Journal of Food Protection

Effect of domestic freezing on the viability of Toxoplasma gondii in raw and dry-cured ham from experimentally infected pigs

--Manuscript Draft--

Manuscript Number:	JFP-21-281R2
Article Type:	Research Note
Section/Category:	Food Microbiology
Keywords:	Toxoplasma gondii; Domestic Freezing; Pork meat; Dry-cured ham
Corresponding Author:	María Jesús Gracia Salinas Universidad de Zaragoza - CITA Zaragoza, SPAIN
First Author:	María Jesús Gracia Salinas
Order of Authors:	María Jesús Gracia Salinas
	REGINA LÁZARO
	CONSUELO PÉREZ-ARQUILLUÉ
	SUSANA BAYARRI
Manuscript Region of Origin:	SPAIN
Abstract:	Toxoplasma gondii is the causative agent of the parasitic disease toxoplasmosis, which is an important food borne zoonosis. Eating undercooked meat of infected animals has been considered the major transmission route of T. gondii to humans. The present study evaluates the efficacy of domestic freezing on the inactivation of T. gondii bradyzoites in raw and dry-cured ham. Meat (raw and dry-cured ham) of a pig experimentally orally inoculated with 4,000 oocysts of T. gondii VEG strain was subjected to domestic freezing of -20 ºC at different days. The effect was evaluated by bioassay in mice followed by qPCR. In raw ham and dry-cured ham, temperature of -20 ºC for 7 and 14 days respectively did not inactivate T. gondii . More studies are needed to find the right temperature and time needed to render the bradyzoites non-infectious for human. Meanwhile, the recommendations of freezing to inactivate T. gondii in raw or dry-cured meats must be revisited considered that it does not reduce the risk of infection.

Received: July 16, 2021; Accepted: December 21, 2021; Published Online Early: December 2021
María J. Gracia, Regina Lázaro, Consuelo Pérez-Arquillué, and Susana Bayarri. 2021. Effect of domestic Freezing on the viability of *Toxoplasma gondii* in raw and dry-cured ham from experimentally infected pigs. https://doi.org/10.4315/JFP-21-281

Freezing meat and *Toxoplasma gondii*. Research Note

Title: Effect of domestic freezing on the viability of *Toxoplasma gondii* in raw and dry-cured ham from experimentally infected pigs.

Author names: MARÍA J. GRACIA*, REGINA LÁZARO, CONSUELO PÉREZ-ARQUILLUÉ, AND SUSANA BAYARRI

Affiliation: Instituto Agroalimentario de Aragón - IA2 (Universidad de Zaragoza - CITA), Veterinary Faculty, 50013 Zaragoza (Spain)

Key words: *Toxoplasma gondii*; Domestic Freezing; Pork meat; Dry-cured ham

***Author for correspondence**: Tel: +34-976-761562; Fax: +34-976-761612; E-mail: mjgracia@unizar.es

ABSTRACT

Toxoplasma gondii is the causative agent of the parasitic disease toxoplasmosis, which is an important food borne zoonosis. Eating undercooked meat of infected animals has been considered the major transmission route of *T. gondii* to humans. The present study evaluates the efficacy of domestic freezing on the inactivation of *T. gondii* bradyzoites in raw and dry-cured ham. Meat (raw and dry-cured ham) of a pig experimentally orally inoculated with 4,000 oocysts of *T. gondii* VEG strain was subjected to domestic freezing of -20 ºC at different days. The effect was evaluated by bioassay in mice followed by qPCR. In raw ham and dry-cured ham, temperature of -20 ºC for 7 and 14 days respectively did not inactivate *T. gondii*. More studies are needed to find the right temperature and time needed to render the bradyzoites non-infectious for human. Meanwhile, the recommendations of freezing to inactivate *T. gondii* in raw or dry-cured meats must be revisited considered that it does not reduce the risk of infection.

HIGHLIGHTS

- Viability of *T. gondii* assessed by bioassay in mice followed by qPCR
- *T. gondii* in raw ham was not inactivated at -20 ºC for 7 days in a domestic freezer
- *T. gondii* in dry-cured ham was not inactivated at -20 ºC for 14 days
- Adequate freezing is a potential tool for risk control of *T. gondii* by consumers

One of the most common parasitic infections is caused by the protozoan *Toxoplasma gondii* (*13*). It is estimated that approximately one-third of the human population worldwide harbour this parasite (*2*). In most adults it does not cause serious illness. However, it exists risk of miscarriage of seronegative pregnant women who acquire toxoplasmosis within the beginning of pregnancy, in congenitally infected children, it can result in blindness and mental retardation; and can produce severe disease in those individuals with depressed immunity (*36*). In addition, *T. gondii* may be associated with an increase in the incidence of many relevant psychiatric disorders (*43*).

People can become infected through consumption of raw or undercooked meat containing tissue cysts or by ingestion of oocysts contaminating food and water. Congenital transmission can occur when a woman becomes primarily infected during pregnancy (*36*). Several studies have identified frequent consumption of undercooked or of cured meat products as main risk factors for food-borne transmission to humans (*5, 6, 27, 28, 40*). Uncooked pork and its derivatives have been the main foods implicated in outbreaks of toxoplasmosis (*35*).

