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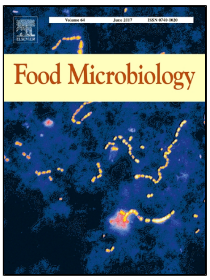

Toxoplasma gondii in raw and dry-cured ham: the influence of the curing process

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High number of seropositive pigs had *Toxoplasma* tissues cysts in raw hams.

The parasite burden in raw hams was low.

Viability of *T. gondii* was significantly influenced by curing periods.

No other variable with influence on *T. gondii* viability in cured ham was determined.

Research is required to validate combinations of salt concentration and curing time.

Toxoplasma gondii in raw and dry-cured ham: the influence of the curing process

Authors

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Abstract

The aim of this work was to analyze *Toxoplasma gondii* in raw hams by mouse bioassay and to evaluate the effect of curing on the viability of the parasite to assess the risk of infection from eating dry-cured ham. After a serology study of 1,200 pigs in Aragón (Spain), forty-one naturally infected pigs with different serological titers against *T. gondii* were selected. Two cured periods (9 and 12 months) were evaluated as well as the influence of the physicochemical composition of hams on *T. gondii* survival. Although the parasite burden was low, a high number of seropositive pigs with *Toxoplasma* tissues cysts in raw hams were found (31.6%). Viability of *T. gondii was* influenced by the curing, with statistically significant differences between fresh and cured hams ($p < 0.001$). The viability was higher in hams cured for 9 months compared to those cured for 12 months. However, this period of curing resulted in the reduction but not in a complete elimination of the risk. Thus, from a public health point of view, under the conditions of this study it is safer to consume dry-cured ham with periods of curing higher than 12 months. Analysis of physicochemical results did not identify any variable with significant influence on the presence and viability of *T. gondii* in cured ham, but loss of viability of *T. gondii* was observed in hams with a lower fat content. Further research is required to validate combinations of salts concentration and time of

curing that can be used as preventive measures in the HACCP system of dry-cured ham industry.

Keywords

Toxoplasma; Pork meat; Cured ham; Mouse bioassay; HACCP

1. Introduction

Toxoplasmosis, caused by the protozoan parasite *Toxoplasma gondii*, is one of the most common parasitic infections of man and other warm-blooded animals. It has been found worldwide and approximately one-third of humanity has been exposed to this parasite (Saadatnia and Golkar, 2012). In most adults it does not cause serious illness, but it can cause blindness and mental retardation in congenitally infected children and devastating disease in immunocompromised individuals (Weiss and Kiss, 2013). Sources of human infection with *T. gondii* are oocysts shed in faeces of infected felines and tissue cysts from infected meat animals. Raw and undercooked meat has thus been considered the main source of infection (Baril et al., 1999; Carme et al., 2002; Choi et al., 1997; Lake et al., 2002; Ross et al., 2001). Pork is one of the major meat sources associated with human *T. gondii* infections and in a recent risk ranking report, the combination of *Toxoplasma* and pork ranked second among 10 pathogen-food combinations (Guo et al., 2015).

T. gondii has a high affinity for neural and muscular tissues. The parasite is located predominantly in the central nervous system (brain), skeletal muscle (tongue or diaphragm) and cardiac muscle (Dubey, 2010). In order to learn more about the role of meat as a source of human infection with *T. gondii*, it is important to have an indication on the prevalence of infectious tissue cysts (Opsteegh et al., 2016). In response to natural infection, most farm animals that are seropositive for *T. gondii* have been shown to harbor infectious parasites in their meat (Opsteegh et al., 2016; Tenter et al., 2000; Zia-Ali et al., 2007). Seropositivity in general is a good indicator of the presence of the parasite in tissues and some authors mention that the level of isolation increases with antibody titer in the pig (Dubey and Jones, 2008; Herrero et al., 2016; Opsteegh et al., 2016).

Some 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 (Bayarri et al., 2010; Dubey et al., 1990; Mie et al., 2008). Technological process of curing with salt could also kill *T. gondii* tissue cysts, although the inactivation of theses cysts depends of the synergistic interaction between salt concentration, maturation time, and temperature of storage (Kijlstra and Jongert, 2008). One of the first reports on the identification of cured meat as a source of *Toxoplasma* infection was conducted by Buffolano et al., (1996) who showed that *Toxoplasma* infection in pregnant women in Naples in the early 1990s was strongly associated with frequency of consumption of cured pork and raw meat; eating these foods at least once a month increased the risk threefold. Cook et al. (2000) also found an association between infection and consumption of cured meats. Buffolano et al. (1996) observed that the conditions in which some pork products are cured are not necessarily lethal to *T. gondii*. In fact, viable parasites were detected in one out of 67 (1.5%) cured meat samples investigated in the United Kingdom (Warnekulasuriya et al., 1998), and in 4.8% of samples in studies carried out in Spain (Gomez-Samblas, et al., 2015). In relation to the time of curing, studies carried out by Bayarri et al. (2010) indicated that it has influence on the inactivation of *T. gondii,* since no viable parasites were found in hams of 14 months *vs* hams of 7 months of curing. However, few studies have been conducted to examine the efficiency of the curing process on the inactivation of *T. gondii* and consequently, there is a need for a risk-based assessment of such products and to evaluate the risk posed by this product category.

