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In vitro assay to determine inactivation of *Toxoplasma gondii* in meat samples

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ABSTRACT

Consumption of raw and undercooked meat is considered as an important source of *Toxoplasma gondii* infections. However, most non-heated meat products contain salt and additives, which affect *T. gondii* viability. It was our aim to develop an in vitro method to substitute the mouse bioassay for determining the effect of salting on *T. gondii* viability. Two sheep were experimentally infected by oral inoculation with 6.5×10^4 oocysts. Grinded meat samples of 50 g were prepared from heart, diaphragm, and four meat cuts. Also, pooled meat samples were either kept untreated (positive control), frozen (negative control) or supplemented with 0.6 %, 0.9 %, 1.2 % or 2.7 % NaCl. All samples were digested in pepsin-HCl solution, and digests were inoculated in duplicate onto monolayers of RK13 (a rabbit kidney cell line). Cells were maintained for up to four weeks and parasite growth was monitored by assessing Cq-values using the *T. gondii* qPCR on cell culture supernatant in intervals of one week and ΔCq-values determined. Additionally, 500 μL of each digest from the individual meat cuts, heart and diaphragm were inoculated in duplicate in IFNγ KO mice. Both sheep developed an antibody response and tissue samples contained similar concentrations of *T. gondii* DNA. From all untreated meat samples positive ΔCq-values were obtained in the in vitro assay, indicating presence and multiplication of viable parasites. This was in line with the mouse bioassay, with the exception of a negative mouse bioassay on one heart sample. Samples supplemented with 0.6 %–1.2 % NaCl showed positive ΔCq-values over time. The frozen sample and the sample supplemented with 2.7 % NaCl remained qPCR positive but with high Cq-values, which indicated no growth. In conclusion, the in vitro method has successfully been used to detect viable *T. gondii* in tissues of experimentally infected sheep, and a clear difference in *T. gondii* viability was observed between the samples supplemented with 2.7 % NaCl and those with 1.2 % NaCl or less.

1. Introduction

Toxoplasma gondii is one of the most important zoonotic parasites in the world and virtually all warm-blooded animals, including humans, mammals and birds can be infected by this pathogen (Dubey, 2009). *T. gondii* is the causative agent of toxoplasmosis, a potentially serious disease in humans. It is estimated that about one third of the world population is infected with this parasite (Tenter et al., 2000). Infections

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are often asymptomatic, or associated with flu-like symptoms and swollen lymph nodes. Sometimes ocular toxoplasmosis with visual disturbances caused by chorioretinitis can develop. However, pregnant women and severely immune-compromised persons are at risk for serious complications due to congenital transmission or disseminated infection, respectively. Globally, *T. gondii* ranked fourth out of 24 foodborne parasites (World Health Organization, 2015). In Europe, *T. gondii* ranked second out of 23 foodborne parasites (Bouwknegt et al., 2018) and in the Netherlands, *T. gondii* ranks second after *Campylobacter* in the annual comparison of the disease burden of 14 foodborne pathogens (Benincà et al., 2022).

T. gondii has a complex life cycle with several infective stages and multiple hosts. There are three infectious stages of *T. gondii*: extra- and intracellular tachyzoites (1), bradyzoites in tissue cysts (2) and sporozoites in oocysts (3) (Dubey, 1998). *Felidae* (including domestic cats) are the definitive host, after a primary infection they can shed millions of oocysts into the environment via their faeces during a period of a few weeks (Dubey, 2010). A wide variety of warm-blooded animals can become infected by ingestion of sporulated oocysts from the contaminated environment. These intermediate hosts do not shed oocysts, but develop infective tissue cysts (Dubey et al., 1998).

There are two main routes of *T. gondii* infection for people. This is by consumption of raw or undercooked meat or meat products from infected animals, containing tissue cysts. The other route is through the ingestion of oocysts, for example while gardening or by eating raw vegetables which are contaminated with oocysts originating from the faeces of the cat. In a study among pregnant women in Europe, 30–60 % of infections could be attributed to meat as the source of infection, and 6–17 % to contact with soil (oocysts) (Cook et al., 2000). In the Netherlands, a quantitative risk assessment (QMRA) for meat-borne *T. gondii* infection predicts that 40 % of total predicted infections were attributed to the consumption of raw meat products (Opsteegh et al., 2011), which increased to >80 % in the updated model (Deng et al., 2020). Freezing meat that is used to produce raw meat products would inactivate the parasites that are potentially present and thereby reduce the risk for consumers (Suijkerbuijk et al., 2019), but because of possible negative effects on the quality of the final meat product (e.g. increased drip and loss of structure), freezing is often not preferred by producers.

In contrast to fresh meat, raw meat products usually undergo processing before distribution in retail. To most non-heated meat products, salt and other additives are added during processing. Salt is known to affect the viability of *T. gondii* (Dubey, 1997; Genchi et al., 2017; Pott et al., 2013). Results from an update of the above described QMRA, made clear that the results for inactivation by salt vary between studies and that salting strongly influences the predicted risk per portion (Deng et al., 2020). To better estimate the risk for salted products and to evaluate the possibilities to reduce the risk by modifying the salting parameters, it is important to obtain *T. gondii* inactivation data using salting experiments that are in line with current production. However, the possibilities for obtaining information about the effect of processing methods on the viability of *T. gondii* are limited. Currently animal experiments (e.g. mouse bioassay) are the standard method to determine whether *T. gondii* in meat is infectious. However, use of the mouse bioassay is costly, time consuming and ethically undesirable. The current non-animal methods for detecting *T. gondii* are usually PCR-based or determine the presence of antibodies to *T. gondii* and cannot distinguish between live and dead parasites. For that reason we started with the development of an in vitro assay as an alternative for the mouse bioassay. Promising results were obtained using a cell culture system to determine the presence of viable *T. gondii* bradyzoites in meat samples, however further optimization was needed (Opsteegh et al., 2020). In this study, we used the cell culture system successfully to detect viable *T. gondii* in tissues of experimentally infected sheep. Moreover, using this cell culture system we were able to detect differences in *T. gondii* viability between the meat samples supplemented with 2.7 % NaCl and those with 1.2 % NaCl or less.