Prevention of toxoplasmosis currently focuses on pregnant women or immunocompromised and transplant patients. Currently, pigs are not tested for *T. gondii* infection at slaughter but several methods can be applied to decontaminate or reduce risk from infected meat. These methods should be an essential practice in the food industry. Different studies have indicated that *T. gondii* tissue cysts in meat are susceptible to various physical procedures such as heat treatment, freezing, irradiation, high-pressure, acidity and enhancing solutions (*18, 24, 29).*

Traditionally, freezing has been considered an effective process that can inactivate *T. gondii* tissue cysts. According to the data in the literature, raw meat tissue cysts could be killed by freezing at -12 ºC (*10, 30, 31)* or -20 ºC (*9, 16, 32, 38)* for 2-4 days. On the basis of these studies, different review articles (*18, 29, 33)* and food safety organizations (Agencia Española de Consumo, Seguridad Alimentaria y Nutrición, European Food Safety Authority, European Centre for Disease Prevention and Control, United States Department of Agriculture and Food Safety and Inspection Service) (*1, 15, 41*) consider that *T. gondii* in raw meat is not resistant to freezing. Therefore, to protect the general

population, authors recommend the freezing of meat destined for raw or undercooked consumption as the most readily applicable option (*29,* 33). In this respect, food safety organisations provide adequate temperatures and times to inactivate *T. gondii* tissue cysts (-20.6 ºC / 82 hours or -17.8 ºC / 106 hours (*41*) and -12 °C for 2 days (*15*)). Nevertheless, some review studies suggest the contrary, that freezing does not achieve total inactivation of the parasite (*2, 12, 39*). In this sense, it has been suggested that some strains of *T. gondii* may be resistant to freezing (*9,19, 30, 39*). An analysis of the review papers (*18, 29, 33*) and recommendations by food safety organisations (*1, 15, 41*) regarding the effectivity of freezing reveals that these are based on the same original papers, with more recent studies lacking for *T. gondii*. The use of more sensitive methodologies developed in recent years could possibly provide more accurate information of the effect of freezing on the viability of *T. gondii*.

Dry-cured ham, one of the meat derivatives made from pork, is a ready-to-eat product widely consumed in the Mediterranean area being Spain one of its main producers, consumers, and exporters. Dry-cured ham is a nonsmoked pork meat product manufactured according to two basic principles: curing with salt and nitrites, and stabilization through decreased water activity (a_w). The entire process takes at least 7 months, although in some cases the hams may be aged for more than 1 year. Curing has traditionally been regarded as an effective technology against *T. gondii*, but data on the efficacy of curing at inactivating *T. gondii* bradyzoites in meat products are limited. Some studies have pointed out that tissue cysts of *T. gondii* are killed by commercial procedures of curing (*21, 23*). However, other studies indicate that curing may not be enough to inactivate *T. gondii* bradyzoite cysts (*22, 26, 42*). Ultimately, unless the salting procedures have been defined and tested for *T. gondii* infectivity, one should assume that salting is not enough to kill all tissue cysts (*12).* Due to concerns that curing may not be effective in eliminating *T. gondii*, procedures involving a combination of treatments may be implemented to assure non-infectivity of dry-cured meat. In this sense, paediatricians and gynaecologists (*3,* 37) recommend to seronegative pregnant women the freezing of dry-cured ham before intake. The temperatures and times for effective inactivation of *T. gondii* has been shown in only one study which indicates that freezing at -20 ºC for 3 days inactivates the parasite (*23*).

The goal of this study was to evaluate the use of a domestic freezer, under conditions used by the consumer, to inactivate *T. gondii* in raw and dry-cured ham. For this purpose, we evaluated, by bioassay in mice followed by qPCR, the effects of domestic freezing on the viability of *T. gondii* bradyzoites infecting raw and dry-cured ham after experimental pig infection.

MATERIAL AND METHODS

Experimental pig infection. Two *T. gondii* seronegative white pigs were used (~50 kg, 5 months of age). One pig was left uninfected and was used as the negative control. The other pig was infected orally with 4,000 sporulated *T. gondii* oocysts (VEG strain). Sporulated oocysts of the VEG strain of *T. gondii* were provided by Dr. J. L. García (Laboratory of Animal Protozoology, Department of Preventive Veterinary Medicine, Universidade Estadual de Londrina, Brasi) and stored in 2% sulfuric acid at 4 °C until used. The inoculation, maintenance and euthanasia of the pigs were carried out under the regulations of the Regional Ministry of Environment, Local Administration and Territorial Planning of the Community of Madrid (Ref. PROEX 415/15). These guidelines are in accordance with the Protocol of International Guiding Principles for Biomedical Research Involving Animals (Directive 2010/63/EU). The pigs were housed and maintained under Biosafety Class III conditions (in the VISAVET facilities of the Veterinary Faculty of Complutense University of Madrid) until the study was completed. Sixty days post infection (*20*), the infected and uninfected animals were slaughtered and both haunches of each were collected. One haunch was analysed on day zero, and the other was cured for 12 months and analysed at the end of the curing period. The technological treatment of the infected and uninfected hams was carried out in the facilities of a commercial dry-cured ham industry as described previously (*25*, *26*). Boneless raw and dry-cured ham were cut into small pieces, minced, and homogenized. Fifty grams of prepared samples were stored in refrigeration until analysis and freezing treatment.