Dry-cured ham is a product manufactured according to the following basic principles: curing with salt and nitrites and stabilization through decreased water activity. The whole process takes at least 7 months, although in some cases the hams may be aged for more than 1 year. It does not require heat treatment before consumption. Dry-cured ham is an important food in the Mediterranean area, and Spain is one of the major producers. It is a high-quality product with increasing economic relevance, widely consumed in Spain and exported to other countries. During the period 2014-2015 Spanish exports have grown by 131 %, reaching 40.436 tons. The leading destinations in Europe are France, Germany, Italy, Portugal, Belgium and Switzerland. Significant non-European importers include Mexico, United States, Japan and China (Cruz, 2015).

Demands of consumers for pathogen-free meat products have led the meat industry to focus attention on food safety and on the necessity to produce wholesome, safe, and high quality meat, using the appropriate technological treatments. At the moment, the detection of *T. gondii* by currently practiced meat inspection is not possible, but serological screening could be used to detect *T. gondii*-infected meat (Herrero et al., 2016; Opsteegh et al., 2016). This tool, together with an effective control of technological process, could be used to reduce the final product risk.

The aim of this work was to determine the presence and viability of *Toxoplasma gondii* in raw hams of selected pigs with different serological titers, and to evaluate the influence of processing of cured ham on the viability of the parasite to assess the risk of infection from eating this meat product. As previous studies lacked detailed information on meat product characteristics, another goal of this study was to determine physical and chemical parameters such as water activity, pH, content of salt (NaCl, nitrate, nitrite) and fat to evaluate their influence on *T. gondii* bradyzoites survival.

2. Materials and Methods

2.1. Selection of naturally infected pigs

After a serological study of 1,200 pigs from 60 swine farms in Aragón, northeast of Spain (Herrero et al., 2016), forty-one Pietrain x Landrace pigs (6 to 7 months old and 120 kg body weight) with different serological titers against *T. gondii* detected by Indirect Immunofluorescence assay (IFA) (bioMérieux, Marcy l'Etoile, France) were selected for the study.

2.2. Selection and sampling of hams

Selected animals were distributed into three batches: three serum negative pigs (<1:20) (Batch I: negative control), 14 pigs with serological titer 1:20 to <1:80 (Batch II) and 24 seropositive pigs with titer ≥1:80 (Batch III). Pigs were slaughtered in a commercial abattoir and both haunches were collected.

Forty-one haunches (one from each pig) were analyzed at day 0 and the other forty-one haunches were cured according to usual industry practices. After 9 months of curing, 21 hams were analyzed, and the 20 remaining hams continued the curing

process until 12 months, when samples were also analyzed for viable forms of *Toxoplasma* (Table 1). The number of hams analyzed was greater when the possibility of risk was higher (haunches from pigs with high serological titers or a shorter cure time).

Boneless fresh hams were cut into small pieces, minced, vacuum packaged and stored in refrigeration until analysis.

2.3. Curing of the hams

The technological treatment of the hams was carried in a cured ham processing industry, and included different steps: salting, post-salting or resting-period, and drying and maturation. For salting, the fresh haunches (8.59 to 14.76 kg) were surface salted with a commercial mixture of 7% nitrates, 4% nitrites, sodium ascorbate, and sodium chloride (10 g/kg of ham). Then, hams were covered with sodium chloride, and conducted to a salt chamber at 2.8 to 3.5ºC and 85%-95% relative humidity, in which remained a variable time depending on the weight of each ham (~2 days for each kilogram of ham).

Then, after cleansing the salt from the ham surface, the resting period began. Hams were kept for at least 90 days at 3ºC±1ºC and 70 to 95% relative humidity. Temperature was gradually increasing up to 16ºC until the final 120 days. In this step the salt slowly and progressively diffuses internally, gradually drawing much of the original moisture from the ham.

During drying and maturation, for at least 110 days at 6-16ºC and 60-70% relative humidity, the hams continued to lose moisture under conditions of increased temperature and decreased relative humidity. Weight loss during drying was controlled. In this final step, the curing process is completed, and the ham slowly shores up its sensory properties before being marketed. In our study, the curing process lasted 9 or 12 months.

Sample preparation for analysis was performed as described in 2.2.

