2.5. Sample collection for T. gondii distribution study

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

2.6. 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. All available muscle tissues, including shoulder, breast, and ham, were collected from the three pigs, totalling 72.8 kg of muscles for the dry sausage production. No organs were used in the production of dry sausage and processed pork.

Three dry sausages were produced for each of the seven corresponding recipes and the six collection days (D) (D1, D6, D13, D20, D30, D50). The mixture was encased in natural intestine casings. 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 physicochemical analysis (pH, nitrates/nitrites, and NaCl measurements) on each of the sampling days (D0, D1, D6, D13, D20, D30, and D50).

The muscle tissues ($n = 72.8$ kg) intended for the dry sausage production, retrieved from oocysts-infected pigs, were 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 1. 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 (Table 1). 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 for 10 min and filled into a natural casing (pork small intestine, previously desalted in spring water for 24 h, rinsed and drained) using a tabletop filler PSV VILLA ST13 (PSV, France). Encased products of a minimum of 300 g per piece (± 10 g) were secured using a clipper and soaked in a suspension of *Penicillium nalgiovense* (Mold 600 – Chr. Hansen, Denmark) spores. Subsequently, dry sausages were suspended in a ripening

Table 1
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

chamber with a specified controlled environment for the production of traditional French dry sausages with parameters specified in Supplementary Table 1. 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).

Processed pork was manufactured using the muscles of animals infected with *T. gondii* tissue cysts. The collected muscle tissues (n = 12 kg) were processed similarly as in the dry sausage production, except for the encasing step, in which the processed pork was bagged into sterile plastic bags instead. Two bags of processed pork were manufactured for each of the four different corresponding recipes (control recipe = FT, minced meat = VH, recipes F2 and F4) and collection days (D0, D1, D6) using the concentrations of salt and nitrites within the recipes according to the corresponding recipes in Table 1. Processed pork samples were transferred into sterile plastic bags and left open during a six-day-long incubation process, including 24 h at 24 °C (ripening simulation) followed by five days at 13 °C (shortened drying simulation).

2.7. Flotation and sedimentation of faecal samples

Faecal samples were collected daily for the first week following the experimental infection for coproparasitological monitoring of common pig parasites. Five grams of faeces per pig were processed by the magnesium sulphate flotation technique described by Quinn et al. (1980), with another five grams per pig processed by the 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).

2.8. Serological analyses

Blood samples from pigs were collected from the jugular vein into serum separator tubes twice a week for the first two weeks post-inoculation (p.i.) and on a weekly basis afterwards until the euthanasia of the pigs. Sera of mice were collected from orbital sinus four weeks p.i. Both the sera of pigs and mice were tested by MAT and sera of the pigs were additionally tested by ELISA to quantify their immunological response.

2.8.1. Modified agglutination test (MAT)

Sera of pigs and mice were obtained by centrifugation of clotted blood, at 1500*g* for 10 min and analysed by MAT for the detection of *T. gondii*-specific immunoglobulin G (IgG), according to Dubey and Desmonts (1987). 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 (Djokic et al., 2016).

2.8.2. Enzyme-linked immunosorbent assay (ELISA)

Sera of pigs were analysed by ID Screen® Toxoplasmosis Indirect Multi-species (IDVet, France) for the detection of anti-*T. gondii* antibodies according to the manufacturer's instructions. The sera samples were tested at a dilution of 1:5 and results were expressed as the S/P percentage calculated: [(OD sample – OD negative control) / (OD positive control - OD negative control)] * 100. Samples with an S/P% >50% were considered positive.

2.9. 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 1000/3 M, both placed in the centre of a sausage. Data were recorded using a Mettler Transmitter M200 easy with the help of the ALMEMO 5690-1 M 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).

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 the 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. Another two samples, collected on these dates, were used to determine pH values, as well as levels of nitrites, nitrates, and NaCl, using the same methods as described previously.

2.10. Molecular detection, load estimates, and viability of T. gondii

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 2.10.3). The obtained pellet was used for qPCR analysis and mouse bioassay. The remaining parts of each, 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.

2.10.1. MC-qPCR

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) were used for detection of *T. gondii* DNA by MC-qPCR. For the dry sausages ($n = 42$ samples), each dry sausage was divided into three equal parts and one of the three parts of each of the three sausages per recipe were pooled to form a single one-hundred-gram sample for the detection. A hundred grams of the processed pork was collected directly from the sterile plastic bags and processed.

All 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 529 bp repetitive element, according to Opsteegh et al. (2010) with minor modifications (25 μL total reaction mixture volume instead of 20 μL, 2× Premix Ex Taq™ (Takara Bio) instead of 5 × concentrated Taqman master mix (Roche) and 5 μL instead of 10 μL of DNA as template) and the competitive internal amplification control (CIAC) probe modification by Deng et al. (2021), using LightCycler® 480 System 96-plate thermocycler (Roche, Germany).

