Veterinary Research

RESEARCH

Open Access

A safe and efficacious inactivated vaccine aids prevent reproductive failure associated with congenital toxoplasmosis in ovine

Javier Regidor-Cerrillo[1*], Andrea Largo-de la Torre[1], Roberto Sánchez-Sánchez[2], Ignacio Ferre[2], Javier Moreno-Gonzalo[2,3] and Luis Miguel Ortega-Mora[2*]

Abstract

Toxoplasma gondii is an apicomplexan parasite causing reproductive failure in small ruminants. In this study, we evaluated the capacity of an inactivated vaccine to prevent reproductive failure caused by congenital toxoplasmosis in sheep. The vaccine is based on an antigen extract obtained from a low passage *T. gondii* Type III isolate (TgPigSp1) preserving the ability to spontaneously produce cysts in vitro and following a procedure involving parasite inactivation via hyperosmotic shock and membrane proteins solubilisation. The vaccine prototype, consisting of 40 µg of parasite extract adjuvanted with QuilA®, was evaluated in two different trials using a pregnant ovine model of *T. gondii* infection based on orally challenging sheep at 90 days of gestation with 10 sporulated oocysts of the heterologous Type II isolate TgShSp1. Two subcutaneous immunizations at days 55 and 76 of pregnancy caused mild and transient local reactions and had no discernible impact on gestation. Vaccination triggered both specific cellular and humoral immune responses. The proportion of viable gestations resulted in 100% (vaccine trial 1) and 78% (vaccine trial 2) in vaccinated/challenged ewes *versus* 50% in unvaccinated/challenged sheep. This increase in viable gestations was associated with a significant increase of lambs born viable for vaccine trial 1 (62.5%) and a decrease of early foetal losses (i.e. abortion) for vaccine trial 2 (70%), associated with control of *T. gondii* multiplication in the cotyledons. This inactivated vaccine could be a suitable and practical tool to mitigate economic losses in sheep caused by *T. gondii* outbreaks.

Keywords Sheep, congenital toxoplasmosis, multistage vaccine, reproductive failure, improvement of reproductive losses

Handling editor: Frank Katzer

*Correspondence:
Javier Regidor-Cerrillo
jregidor.saluvetinnova@gmail.com
Luis Miguel Ortega-Mora
luis.ortega@ucm.es
[1] SALUVET-Innova, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain
[2] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain
[3] Animal Medicine and Surgery Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

Introduction

The apicomplexan parasite *Toxoplasma gondii* infects virtually all warm-blooded animals, including humans and livestock, and is distributed worldwide. It has a complex life cycle in which three infective stages have been identified: tachyzoites, involved in the acute phase of infection; bradyzoites, contained within tissue cysts and responsible for the chronic phase of infection in the intermediate hosts; and sporozoites, which are found within sporulated oocysts—the environmental resistant stage- formed after non-sporulated oocysts are shed in the faeces of feline definitive hosts [1]. Hosts can acquire infection

horizontally by ingestion of feed or water contaminated with sporulated oocysts or tissue cysts present in raw or undercooked muscle tissues from intermediate hosts. Following infection, released bradyzoites or sporozoites convert to tachyzoites in the host's intestinal tissues and disseminate throughout the body during the acute phase of infection [2]. Once the host develops a cell-mediated immune response, tachyzoites convert into bradyzoites within tissue cysts, mainly located in the brain, heart, and skeletal muscles, where they can persist and evade the host's immune response [1]. *Toxoplasma gondii* can also be transmitted vertically when infection occurs during pregnancy, as tachyzoites may reach the placenta and foetus during the acute phase of infection [1].

Congenital toxoplasmosis is a well-known cause of abortions and foetal malformations in humans and small ruminants [1, 3]. In sheep, primary *T. gondii* infection due to oocyst ingestion during gestation may cause abortion, foetal mummification, stillbirths, and the birth of weak offspring. The pregnancy outcome is largely dependent on the gestational stage at which infection occurs [4–7]. During early gestation, when the foetal immune system is immature, congenital transmission often leads to foetal death, sometimes resulting in resorption. Infection at mid-gestation usually leads to abortion or stillborn lambs, while infection late in gestation may result in stillborn lambs or the birth of weak or clinically normal but infected lambs. *Toxoplasma gondii* infection in sheep has a worldwide distribution, with prevalence varying widely between regions [7, 8]. Abortions caused by *T. gondii* represent 10–23% of ovine abortions in Europe and the USA, and 3–54% in the Middle East and South America [1, 8]. *Toxoplasma gondii* abortion outbreaks can lead to significant economic losses for sheep producers [9]. Furthermore, the consumption of *T. gondii*-infected lambs may serve as a source for human infection [10].

Vaccination is regarded as a promising and cost-effective approach to mitigate the impacts of *T. gondii* infection in sheep, particularly when combined with cat population management and improved flock hygiene [11]. Currently, only one commercially available vaccine, Toxovax® (MSD), is registered in certain European countries and New Zealand to reduce abortions in sheep. Toxovax® is a live attenuated vaccine derived from tachyzoites of the "incomplete" S48 strain, which, after prolonged serial passage in mice, lost its capacity to form tissue cysts or oocysts [12]. S48 protection achieved against reproductive losses was high and produced 76.8% of viable offspring in vaccinated compared with the 17.8% in unvaccinated (>66%) [12]. The main limitations of the Toxovax® vaccine are related to safety, as it cannot be used in pregnant sheep and poses a risk of infection to operators handling it, together with a short shelf life. Although other live attenuated vaccines have been evaluated in sheep, they share the limitations of the Toxovax® vaccine [13]. The detailed characteristics of vaccine development approaches against toxoplasmosis in sheep and goats have recently been reviewed [14].

Focusing on inactivated vaccines, earlier studies reported that the use of vaccine preparations based on killed whole tachyzoites (non-adjuvanted or adjuvanted with Freund's incomplete adjuvant) did not protect sheep against reproductive losses [15, 16]. A vaccine based on a tachyzoite membrane fraction (four major proteins adjuvanted with QuilA® as ISCOMs) achieved partial protection of 56% against lamb mortality (36.4% lamb mortality in vaccinated *versus* 64.7% in unvaccinated) [17]. Nonetheless, the number of studies with inactivated vaccines in sheep is very limited.

Recently, an inactivated vaccine prototype based on the TgPigSp1 isolate has been evaluated against chronic *T. gondii* infection in both a harmonized proof-of-concept mouse model and in a piglet model. In this study, TgPigSp1 demonstrated the ability to spontaneously form tissue cysts in vitro and it provides both tachyzoite and bradyzoite antigens for the inactivated vaccine prototype [18, 19]. Furthermore, mouse vaccination led to a reduction higher than 80% of parasite loads in brain and muscle tissues. In piglets, vaccination conferred strong protection achieving a 95% reduction in parasite load and over an 88% decrease in detection frequency in target muscles [20]. In the present study, we evaluated the capacity of this inactivated vaccine prototype to protect against reproductive failure caused by congenital toxoplasmosis in an ovine model based on oocyst infection at mid-gestation.

Materials and methods

Parasites

For vaccine antigen production, the Type III *Toxoplasma gondii* isolate TgPigSp1 (ToxoDB genotype #2) was used, whereas pregnant sheep were challenged with oocysts of the Type II isolate TgShSp1 (ToxoDB genotype #3). These isolates were obtained from an adult pig myocardium (TgPigSp1) and the brain of an ovine abortion case (TgShSp1) [21, 22]. The TgPigSp1 and TgShSp1 isolates were maintained in Vero cells (ATCC® CCL81™) in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1% foetal bovine serum (FBS) (Gibco), penicillin (100 U/mL), streptomycin (100 g/mL}, and amphotericin B (0.25 g/mL) (Lonza Group, Basel, Switzerland) at 37 °C and 5% CO_2. Both isolates were grown for a controlled number of passages from isolation to minimize adaptation-related changes, and both resulted in spontaneous cyst formation in vitro [21, 22].