2.4. Mouse bioassay of tissues for T. gondii

A concentration bioassay technique with an acid pepsin digestion procedure was used to demonstrate presence and viability of bradyzoites of *T. gondii* in fresh and dry-

cured hams, as described previously (Bayarri et al., 2010; Dubey, 1998a). A 0.5 ml aliquot of digestion extract was inoculated intraperitoneally into each of eight 20-25 g CD1 Swiss female mice per sample (Janvier Labs, Le Genest-Saint-Isle, France). Digestion and inoculation were done in quintuplicate for each sample (250 g total). All experiments included negative control mice. An aliquot of the digestion extract (fresh and dry-cured ham) was stored at −20°C to posterior analysis by PCR to confirm the presence of *T. gondii* when serology in mouse was negative.

Mice were received with a health certificate showing that the animals were free from pathogens. They were maintained at the Centro de Investigación Biomédica de Aragón (CIBA), in Zaragoza (Spain). The inoculation, maintenance and euthanasia of mice were performed under the standards of the Ethics Advisory Commission for Animal Experimentation and the Biosecurity Commission of the University of Zaragoza, as granted by Opinion No PI07/12. These guidelines are in accordance with the Protocol of International Guiding Principles for Biomedical Research Involving Animals (Directive 2010/63/EU).

2.4.1. IFA of mouse sera

Blood samples were drawn from mice that survived 60 days after inoculation. Sera samples of mice were analyzed by IFA to detect antibodies against *T. gondii* with polyclonal rabbit anti-mouse immunoglobulins (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 titer of ≥1:10 were considered positive.

2.4.2. DNA extraction and identification of T. gondii

Analysis of *T. gondii* DNA from brains of serologically positive mice was performed by real time-PCR to determine viability of the parasite. When serology in mouse was negative to *T. gondii*, the digestion extracts of raw and cured ham were analyzed by PCR to confirm the presence of *T. gondii*. The number of positive samples

by bioassay and/or the number of positive samples by PCR give us the number of total positive samples.

The DNA extraction was performed from 15 mg of brain or 100 µl of extracts of digestion, using UltraClean® Tissue & Cells DNA Isolation Kit Sample Catalog No. 12334-S (Mobio Laboratories, Inc.) according to the manufacturer's instructions. DNA amplification targeting specific sequence of 529 repeat element and SAG genes were performed. CFX Connect (Bio-Rad Laboratories) real time PCR instrument was used for the amplification and detection of *T. gondii*. The reaction volume was 20 µl and samples were run in triplicates. The protocol consisted of 7 minutes at 94°C for enzyme activation (Hot start), and 40 cycles of denaturation at 94°C for 5 s, annealing at 55°C for 30 s and extension at 72°C for 10 s. The program ended with a dissociation curve from 60 to 94ºC with a 0.5ºC increase interval. Each PCR 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 positives with both markers. The Ct value used was indicated by the marker 529 repeat element (Ct must be lower than 38) and the marker SAG served to confirm the result. Calibration curves were prepared using 15 mg of homogenate negative tissues spiked with a known number of tachyzoites (10^4, 10^3, 10^2, 10^1 and 10^0). Subsequently, the samples were homogenized and processed with the commercial DNA extraction kit following the manufacturer's instructions in the same way as the rest of the samples. *T. gondii* parasites present in tissues were estimated using the formula obtained from the calibration curve and the Ct value obtained for each sample. The measure of the adjustment performed in the curve was determined with the coefficient of determination, which was $R^2 = 0.992$. An efficiency of 99.7% was obtained, calculated through the slope of the curve (3.329).

2.5. Determination of chemical and physical parameters of dry-cured hams

Loss of weight in the final product was monitored by gravimetry; nitrate and nitrite contents were analyzed according to ISO 3091:1975 and ISO 2918:1975 standards respectively; moisture content was determined according to ISO 1442:1997; pH and water activity (a_w) were measured according to ISO 2917:1999 and ISO

21807:200 standards, respectively. Finally, sodium chloride content was determined according to ISO 1841-1:1996, and fat content according to ISO 1444:1996.

2.6. Statistical analysis

Serological results, presence and viability of *T. gondii* in fresh and cured ham were compared using Pearson's Chi-square test (or Likelihood Ratio test when Pearson's Chi-square test was not valid). This test was also used to establish a statistical relationship between results obtained in the different months of curing and the presence and viability of *T. gondii* cysts in tissues.

To determine the relationship between the physicochemical parameters of cured hams and the presence and viability of *T. gondii*, the following tests were used. Firstly the association between quantitative and qualitative dichotomous variables was studied to determine the normality of the quantitative variable by Kolmogorov-Smirnov test and Shapiro-Wilk test. In the case that the variables showed a normal distribution they were compared using the Student's t test for independent samples, and when the normality assumption was declined, Mann-Whitney test as a nonparametric alternative was used.

Statistical analyses were performed with IBM SPSS 19.0 for Windows. Differences were considered statistically significant when $p < 0.05$.