2.10.2. Parasite load estimates

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 negative reactions were not considered in the calculation of the final mean Cq value and the subsequent parasite burden estimates in samples. The standard curve was obtained as follows: 100 g of *T. gondii*-negative pork was spiked with a counted number of parasites ranging from 1 to 10,000 tachyzoites in tenfold dilutions and quantified by MC-qPCR. The curve fitted to MC-qPCR Cq-values by parasite number for the spiked samples was used to estimate parasite burden in samples.

2.10.3. Trypsin artificial digestion

Two hundred grams of each of the dry sausage and processed pork samples ($n = 54$ samples) were each blended and incubated (90 min at 37 °C, 200 RPM on a shaking plate) in trypsin solution (Trypsin (1:250), powder, ThermoFisher Scientific, final concentration of 4*g*/L). The mixture was filtered through a double layer of gauze, transferred to 50 mL centrifuge tubes, and centrifuged at 1800 *g* for 10 min. 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 were stored at −20 °C until DNA extraction and subsequent qPCR analysis. The leftover pellet was used for mouse bioassay.

2.10.4. qPCR

For dry sausages and processed pork, DNA was extracted from a total of 300 μL of the trypsin artificial digestion pellet using QIAamp DNA mini kit (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 529 bp repetitive element (Lélu et al., 2011). The negative qPCR reactions were not considered in the calculation of the Cq mean.

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

Note that 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.

2.10.5. Mouse bioassay

Following the trypsin digestion of the dry sausages and processed pork, penicillin-streptomycin (Fisher Scientific, France) was added to the pellet homogenate as described in Opsteegh et al. (2016). 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 sera of the mice were tested for the presence of *T. gondii*-specific immunoglobulin (IgG) antibodies by MAT (see 2.7.1). 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. 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 10509–2,017,062,614,316,905.

2.11. Statistical analyses

All statistical analyses were performed using RStudio (Team, 2009). Arithmetic mean and standard deviation were calculated for numerical data. The normality of the data distribution was assessed by visualization in a Q-Q 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.

3. Results

3.1. Experimental infection

The infectivity of the used *T. gondii* oocysts and tissue cysts was confirmed by positive MAT result (titre 1:6) in all the inoculated mice fed *T. gondii* oocyst ($n = 15$) and tissue cysts ($n = 15$).

The seroconversion was achieved in all pigs experimentally inoculated with *T. gondii* oocysts and tissue cysts, as confirmed by MAT and ELISA (Supplementary Fig. 2).

Following the analysis of faeces by the flotation and sedimentation methods, no gastro-intestinal parasitic infection was detected. 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 2).

Seventeen out of thirty-two tissues (53.1%) tested positive for the presence of *T. gondii* DNA by MC-qPCR (Table 2). 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 lowest parasite per gram estimates ($M = 1$, $SD = 2$) (Table 3).

3.2. 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.

Table 2
Toxoplasma 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 3
Arithmetic mean of *T. gondii* parasite burden estimates by MC-qPCR in the individual tissues and groups of pigs based on the type of infection or stage of *T. gondii*. Standard deviation values are provided in round brackets. All values are rounded to a whole number.

	Pig group			
Tissue	Oocyst-infected	Tissue-cyst-infected	Naturally infected	Total
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	64 (105)	126 (100)	124 (60)	105 (136)

3.3. pH level measurements in dry sausages and processed pork

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 (Supplementary Table 2, Supplementary Fig. 3). 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) (Supplementary Table 2). Continuous pH monitoring in one of the sausages from each recipe during the ripening process is shown in the supplementary files (Supplementary Table 2, Supplementary Fig. 3).

3.4. NaCl, nitrates, and nitrites in dry sausages and processed pork

Nitrates ($NaNO_3$), nitrites ($NaNO_2$), and salt (NaCl) levels measured at D0 and at the end of ripening (D50) are presented in supplementary files (Supplementary Table 2). The lower limit of quantification of nitrites for the applied method is 2 mg/kg. For this reason, residual nitrite doses in dry sausages ranged between 2 and 15 mg/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 (Supplementary Table 2). 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 148.5 mg/kg to 15 mg/kg. The only noteworthy residual nitrite dose was measured in recipe F4, the only recipe containing added nitrite salt, at 6.5 mg/kg (Supplementary Table 2).

3.5. T. gondii molecular detection and parasite burden estimates in 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 Table 2. 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).

3.6. T. gondii molecular detection and parasite burden estimates in processed pork

The 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 Table 2. The highest parasite burden was estimated for the recipe FT, collected on D6 (241 ppg).

3.7. T. gondii viability analyses in dry sausages and processed pork

Three mice were used for the mouse bioassay for each of the six dry sausage 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. by MAT (titre 1:6).