Confirmation of experimental pig infection. In order to verify the presence of *T. gondii* in the infected pig, raw and dry-cured ham samples were analysed. Samples of uninfected pig were also analysed in order to confirm the absence of parasite. A total of 50 g from each sample was used and a

concentration technique with an acid pepsin digestion procedure was applied, as described elsewhere (*4,11*). An intercalating dye (Sybr Green) qPCR assay was performed to analysis the presence of *T. gondii* DNA from meat A 200 µL volume of the concentrated samples was added to 300 µL of lysis buffer and homogenated using a pestle with rotating plastic plungers in a microcentrifuge tube. The DNA extraction was performed using an UltraClean Tissue & Cells DNA Isolation Kit Sample (Catalog No. 12334-S Mobio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. Two sets of primers for DNA amplification, ToxoRoc and ToxoRepeat 500, targeting specific sequence of 529 repeat element were used (ToxoRoc F 5´-TAGACGAGACGACGCTTTCC-3´, ToxoRoc R 5´-TCGCCCTCTTCTCCACTCT-3´, and ToxoRepeat 500 F 5´-CGCTGCAGGGAGGAAGACGAAAGTTG-3´, ToxoRepeat 500 R 5´-CGCTGCAGACACAGTGCATCTGGATT-3´) and another set of primers for the surface antigen protein-1 gene (SAG1) (ToxoSG1 F 5´-TCATCGGTCGTCAATAA-3´, ToxoSG1 R 5´-CTTTGACTCCATCTTTCC-3´). A CFX Connect real time PCR instrument (Bio-Rad Laboratories, Hercules, CA, USA) was used for the amplification and detection of *T. gondii* using Gotaq Sybr Green Master Mix (catalog # A6002 from Promega, Madison, WI, USA). Each reaction was performed in a final volume of 20 µL containing 10 µL of Master Mix and 2.5 µL of DNA template. The forward and reverse primers concentrations were adjusted to 0.4 µmol l-1. All samples were run in triplicate. The amplification protocol consisted of an initial incubation at 95°C for 7 min, followed by 40 cycles of 94°C for 5 s (denaturation), 55°C for 30 s (annealing) and 72 °C for 10 s (extension). The program ended with a dissociation curve from 60 to 94 °C with a 0.5 °C increase interval. Each qPCR run included a negative control, a positive control, and a separate reaction for Actin DNA copies as internal control (IC). A sample was considered positive if at least two of the triplicates were positive with both markers. The threshold cycle (Ct) value used was indicated by the marker 529 repeat element (Ct < 38 was considered positive), and the SAG marker served to confirm the result. To calculate the efficiency of the qPCR calibration, curves were performed using 15 mg of homogenate negative tissues spiked with a known number of tachyzoites (Serial dilutions from 10^2 to 10^6 copies of DNA/0.01 mL were achieved). The samples were subsequently homogenized and processed with the commercial DNA extraction kit

following the manufacturer's instructions in the same way as the rest of the samples. Efficiency and the correlation fit value were calculated by the CFX Master Manager 2.0 software.

Freezing treatment. The samples were placed in sealable plastic bags, then vacuum packed and a thickness of approximately 2-4 cm was obtained. Samples were stored chilled prior to freezing. Freezing process was performed in a domestic freezer (Balay 3GF8601B, A++). Different freezing times were carried out. Freezing was applied on raw meat at -20 ºC for 1, 2, 3, 6 and 7 days. While in dry-cured ham, freezing was applied at the same temperature for 5, 7 and 14 days. The temperature of the freezing chamber as well as the centre of the meat was measured with a temperature sensor (ALMEMO 2590-3S v5) during the assay. Each freezing treatment was replicated three times. A negative control and a positive control were included for each assay (raw and dry-cured ham). The negative control did not contain *T. gondii* cysts (raw and dry-cured ham from non-infected pig), while the positive control did (raw and dry-cured ham from infected pig). Both controls were not subjected to any freezing treatment.

Mouse bioassay of tissues for *T. gondii*. For each freezing treated or control (negative and positive) sample of raw and dry-cured ham, a concentration bioassay technique with an acid pepsin digestion procedure was applied, as described elsewhere (*4*, *11*). A 0.5 ml aliquot of digestion extract was inoculated intraperitoneally into each 20-25 g CD1 Swiss female mice (Janvier Labs, Le Genest-Saint-Isle, France). Three replicates were carried out for each freezing treatment (5 mice per replicate, 15 mice per treatment) while only one replicate was carried out for each control (5 mice per replicate). The mice came with a health certificate attesting that they were free from pathogens. They were kept at the Centro de Investigación Biomédica de Aragón (CIBA) in Zaragoza (Spain). The inoculation, maintenance, and euthanasia of the mice were performed under the standards of the Ethics Advisory Commission for Animal Experimentation and the Biosecurity Commission of the University of Zaragoza (Ref. PI55/14). After the inoculation of the sample to be analysed, mouse seroconversion occurs if the parasite is contained in it. If the parasite is viable, the cysts infecting the brain can be confirmed by qPCR.