Generation of *T. gondii* sporulated oocysts

Toxoplasma gondii oocysts of the TgShSp1 isolate used for sheep challenge were obtained as previously described by [23]. Briefly, CD1 mice (Janvier-Labs, Laval, France) were inoculated intraperitoneally with 10^5 tachyzoites. Forty days post-infection mice were euthanized and the brains were collected. One kitten (Isoquimen S.L., Barcelona, Spain) was fed with a pool of brains, and faeces were examined until *T. gondii* oocysts were detected. Purified unsporulated oocysts from faeces were sporulated in 2% H_2SO_4 at room temperature and kept at 4 °C until use. Two batches of sporulated oocysts, used in the two successive vaccine trials (vaccine trial 1 and vaccine trial 2), were preserved at 4 °C for thirteen and three months, respectively.

Production and quality control of the antigen and vaccine formulation

Vaccine antigen production

For vaccine antigen preparation, 24 h semi-confluent cell monolayers (80–90% of the T75 flask surface) were inoculated with TgPigSp1 tachyzoites of passages 28, 29, and 30 with a multiplicity of infection (MOI) of 3:1 or 4:1. Inoculated cultures were maintained for 3.5–4 days until parasite egress was observed by light microscopy (40X), after which parasites were collected, passed through a 25G needle, and purified using 10 µm polycarbonate membranes (Whatman™ Cyclopore™, Buckinghamshire, UK). Parasites were recovered by centrifugation at 1350×*g* for 10 min and resuspended in PBS. Tachyzoites were counted using a Neubauer chamber, distributed in 1×10^8 tachyzoite aliquots, collected by centrifugation (1350×*g*, 10 min, at 4 °C), and maintained at −80 °C until use.

Each pellet containing 1×10^8 tachyzoites was re-suspended in 600 µL of PBS with 0.5% (v/v) protease inhibitor (Protease inhibitor cocktail, Sigma-Aldrich, St. Louis, Missouri, USA). Once re-suspended, 300 µL of 60% sucrose (w/v) in PBS were added to the suspension to obtain a final concentration of 20% (w/v) sucrose in the mixture. The parasite pellet was then recovered through centrifugation at 10 000×*g* for 60 min at 4 °C. The obtained pellet was re-suspended in 0.1 mL of 1% Igepal® Ca-630 (Sigma) solution (v/v) in ultrapure water, supplemented with 0.5% of the same protease inhibitor as above, for the solubilisation of the protein antigens. This suspension was maintained under constant agitation for 18–24 h at 4 °C to complete homogenization. The protein concentration in the parasite extract was quantified using the Bradford method, aliquoted, and kept at −80 °C for quality controls and vaccine formulation. Each dose of the vaccine formulation contained 40 µg of the protein extract mixed with 300 µg of QuilA® (InvivoGen, Toulouse, France) in a total volume of 1 mL of PBS.

Vaccine quality controls

Parasite inactivation in the new batch of antigen intended for the sheep vaccine formulation was verified by in vitro culture in Vero cells, following the methodology previously described [20]. After sucrose treatment, a portion of parasites from each production batch was resuspended in PBS instead of Igepal® Ca-630 solution, and a volume containing 10^7 tachyzoites was inoculated onto a Vero cell monolayer in a T25 flask at MOI 10:1. The culture was then maintained through three successive blind passages in supplemented DMEM at 37 °C and 5% CO_2. After the third blind culture passage, the cell monolayer was harvested using a cell scraper, centrifuged at 1350×*g* for 10 min, and the resulting pellet was used for DNA extraction and parasite detection by real-time quantitative PCR (qPCR) (see parasite quantification section).

The protein profile of the vaccine antigen was studied by Coomassie-stained SDS-PAGE gels to confirm the consistency among production batches. For Coomassie-stained gels, 40 µg of vaccine antigen protein from each production batch was mixed with 2X protein lysis buffer (4% SDS, 10% glycerol, 60 mM of Tris–HCl pH 6.8, 100 mM of dithiothreitol, and 0.048% of bromophenol blue), boiled for 5 min, and subsequently resolved by SDS-PAGE. All samples were run in parallel with the Precision Plus Protein Standards Kaleidoscope™ marker (Bio-Rad, Hercules, California, USA) to determine the relative molecular weight of the protein bands. The samples were resolved at a constant 100 V for 6 h on a 4% bis/acrylamide stacking gel (pH 6.8), followed by a 10% acrylamide/bisacrylamide separating gel, using Tris–Glycine-SDS electrophoresis buffer and a PROTEAN II system (Bio-Rad). After electrophoresis, the acrylamide gels were stained with Coomassie solution and subsequently rinsed in an ethanol-acetic solution until the protein band patterns became clearly visible. Protein profile analyses were carried out by comparing the relative molecular weights of the stained bands determined using Quantity One software (Bio-Rad).

The presence of specific cyst and bradyzoite proteins in the vaccine antigen was confirmed using a double assay approach. Spontaneous cyst formation and tachyzoite proliferation were assessed in *T. gondii* cell cultures maintained in parallel to antigen production, as previously described [18]. Briefly, parasite *inocula* used for antigen production, were also seeded onto Vero cell monolayers grown to confluence on circular glass coverslips (12 mm diameter) in 24-well plates, and incubated at 37 °C, 5% CO_2. Prior to harvesting parasites from cultures for vaccine antigen production, the coverslips were fixed in

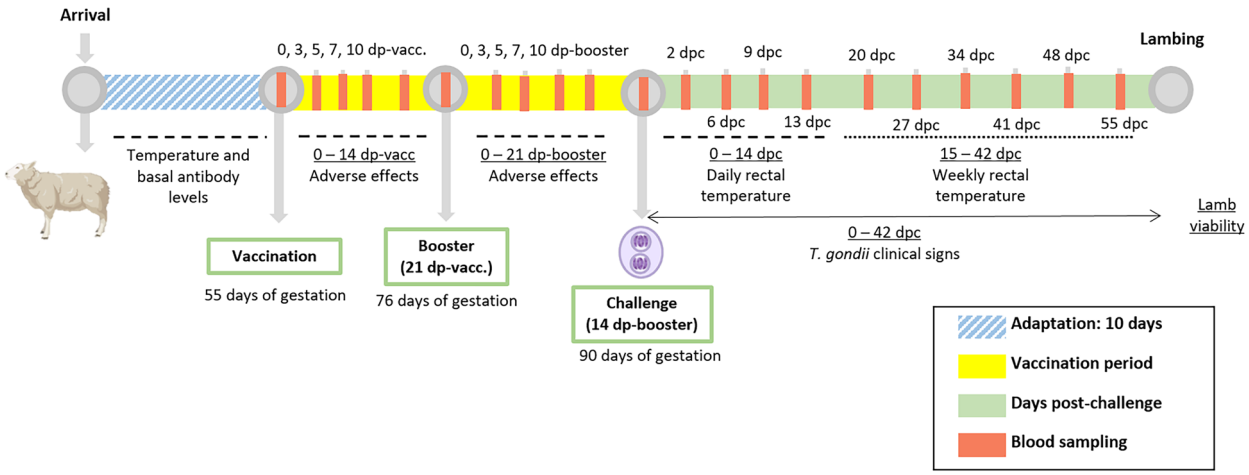

Figure 1 Experimental design for the vaccine trials against *T. gondii*. The figure outlines the timeline, including the adaptation period, immunization schedule, challenge with *T. gondii* and all procedures performed during the experiment. Dp-vacc: days post-vaccination, dp-booster: days post-booster, dpc: days post-challenge.

ice-cold methanol, permeabilized with 0.25% Triton X-100, stained with FITC-conjugated *Dolichos biflorus* lectin (DBL-FITC, Vector Laboratories, Newark, California, USA), and labelled with a hyperimmune mouse antiserum against *T. gondii*, followed by a goat anti-mouse IgG conjugated to Alexa Fluor®594 ("red", Thermo Fisher Scientific). Finally, the coverslips were washed, mounted, and visualized using an inverted fluorescence microscope (Nikon Eclipse TE 200, Chiyoda, TYO, Japan) at 40X for cyst visualization. The presence of the specific bradyzoite protein *Tg*BAG1 was assessed by immunoblotting. For this purpose, 10 µg of vaccine antigen protein from different production batches were subjected to SDS-PAGE electrophoresis as described above, and the proteins subsequently transferred to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in TBS-0.05% Tween 20 and subsequently incubated with a monoclonal mouse serum against *Tg*BAG1 at 1:100 dilution (Genscript, The Netherlands), followed by an anti-mouse IgG2a conjugated HRP (SouthernBiotech, Birmingham, USA) at 1:1000 dilution, and revealed by chemiluminescence using the ECL substrate (Thermo-Fisher Scientific).