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 ppg, one mouse died on D2 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 (titre 1:6). All other mice were negative for *T. gondii*-specific antibodies by MAT.

3.8. Statistical analyses

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 regions tested (shoulder, breast, ham, heart) or the parasite stages (oocyst, tissue cyst) on the parasite burden estimate.

4. Discussion

Pork is the most consumed meat in France with approximately three-quarters of the consumed pork eaten in the form of charcuterie (FranceAgriMer, 2022). For this reason, the present study aimed to investigate *T. gondii* distribution 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. Animal welfare and the 3R principle (Russell and Burch, 1992) were taken into account in the study design, reflected amongst other things in the lowest possible number of animals included in the study.

The sample collection from the tissue-cyst-infected pigs was largely impacted by a national lockdown during the COVID-19 pandemic. 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 the tissue-cyst-infected pigs were culled with a ten-day delay (D68 p.i.) compared to the oocysts-infected pigs. The delay in culling led to an overall increased size of the pigs which may have impacted the raw number of parasites per gram of tissue but it should not have influenced the distribution patterns of the parasite in tissues between the two experimentally infected groups as the average tissue cyst burden remains relatively stable past the fifth week p.i. (Watts et al., 2015).

Our results show that no *T. gondii* DNA was found by MC-qPCR 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 in the shoulder muscles of pigs of the tissue cyst group, and naturally infected pigs in particular. The low parasite burden in hams in the current study is compatible with results reported in pigs naturally infected with *T. gondii,* regardless of the frequent parasite occurrence in these hams (Herrero et al., 2017). Comparable results with a relatively low parasite burden along with frequent detection of *T. gondii* DNA by MC-qPCR in both hams and shoulders were previously observed 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). 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 were observed in studies providing a detailed comparison of *T. gondii* distribution 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 detection 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. This difference in the parasite distribution 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 MC-qPCR, thus confirming one of the predilection sites for the parasite (Dubey, 1986; Opsteegh et al., 2016). 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 (Algaba et al., 2018; Juránková et al., 2014b). Parasite distribution and load in tissues varied at the anatomical and individual level, suggesting a random tissue cyst distribution amongst organs and muscles, with the heart being more heavily infected than other tissues, as observed previously (Algaba et al., 2018; Rani et al., 2019). Overall, there was no significant difference in the number of positive tissues and *T. gondii* load in tissues between the oocyst and tissue cyst-infected pig groups, despite the initial difference in the amount of the parasites administered. This outcome may be due to the lack of research on how the infectious dose affects parasite load in tissues, as some studies suggest that there could be an independent effect of the dose (Jennes et al., 2017).

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) due to the improved sensitivity over commercial DNA isolation followed by qPCR (Opsteegh et al., 2010; Juránková et al., 2014b). 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 g of a sample proven over time (Opsteegh et al., 2010; Juránková et al., 2013, 2014b; Gomez-Samblas et al., 2015, 2016), this option seems unlikely. The specific magnetic capture protocol (Opsteegh et al., 2010) with the adjusted CIAC design

(Deng et al., 2021) 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 slightly higher number of observed qPCR positive results compared to the magnetic capture qPCR method in the dry sausage testing can be explained by the input of 200 g of sample in the trypsin digestion, followed by the commercial DNA extraction kit, and only half of the sample weight (100 g) 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 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., 2014a), however, with the magnetic capture method coming out as the more sensitive of the two.

Given the relatively low weight of the pigs and the seven different sausage recipes to be produced, 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 our results shown that *T. gondii* distribution in the tested samples varies more on an individual level than between muscle tissues of the pigs. 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 sausage samples 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). Ideally, all sausages included in the testing of the effect of NaCl, nitrates, and nitrites would contain a similar high parasite burdens.

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* inactivation, 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.

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 (Supplementary Table 2). 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 $\leq$ 5.2), NaCl content $\geq$1.3% and $>$ 4 h of fermentation (Fredericks et al., 2019).

The nitrate, nitrite, and salt (NaCl) levels measured were generally in line with those expected (Supplementary Table 2), 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. (2018) showed that the concentration of nitrites in dry fermented sausages of $\geq$80 ppm represented an essential barrier in the growth of bacterial pathogens like *Listeria monocytogenes* and *Salmonella* spp., 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. Anses - Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnement et Du Travail,* 2022). Overall, $>$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).

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 (Navarro et al., 1992; Dubey, 1997; Hill et al., 2004, 2006). 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 (Gomez-Samblas et al., 2016; Fredericks et al., 2019; Hill et al., 2018; Fredericks et al., 2020).

According to our findings, the processing with 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 bioassay using untreated processed pork were not consistently positive. 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.

Declaration of Competing Interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

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