IFA of mouse sera. Blood samples were drawn from mice that survived 60 days after inoculation. The blood was collected by puncture of the mandibular vein. Sera samples of mice were analysed by IFA to detect antibodies against *T. gondii* with polyclonal rabbit antimouse immunoglobulins/FITC (DakoCytomation). Serum from each mouse was diluted 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320. A positive and a negative control serum from previous studies in our laboratory were included in each test. Final preparations were examined with an Eclipse 80i fluorescence microscope (Eclipse 80i, Nikon instruments INC, Netherlands). Sera samples with a titre of 1:10 were considered positive (*26*).

DNA extraction and identification of *T. gondii.* Analysis of *T. gondii* DNA from brains of mice was performed by qPCR to determine viability of the parasite. Fifteen mg piece of each brain were homogenated through pellet pestles in a microcentrifuge tubes. DNA extraction and identification of *T. gondii* by qPCR was performed using the same protocol and primers indicated in the confirmation of experimental pig infection section.

RESULTS AND DISCUSSION

Prior to the freezing treatment, experimental pig infection was confirmed in raw (Ct = 35.09) and dry-cured-ham (Ct = 33.74) samples from the positive control pig, while the analysis of samples from the negative control pig revealed the absence of the parasite (Ct ≥38). It is well-known that the distribution of *T. gondii* cysts within the muscle is completely random; it has been estimated that the number of *T. gondii* cysts per gram of tissue from food animals such as pigs may be less than 1 cyst/50 g of tissue, and that one cyst may contain a few to 1000 bradyzoites (*17, 34*). The natural transmission of parasites would be through ingestion of contaminated meat; however inoculation is a very effective route of infection used in mice bioassays. Although the exact number of parasites eventually inoculated into the mice cannot be known, and maybe the dose used could be above of natural infection, we were able to confirm the viability of *Toxoplasma* as the inoculated mice with positive samples of raw or dry-cured ham (positive control) were infected (titre value ≥1:10) and viable parasites were detected in mice brain (Ct = 33.83-35.61 in raw inoculated mice and Ct = 28.77-30.58 in dry-cured ham inoculated mice).

While mice inoculated with negative samples of raw or dry-cured ham (negative control) did not show seroconversion (titre value ≤1:10) nor the presence of parasites in their brains (Ct ≥38) (Tables 1 and 2).

The results of the effect of domestic freezing on *T. gondii* tissue cysts in raw ham are presented in Table 1. Freezing of raw meat at -20 ºC with holding duration of 1, 2, 3, 6 and 7 days was not effective in reducing infectivity of *T. gondii*. Mice seroconverted and the Ct in the brains of mice infected with frozen samples ranged from 27.69 to 35.22.

When we compare our results with others, we observe discrepancies with studies carried out in raw meat, where freezing was effective in inactivating *T. gondii*. It is especially striking that in our study it was not possible to inactivate *T. gondii* with the use of lower temperatures (-20 ºC) and longer freezing time (7 days) than those used in other studies (-12 ºC / 2-4 days) (*10, 30, 31*). We also observe differences with studies that inactivate the parasite with temperatures similar to ours, but where the times were much lower (2-3 days) (*9, 16, 32, 38).* Unlike other review studies (*2, 12, 39*), in our work it does not appear as there has been a decrease in the viability of the parasite as a result of freezing. That is to say, we cannot assure that freezing times diminish the infectivity of the sample as it happens with the study carried out by Djurković-Djaković, and Milenković (*9*). In any case, although freezing could have reduced the parasitic load, the samples after 7 days of freezing at -20 ºC still contained viable parasites.

A review of the published literature shows that most of the studies are quite old and that there are no recent studies evaluating the effect of freezing against *T. gondii* tissue cyst. Probably, the use of more sensitive methodologies developed in recent years could provide more accurate information on the effect of freezing on the viability of *T. gondii*. In published studies, although the viability confirmation is performed by bioassay, the presence of cysts in the brain is performed by histology and not by qPCR. Because of that, since the tissue cysts are not uniformly distributed in the edible tissues, a negative result obtained by a direct detection method cannot exclude the presence of infectious cysts in other edible portions. PCR has more specificity and sensitivity to detect the presence of *T. gondii* than histological detection (*7, 17, 34*). In our study, highly sensitive methods for the detection of the parasite within

freezing tissues were applied (mouse bioassay and qPCR). Bioassay is regarded as gold standard for evaluation of treatment efficacy of parasites in food (*18*). The mouse bioassay results were based on two criteria: detection of anti-*T. gondii* antibodies in the mice and positive qPCR results for parasite DNA in mouse brains. This is the first study as far as we know that uses qPCR to confirm the viability of *T. gondii* after subjecting the raw meat to a domestic freezing.

Although there is no evidence that strains of *T. gondii* differ in regard to susceptibility to freezing (*19*), some authors point out the possibility of freezing resistant strains (*39*). There is very little information on studies of strains resistant to freezing and we are only aware of the study of Kotula et al., (*30*) where working with the Aldrin strain indicates that its survival at -20 ºC for 16 days was probably related to chance rather than the strain. Another study conducted by Djurković-Djaković, and Milenković (*9*) working with the Me49 strain observed its inactivation at -20 ºC in 72 hours. In our work the VEG strain was used, but there are no published data on whether this strain has a greater resistance to freezing than other strains that could support our results.