3. Results

Presence of *T. gondii* of 68.4% (26 of 38) and viability of 31.6% (12 of 38) were determined in fresh hams from seropositive animals; all samples from the negative control animals were negative by bioassay in mice and real-time PCR (Table 2). Lower presence and viability of *T. gondii* (28.6% and 0% respectively) was found in hams from pigs with titers < 1:80 than in those hams from pigs with higher titers (91.7% and 50% respectively). The presence ($p < 0.001$) and viability ($p = 0.001$) of *T. gondii* in fresh hams were significantly associated with pigs whose serological titers were ≥1:80. Pigs with a serologic titer ≥1:80 have 3.21 times more probability of finding the parasite in the fresh ham. No information regarding the viability can be obtained because no pig with a serological titer lower than 1:80 showed viable parasites in their hams.

Real time PCR results (mouse brain and extracts) from raw hams of pigs naturally infected with *T. gondii* are presented in Table 2. Mean values equivalent to

69.44 (SD ± 57.61) parasites per gram of tissues were detected. The lowest Ct value of 33.44 (corresponding to 250.7 parasites per gram) and the highest Ct value of 37.51 (corresponding to 15 parasites per gram) were detected in brain tissue of mouse inoculated with raw ham of pigs with a serological titer of 1:80.

After performing the technological process of curing, from the 26 fresh hams in which the presence of *T. gondii* was confirmed, the parasite was detected in 11 cured hams (42.3%) and showed viability in 4 (15.4%) of them. The curing process reduced the presence and viability of *T. gondii*, and statistically significant differences ($p<0.001$) of both parameters between fresh and cured hams could be observed.

Regarding the curing time (9 months *vs.* 12 months), presence of *T. gondii* in cured ham is quite similar in both curing times. The parasite was detected in 6 of 21 (28.6%) 9 months cured hams and, excluding negative controls, in 5 of 17 (29.4%) 12 months cured hams. Concerning viability, it was higher in hams cured for 9 months compared to those cured for 12 months; the parasite was viable in 3 of 21 hams that had been cured for 9 months (14.3%) and, excluding negative controls, in one of 17 cured hams for 12 months (5.9%). In relation with the samples in which the parasite was detected, it was viable in 3 of the 6 positive hams cured for 9 months and only in one of the 4 positive cured hams cured for 12 months.

Real time PCR results (mouse brain and extracts) from cured hams are presented in Table 2. Median values (± SD) equivalent to 85.9 ± 40.63 parasites per gram of tissues were detected. The lowest Ct value of 34.15 (corresponding to 153.3 parasites per gram) and the highest Ct value of 36.85 (corresponding to 23.3 parasites per gram) were detected in brain tissue and extract of mouse, respectively.

The final mean composition (± SD) of dry-cured hams at the end of the curing process was 35.6 ± 3.5 % moisture content, 0.92 ± 0.02 water activity (aw), 5.1 ± 1.3 % fat, 3.1 ± 0.5% NaCl, 68.3 ± 15.6 mg/kg nitrate, 11.76 ± 26.81 mg/kg nitrite, and 5.8 ± 0.1 pH. Weights of dry-cured hams were 5.1-10.4 kg, and loss of weight in the final products was from 26.8 to 45.9% (Table 3). Analysis of these results did not identify any variable with significant influence on the presence and viability of *T. gondii* in cured ham ($p>0.05$), but a loss of viability of *T. gondii* was observed in hams with a lower fat content ($p=0.039$).

4. Discussion

T. gondii has high affinity for neural and muscular tissues including brain, eyes, and skeletal and cardiac muscles (Dubey et al., 1986, 1998b, 2009; Guo, et al., 2015; Juránková et al., 2014). Muscular tissue is of interest due to its use in small goods production (Kijlstra and Jongert, 2008), but there are limited data on the prevalence of *T. gondii* in raw ham (Opsteegh et al., 2016) and studies carried out by Juránková et al., (2014) indicate low *T. gondii* infection of muscular tissues in pigs. However, our results show that nearly 70% of raw ham from seropositive pigs has *Toxoplasma* tissues cysts. Probably, selection of tissues from seropositive pigs increased the efficiency of isolation *versus* bioassays of tissues irrespective of antibody status (Dubey, 2009). Nevertheless, the mouse positivity and the PCR results in meat samples detect both viable and non-viable *T. gondii*, which leads to an overestimate of the infectivity of meat. Finding a *T. gondii* cyst in mouse brain is dependent upon the level of the parasite in the tissue (Guo et al., 2015); the PCR assay in mice brain, could detect viable cysts in the 31.6% of raw hams. Nevertheless, the bioassay results suggested that pork tissues contained low levels of infective organisms since titers in mice were low and PCR results in mouse brain and extracts show a low parasite burden (equivalent to [median value] <69.44 parasites per gram of tissues). However, this parasite burden is higher than that found by Juránková et al. (2014) where a very low parasite burden was estimated for fore and hind limb muscles, equivalent to [median] 0.2 parasites per gram of tissues.