2. Materials and methods

2.1. *T. gondii* oocysts for experimental infection of sheep

2.1.1. *T. gondii* isolate

Oocysts of *T. gondii* Savannah strain were originally obtained from the faeces of a Savannah cat (cross between domestic cat and African wild cat serval) imported from the Netherlands to the Czech Republic in October 2018. Nonsporulated *T. gondii* oocysts were found in the faeces of this Savannah cat during routine coprological examination shortly after import to the Czech Republic and this *T. gondii* oocyst isolate had been later passaged two times between outbred ICR mice and cats.

Animal procedures for the *T. gondii* infection in cats were approved by the Animal Welfare Committee of the Bioveta, Ivanovice na Hané, Czech Republic and authorized by Czech Ministry of Agriculture (PP 5-2018). The experiments were carried out at the University of Veterinary Sciences Brno (Czech Republic). All animals used were handled in strict accordance with good clinical practices, and all efforts were made to minimize suffering.

2.1.2. *Viability assay of T. gondii oocysts in mice*

Fresh cat faeces with oocysts of the third passage of *T. gondii* Savannah strain, obtained by feeding infected tissues of ICR mice to cats, were sporulated in 2.5 % *w/v* aqueous potassium dichromate solution and stored at 4 °C for 11 months. Oocysts used for this study were recovered by using the method described by Wainwright et al. (2007) and the number of oocysts was determined by using a Bürker haemocytometer counting chamber. The viability of the *T. gondii* oocysts was confirmed by bioassay in ICR mice. Oocysts were diluted 10-fold from 1000 oocysts to one oocyst per ml of distilled water. These suspensions were inoculated orally into two ICR mice each (one ml/mouse) via a gastric needle with a blunt bulb. All mice were inoculated at the same time and inoculated mice were housed in autoclaved rodent cages. Mice inoculated with 100 and 1000 oocysts developed clinical symptoms such as apathy and shaggy hair during the second week after inoculation. All inoculated mice were euthanized and examined for *T. gondii* infection 6 weeks after inoculation. Their sera were examined in 1:40 dilutions for *T. gondii* antibodies using a commercial modified agglutination test (MAT, Toxo-Screen DA, bioMérieux, France). Furthermore, impression smears of brains and touch imprint slides of lung tissue stained with Giemsa were microscopically examined for presence of *T. gondii* tissue cysts or tachyzoites. Positive serological examinations and positive microscopical findings of mice inoculated with 10, 100 and 1000 oocysts confirmed the viability of the oocysts.

Animal procedures for the *T. gondii* infection in mice were approved by the Animal Welfare Committee of the University of Veterinary Sciences Brno, Czech Republic and authorized by the Czech Ministry of Education, Youth and Sports (PP 30–2018, PP 11–2021). The experiments were carried out at the University of Veterinary Sciences Brno (Czech Republic).

2.2. *T. gondii* positive meat samples

2.2.1. *Experimental infection of sheep with T. gondii oocysts*

To obtain *T. gondii* negative sheep, a conventional sheep herd in the Netherlands with no recent *T. gondii* abortion history, no use of a vaccine against *T. gondii* and no cats present was selected. Fifty-one days before the start of the experiment, blood samples were taken from ten sheep of this farm and tested for IgG antibodies against *T. gondii*, using an indirect ELISA as recommended by the manufacturer of the ELISA (ID Screen® Toxoplasmosis Indirect Multi-species; IDvet, France). All ten sheep proved seronegative. Two of these sheep were selected for the experimental infection. Both sheep were Texel cross ewes and were at an age of seven months. This age was chosen because *T. gondii* seroprevalence in sheep is significantly higher in sheep over one year old (Opsteegh et al., 2010) and sheep at the age of seven months had sufficiently developed

muscles to have enough meat for the experiments.

Nine days before the start of the experiment, blood samples of these two sheep were tested to confirm their seronegative status. Seven days before the start of the experiment, the sheep were transported to the experimental animal facilities of Wageningen Bioveterinary Research (Lelystad, the Netherlands). At day 0 p.i. (post infection) the sheep were infected by oral administration of 6.5×10^4 sporulated oocysts in a final volume of 6.5 ml of phosphate-buffered saline (PBS) using a curved needle with a blunt bulb. Health, behavior and respiratory rate of the sheep were examined two times a day during the first 16 days p.i., and one time a day from day 17 p.i. until the end of the experiment at day 72 p.i.. Rectal temperatures were recorded daily from day 0 to16 p.i.; rectal temperatures of 40 °C or higher were considered to indicate fever. In case rectal temperatures were above 41 °C for more than one day (24 h), the sheep received daily, until rectal temperatures were below 41 °C, an intramuscular injection with meloxicam (1 mg/50 kg; Novem 20, Boehringer Ingelheim Vetmedica GmbH, Germany), a non-steroidal anti-inflammatory drug (NSAID). To monitor the antibody response of the sheep to *T. gondii*, blood samples were taken from the jugular vein at days 0, 14, 42, 63 and 72 p.i. and the serum samples were tested by ELISA (ID Screen® Toxoplasmosis Indirect Multi-species; IDvet, France). The sheep were euthanized at day 72 p.i. using an intravenous pentobarbiturate overdose and subsequently examined at post mortem. From each sheep the heart, diaphragm and four meat cuts (thick flank, strip loin, topside and silverside) were sampled, placed in sealable plastic bags, vacuum packed and stored at 4 °C until use.