Current regulations do not establish criteria for detection of *T. gondii* during meat inspection (*14*). Therefore, the information provided to the consumer on preventive measures is of particular importance to avoid toxoplasmosis. In this respect, food safety organisations pronounce different guidelines considered effective in inactivating *T. gondii* (*1, 15, 41*). However, in view of our results it is essential and of particular relevance to know exactly the temperatures and times that are necessary to inactivate the parasite and to provide the consumer with adequate information so that they can apply this technology with guarantees. Whilst consumption of raw meat occurs in several culinary cultures, more common is consumption of rare meat (cooked briefly to a temperature below 60 °C). This may be insufficient to inactivate tissue cyst of *T. gondii*. For this reason, at the moment, complete heat treatment of parasitized meat is the safest method to inactivate *T. gondii* (*1, 15, 18, 41*).

With regard to the freezing treatment in dry-cured ham and in view of our results obtained in raw meat, it was decided to increase the freezing times in the treatment. In relation to freezing treatment in dry-cured ham, freezing at -20 ºC for 5, 7, and 14 days did not eliminate *T. gondii* infectivity for mice

(Table 2). Mice seroconvert and the Ct in the brains of mice infected with frozen samples ranged from 32.52 to 34.77.

As was the case with raw meat, in the freezing of dry-cured ham it does not seem that the parasitic load decreases as times increase. There does not appear to be a decrease in the number of seroconverted mice or the number of mice in which the parasite is detected in the brain. In any case, although freezing could have reduced the parasitic load, the samples after 14 days of freezing at -20 ºC still contained viable parasites.

In our trial we observed a higher resistance of the parasite to freezing than the results reported by Gómez-Samblas et al (*23*), which indicate that the traditional curing, followed by freezing at -20 ºC for 3 days, inactivates *T. gondi* in only 3 months of curing. This discrepancy is surprising since, although the temperature applied was the same in both studies, both the freezing times (14 days) and especially the curing times (> 12 months) are much higher in our study than those used by these authors. Both studies used experimentally infected meat and used qPCR so we believe that the difference in results could be due to the different procedure in sampling. In our work the freezing treatment is performed on samples obtained from the homogenization of the whole ham while in the work of Gomez-Samblas et al., (*23*) the samples are obtained from a small portion of it (100 grams). As noted above, since the tissue cysts of *T. gondii* are not uniformly distributed in the edible tissues, a negative result obtained from a small sample cannot exclude the presence of infectious cysts in other edible portions.

Our study demonstrated that *T. gondii* is resistant to the dry-cured processed as well as the freezing temperatures and times used in the experiments. Because of that, it is necessary to continue evaluating the temperatures and times needed to inactivate *T. gondii* in dry-cured ham for safe consumption. A controlled freezing step and a period of curing >12 months (*25*) could ensure consumers the consumption of safe products for people in whom toxoplasmosis may pose a serious risk to their health.

In conclusion, domestic freezing at -20 °C for times less than 7 or 14 days in raw meat or cured ham respectively is not effective in inactivating tissue cysts of *T. gondii*. Although the infective dose for

T. gondii has not been established for humans, there is likelihood that freezing of raw and cured meat under the conditions that the present study has been carried out contains viable cysts and, therefore, poses a risk to consumers. Freezing could inactivate the *T. gondii* tissue cysts, but more studies are needed to find proper timing and temperature for a 100% parasite killing efficiency in fresh and cured meat. Meanwhile, we must be cautious in the recommendations made for the use of this technology in at-risk populations.

ACKNOWLEDGMENTS

Special thanks to Grupo VISAVET for having housed the pigs in their biosafety facilities. We also want to thank Joao Luis García for providing us with the VEG strain. This project has been cofinanced by the Spanish National Institute for Agriculture and Food Research and Technology (INIA), the Spanish Ministry of Economy and Competitiveness (MINECO), the European Regional Development Fund (FEDER) (Project RTA2014-00024-C04-02) and the Government of Aragón - Spain (Grant Grupo A06_20R). All authors report no conflicts of interest.

REFERENCES

1. Agencia Española de Consumo, Seguridad Alimentaria y Nutrición (AECOSAN). 2014. Informe del Comité Científico de la Agencia Española de Consumo, Seguridad Alimentaria y Nutrición (AECOSAN) en relación con los riesgos microbiológicos asociados al consumo de determinados alimentos por mujeres embarazadas. *Revista del Comité Científico de la AECOSAN,* 19:11-49.

2. Alizadeh, A. M., S. Jazaeri, B. Shemshadi, F. Hashempour-Baltork, Z. Sarlak, Z. Pilevar, and H. Hosseini. 2018. A review on inactivation methods of *Toxoplasma gondii* in foods. *Pathog. Glob. Health.,* 112: 306-319. doi: 10.1080/20477724.2018.1514137.