Although these results suggest a low *T. gondii* burden of muscular tissues in pigs, there are raw hams that contain viable cysts and, therefore, pose a risk to consumers. *T. gondii* transmission risk is also associated with consumers' cooking behaviors. According to a 2007 survey, approximately 9% of consumers cooked their pork products to a temperature of less than 48ºC (Ecolab, 2008), which may not be sufficient to inactivate *T. gondii* cysts. Tissue cysts were generally rendered nonviable by heating to 61ºC or higher temperature for 3.6 minutes (Dubey et al., 1990). It should be noticed that dry-cured ham is consumed without heat treatment and the consequences of consumption of viable *T. gondii* cysts could be severe, especially for the pregnant and immunocompromised people.

Considering the ecology of *T. gondii* infection and formation of tissue cysts as the cause of the immunological response, it may be assumed that seropositive animals will have tissue cyst to a greater level (Dubey, 2009). However, presence of *T. gondii* in various tissues can be dependent on the duration of the infection. Verhelst et al. (2011) observed a clearance of the parasite in pig tissues after 6 months of experimental

infection. Indirect detection showed relation with the detection of parasites by mouse bioassay, and the isolation of *T. gondii* was found to increase with antibody titer. At the moment, the detection of *T. gondii* is impossible by currently practiced meat inspection, the serology could be used to identify potentially infected pork. Titer ≥1:80 may represent a first line selection that could be used as a screening tool to detect more risk raw material in order to apply a specific technological treatment to reduce the risk.

The inactivation of *T. gondii* during the curing process depends on the synergistic interaction among salt concentration, maturation time, and temperature of storage (Kijlstra and Jongert, 2008; Mie et al., 2008).

The main objective of this investigation was to evaluate the efficiency of the curing process for the inactivation of the parasite *T. gondii*. The technological process of curing clearly has evidenced an effect on the inactivation of *T. gondii*; differences between fresh and cured ham are significant both regarding the presence and the viability of *T. gondii*. However, the process of curing does not completely inactivate *T. gondii*, as the parasite has been detected in 11 of 26 hams analyzed and remained viable in 4 of them. The viability of *T. gondii* found in our study was higher than data previously obtained (1.5%-4.8%) (Gómez-Samblas et al., 2015; Warnekulasuriya et al., 1998). However, it must be taken into account that the mentioned studies were performed with commercial samples whose previous serological status was unknown.

Some studies indicate that inactivation of *T. gondii* may be dependent on the time of curing (Bayarri et al., 2010; Gómez-Samblas et al., 2016). In this assay, the viability seems higher in hams of 9 months of curing than in those of 12 months, in which the parasite viability was confirmed only in one ham. The risk assessment depends not only on the viability of *T. gondii* but also on the quantity of the parasite present in the cured product. Levels detected in cured meats in the United Kingdom were estimated at 5 x 10^3 bradyzoites per gram, (Warnekulasuriya et al., 1998) and the researchers concluded that there were sufficient *T. gondii* bradyzoites to cause infection after consumption of a normal portion of cured meat. Levels detected in cured hams in our work were estimated between 23.3 and 153.3 parasites per gram, that is a lower burden. Nevertheless, there is a reduction of the risk but not a complete elimination of risk and there is likelihood of cured meat drying for 9 or 12 months containing viable cysts. Our results suggest that, from a food safety point of view, it is better to consume cured ham with periods of curing higher than 12 months. Moreover, according to results

368 reported by Bayarri et al. (2010), 14 months of curing time matches the loss of *T. gondii*
369 viability.

370 Another goal of this study was to test the physical and chemical parameters such
371 as water activity, content of salt (NaCl, nitrate, nitrite), pH and fat content, in order to
372 evaluate their influence on *T. gondii* bradyzoites survival. As far as we know previous
373 studies lacked or reported limited information on meat product characteristics and their
374 relationship with the viability of *T. gondii* in cured ham. In this context, the study of
375 Gómez-Samblas et al. (2016) should be mentioned as they analyzed some
376 physicochemical parameters (water activity, salt content, and pH).

377 Raising the salt concentration or the temperature leads to inactivation of the
378 parasite (Kijlstra and Jongert, 2008; Mie et al., 2008). However, occasionally some
379 tissue cysts may survive the curing process (Gómez-Samblas et al., 2015;
380 Warnekulasuriya et al, 1998). It has also been reported that salting does not necessarily
381 kill tissue cysts; in fact, the presence of viable *T. gondii* organisms in commercially
382 cured meat was recently described in a study carried out in Spain (Gomez-Samblas et
383 al., 2015). However, the lack of information about the salt concentration, period of
384 curing and other commonly used parameters in meat industry, such as water activity and
385 pH, make the comparison rather difficult.