The use of the sheep was approved by the governmental Central Authority for Scientific Procedures on Animals (CCD) in the Netherlands (AVD40100202115002).

2.2.2. Meat processing

From each sheep, samples of 50 g were prepared from heart, diaphragm and four meat cuts (thick flank, strip loin, topside and silverside) and grinded individually with a meat grinder (Meat Mincer 198, Hendi, the Netherlands) through a grinder plate with 3 mm perforations. Tissues of sheep 1 were processed on the same day as the euthanasia took place and tissues of sheep 2 were stored vacuum packed at 4 °C and processed one week later. A pooled grinded sample was prepared from the four meat cuts, divided in 50 g portions and either kept untreated (positive control), frozen overnight at −20 °C (frozen control) or supplemented with 0.6 %, 0.9 %, 1.2 % or 2.7 % (*w*/w) NaCl (final concentrations). Finally, a meat sample of 50 g was prepared from a store-bought leg of a lamb (presumed negative for *T. gondii*), which was also frozen overnight at −20 °C and which was used as a negative control. All samples were stored for 20 h at 4 °C and subsequently digested in pepsin-HCl solution as follows. In short, grinded meat portions were added to 250 ml of pepsin-HCl solution (9.2 ml of 9.5 % (*v*/v) HCl), 15 g of pepsin (Sigma-Aldrich P7125, ≥400 units/mg protein, the Netherlands) and up to 2.5 g of NaCl. NaCl added to the pepsin-HCl solution was adjusted depending on the NaCl added the previous day to the meat sample to come to the same final concentration during digestion. The suspension was incubated on a magnetic stirrer at 37 °C for 1 h. Subsequently, the digest was filtrated (180 μm sieve) and approximately 250 ml of 1.2 % $NaHCO_3$ (pH 8.3) was added up to a total volume of 500 ml for neutralization of pH. Digest was centrifuged for 10 min at 1500*g* and the pellet was resuspended in 10 ml PBS. The pH of the suspension was checked again, in case this was <6.5 then 10 ml of 1.2 % $NaHCO_3$ (pH 8.3) was added. The suspension was centrifuged for 10 min at 1500*g* and the pellet was suspended 2:1 in RPMI-1640 cell culture medium (VWR, the Netherlands) with 10 % Fetal Calf Serum (Gibco, USA) and 1 % penicillin/streptomycin/amphotericin B (Biowest, France) and stored at 4 °C.

2.3. In vitro testing of meat samples for T. gondii

The next day, from each sample, one ml of the suspended digest was inoculated in duplicate onto 80–100 % confluent monolayers of RK13 (a rabbit kidney cell line) in T25 flasks. Digests were incubated on the cells for 2 h at 37 °C. Afterwards, the digests were removed from the cells, cells were washed twice with prewarmed cell culture medium (RPMI-1640) and 5 ml of new cell culture medium was added. Cells were maintained either three or four weeks; i.e., three weeks for determination of the anatomical distribution of *T. gondii* in selected meat samples (Fig. 3) and four weeks for determination the influence of salting on viability of *T. gondii* in meat samples (Fig. 4). Two times per week 50 % of the cell culture medium (2.5 ml) was replaced. From the culture medium that was taken off, 1.5 ml was centrifuged at 1500 *g* for 10 min, the pellet was resuspended in 200 μl PBS and stored at −20 °C until DNA isolation. DNA was isolated using DNeasy Blood and Tissue kit and tested by *T. gondii* qPCR as described previously (Opsteegh et al., 2020). A decrease in Cq-values for DNAs from subsequent sampling dates (i.e. a positive ΔCq) indicated multiplying parasites.

2.4. Mouse bioassay

To confirm the viability of the parasites in untreated meat samples, mouse bioassays were performed at the facilities of the Friedrich-Loeffler-Institut (FLI, Greifswald, Germany). Digests of the individual meat cuts, heart and diaphragm from each sheep were transported refrigerated. On the same day as inoculation on cell culture, 500 μL of each digest was intraperitoneally inoculated in duplicate in IFNγ-knockout mouse (GKO, IFNγ-/-C.129S7(B6)-Ifngtm1Ts/J) as already described (Schares et al., 2017). Mice were euthanized when developing signs of toxoplasmosis or kept for six weeks and tested by qPCR for *T. gondii* in DNA extracted from 25 mg of lung tissue (Schares et al., 2017), including an internal control as described (Schares et al., 2021).

Mouse experiments (bioassays) were approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei of the German Federal State of Mecklenburg-Vorpommern (Permission 7221.3-2-023/17).