3. Baquero-Artigao, F, F. del Castillo Martín, I. Fuentes Corripio, A. Goncé Mellgren, C. Fortuny Guasch, M. de la Calle Fernández-Miranda, M. I. González-Tomé, J. A. Couceiro Gianzo, O. Nethh, and J. T. Ramos Amador. 2013. The Spanish Society of Pediatric Infectious Diseases Guidelines

for the diagnosis and treatment of congenital toxoplasmosis. *An. Pediatr. (Barc).* 79:116.e1-116.e16. doi:10.1016/j.anpedi.2012.12.001

4. Bayarri, S., M. J. Gracia, R. Lázaro, C. Pérez-Arquillué, M. Barberá, and A. Herrera. 2010. Determination of the viability of *Toxoplasma gondii* in cured ham using bioassay: influence of technological processing and food safety implications. *J. Food Prot.* 73(12):2239-2243. doi:10.4315/0362-028X-73.12.2239

5. Buffolano, W., R. E. Gilbert, F. J. Holland, D. Fratta, F. Palumbo, and A. E. Ades. 1996. Risk factors for recent *Toxoplasma infection* in pregnant women in Naples. *Epidemiol. Infect.* 116: 47-351. PMCID: PMC2271422 PMID: 8666080

6. Cook, A. J. C., R. E. Gilbert, W. Buffolano, J. Zufferey, E. Petersen, P. A. Jenum, W. Foulon, and A. E. Semprini. 2000. Sources of *Toxoplasma* infection in pregnant women: European multicentre case-control study. *BMJ,* 321:142-147. doi:10.1136/bmj.321.7254.142

7. Da Silva, A. V., and H. Langoni. 2001. The detection of *Toxoplasma gondii* by comparing cytology, histopathology, bioassay in mice, and the polymerase chain reaction (PCR). *Vet. Parasitol.* 97:191-198. doi:10.1016/s0304-4017(01)00404-6

8. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union L 276 of 20 October 2010.

9. Djurković-Djaković, O., and V. Milenković. 2000. Effect of refrigeration and freezing on survival of *Toxoplasma gondii* tissue cysts. *Acta Vet. Beograd.* 50:375-380. doi: 20013018250

10. Dubey, J. P. 1988. Long-term persistence of *Toxoplasma gondii* in tissues of pigs inoculated with *T. gondii* oocysts and effect of freezing on viability of tissue cysts in pork. *Am. J. Vet. Res.* 49:910–913. doi: 1988 49 6 910-913

11. Dubey, J. P. 1998. Refinement of pepsin digestion method for isolation of *Toxoplasma gondii* from infected tissues. *Vet. Parasitol.* 74(1):75-77. doi:10.1016/S0304-4017(97)00135-0

12. Dubey, J. P. 2000. The scientific basis for prevention of *Toxoplasma gondii* infection: studies on tissue cyst survival, risk factors and hygiene measures. In P. Ambroise-Thomas, and E. Petersen, Congenital toxoplasmosis: scientific background, clinical management and control, Springer, Paris, p. 271-275. doi: 10.1007/978-2-8178-0847-5

13. Dubey, J. P. 2010. Toxoplasmosis of animals and humans (Second edition). Boca Raton: CRC press. doi:10.1186/1756-3305-3-112

14. European Food Safety Authority (EFSA). 2011. Scientific Report on Technical specifications on harmonised epidemiological indicators for public health hazards to be covered by meat inspection of swine. *EFSA J.* 9:1-125. doi: 10.2903/j.efsa.2011.2371

15. European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC). 2018. Public health risks associated with food-borne parasites. *EFSA J.* 16:1-123. doi: 10.2903/j.efsa.2018.5495

16. El-Nawawi, F. A., M. A. Tawfik, and R. M. Shaapan. 2008. Methods for inactivation of *Toxoplasma gondii* cysts in meat and tissues of experimentally infected sheep. *Foodborne Pathog. Dis.* 5:687-690. doi: 10.1089/fpd.2007.0060.

17. Esteban-Redondo, I., S. W. Maley, K. Thomson, S. Nicoll, S. Wright, D. Buxton, and E. A. Innes. 1999. Detection of *T. gondii* in tissues of sheep and cattle following oral infection. *Vet. Parasitol.* 86:155-171. doi: 10.1016/s0304-4017(99)00138-7

18. Franssen, F., C. Gerard, A. Cozma-Petruţ, M. Vieira-Pinto, A. R. Jambrak, N. Rowan, P. Paulsen, M. Rozycki, K. Tysnes, D. Rodríguez-Lázaro, and L. Robertson. 2019. Inactivation of parasite transmission stages: Efficacy of treatments on food of animal origin. *Trends Food Sci. Technol.* 83:114-128. doi:10.1016/j.tifs.2018.11.009

19. Gamble, H. R. 1997. Parasites associated with pork and pork products. *Rev. Sci. Tech. Off. Int. Epiz.* 16:496-506. doi: 10.20506/rst.16.2.1032

20. García, J. L., S. M. Gennari, R. Z. Machado, and I. T. Navarro. 2006. *Toxoplasma gondii*: Detection by mouse bioassay, histopathology, and polymerase chain reaction in tissues from experimentally infected pigs. Exp. Parasitol. 113:267–271. doi:10.1016/j.exppara.2006.02.001

21. Genchi, M., A. Vismarra, C. Mangia, S. Faccini, N. Vicari, S. Rigamonti, P. Prati, A. M. Marino, L. Kramer, and M. Fabbi. 2017. Lack of viable parasites in cured 'Parma Ham' (PDO), following experimental *Toxoplasma gondii* infection of pigs. *Food Microbiol.* 66:157-164. doi:10.1016/j.fm.2017.04.007