386 Sodium chloride is used in the curing process for its effect on flavor, water
387 activity, and preservation of final product. The salt concentration will always increase
388 during maturation due to the loss of water. Sodium nitrate and potassium nitrate induce
389 color development in cured meat products and are used when drying is a long term
390 process. Nitrates also reduce water activity. To be effective, reduction of nitrates to
391 nitrites under the influence of bacterial enzymes is required, and this is a time-
392 consuming process. Nitrites affect the color and flavor of the product and aid in its
393 preservation (Guo et al., 2015). There is currently little knowledge about the effects of
394 the nitrates and nitrites on the inactivation of *T. gondii*. These may, themselves or in
395 combination, provide further interventions for the reduction of *T. gondii* in the final
396 product (Mie et al., 2008). The loss of viability of *T. gondii* during the curing process
397 may be due to the dehydration suffered. Meat loses weight during drying and the
398 increase in the concentration of NaCl and other electrolytes may affect the viability of
399 the protozoan cysts (Dubey, 1997; Mie et al., 2008; Sommer et al., 1965; Work, 1968).
400 Likewise, the buildup of peroxides, together with the free fatty acids and their detergent
401 effect (Ventanas, 2001), may alter the membrane of cysts and protozoan bradyzoites

resulting in a loss of infectivity. The dehydration, the dry environment and the buildup of free fatty acids reach their maximum levels after around 14-15 months in the drying shed (Gómez-Samblas et al., 2015). This could explain the need of curing for times longer than 12 months to ensure the inactivation of *T. gondii*.

Bayarri et al. (2010) evaluated cured ham made in the traditional manner, and indicated that the final curing salt concentration of 3.9% NaCl, 25 mg/kg nitrate, and 3 mg/kg nitrite as well as the duration of the curing period (14 months) inactivate *T. gondii*. Recently, Gomez-Samblas et al. (2016) studied different ham curing treatments, and observed that the parasite was still viable after 5 months in the samples with the lowest NaCl content. Neumayerová et al. (2014) analyzed vacuum packed goat meat and concluded that 2.5% initial amount of sodium nitrite was effective in killing *T. gondii* cysts in 14 days; this time is much shorter than the usual curing time. These authors also report that the following physical and chemical parameters are lethal for the parasite: 0.960 ± 0.002 meat a_w, 1.856% ± 0.70% salt content and 5.825 ± 0.008 pH. However, in our study we detected viable parasites in a final product with a lower a_w (0.903-0.942) and a higher salt concentration (NaCl 2.7-2.9), and no relation was found between viability of *T. gondii* and water activity and content of salt (NaCl, nitrate, nitrite). Neither relation was found between viability and pH. During curing, the pH usually falls, while dehydration is achieved by keeping the product under controlled temperature and humidity, this drop in pH is unlikely to have an impact on the viability of the tissue cyst, which has been reported to be resistant at pH < 1.0 (Dubey, 1998a). Apparently, this product's high salt content, pH and low water activity is not completely amenable for *T. gondii* bradyzoite survival. When the hams are rubbed with the salting mix we assume that salt is able to penetrate the raw meat effectively and results in a homogeneous concentration lethal to *T. gondii* bradyzoites. However, there may be areas where lethal concentrations of salts have not been distributed evenly or were unable to exert its effect on the bradyzoites because they are protected by the cyst wall. An important function of the cyst wall and matrix is to protect bradyzoites from harsh environmental conditions such as dehydration (Weiss and Kim, 2007). In addition, fat may protect tissue cysts from the effects of salt, and in this sense, it should be noted that in cured hams an association between loss of viability of *T. gondii* and lower fat content was found. To date no investigation of this specific issue has been published.

A possible limitation of our study is that although we use both hind legs, we cannot guarantee that both pieces are identical in parasite load. In fact, detection of the

436 parasite in a leg does not ensure its detection in the other and, reciprocally no parasite
437 detection in the fresh ham does not ensure that the cured ham will be free of the
438 parasite. This is especially important in studies, such as ours, in which naturally infected
439 animals are used and, particularly, when parasitism levels are low.

440 In conclusion, a high number of seropositive pigs had *Toxoplasma* tissues cysts
441 in the raw ham although the parasite burden was low. We observe that time of curing
442 significantly contributed to *T. gondii* cyst survival; nevertheless curing during 12
443 months resulted in the reduction but not in a complete elimination of the risk. Thus,
444 from a public health point of view, under the conditions of this study it is safer to
445 consume dry-cured ham with times of curing higher than 12 months. Furthermore, we
446 did not identify any variable with significant influence on the presence and viability of
447 *T. gondii* in cured ham and further research is required to validate combinations of salt
448 concentration, and time of curing as a critical control points. We can also conclude that
449 there is urgent need for further development and standardization of methods for the
450 testing of the pathogen survival in dry-cured meat products. It is desirable to avoid
451 animal experimentation and development of sensitive viability assays that are not based
452 on the use of experimental animals would be valuable.
453