Vaccine trials

A pregnant sheep model, based on challenge with 10 TgShSp1 oocysts, was used for vaccine trials [21, 23]. Vaccine trials were conducted with pure Rasa Aragonesa breed female ewes, 18 months of age, selected from the SALUVET-Innova flock (SALUVET-Innova, Madrid, Spain). All animals were seronegative for *T. gondii*, *Neospora caninum*, Border Disease Virus (BDV), Schmallenberg Virus (SBV), *Coxiella burnetii*, and *Chlamydia abortus*, as determined by Enzyme-Linked Immunosorbent Assay (ELISA). Ewes had been subcutaneously vaccinated against bluetongue (Syvazul BTV serotype 4 vaccine, Syva laboratories, León, Spain) five months prior to *T. gondii* vaccination. The ewes were oestrus-synchronized and mated for 2 days with pure-bred Rasa Aragonesa tups. Pregnancy was confirmed by ultrasound examination on day 40 post-mating on ewes involved in the vaccine trials. The experimental design for vaccine trials is represented in Figure 1.

Vaccine trial 1

The first vaccine trial was conducted to assess the absence of severe adverse effects associated with vaccination, as well as the immunogenicity and efficacy of the vaccine. Fourteen pregnant ewes were selected and randomly assigned to three groups: G1a (vaccinated and challenged; n = 3), G1b (unvaccinated and challenged; n = 8), and G1c (unvaccinated and unchallenged; n = 3). Sheep in G1a were vaccinated on day 55 of gestation and received a booster 21 days later, on day 76 of gestation. The vaccine was administered subcutaneously in the right side of the neck, and the booster was given in the left side. Fourteen days after the booster (day 90 of gestation), ewes from G1a and G1b were orally challenged with 10 sporulated TgShSp1 oocysts, while the G1c group received PBS as unchallenged control.

Vaccine trial 2

Twenty-six pregnant ewes were selected for the second vaccine trial and randomly assigned to three groups: G2a (vaccinated and challenged; n = 10), G2b (unvaccinated and challenged; n = 10), and G2c (vaccinated and unchallenged; n = 6). Sheep from G2a and G2c were vaccinated

on day 55 of gestation and received a booster 21 days later (76 days of gestation) as above; vaccination was subcutaneously in the right side of the neck, and the booster was given in the left side. Fourteen days after the booster (day 90 of gestation), ewes from G2a and G2b were orally challenged with 10 sporulated TgShSp1 oocysts. G2c was orally inoculated with the same volume of PBS.

Clinical monitoring

Local reactions at the vaccine inoculation site were examined daily from day 0 until day 14 following vaccination and booster and scored from 0 to 9 based on lesion type and associated pain (Additional file 1). Rectal temperatures were recorded daily from day 0 to day 14 following vaccination, booster, and challenge. Thereafter, rectal temperatures were recorded once weekly until the end of the trial. Temperatures above 40 °C were considered indicative of hyperthermia.

Foetal viability was assessed weekly in all groups after vaccination and challenge by monitoring foetal heartbeat, movements, and the presence of hyperechoic amniotic fluid by ultrasound scanning). In the group G2a, one ewe with a quintuplet gestation experienced abortion of two foetuses at 37 days post-challenge, in addition to three mummified foetuses estimated to be at 80–90 days of gestation (before challenge) and was subsequently excluded from the study. Deliveries occurring at or before 142 days of gestation were considered premature [24]. After birth, lambs were clinically examined using an adapted Apgar method assessing mucous membrane colour, response to stimulation in the nasal zone, eye zone, hoof, and respiration (Additional file 2). Key times in the neonatal period of the lambs (time-to-*decubito prono* position, time-to-stand, and time-to-suckle) were also considered in vaccine trial 2 for assessing newborn viability. Additionally, the birth weight of the lambs was recorded and normalized according to type of gestation (single, twin, etc.) [26].

According to the period of gestation and delivery, reproductive failure was classified into three categories; (1) early reproductive failure: for foetuses that died (aborted or not) up to day 130 of gestation (i.e., 40 days post-challenge) and mummified foetuses (i.e., estimated to have died before 130 days of gestation but retained in utero until delivery); (2) late reproductive failure: foetuses that died from day 131 of gestation until two days after birth, including dead foetuses (aborted or not), stillborn lambs and weak lambs (i.e., born dead or dying within the first two days after birth); and (3) viable lambs: lambs born alive and showing no clinical signs during the first two days after birth (Additional file 2). Viable pregnancies were defined as those that reached term with at least one viable lamb born.

Two days after delivery, dams and lambs were initially sedated with 0.1 mg/kg of xylazine administered intravenously (Rompun, Bayer, Mannheim, Germany) and subsequently euthanized by intravenous overdose of embutramide and mebezonium iodide (T61, Intervet, Salamanca, Spain).

Sample collection

To evaluate immune responses in the dams during vaccine trial 1, blood samples were collected on day 0 prior to vaccination, on days 1, 8, and 14 after the booster, on days 0, 5, 7, 10, 14 post-challenge, and weekly thereafter. In vaccine trial 2, blood samples were collected on days 0, 3, 5, 7, and 10 post-vaccination and booster, and on days 0, 2, 6, 9, 13, and then weekly post-challenge (Figure 1). Blood was collected via jugular venipuncture using vacuum devices with and without heparin anticoagulant (BD Vacutainer® Plus Plastic Serum, Franklin Lakes, NJ, USA). Precolostral serum was also collected from lambs immediately after birth. To prevent the transfer of maternal colostral antibodies, the ewes' udders were covered with a cloth one week prior to the expected date of delivery as a preventive measure. Blood samples collected without anticoagulant were allowed to clot and then centrifuged 1200×*g*, 10 min, 4 °C to obtain serum. Thoracic and abdominal fluids were also collected from dead foetuses and stillborn lambs. Sera and foetal fluids were stored at −80 °C until further analysis.

For tissue samples, six cotyledons were randomly selected from each placenta of aborted dams and dams that gave birth and stored at −80 °C for subsequent DNA extraction and PCR analyses. In addition, for vaccine trial 2, a portion of each cotyledon was transversally sliced into 2–3 mm-thick sections and fixed in 10% formalin for histopathological examination. Regarding tissue samples from foetuses and lambs, a portion of brain and lungs were stored at −80 °C for DNA extraction. Additionally, in vaccine trial 2, foetal brain sections were fixed in 10% formalin for histopathological analysis. The histopathological and PCR analyses were performed blinded.

Humoral and cellular immune responses

Humoral immune response was evaluated by measuring specific IgG levels in serum using a previously normalized in-house TgSALUVET ELISA 2.0. (RIPC cut-off 19.18) [25]. Ewe and precolostral sera, diluted 1:100, were analysed by ELISA using an anti-sheep IgG conjugate diluted 1:6000 (Sigma-Aldrich). Foetal fluids diluted 1:4 and 1:8 were also tested by TgSALUVET ELISA 2.0. Positive precolostral sera and foetal fluids were confirmed by immunoblotting for antigen profiling using a nitrocellulose membrane with *T. gondii* antigens transferred from

12.5% polyacrylamide gels under reducing conditions, and anti-sheep IgG diluted 1:600 [25].