22. Gómez-Samblas, M., S. Vilchez, J. C. Racero, M. V. Fuentes, and A. Osuna. 2015. Quantification and viability assays of *Toxoplasma gondii* in commercial "Serrano" ham samples using magnetic capture real-time qPCR and bioassay techniques. *Food Microbiol.* 46:107-113. doi:10.1016/j.fm.2014.07.003

23. Gomez-Samblas, M., S. Vilchez, J. C. Racero, M. V. Fuentes, and A. Osuna. 2016. *Toxoplasma gondii* detection and viability assays in ham legs and shoulders from experimentally infected pigs. *Food Microbiol.* 58:112-120. doi: 10.1016/j.fm.2016.04.005

24. Gracia, M. J., R. Lázaro, C. Pérez-Arquillué, R. Pagán, S. Ramos, J. L. García, and S. Bayarri. 2020. High-Pressure Processing (HPP) of raw and dry-cured ham from experimentally infected pigs as a potential tool for the risk control of *Toxoplasma gondii*. *Innov. Food Sci. Emerg. Technol.* 61:102315. doi: 10.1016/j.ifset.2020.102315

25. Herrero Querol, L. 2019. Evaluación y control del riesgo de *Toxoplasma gondii* en el proceso integral de elaboración del jamón curado. Tesis doctoral. Universidad de Zaragoza, 232pp.

26. Herrero, L., M. J. Gracia, C. Pérez-Arquillué, R. Lázaro, A. Herrera, and S. Bayarri. 2017. *Toxoplasma gondii* in raw and dry-cured ham: The influence of the curing process. *Food Microbiol.* 65:213-220. doi:10.1016/j.fm.2017.02.010.

27. Jiang, R. L., L. H. Ma, Z. R. Ma, G. Hou, Q. Zhao, and X. Wu. 2018. Seroprevalence and associated risk factors of *Toxoplasma gondii* among Manchu pregnant women in northeastern China. *Microb. Pathog.* 123:398-401. doi:10.1016/j.micpath.2018.07.041

28. Kapperud, G., P. A. Jenum, B. Stray-Pedersen, K. K. Melby, A. Eskild, and J. Eng. 1996. Risk factors for *Toxoplasma gondii* infection in pregnancy. Results of a prospective case-control study in Norway. *Am. J. Epidemiol.* 144(4):405-412. doi:10.1093/oxfordjournals.aje.a008942

29. Kijlstra, A. and E. Jongert, E. 2008. Control of the risk of human toxoplasmosis transmitted by meat. *Int. J. Parasitol.* 38:1359-1370. doi: 10.1016/j.ijpara.2008.06.002.

30. Kotula, A. W., J. P. Dubey, A. K. Sharar, C. D. Andrews, S.K. Shen, and D. S. Lindsay. 1991. Effect of freezing on infectivity of *Toxoplasma gondii* tissue cysts in pork. *J. Food Prot.* 54:687-690. doi: 10.4315/0362-028X-54.9.687.

31. Kuticic, V., and T. Wikerhauser. 1996. Studies of the effect of various treatments on the viability of *Toxoplasma gondii* tissue cysts and oocysts. *Curr. Top. Microbiol.* 219:261–265. doi: 10.1007/978-3-642-51014-4_23

32. Lundén, A., and A. Uggla. 1992. Infectivity of *Toxoplasma gondii* in mutton following curing, smoking, freezing or microwave cooking. *Int. J. Food Microbiol.* 15:357-363. doi: 10.1016/0168-1605(92)90069-F

33. Opsteegh, M., T. M. Kortbeek, A. H. Havelaar and J. W. B. van der Giessen. 2015. Intervention strategies to reduce human *Toxoplasma gondii* disease burden. *Clin. Infect. Dis.* 60:101-107. doi: 10.1093/cid/ciu721

34. Opsteegh, M., G. Schares, and J. van der Giessen on behalf of the consortium. 2016. Relationship between seroprevalence in the main livestock species and presence of *Toxoplasma gondii* in meat (GP/EFSA/BIOHAZ/2013/01) An extensive literature review. Final report. EFSA supporting publication 2016: EN-996, 294 pp. efsa.europa.eu/publications

35. Pereira, K. S., R. M. B. Franco, and D. A. G. Leal. 2010. Transmission of Toxoplasmosis (*Toxoplasma gondii*) by foods. *Adv. Food Nutr. Res.* 60:1-19. doi: 10.1016/S1043-4526(10)60001-0

36. Robert-Gangneux, F., D. Aubert, and I. Villena. 2015. Toxoplasmosis: A widespread zoonosis diversely affecting humans and animals. In: Zoonoses-Infections affecting humans and animals. Focus on Public Aspects. Editor: Andreas Sing, Springer. pp: 355-376. doi: 10.1007/978-94-017-9457-2_14

37. Sociedad Española de Ginecología y Obstetricia. 2018. Guía de Asistencia Práctica. Prenatal control of normal pregnancy. *Prog. en Obstet. y Ginecol.* 61:510-527. doi: 10.20960/j.pog.00141

38. Sommer, R., M. Rommel, and R. Levetzow. 1965. Die Uberlebensdauer von Toxoplasmazysten in fleish und fleishzubereitungen. *Fleischwirtschaft,* 5:454-457.