2.5. T. gondii strain determination

For each of the two sheep, the identity of the strain was confirmed in one of the inoculated mice. To this end, *T. gondii* was in vitro re-isolated from two mice using cell-culture (MARC-145 cells; (Schares et al., 2017)). These mice had been inoculated by samples originating from sheep 1 (Silverside, V25-8) or sheep 2 (Thick flank, V25-18). To confirm that *T. gondii* isolated from sheep originated from the oocysts used for inoculation, the *T. gondii* microsatellite pattern was determined as described (Andreopoulou et al., 2023; Joeres et al., 2023) using DNA extracted (Nucleo-Spin Tissue Kit) from both in-vitro isolates three weeks (V25-8) or two weeks (V25-18) after inoculation of lung homogenates of GKO mice.

3. Results

3.1. Clinical results of T. gondii experimentally infected sheep

Following oral infection with 6.5×10^4 sporulated oocysts of *T. gondii* Savannah strain, at day 5 p.i. both sheep developed fever (41.5 °C and 42 °C) and at days 5 and 6 p.i. the rectal temperature reached a maximum for both sheep (Fig. 1). To inhibit the inflammatory response, the sheep were treated with meloxicam (Novem 20), until the rectal temperature was below 41 °C. Sheep 1 was treated with Novem 20 from days 6–10 p.i. and sheep 2 from days 6–12 p.i. as described above. At days 11 and 13 p.i. the rectal temperature returned to normal (between 38.5 °C and 40 °C; Fig. 1). In addition to the fever, both sheep showed during the first two weeks p.i. mild clinical signs such as decreased appetite, diarrhea and nasal discharge. The respiratory rate was between 20 and 32 breaths per minute, which is a normal rate for sheep.

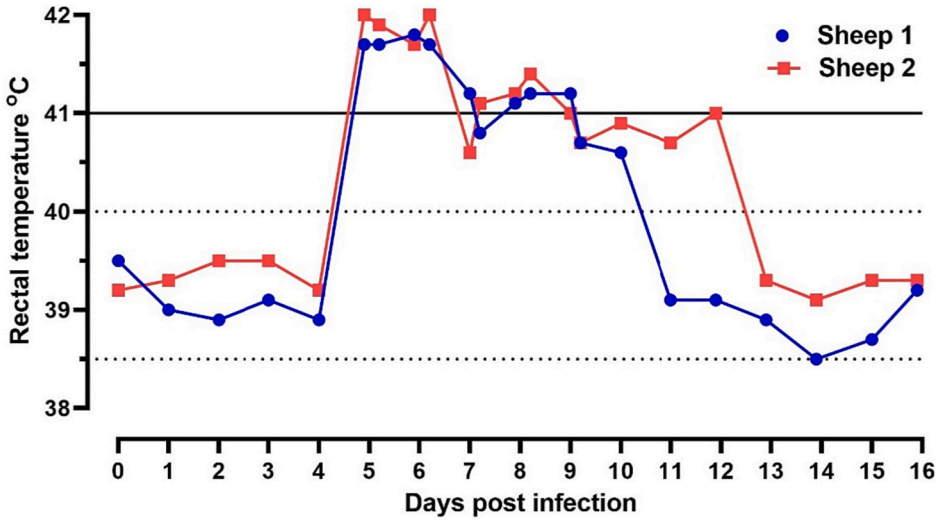

Fig. 1. Rectal temperatures of two sheep, experimentally infected with *T. gondii* oocysts. Rectal temperatures between 38.5 °C and 40 °C were considered as normal for sheep and when rectal temperatures were more than one day (24 h) above 41 °C the sheep were treated with meloxicam (Novem 20), an antipyretic drug.

3.2. Serological results of T. gondii experimentally infected sheep

Using ELISA for detection of IgG antibodies to *T. gondii*, seroconversion was detected in both sheep at 14 days p.i., which was the first blood sampling moment after inoculation with oocysts. At that day the sample-positive control ratio (S/P%) was 187 and 188 for the two sheep, which is above the cut off of 50 %. The S/P ratio of *T. gondii* IgG antibodies increased in the period thereafter and reached 248 and 269 at day 72 p.i., the day when the sheep were euthanized (Fig. 2).

3.3. Pepsin-acid digestion

Each sample for pepsin-acid digestion consisted of a 50 g portion of sheep tissue, either diaphragm, heart or a meat cut (series A), or a pooled sample of the meat cuts with or without (frozen tissue and untreated) added NaCl (series B). These samples were prepared from sheep 1 in the first week and sheep 2 in the second. In both weeks, also a negative tissue sample was used in each series, these four samples were prepared from one portion of store-bought frozen lamb. In the second week a new batch of the pepsin had to be used. Digestion efficiency differed between samples, with a higher amount of tissue remaining on the sieve for samples processed with a higher NaCl concentration (Table 1).

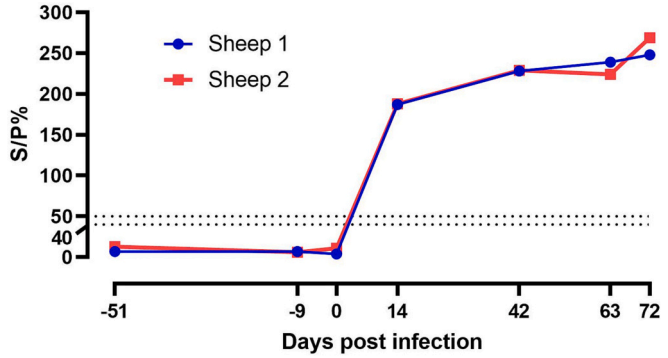

Fig. 2. Detection of anti-*Toxoplasma gondii* antibodies using an indirect ELISA in two sheep, experimentally infected with *T. gondii* oocysts. According to the manufacturer's instructions, sera with a sample-positive control ratio (S/P%) <40 % were considered negative, between 40 % and 50 % inconclusive, and >50 % positive.