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455

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460

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Table 1: Sampling description

	Haunches	Dry-cured hams	
	Day 0	9 months	12 months
Batch I Negative Control (Titer <1:20)	3	0	3
Batch II (Titer 1:20 to <1:80)	14	6	8
Batch III (Titer ≥1:80)	24	15	9
Total	41	21	20

Table 2. *T. gondii* in raw ham and dry-cured ham (detection by mouse bioassay and PCR)

Pig titer	Raw ham			Dry-cured ham			Time of curing
	Presence		Viability[c]	Presence		Viability[c]	
	Bioassay [a]	PCR extract[b]		Bioassay [a]	PCR extract[b]		
Batch I Negative Control (Titer <1:20)							
1	0/8	-	0/0	0/6	-	0/0	12
2	0/8	-	0/0	0/6	-	0/0	12
3	0/8	-	0/0	0/8	-	0/0	12
Batch II (Titer 1:20 to <1:80)							
4	0/8	34.5 ± 0.25 (118)	0/0	0/8	-	0/0	9
5	1/8		0/1	0/8	-	0/0	9
6	0/8	-	0/0	0/8	-	0/0	9
7	0/8	-	0/0	0/8	-	0/0	9
8	1/8		0/1	0/8	-	0/0	9
9	2/7		0/2	0/8	-	0/0	9
10	0/8	-	0/0	0/8	-	0/0	12
11	0/8	-	0/0	0/8	-	0/0	12
12	0/7	-	0/0	0/8	-	0/0	12
13	0/8	-	0/0	0/8	-	0/0	12
14	0/8	-	0/0	0/8	-	0/0	12
15	0/8	-	0/0	0/8	-	0/0	12
16	0/8	-	0/0	0/8	-	0/0	12
17	0/7	-	0/0	0/8	-	0/0	12

Batch III (Titer ≥1:80)						
18	5/8	0/5	0/4	-	0/0	9
19	7/8	3/7 35.80 ± 0.34 (49.2) 35.92 ± 0.51 (45.3) 36.09 ± 0.19 (40.1)	0/7	-	0/0	9
20	4/8	2/4 35.48 ± 0.62 (61.4) 36.92 ± 0.82 (22.6)	1/8		1/1 34.52 (119.3)	9
21	4/8	0/4	0/6	35.12 ± 0.35 (78.7)	0/0	9
22	3/8	1/3 34.44 ± 0.48 (126)	2/7		2/2 35.02 ± 0.64 (84) 34.34 ± 0.21 (134.7)	9
23	6/8	4/6 33.44 ± 0.20 (250.7) 34.68 ± 0.46 (106) 34.05 ± 0.75 (165.5) 33.83 ± 0.13 (193)	0/8	35.61 ± 0.29 (55.9)	0/0	9
24	5/8	1/5 37.51 ± 0.64 (15)	0/8	-	0/0	9
25	4/8	2/4 35.90 ± 0.45 (45.9) 35.54 ± 0.34 (58.9)	1/8		1/1 34.15 ± 0.41(153.3)	9
26	5/7	1/5 34.5 ± 0.65 (120.7)	0/8	-	0/0	9
27	4/8	1/4 36.72 ± 0.87 (25.9)	0/8	-	0/0	9
28	2/8	0/2	0/4	-	0/0	9

29	6/8		3/6 34.33 ± 0.39 (136) 35.29 ± 0.52 (70) 36.17 ± 0.24 (37.9)	0/7	-	0/0	9
30	8/8		3/8 37.27 ± 0.63 (17.7) 35.12 ± 0.18 (78.7) 35.07 ± 0.79 (81.3)	0/7	-	0/0	9
31	5/8		1/5 36.35 ± 0.50 (33.5)	0/8	36.85 ± 0.84 (23.3)	0/0	9
32	6/8		0/6	0/8	-	0/0	9
33	3/8		3/3 37.48 ± 0.43 (15.3) 36.66 ± 0.17 (27) 36.73 ± 0.36 (25.7)	1/7		1/1 36.02 ± 0.17 (42)	12
34	0/8	-	0/0	0/8	-	0/0	12
35	0/8	-	0/0	0/7	-	0/0	12
36	1/8		0/1	2/8		0/2	12
37	0/8	36 ± 0.64 (42.7)	0/0	0/7	-	0/0	12
38	0/8	36.76 ± 0.14 (25.2)	0/0	1/8		0/1	12
39	0/8	36.77 ± 0.35(25)	0/0	1/8		0/1	12
40	0/8	36.89 ± 0.40 (23)	0/0	0/8	-	0/0	12
41	1/8		0/1	0/8	35.06 ± 0.48 (82)	0/0	12
Total[d]		**26/38**	**12/38**		**11/38**	**4/38**	