Cellular immune responses were assessed by measuring IFN-γ production in a peripheral blood stimulation assay. For this purpose, heparinized blood samples were processed within 2 h of collection by mixing 500 µL blood with 500 µL RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, USA) and 1% antibiotic/antimycotic solution (Lonza, Basel, Switzerland). Blood cells were cultured in 24-well plates (Thermo Fisher Scientific, Waltham, USA) in the presence of 10^6 lyophilized *T. gondii* tachyzoites of the TgShSp1 isolate or 5 µg of soluble antigen extract. In each assay, concanavalin A (ConA, Sigma-Aldrich), at a final concentration of 5 µg/mL, was used as a positive control, and PBS as a negative control. The plates were incubated in at 37 °C and 5% CO_2 atmosphere for 24 h and subsequently centrifuged at 1000×*g* for 10 min at 4 °C. Finally, cell-free culture supernatants were recovered and stored at -80 °C until IFN-γ determination. IFN-γ levels in the supernatants were determined using a commercial ELISA (Mabtech AB, Nacka, Sweden) validated for sheep and with a limit of detection of 7.8 pg/mL.

Quantification of Toxoplasma gondii DNA in placental and lamb tissues

Genomic DNA was extracted from 50 to 100 mg of cotyledon tissue (six samples per ewe), as well as from brain and lung (three samples from each foetus/lamb), using the commercial Maxwell® RSC Tissue DNA Kit (Promega, Wisconsin, USA) under the manufacturer's recommendations. *Toxoplasma gondii* DNA quantification was performed by real-time quantitative PCR (qPCR) using primer pairs targeting the 529-bp repetitive element for parasite quantification and primer pairs targeting the 28S rRNA gene to quantify host cell DNA, under the conditions previously described [18]. Standard curves for *T. gondii* DNA (DNA of 10^5 to 0.1 tachyzoites per each reaction) showed average slopes ranging from −4.1 to −3.30 and correlation coefficients (R^2) > 0.99. DNA samples from uninfected animals were included in each round of DNA extraction and qPCR as negative controls.

Histopathological analysis

After a 5-day fixation period, tissue samples from vaccine trial 2 were trimmed to obtain one section per cotyledon and four sections from each foetal brain (frontal lobe, corpus callosum, midbrain, and cerebellum). The sections were then embedded in paraffin wax and processed by standard histological procedures for haematoxylin and eosin (HE) staining. Conventional histological evaluation was carried out, and lesions in each section were scored as follows: (0) no lesion; (1) non-specific lesion (haemorrhage, oedema, and non-purulent infiltration); (2) compatible lesion with *T. gondii* infection (necrosis, gliosis, and leukomalacia); (3) lesion characteristic of *T. gondii* infection (necrotic placentitis with calcification and foci of necrosis or gliosis in the foetal brain).

Statistical analysis

Rectal temperatures and cellular and humoral immune responses results in the dams were analysed using a Two-way ANOVA of repeated measures test. The number of viable pregnancies (i.e., at least one viable lamb born), early reproductive failure before 130 days of gestation (i.e., abortions and mummified foetuses), late reproductive failure after 130 days of gestation (i.e., stillbirths and weak lambs), the number of viable lambs, as well as the frequencies of parasite DNA detection in placental and foetal-lamb tissues were compared among the different groups using the Chi-square test (Chi^2-test). When significant differences were detected, the Fisher's exact F-test was used to examine all possible pairwise comparisons. A value of $p < 0.05/$ (K-1) was considered statistically significant for pairwise comparisons, where K corresponds to the number of groups. Birthweights of the lambs were analysed using a One-way ANOVA test, followed by Tukey's multiple range test. Differences in the scored parameters (i.e., local reactions, viability of the lambs using the Apgar score, and histopathology) and parasite loads in placental and foetal tissues were evaluated using the Kruskal–Wallis test, followed by Dunn's multiple range test or the Mann–Whitney test. A non-parametric Spearman correlation analysis was also carried out between median parasite loads and mean lesion score in cotyledon and brain tissues. Statistical significance was set at $p < 0.05$ for all analyses. All statistical analyses were conducted using GraphPad Prism 8.0.1 software (San Diego, CA, USA).

Results

Quality controls of the vaccine

Inactivation was confirmed by the absence of parasite growth and the undetectable levels of *T. gondii* DNA via PCR, following three blind passages of the antigen extract in Vero cell cultures.

Coomassie-stained gels demonstrated the integrity of the vaccine antigen's proteins and the consistency of protein profiles across production batches (passage 28 to 33; data not shown). Because the antigen was derived from the Type III TgPigSp1 isolate, which spontaneously produces cysts in Vero cell cultures, the presence of bradyzoite-specific antigens was verified. Formation of structures consistent with cysts was observed in the parasite production cultures and the bradyzoite-specific

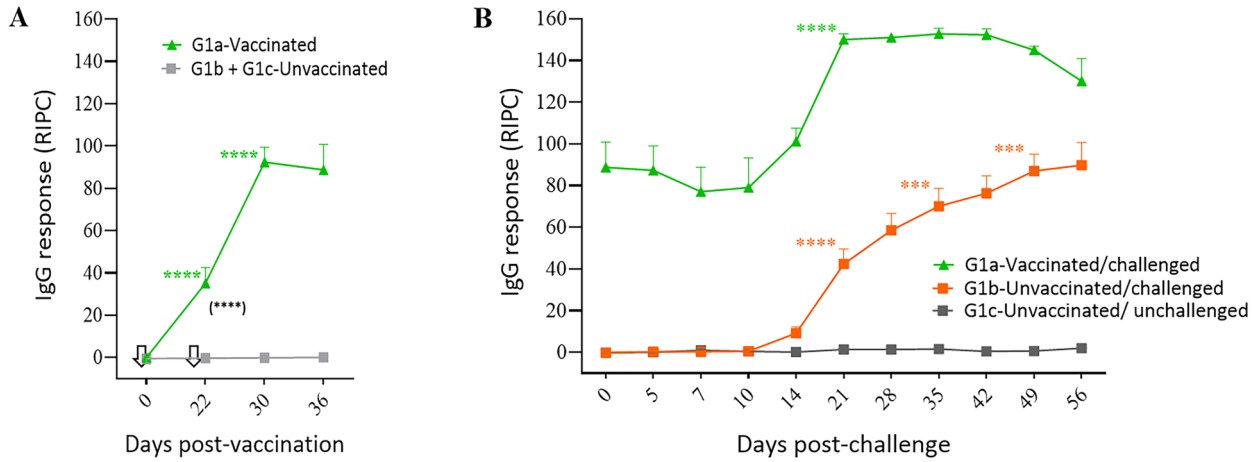

Figure 2 Kinetics of anti-*T. gondii* IgG response in vaccine trial 1 after vaccination (A) and after challenge (B). The Y-axis represents IgG levels expressed as Relative Index Percent (RIPC). Each data point indicates the mean, and the error bars represent the Standard Error of the Mean (S.E.M.). **A** Response post-vaccination. White arrows above the X-axis indicate the days of vaccination and booster dose administration. Data for the unvaccinated groups, G1b and G1c are pooled for comparison. Green and orange asterisks above each data point denote a significant increase of IgG levels relative to day 0 within that specific group. Black asterisks (in brackets) mark the days when IgG levels were significantly higher in the vaccinated group G1a compared to the pooled unvaccinated groups (G1b + G1c). IgG levels were significantly higher in G1a vs G1b + G1c starting from day 22 post-vaccination. **B** Response post-challenge. IgG levels are compared among the three groups. IgG levels were significantly higher in G1a (vaccinated/challenged) compared to G1c (unvaccinated control) post-challenge. Furthermore, IgG levels in G1b (unvaccinated/challenged) were significantly higher than G1c (unvaccinated control) after day 21 post-challenge (data for G1b vs G1c not indicated in the graphic). Significance levels are indicated as ****$p < 0.0001$; ***$p < 0.001$ (Two-way ANOVA test).

protein *Tg*BAG1 was detected by immunoblotting (Additional file 3).