Table 1
Amount of undigested tissue remaining on the sieve after pepsin-HCl digestion for 50 g samples of heart, diaphragm, four meat cuts and pooled meat cut samples supplemented with NaCl.

Sample	Series[a]	Undigested tissue	
		Sheep 1	Sheep 2
Negative tissue	A	20.0 g	12.8 g
Heart	A	22.2 g	19.5 g
Diaphragm	A	17.4 g	6.9 g
Thick flank	A	4.8 g	12.2 g
Strip loin	A	9.5 g	12.3 g
Topside	A	5.3 g	11.8 g
Silverside	A	6.5 g	12.3 g
Negative tissue	B	22.5 g	17.8 g
Frozen tissue	B	3.3 g	3.9 g
2.7 % NaCl	B	26.7 g	26.7 g
1.2 % NaCl	B	12.7 g	18.6 g
0.9 % NaCl	B	10.6 g	11.9 g
0.6 % NaCl	B	5.7 g	NA
Untreated	B	11.2 g	12.7 g

[a] Digestions were performed in two series per week (A and B), with tissues of sheep 1 in the first week and sheep 2 in the second week.

3.4. Detection of T. gondii in tissues of experimentally infected sheep by qPCR and mouse bioassay

Following pepsin-acid digestion of the heart, diaphragm and four meat cuts of the two *T. gondii* experimentally infected sheep, DNA extracts of the digests were amplified by qPCR, targeting the 529 bp repeated element, as described in Section 2.4. For both experimentally infected sheep, in all six digest samples *T. gondii* DNA was detected with Cq-values ranging from 26.2 to 29.1 (Table 2).

To test for the infectivity of *T. gondii* present in the samples, mice were inoculated with 500 µL of the digest of these samples. All except two mice inoculated with the tissues of experimentally infected sheep became infected (Table 2). That is, in addition to the mice inoculated with negative control meat digest, also the two mice inoculated with digest of heart tissue from sheep 2 did not develop disease and were negative by qPCR.

3.5. T. gondii strain determination

The pattern (15/15 markers, Supplementary Table 2) obtained for the V25-8 isolate (Sheep 1) and the V25-18 isolate (Sheep 2) resembled

Table 2
Detection of *Toxoplasma gondii* DNA by qPCR in digest samples of heart, diaphragm and four meat cuts of two sheep, experimentally infected with *T. gondii* oocysts and confirmation of the infectivity of the tissue samples in the mouse bioassay[a].

Tissue samples		Sheep 1			Sheep 2		
Meat cuts	Anatomical parts	qPCR result (Cq-values)	Mouse bioassay (No. of mice positive/tested)		qPCR result (Cq-values)	Mouse bioassay (No. of mice positive/tested)	
			Clinical (dpi)	qPCR (Cq)		Clinical (dpi)	qPCR (Cq)
Heart	Heart	29.1	2/2 (36, 28)	2/2 (17.7, 17.9)	28.5	0/2 (NA, NA)	0/2 (Negative, Negative)
Diaphragm	Musculus diafragmaticus	28.3	2/2 (21,22)	2/2 (15.7, 16.3)	27.4	2/2 (22,23)	2/2 (16.3, 15.6)
Thick flank	Musculus sartorius Musculus tensor fascia latae	26.3	2/2 (18, 21)	2/2 (23.6, 17.1)	26.3	2/2 (18, 18)	2/2 (18.9, 25.3)
Strip loin	Musculus longissimus	26.2	2/2 (21, 18)	2/2 (16.8, 19.2)	25.6	2/2 (25, 21)	2/2 (15.5, 17.3)
Topside	Musculus adductor Musculus gracilis Musculus pectineus	27.9	2/2 (18, 18)	2/2 (23.1, 28.1)	27.4	2/2 (21, 28)	2/2 (19.0, 14.9)
Silverside	Musculus biceps femoris Musculus semimembranosus Musculus semitendinosus	26.3	2/2 (18, 18)	2/2 (15.2, 14.9)	27.0	2/2 (23,21)	2/2 (14.1, 14.4)

[a] A negative control (digest from store bought lamb leg) was analysed alongside samples from Sheep 1 and Sheep 2 and tested negative in the qPCR of digest and in the mouse bioassay.

exactly the pattern of DNA obtained from *T. gondii* in vitro isolated from GKO mice inoculated by CZ-Savannah oocysts used to inoculate the sheep (V12-1, Supplementary Table 2).

3.6. Detection of viable T. gondii in tissues of experimentally infected sheep by cell culture

Growth of *T. gondii* in cell culture was shown for all six tissues of both sheep (Fig. 3). There is little difference in Cq-values after three weeks between tissues of sheep 1, whereas for sheep 2, with the exception of strip loin, the differences in Cq-values at the start are still reflected in the Cq-values after three weeks. For sheep 1, Cq-values decrease steeply in week 1 and remain relatively stable after 2 and 3 weeks. For sheep 2 the Cq-values do not decrease as steeply in week 1, but continue decreasing in week 2 and 3. After three weeks it differs per tissue which sheep has shown the largest decrease in Cq-value.