39. Tenter, A. M. 2009. *Toxoplasma gondii* in animals used for human consumption. *Mem. Inst. Oswaldo Cruz.* 104:364-369. doi: 10.1590/s0074-02762009000200033

40. Thaller, R., F. Tammaro, and H. Pentimalli. 2011. Risk factors for toxoplasmosis in 492 pregnant women in central Italy. *Infez. Med.* 19:241-7. PMID: 493 22212163

41. United States Department of Agriculture, Food Safety and Inspection Service (USDA FSIS). 2018. Compliance Guideline for the Prevention and Control of *Trichinella* and Other Parasitic Hazards in Pork and Products Containing Pork. pp: 34.

42. Warnekulasuriya, M. R., J. D. Johnson, and R. E. Holliman. 1998. Detection of *Toxoplasma gondii* in cured meats. *In. J. Food Microbiol.* 45:211-215. doi:10.1016/s0168-1605(98)00158-5

43. Webster, J. P., M. Kaushik, G. C. Bristow, A. Glenn, and G. A. McConkey. 2013. *Toxoplasma gondii* infection, from predation to schizophrenia: can animal behaviour help us understand human behaviour?. *J. Exp. Biol.* 216:99-112. doi:10.1242/jeb.074716

TABLE 1. Results of freezing on *Toxoplasma gondii* tissue cysts in raw ham.

Treatment	**Freezing time (days)**	**Seropositive mice (positive/total)** [a]	**Viability of *T. gondii* (positive/total)** [b]	**Infectivity of the sample** [c]
Negative control [d]	0	0/5	-	-
Positive control [e]	0	4/5	4/4	+
		(1:40, 1:40, 1:180, 1:10)	35.61	
			33.83	
			34.23	
			35.02	
-20ºC	1	1/5	1/1	+
		(1:10)	33.31	
		4/5	1/4	
		(1:10. 1:20, 1:20, 1:40)	31,56	
		2/5	2/2	
		(1:80, 1:40)	33.53	
			33.49	
-20ºC	2	2/5	2/2	+
		(1:10, 1:20)	33.89	
			35.01	
		3/5	1/3	
		(1:10, 1:20, 1:20)	31.48	
		5/5	1/5	
		(1:40, 1:20, 1:20, 1:10, 1:40)	27.69	

-20ºC	3	5/5	5/5	+
		(1:20, 1:40, 1:40, 1:80, 1:20)	31.19	
			31.09	
			31.18	
			30.40	
			28.91	
		0/5	0/0	
		4/5	4/4	
		(1:10, 1:10, 1:10, 1:20)	32.64	
			30.44	
			31.78	
			33.16	
-20ºC	6	3/5	2/3	+
		(1:20, 1:40, 1:20)	30.66	
			34.44	
		1/5	1/1	
		(1:80)	30.83	
		4/5	3/4	
		(1:20, 1:80, 1:20, 1:10)	35.22	
			32.47	
			29.67	
-20ºC	7	3/5	3/3	+
		(1:80, 1:10, 1:40)	30.33	
			29.67	
			31.69	
		2/5	1/2	
		(1:40, 1:80)	31.31	

	1/5	1/1
	(1:40)	30.13

[a] Seropositive mice by IFA/Total of mice (Serological titers).

[b] Positive mice brain by PCR/Total of seropositive mice by IFA; Ct values of positive mice brains (< 38).

[c] Infectivity of the sample was scored as + or -; + indicates that the procedure did not kill all parasites and - indicates a 100% killing effect of the procedure on the parasite

[d] Inoculated with raw meat from non-infected pig

[e] Inoculated with raw meat from infected pig

TABLE 2. Results of freezing on *Toxoplasma gondii* tissue cysts in dry-cured ham.

Treatment	**Freezing time (days)**	**Seropositive mice (positive/total)** [a]	**Viability of *T. gondii* (positive/total)** [b]	**Infectivity of the sample** [c]
Negative control [d]	0	0/5	-	-
Positive control [e]	0	5/5 (1:10, 1:10, 1:20, 1:40, 1:80)	3/5 28.77 29.77 30.58	+
-20ºC	5	1/5 (1:20) 3/5 (1:20, 1:20, 1:10) 1/5 (1:10)	1/1 32.52 1/3 34.18 0/1	+
-20ºC	7	3/5 (1:20, 1:20, 1:10) 4/5 (1:20, 1:40, 1:40, 1:10) 1/5 (1:10)	2/3 33.91 33.97 2/4 33.32 33.09 0/1	+
-20ºC	14	3/5	1/3	+

	(1:10, 1:10, 1:20)	34.77
	4/5	1/4
	(1:10, 1:20, 1:20, 1:40)	33.34
	1/5	0/1
	(1:10)	

[a] Seropositive mice by IFA/Total of mice. (Serological titers)

[b] Positive mice brain by PCR/Total of seropositive mice by IFA; Ct values of positive mice brains (< 38).

[c] Infectivity of the sample was scored as + or -; + indicates that the procedure did not kill all parasites and - indicates a 100% killing effect of the procedure on the parasite

[d] Inoculated with dry-cured ham from non-infected pig

[e] Inoculated with dry-cured ham from infected pig