Evaluation of vaccine safety, immunogenicity and efficacy against reproductive failure in vaccine trial 1

Vaccine safety

No relevant systemic, pregnancy-related adverse effects, or local reactions attributable to the vaccine were recorded in the sheep. The only observed adverse effect was a transient and significant elevation in rectal temperature in group G1a (vaccinated) on day 1 after both primary vaccination and booster ($p < 0.05$). Two out of three ewes exhibited hyperthermia (> 40 °C), which returned to physiological levels by day 2 (data not shown).

Vaccine immunogenicity

Strong vaccine immunogenicity was evident by measuring the humoral response. Two out of three ewes from G1a seroconverted by day 1 post-booster, with all G1a ewes achieving high IgG responses by day 8 post-booster, which were maintained until the challenge ($p < 0.0001$) (Figure 2A).

Following challenge, IgG levels in G1a increased significantly starting on day 21 post-challenge and remained elevated until the end of the trial ($p < 0.001$) (Figure 2B). All G1b ewes seroconverted by day 21 ($p < 0.001$). G1c ewes remained seronegative (Figure 2B). IgG levels in G1a were significantly higher than those in G1b from day 21 post-challenge and throughout the trial ($p < 0.001$).

Vaccine efficacy

Post-challenge G1b (unvaccinated/challenged) ewes were febrile from day 5 to 10, reaching a maximum mean temperature of 41.1 °C on day 7. G1a (vaccinated/challenged) ewes were febrile from day 7 to 10, peaking at 40.5 °C on day 7 ($p < 0.0001$) (Figure 3A). No increase in rectal temperature was observed in G1c (unchallenged control).

Gestation outcomes are summarized in Table 1, with indication of statistical differences detected among groups, and Figure 3B (see individual details in Additional file 4). All gestations in groups G1a (vaccinated/challenged) and G1c (unchallenged control) were viable and progressed to term each resulting at least in the birth of one healthy lamb. In G1a (vaccinated/challenged) and G1c (unchallenged control) groups, only one ewe with a multi-foetal gestation delivered a single stillborn lamb. In sharp contrast, only 50% of pregnant ewes (4 out of 8) in G1b (unvaccinated/challenged) delivered a viable lamb. Significant differences on the ratio of viable gestations were detected among these three groups ($p < 0.05$). Detailed analysis of G1b showed an increase in reproductive failure, including the abortion of two foetuses and one mummified foetus before day 130 of gestation. Overall, only five out of 16 (31.2%) expected lambs in G1b were born viable. The percentage of viable lambs born

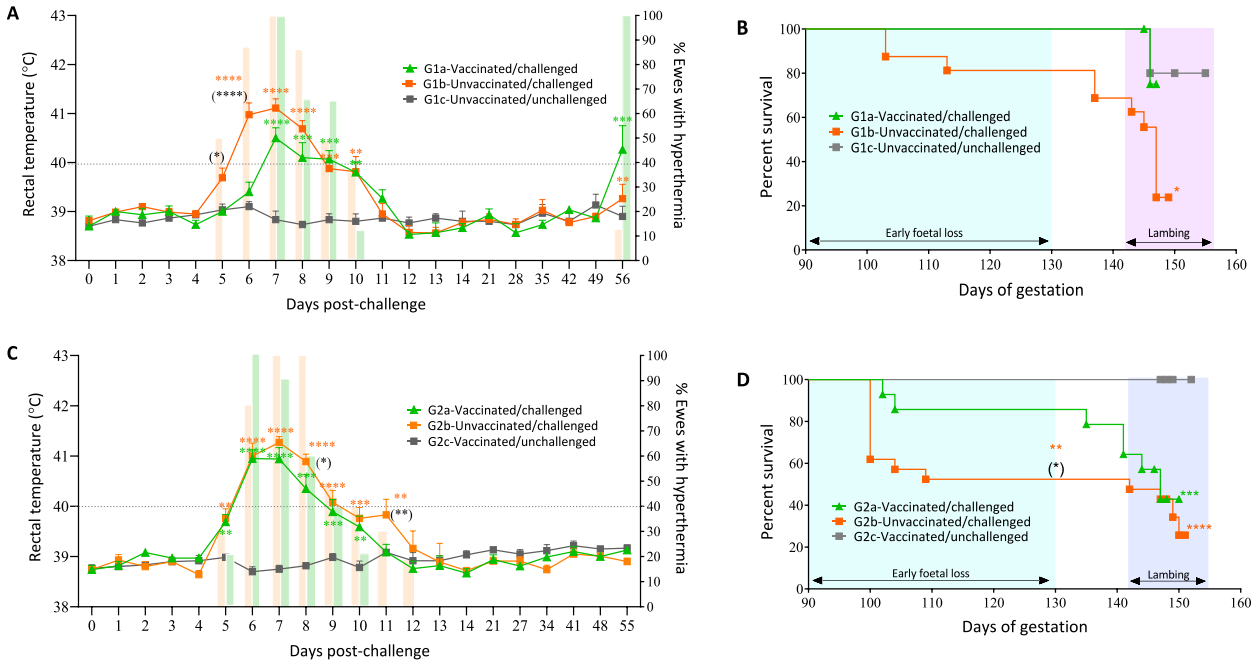

Figure 3 Rectal temperature kinetics (A and C) and foetal/lamb survival curves (B and D) after *T. gondii* challenge in vaccine trials 1 and 2. Panels **A** and **C** display the mean rectal temperature (± S.E.M.) of ewes over time in trial 1 (**A**) and trial 2 (**C**). The left Y-axis represents the mean rectal temperature. The discontinuous horizontal line indicates the threshold for hyperthermia (≥ 40 °C). Columns over the X-axis (read against the right Y-axis) show the percentage of ewes in each group that experienced hyperthermia daily. Green and orange asterisks indicate a significant temperature increase in the challenged groups G1a (vaccinated/challenged)-G1b (unvaccinated/challenged) in (**A**), and G2a (vaccinated/challenged)-G2b (unvaccinated/challenged) in (**C**) versus their respective unchallenged control groups G1c (unvaccinated/unchallenged) and G2c (vaccinated/unchallenged). Black asterisks (in brackets) indicate a significant increase in rectal temperature between the vaccinated groups G1a and G2a vs unvaccinated G1b and G2b (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$; Two-way ANOVA test). Panels **B** and **D** display the percentage of surviving foetuses/lambs over days post-challenge in trial 1 (**B**) and trial 2 (**D**), respectively. Each data point represents the percentage of surviving, and vertical downward steps correspond to an observed foetal/lamb death event. Green and orange asterisks indicate significant differences in foetal survival in challenged groups (G1a-G1b) or (G2a-G2b) vs their respective control groups G1c and G2c. Black asterisks (in brackets) significant differences between groups G2a (vaccinated/challenge) and G2b (unvaccinated/challenge) at 130 days of gestation and after lambing (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ and ****$p < 0.0001$, Log-rank test).

was 83.3% for G1a (vaccinated/challenged) (Figure 3B). Reproductive losses were significantly different among groups ($p < 0.05$), with the lowest number of lambs born viable in G1b (Table 1). The statistical differences were only found on comparisons among groups, likely due to limited number of ewes included in G1a (Table 1). Analysis of foetal/lamb survival curves showed a significant decrease in median survival time in G1b compared with G1c ($p < 0.05$; Figure 3B). No differences were observed in mean gestation period, body weight, or Apgar score of viable lambs across the groups.