3.7. Influence of salting on viability of T. gondii in meat samples

Additions of 0.6 %, 0.9 % and 1.2 % NaCl to 50 g of mixed meat cut

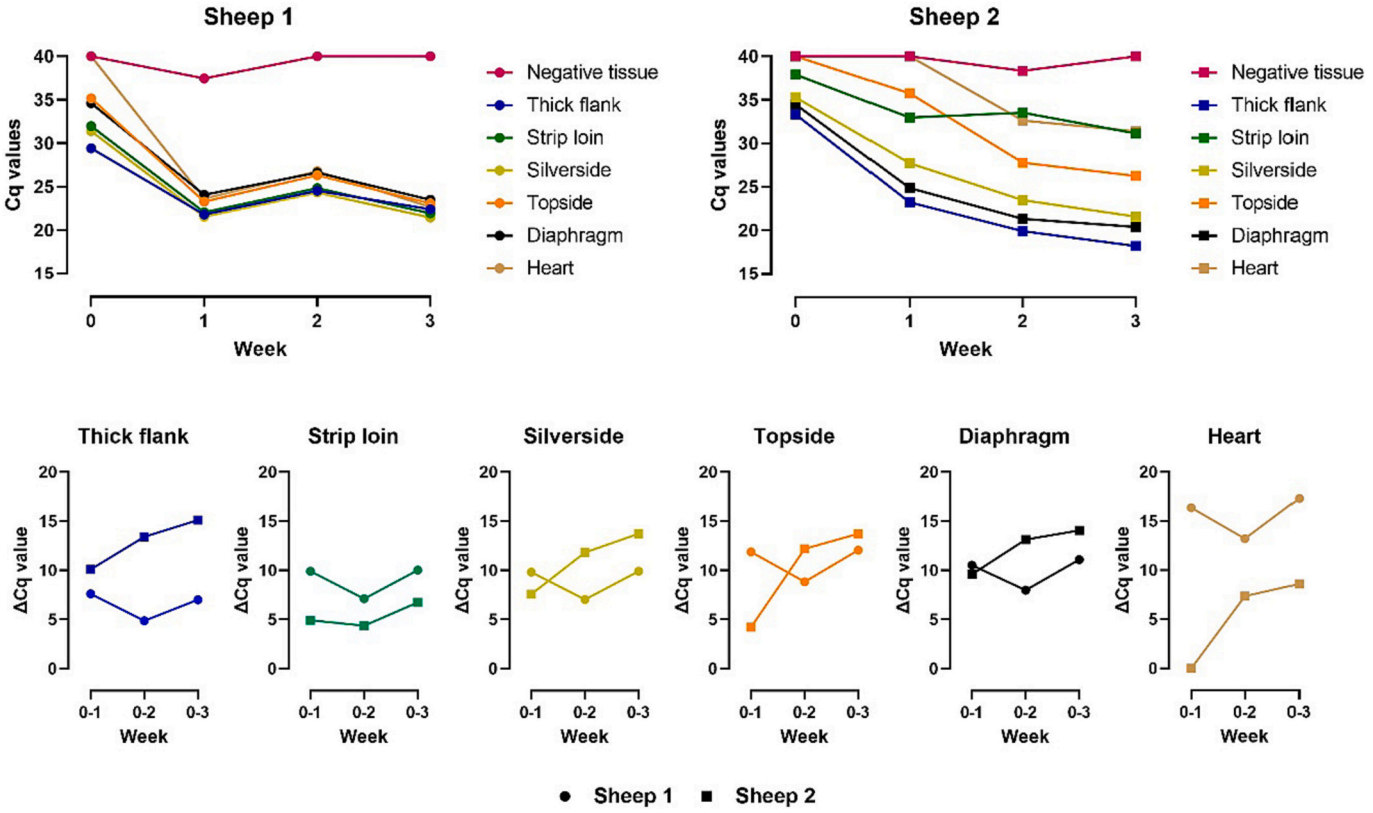

Fig. 3. Detection of viable *Toxoplasma gondii* by cell culture in digests of heart, diaphragm and four meat cuts of two sheep, experimentally infected with *T. gondii* oocysts. Cells were maintained for three weeks and parasite growth was monitored by assessing Cq-values using the *T. gondii* qPCR on cell culture supernatant in intervals of one week.

samples were tested. Salting at 2.7 % was included since this was identified as the maximum salt concentration used in raw minced meat products produced by the project partners. No growth was observed from the sample supplemented with 2.7 % NaCl, indicating inactivation, whereas the other samples clearly showed growth of *T. gondii* (Fig. 4). Growth in 0.6 %, 0.9 %, 1.2 % and untreated samples were quite comparable. The curve for the sample with 2.7 % NaCl was comparable to the frozen and the negative control. The negative control run alongside samples of sheep 2 showed some unexpected decrease in Cq-value after four weeks of culture (Fig. 4).

4. Discussion

The aim for this study was to develop an in vitro method to replace the mouse bioassay in experiments to determine the effect of salting on *T. gondii* viability. Previous experiments showed promising results of a cell culture method to test meat samples for viable *T. gondii* parasites (Opsteegh et al., 2020). In that study, spiking experiments with tachyzoites and bradyzoites in culture medium and meat samples resulted in detectable growth of the parasite in the cell culture method. However, in that study we were not successful in using hearts of *T. gondii* naturally infected sheep as a source of infected tissue for our salt inactivation experiments (Opsteegh et al., 2020). For this follow-up study, it was decided to use experimentally *T. gondii* infected sheep as source of positive *T. gondii* tissue.