[a] Seropositive mice by IFA/Total of mice
[b] Presence by PCR of extracts. Ct values (parasites/g)
[c] Positive mice brain by PCR/Total of seropositive mice by IFA. Ct values (parasites/g)
[d] Positive samples/total seropositive pigs
Blank boxes: not analyzed (because the sample was positive by bioassay)

Table 3. Physicochemical parameters of cured hams

Pig	Haunch weight (Kg)	Cured ham weight (Kg)	Weight loss (%)	Curing time (months)	Nitrates (mg/Kg)	Nitrites (mg/Kg)	Moisture content (%)	a_w	pH	NaCl (%)	Fat content (%)
1	12.98	8.66	33.28	12	82	74	51.1	0.916	5.80	3.6	4.5
2	12.6	8.17	35.16	12	<50	90	53.2	0.894	5.90	3.7	4.7
3	13.16	8.42	36.02	12	<50	42.6	53.1	0.914	5.85	3.2	5.1
4	14.76	10.14	31.30	12	53	53.8	55.2	0.909	5.95	2.7	5.7
5	13.66	8.96	34.41	12	<50	54.2	55.1	0.897	5.70	2.8	7.4
6	12.64	8.21	35.05	9	83	<6	58.0	0.933	5.90	2.5	5.5
7	10.57	6.44	39.07	9	81	<6	55.7	0.945	5.55	3.9	3.4
8	13.72	10.05	26.75	9	48	<6	59.2	0.939	5.80	2.4	5.9
9	12.3	7.58	38.37	12	<50	58.6	55.4	0.889	5.85	2.8	4.5
10	14.76	9.8	33.60	12	<50	79	53.3	0.898	5.80	3.1	5.0
11	12.76	8.24	35.42	12	<50	66	55.1	0.901	5.80	2.6	3.8
12	12.9	8.32	35.50	12	<50	73	54.6	0.902	6.00	2.5	3.9
13	12.07	7.05	41.59	12	74	34.6	50.3	0.893	5.65	3.0	7.3
14	14.24	7.83	45.01	12	<50	63	54.3	0.887	6.05	4.6	3.8
15	15.01	9.97	33.58	9	59	<6	59.9	0.950	5.75	2.5	3.3
16	11.62	7.84	32.53	9	70	<6	59.1	0.948	5.75	3.4	5.1
17	12.2	7.18	41.15	9	73	<6	49.3	0.948	5.75	3.4	4.9
18	12.89	8.46	34.37	12	69	<6	54.8	0.929	5.90	2.9	3.9
19	11.14	6.67	40.13	9	81	<6	54.2	0.926	5.95	3.0	5.3
20	8.59	5.23	39.12	9	108	<6	49.5	0.932	5.90	3.8	6.4
21	11.36	7.11	37.41	9	77	7.8	53.9	0.934	5.90	2.9	5.3
22	12.13	7.72	36.36	9	82	<6	55.5	0.936	5.65	3.2	6.2
23	12.01	7.37	38.63	9	78	<6	53.9	0.940	5.75	2.9	5.2
24	12.92	8.76	32.20	9	70	6.7	57.6	0.942	5.75	3.3	5.4
25	12.72	7.83	38.44	9	74	<6	50.5	0.931	6.00	3.4	6.4

26	12.04	8.07	32.97	9	64	<6	58.6	0.942	5.75	2.7	3.6
27	11.85	6.78	42.78	12	84	<6	47.4	0.903	5.90	3.8	3.3
28	12.75	7.35	42.35	12	92	<6	49.1	0.920	5.80	4.0	4.2
29	9.49	5.13	45.94	12	74	<6	46.3	0.879	5.80	3.9	3.5
30	13.14	8.83	32.80	12	72	<6	50.2	0.882	5.95	3.1	8.5
31	11.48	7.0	39.02	12	68	<6	54.3	0.918	5.95	3.3	3.8
32	12.05	7.89	34.52	12	86	<6	54.8	0.925	5.80	2.9	7.5
33	12.76	8.35	34.56	12	<50	58.7	52.0	0.903	5.85	2.9	6.3
34	10.4	6.58	36.73	9	83	<6	53.0	0.937	5.75	2.9	3.2
35	14.76	10.36	29.81	9	52	<6	55.9	0.942	5.80	2.2	5.3
36	11.93	8.10	32.10	9	79	<6	49.7	0.947	5.80	3.3	4.9
37	12.2	7.88	35.41	9	77	10.4	54.3	0.934	5.80	3.1	5.2
38	11.09	7.83	29.40	9	58	24	52.6	0.937	5.95	3.6	5.8
39	14.3	9.98	30.21	9	85	<6	48.5	0.948	5.75	2.3	4.3
40	12.86	9.0	30.02	9	91	<6	59.4	0.949	5.60	2.1	5.4
41	12.5	8.14	34.88	12	97	<6	55.4	0.927	5.85	3.0	6