Foetal and precolostral serology demonstrated parasite exposure in all stillborn and lambs from G1a (vaccinated/challenged), and in 50% (unvaccinated/challenged) of stillborn and lambs from G1b (Table 1, Additional file 4). Parasite DNA was detected in all cotyledons and almost all foetal/lamb tissues from both G1a and G1b, confirming a high rate of vertical transmission in both challenged groups (Table 1, Additional file 4). Focusing on parasite loads in cotyledons from ewes with either all lambs viable or with stillborns, parasite loads were lower in G1a (vaccinated/challenged) than G1b (unvaccinated/challenged) in both categories (average parasite loads in G1a and G1b with all lamb viable: 47.2 and 137.7 tachyzoites/mg of tissue, respectively; average parasite loads in G1a and G1b with stillborn: 520.5 and 2657 tachyzoites/mg of tissue, respectively), although the differences were not significant. Additionally, no significant differences were observed between G1a and G1b in parasite loads in the brains and lungs of the offspring with all lamb viable or with stillborns (Additional file 4). As expected, parasite DNA was not detected in tissue samples from the G1c control group.

Table 1 Clinical outcome and parasite transmission to offspring in vaccine trials 1 and 2

	Group	Clinical outcome in dams					Clinical outcome in lambs					Parasite transmission			
		Viable gestations (%)[a]	Mean gestation period[b]	*T. gondii* PCR detection in placenta (%)[c]	Placental histology lesions (% & score)[d]	Expected lambs[e]	Early foetal loss < 130 dg (%)[f]	Late foetal loss > 130 dg (%)[g]	Lambs born viable (%)[h]	Mean weight (g)[i]	Apgar score (0–10)[j]	Foetal/ lamb IgG[k]	*T. gondii* PCR detection in brain (%)[l]	*T. gondii* PCR detection in lung (%)[m]	Brain's histology lesions (% & score)[n]
Vaccine Trial 1	G1a	3/3* (100)	146	2/2* (100)	NA	6	0/6 (0)	1/6 (16.7)	5/6* (83.3)	3369	9.8	4/4*,[1] (100)	2/6 (33.3)	5/6*,[1] (83.3)	NA
	G1b	4/8 (50)	146	7/8[1] (87.5)	NA	16	4/16 (25)	7/16 (43.8)	5/16 (31.2)	3540	9	7/14 (50)	9/16 (56.2)	13/16[1] (81.2)	NA
	G1c	3/3 (100)	150.3	0/3[2] (0)	NA	5	0/5 (0)	1/5† (20)	4/5 (80)	4464	9.8	0/5[2] (0)	0/5 (0)	0/5[2] (0)	NA
Vaccine Trial 2	G2a	7/9 (78)‡	146.4	6/6*,[1] (100)	4/6* (66.6; 2.8)	14	2/14* (14.3)	5/14 (35.7)	7/14*,[1] (50)	3237[1]	10	7/13*,[1] (58.3)	8/14*,[1] (57.1)	11/14*,[1] (78.5)	7/12 (58.3; 1.6)
	G2b	5/10 (50)	148.6	7/9[1] (77.8)	6/7[1] (85.7; 2.4)	21	10/21[1] (47.7)	4/21 (19)	7/21[1] (33.3)	3344	10	6/17 (54.6)	7/21[1] (33.3)	8/21[2] (38)	4/11 (36.4; 1.2)
	G2c	6/6 (100)	149.1	0/6[2] (0)	0/6[2] (0; 0)	12	0/12[2] (0)	0/12 (0)	12/12[2] (100)	4093[2]	10	0/10[2] (0)	0/12[2] (0)	0/12[3] (0)	0/3 (0; 0)

Statistics results are shown in the table for each evaluated parameter (by column).

*Over the number in first line for each vaccine trial indicates statistical differences among groups ($p < 0.05$; Chi^2 test).

[1, 2] and [3] Indicates statistical differences between groups (adjusted $p < 0.025$; Fisher exact test or One-way ANOVA test, followed by Tukey's multiple range test)

NA: not analysed, dg: days of gestation.

[a] Number of ewes per group with viable gestations (with at least one lamb born viable and healthy; Apgar score > 8): number of viable gestations/numbers of pregnant ewes and percentage.

[b] Mean of days of gestation until lambing for each group.

[c] Parasite DNA detection in six samples of placental cotyledon per ewe; number of PCR positive pregnant ewes/number of analysed pregnant ewes for each group (percentage).

[d] Number of ewes with characteristic lesion of *T. gondii* infection in cotyledons: number of examined ewes (percentage; median histopathological score (0–3) for each group).

[e] Number of expected lambs for each group.

[f] Number of abortion/foetal death before 130 days of gestation and delivered mummified /number of expected lambs (percentage).

[g] Number of abortion/foetal death after 130 days of gestation and stillborn /number of expected lambs (percentage).

[h] Number of viable lambs/number of expected lambs (percentage).

[i] Adjusted mean weight of lambs born live for each group. Correction factors of 1, 1.25, 1.75 and 2.11 were applied to lambs born from single, twin, triplet and quadruplet gestations, respectively, according [26].

[j] Median Apgar score determined in viable lambs (Additional file 2).

[k] Number of IgG seropositive abortion > 130 days of gestation, stillborn and lambs/number of abortions > 130 days of gestation, stillborn and lambs. Abortions and death foetuses < 130 days of gestation and lambs feed with colostrum are excluded.

[l] Parasite DNA detection in three samples of brain per foetus/lamb; number of PCR positive foetuses-lambs/number of analysed foetuses lamb for each group (percentage).

[m] Parasite DNA detection in three samples of lung per foetus-lamb; number of PCR positive foetuses-lambs/number of analysed foetuses-lambs for each group (percentage).

[n] Number of stillborn and lambs with characteristic lesion of *T. gondii* infection in brain: number of examined stillborn and lambs, percentage; median histopathological score (0–3) for each group.

† Lamb death was associated to dystocia.

‡ Ewe with a quintupled gestation with abortion at day 80 of gestation was discarded from the study; G1 n = 9.

Evaluation of vaccine safety, immunogenicity and efficacy against reproductive failure in vaccine trial 2.

Vaccine safety

Rectal temperatures increased significantly in the vaccinated groups G2a and G2c on day 1 after vaccination (> 40 °C in 7 out of 16 ewes) and booster (> 40 °C in all ewes from the groups G2a and G2c) ($p < 0.0001$). Temperatures returned to basal levels by day two following both administrations (Additional files 5A and B). Apart from the transient increase of the rectal temperatures, no other systemic or pregnancy-related adverse effects were observed in vaccinated ewes.

Mild lesions in the inoculation sites were recorded after the vaccination and the booster (Additional files 5C and D). Lesions consisted in oedematous swelling at the inoculation sites during the first days post-vaccination, which progressed to 2 × 2 cm nodules and resolved by 14 days after both administrations in majority of sheep (6 out of 8) (maximum score of 3 out of 9). No pain associated with lesion was detected (Additional files 5C and D).

Vaccine immunogenicity

By day 8 post-vaccination, specific IgG levels significantly increased in ewes from vaccinated groups G2a and G2c, and were maintained by day 21 (day of booster) ($p < 0.01$). A significant increase was also observed on day 6 after the booster (day 27 post-vaccination) with IgG levels maintained until challenge (Figure 4A). Oral challenge with *T. gondii* oocysts significantly increased the IgG response in G2a (vaccinated/challenged) starting from day 13 post-challenge, and in G2b (unvaccinated/challenged) from day 20 post-challenge (Figure 4B). IgG levels remained significantly higher in G2a *vs* G2b until lambing (day 55 post-challenge) ($p < 0.0001$). In G2c (vaccinated/unchallenged), IgG levels remained stable until day 50 after the booster (day 34 post-challenge, Figure 4B), when they declined ($p < 0.001$, Two-way ANOVA test). Nevertheless,