To obtain *T. gondii* negative sheep for the experimental infection, we selected a conventional sheep herd in the Netherlands. Even though we selected a herd where no vaccination was performed, where no recent history of abortions due to *T. gondii* infections was and where no cats were present, on the farm initially selected, seropositive sheep were found (data not shown). Seroprevalence in sheep in the Netherlands is high (Opsteegh et al., 2010), therefore selection of a herd with *T. gondii* negative sheep needs attention.

Sheep can be easily infected by oral inoculation with oocysts and a few live oocysts may be enough to infect sheep (Dubey, 2009). However, the parasite concentration in sheep tissue appears to be dose-dependent as *T. gondii* was recovered more frequently from sheep infected with a dose of 1×10^5 than from sheep infected with 1×10^3 oocysts (Esteban-Redondo et al., 1999). A high starting concentration of *T. gondii* in meat was necessary for our aims to obtain consistent positive results for untreated portions and to be able to assess and compare inactivation at different salt concentrations. Therefore, we used a relatively high inoculation dose (6.5×10^4) to realize a high concentration of *T. gondii* tissue cysts in sheep tissue.

Both sheep developed high fever at day 5 days p.i. A febrile response around 3–5 days p.i. in sheep with *T. gondii* is a consistent clinical finding, as described in the literature by others (Dubey, 2009; Thomas et al., 2022). Despite the high fever, the sheep were still eating and drinking but less than prior to inoculation. The effect of the application of meloxicam is difficult to judge, since changes in fever can also be caused by variations in the effects of the infection. In literature, some respiratory distress has been reported occasionally (Dubey, 2009). However, breath rate from both sheep remained normal. Results showed that the experimental infection was successful and the tissues of the sheep were suitable for further use in the experiments.

Most control samples gave expected results in the cell-culture, with the exception of one negative meat sample (Fig. 4, sheep 2). Since this was detected only after four weeks of culture, contamination of the cell culture or the sample for DNA isolation is perceived more likely than presence of viable parasites in the digest inoculated on the cell culture.

The different meat cuts showed little variation in Cq-values for digest or in the detection of viable parasites. The inoculation dose (6.5×10^4)

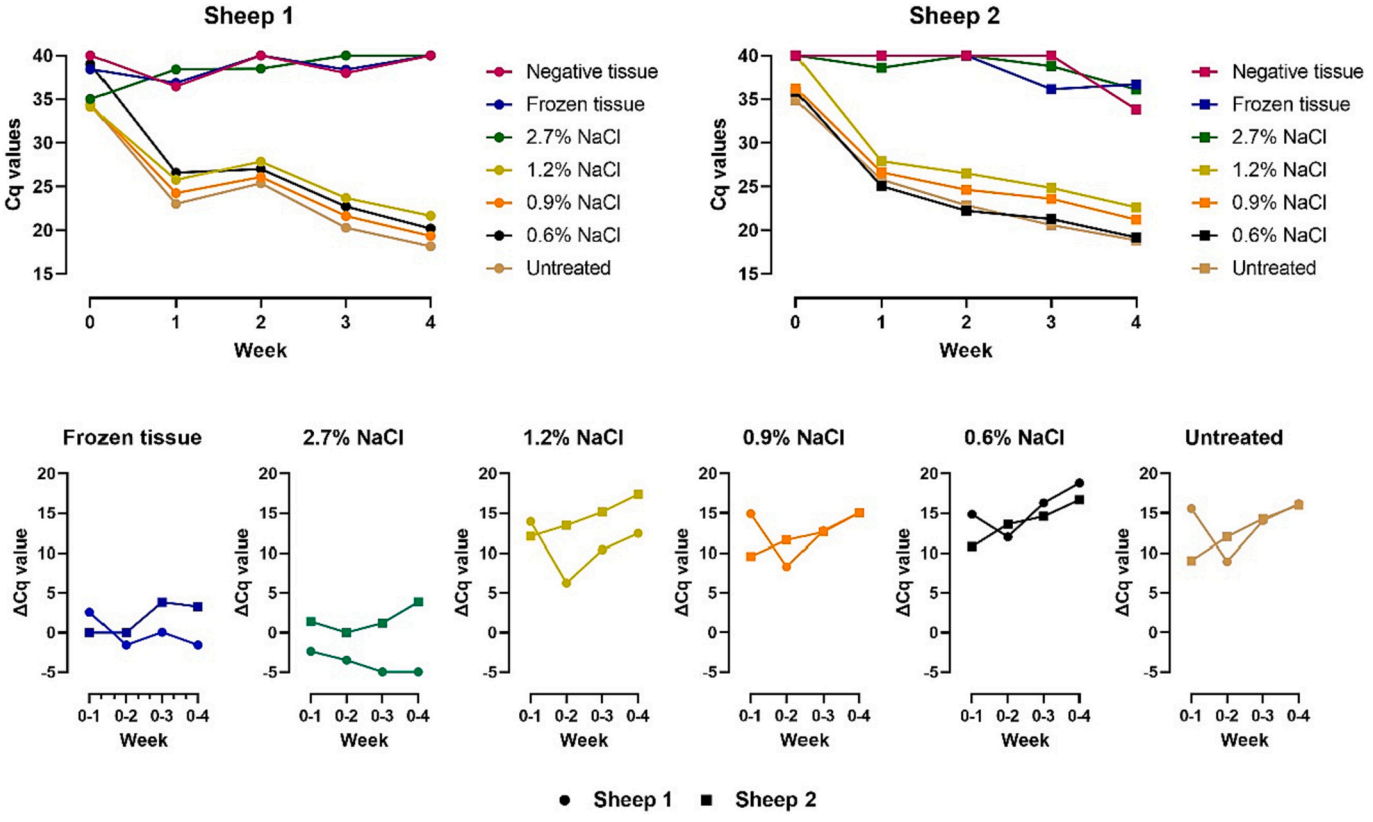

Fig. 4. Detection of viable *Toxoplasma gondii* by cell culture in digests of mixed meat portions of two sheep, experimentally infected with *T. gondii* oocysts. Prior to pepsin-HCl digestion, the meat portions were treated overnight with different NaCl concentrations. Cells were maintained for four weeks and parasite growth was monitored by assessing Cq-values using the *T. gondii* qPCR on cell culture supernatant in intervals of one week.

used here is probably higher than in case of natural infection and therefore not optimal to study differences in the anatomical distribution of parasites. Notably, parasite DNA concentrations were lowest in heart tissue and in the bioassay there was a delay or absence of clinical symptoms in the mice, although heart has often been identified as a predilection site for *T. gondii* in sheep (Esteban-Redondo et al., 1999; Juranková et al., 2015; Silva et al., 2022). The efficiency of pepsin-HCl digestion varied and was lower for heart and diaphragm, which may have attributed to the lower recovery of *T. gondii* from heart tissue.

All tested tissues from both sheep contained infectious parasites. Despite similar parasite concentrations in the digest as determined by qPCR, the development of Cq-value by week differs between the two sheep (Fig. 2). This difference may partly be attributable to differences in the procedure. Firstly, samples from sheep 2 were stored vacuum-packed for one week at 4 °C before processing as the two sheep were euthanized on the same day because animal welfare regulations did not allow single housing of sheep. Previously, in one study *T. gondii* was shown to survive in vacuum-packed goat meat for 42 days at 2 °C (Neumayerová et al., 2014), however, another study showed decreased infectivity in vacuum-packed pork loin aged for 14 days at 0 °C compared to samples used on the day of slaughter, and no infectivity after 21 and 28 days of vacuum-packed storage (Alves et al., 2020). Moreover, a new batch of pepsin had to be used in the second week and more undigested material remained on the sieve for the meat cuts of sheep 2. In conclusion, there are some differences in processing for sheep 1 and sheep 2, but it is unclear whether these differences caused variation in the results.

The lack of infection for the mice inoculated with the heart of sheep 2 compared to growth of this sample in the cell culture, suggests that the mouse bioassay might be less sensitive than the cell culture method. However, a more extensive comparison of these methods is needed and will be included in future experiments.

The results with the different salt concentrations showed differences in growth of *T. gondii* in cell-culture between 2.7 % NaCl compared to low salt concentrations (≤1.2 %). These results indicate that the cell-culture method developed here is suitable for salt-inactivation studies. However, there was a clear relation between digestion efficiency and the concentration of NaCl (Table 1). This was despite the fact that NaCl concentration in the pepsin-acid digest was adapted to the higher NaCl concentrations used in processing. It is known that salting alters protein properties and therefore their digestibility (Choi and Chin, 2021; Li et al., 2017). Therefore, decrease in sensitivity (lower digestion efficiency) coincides with increasing inactivation of viable *T. gondii* and the effect of salt may be overestimated. This effect must be taken into account in modelling of salt-inactivation data.

A drawback of the developed system is that although the mouse bioassay might be replaced, still an animal experiment is needed to obtain infected sheep muscle to perform the inactivation experiments. To completely rule out the experimental infections in animals, experiments are needed to determine if salting experiments carried out on meat spiked with parasites (either tachyzoites or tissue cysts) derived from cell culture give similar inactivation results as experiments with meat from infected animals.

5. Conclusions

It was our aim to develop an in vitro method to substitute the mouse bioassay to determine the effect of salting on *T. gondii* viability. Previous experiments showed promising results with a cell culture method to test meat samples for viable *T. gondii* parasites (Opsteegh et al., 2020). In this study we continued our experiments and used the cell culture system successfully to detect viable *T. gondii* in tissues of experimentally infected sheep. Moreover, using this cell culture system we were able to detect differences in *T. gondii* viability between the meat samples supplemented with 2.7 % NaCl and those with 1.2 % NaCl or less. Taken together, the results of these experiments indicate that the mouse bioassay can possibly be replaced by cell culture to test meat samples for inactivation of *T. gondii* with salt and other additives like acetate and lactate used for the processing of meat products.

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CRediT authorship contribution statement

Marieke Opsteegh: Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Tryntsje Cuperus:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chesley van Buuren:** Methodology, Investigation, Formal analysis, Data curation. **Cecile Dam-Deisz:** Methodology, Investigation, Formal analysis, Data curation. **Conny van Solt-Smits:** Methodology, Investigation, Formal analysis, Data curation. **Bavo Verhaegen:** Writing – review & editing, Methodology, Investigation. **Maike Joeres:** Methodology, Investigation, Formal analysis. **Gereon Schares:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Břetislav Koudela:** Writing – review & editing, Methodology, Investigation, Data curation. **Frans Egberts:** Supervision, Methodology, Funding acquisition, Conceptualization. **Theo Verkleij:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Joke van der Giessen:** Methodology, Funding acquisition, Conceptualization. **Henk J. Wisselink:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

There are no interests to declare for the manuscript “In vitro assay to determine inactivation of *Toxoplasma gondii* in meat samples.”

Data availability

Data will be made available on request.

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