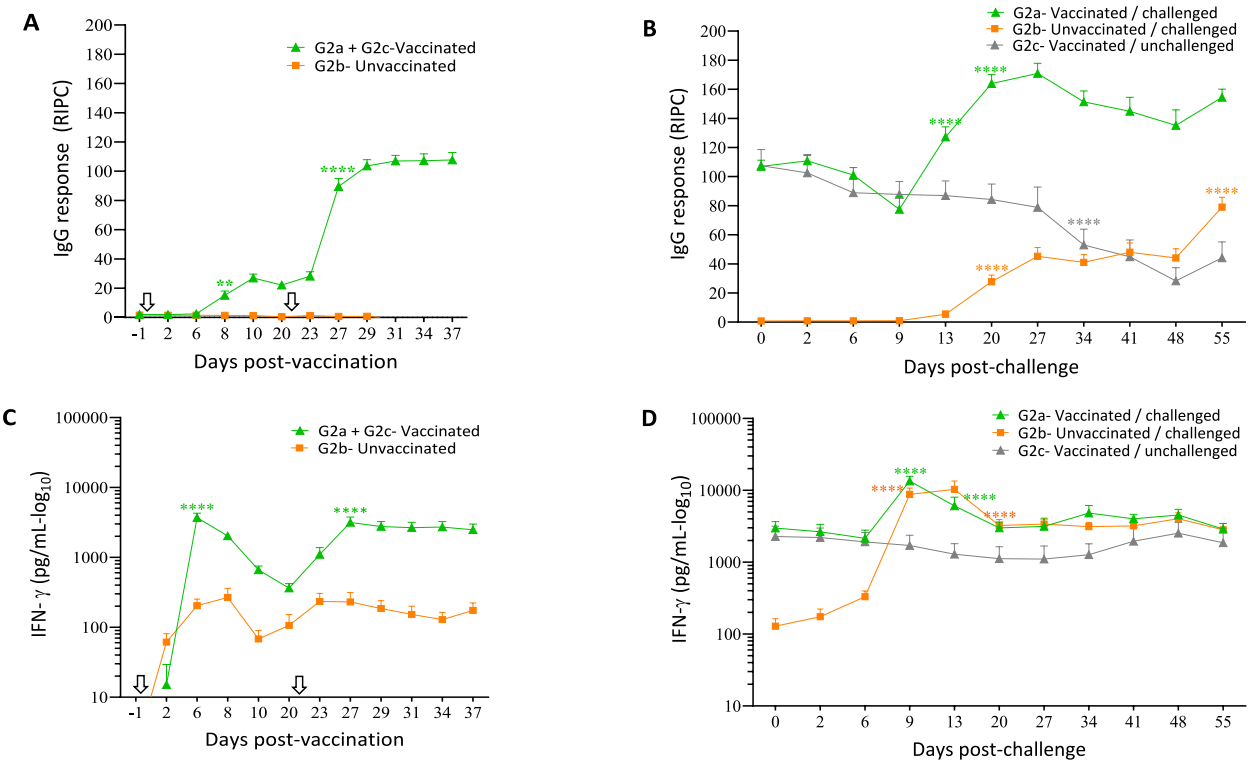

Figure 4 Vaccine Trial 2: Kinetics of anti-*T. gondii* IgG (A and B) and IFN-γ response (C and D). The Y-axis in **A** and **B** shows IgG levels expressed as Relative Index Percent (RIPC). The Y-axis in **C** and **D** shows IFN-γ levels expressed in pg/mL, measured in supernatants from whole blood stimulated with soluble tachyzoite extract. Each data point represents the mean, and error bars indicate the Standard Error of the Mean (S.E.M.). White arrows over the X-axis in **A** and **C** indicate the days of vaccination and booster dose administration (day 0 and day 21, respectively). Panels A and C show the response kinetics before challenge. Data from the two vaccinated groups G2a and G2c are pooled for comparison against the unvaccinated group G2b. Green asterisks over each data point of G2a + G2c, indicate a significant variation of the IgG (A) or IFN-γ (C) level (** $p < 0.01$;**** $p < 0.0001$, Two-way ANOVA test). Panels B and D show the response kinetics after the booster dose and during the challenge period. Green, orange, and grey over each data point of G2a, G2b, and G2c, respectively, indicate a significant variation of the IgG (A) or IFN-γ (C) level within that specific group (** $p < 0.01$;**** $p < 0.0001$, Two-way ANOVA test).

ewes in G2c (vaccinated/unchallenged) remained seropositive until lambing (day 55 post-challenge).

Concerning cellular immune responses, a marked peak of *T. gondii*-specific IFN-γ levels in the supernatants of whole blood stimulated with soluble and lyophilized *T. gondii* was observed in vaccinated ewes (G2a and G2c) by day 6 post-vaccination and by day 6 after the booster. Achieved IFN-γ levels were maintained until challenge ($p < 0.0001$) (Figure 4C). After challenge, IFN-γ levels increased significantly in G2a (vaccinated/challenged) and in G2b (unvaccinated/challenged) from days 9 to 13 post-challenge, when declined ($p < 0.0001$) (Figure 4D). In the group G2c (vaccinated/unchallenged), IFN-γ levels remained stable until lambing (Figure 4D). After day 13 post-challenge IFN-γ levels remained high in all three groups without significant differences (G2a vs G2b vs G2c) (Figure 4D).

Vaccine efficacy

After challenge, ewes in G2a (vaccinated/challenged) and G2b (unvaccinated/challenged) were febrile from days 5 to 10 and days 5 to 12, respectively, with peak mean temperatures observed on day 6 for G2a (41.0 °C) and at day 7 post-challenge for G2b (41.3 °C), when all ewes exhibited hyperthermia (Figure 3C). The number of ewes with hyperthermia notably decreased from day 6 post-challenge in G2a compared with G2b, and rectal temperatures were significantly lower in G2a (vaccinated/challenged) compared with G2b (unvaccinated/challenged) at days 8 and 11 post-challenge ($p < 0.05$) (Figure 3C).

Pregnancy outcomes are summarized in Table 1 and Figure 3D (with individual data provided in Additional file 6). Full term gestations resulting in viable lambs were 100% in group G2c (vaccinated/unchallenged), 78% in group G2a (vaccinated/challenged), and 50% in group G2b (unvaccinated/challenged), without differences among groups (Table 1). Early foetal losses prior to 130 days of gestation were observed in 22% (2 out of 9) of the ewes in group G2a (vaccinated/challenged) and in 50% (5 out of 10) of the ewes in group G2b (unvaccinated/challenged). Therefore, the highest early foetal losses were found in G2b (10 out of 21 expected lambs; 47.7%), followed of G2a (2 out of 14; 14.3%), indicating a 70% of protection of the vaccination against foetal losses prior to day 130 of gestation, although without significant differences between these both infected groups (Table 1). In fact, no differences in early foetal losses were found between G2a (vaccinated/challenged) and G2c (vaccinated/unchallenged) in which all of foetuses remained viable, and only early foetal losses in G2b (unvaccinated/challenged) were significantly higher compared to G2c ($p < 0.025$) (Table 1). Survival foetal curves at 130 days of gestation also revealed significantly higher median foetal survival time in G2a compared to G2b ($p < 0.05$) (Figure 3D). After day 130 of gestation, late foetal losses in group G2a were detected in three ewes with twins, each giving birth to a stillborn and a viable lamb, and in one ewe with triplets, which had two stillbirths and one viable lamb. In G2b, two ewes with twins each gave birth to a stillborn and a viable lamb. Stillborn lambs accounted for 35.7% and 19% of births in G2a and G2b, respectively, without differences on lamb viability between these groups (Table 1). No differences were observed in the mean gestation period or Apgar viability scores of the lambs between G2a and G2b groups. Birthweights of the lambs from G2a, but not those from G2b, were significantly lower than those from the G2c ($p < 0.05$).

Anti-*T. gondii* IgG response was not detected in foetuses that died before day 130 of gestation in G2a (vaccinated/challenged) and G2b (unvaccinated/challenged) (Table 1 and Additional file 6). Precolostral serology indicated parasite exposure in stillborns and lambs from G2a and G2b, with 58.3% and 54.6% seropositive cases, respectively (Table 1 and Additional file 6). Precolostral anti-*T. gondii* IgG response was not detected in all lambs from G2c (vaccinated/unchallenged).

T. gondii DNA was detected in the brain and lungs of all (100%) stillborn and viable lambs in group G2a (vaccinated/challenged) and in 91% (10 out of 11) of lambs in G2b (unvaccinated/challenged), indicating a lack of protection against vertical transmission (Table 1 and Additional file 6). No differences were detected in the parasite loads in brain or lungs from stillborn and viable lambs between G2a and G2b, nor between stillborn and viable lambs within each group (Additional file 6). Characteristic lesions of *T. gondii* infection were mainly observed in the brains of stillborn lambs from G2a and G2b (75% and 80%, respectively) and were less frequent in viable lambs (42.8% and 14.3%, respectively), without significant differences between groups. Three out of four stillborns and viable lambs with characteristic *T. gondii* lesions were seropositive in both G2a and G2b. No correlation was observed between median parasite load and lesion score in foetal and lamb brains.

At placental level, parasite DNA was detected in all analysed cotyledons from ewes of G2a and G2b that carried their pregnancies to full term (100%), giving birth to stillborn or viable lambs. Parasite burdens in cotyledons were significantly lower in ewes from G2a (vaccinated/challenged), whose gestations resulted in all lambs viable than those giving birth to stillborns (Figure 5A) ($p < 0.05$). In addition, parasite burdens from G2b (unvaccinated/challenged) for both categories –gestation with viable and stillborn- were higher than those with all viable lambs in G2a ($p < 0.05$) (Figure 5A). Analysis of histopathological

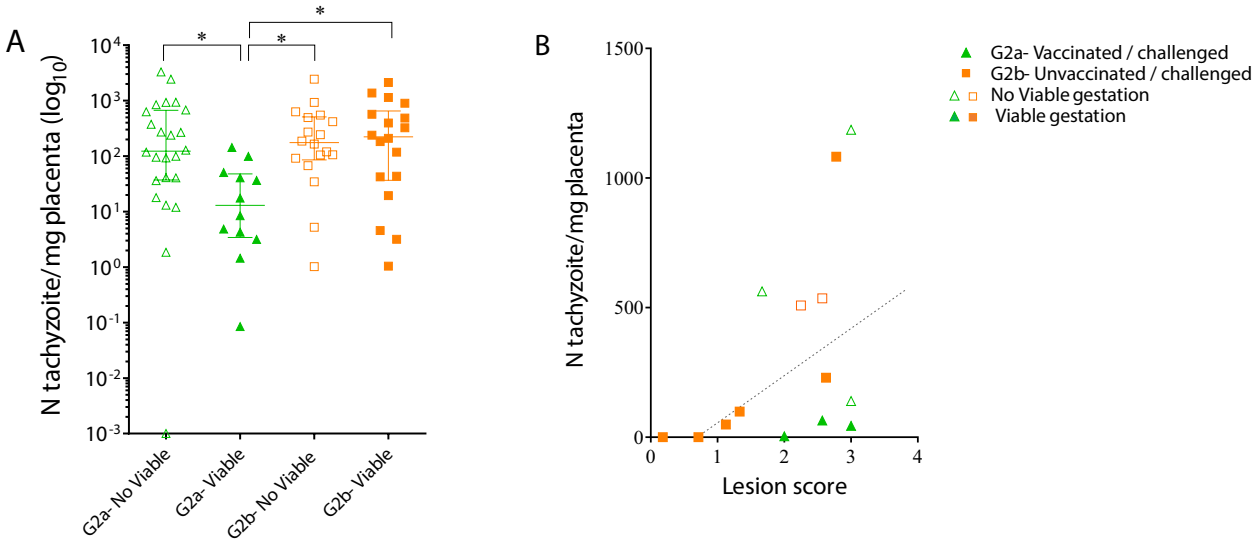

Figure 5 Parasite loads in cotyledons (A) and correlation with histopathological lesions of *T. gondii* infection (B). Samples are grouped by gestational outcome: stillborn lambs (empty symbols) or exclusively viable lambs (solid symbols) for each challenged group G2a (vaccinated/challenged) and G2b (unvaccinated/challenged). Panel **A** displays the individual parasite loads (measured as number of parasites per mg of tissue) detected in cotyledon samples from the challenged groups G2a (vaccinated/challenged) and G2b (unvaccinated/challenged). Brackets indicate significant differences between groups ($*p < 0.05$, Kruskal–Wallis followed by Dunn's multiple comparison test). Panel **B** illustrates the correlation between the mean parasite loads and the median histopathological lesion scores determined in cotyledons for each individual ewe from the challenged groups G2a (vaccinated/challenged) and G2b (unvaccinated/challenged). The discontinuous line represents the line of regression from the correlation analysis.

lesions revealed characteristic lesions of *T. gondii* infection in 66.6% of cotyledons from G2a compared to 85.7% in G2b. A correlation was observed between median parasite loads and mean lesion scores for G2a plus G2b groups (Spearman´s coefficient, r = 0.55; $p < 0.05$) (Figure 5B). The correlation was maintained in the individual analysis of G2b (r = 0.922; $p < 0.003$), but no correlation was observed in G2a samples. No parasite DNA or characteristic lesions of *T. gondii* infection were detected in foetal tissues or placental from the G2c (vaccinated/unchallenged).

Discussion

Live attenuated vaccines developed against ovine toxoplasmosis have demonstrated a high efficacy against reproductive failure, achieving over 60% protection in controlled trials [12, 13]. Toxovax®, based on S48 strain of *T. gondii,* is currently the only commercially available live vaccine in a few regions of the world. Nevertheless, the use of *T. gondii* live vaccines has been limited by safety concerns, such as the risk of reversion to virulence, as well as issues related to commercial feasibility (the shelf-life is limited to only 10 days, and the vaccine is produced upon request due to its restricted availability). Toxovax® vaccination must be administered three months prior to gestation and is contraindicated during pregnancy in sheep to avoid severe gestational consequences. In addition, this vaccine has zoonotic potential and should not be handled by pregnant or immunocompromised individuals. Therefore, the development of novel control strategies is required. These include: (i) vaccines applicable to a broader range of livestock species in which toxoplasmosis has an impact such as; (a) small ruminants, to reduce reproductive losses; (b) pigs, to lower tissue cyst burden and thereby decrease the risk of zoonotic transmission; (c), cats, to block oocyst shedding; and (d) high susceptible species such as New World primates and marsupials, (ii) non-live vaccines (inactivated or recombinant) that eliminate user-related risks and improve availability; (iii) broad protection against the diversity of *T. gondii* lineages circulating worldwide and (iv) compatibility with a DIVA (Differentiation of Infected vs. Vaccinated Animals) strategy.

The development of live vaccines had been preferred over inactivated vaccines for apicomplexan infections because they induce more effective innate and adaptive immune responses [27, 28]. Nevertheless, inactivated or killed vaccines are desirable as a safer and commercially viable alternative to live vaccines. The new prototype vaccine of this study, containing tachyzoite and bradyzoite antigens, has been previously evaluated against acquired toxoplasmosis in mice and piglets for chronic infection, demonstrating high safety and efficacy; with only mild adverse effects and a reduction of parasite loads found in

target muscle tissues by over 90%. Efficacy was directly associated with stable and specific humoral (IgG) and cellular (IFN-γ) responses, which conferred protection against chronic infection [20]. Subsequently, the vaccine prototype was evaluated in this study for its ability to prevent reproductive failure in sheep.

The vaccine formulation was developed as previously described [20], using 40 μg of total parasite extract from recently obtained *T. gondii* TgPigSp1, which contains bradyzoite antigens [18, 19], and a membrane fraction after detergent solubilisation. The role of bradyzoite antigens in the inactivated vaccine against foetal losses is not immediately clear, since tachyzoite is the parasite stage primarily responsible for dissemination in the host and sheep are infected by oocyst consumption. The inclusion of bradyzoite antigens has been aimed at minimizing the persistence of chronic infection in intermediate hosts in previous studies [20]. Whether vaccination was efficient against chronic infection in ewes, limiting or clearing the tissue cysts in target organs was not evaluated in this study. Nevertheless, recent studies have shown that *T. gondii* adapted in the short term in vitro leads to variations on proteome that could affect antigen composition [19]. Thus, we hypothesized that using recently obtained *T. gondii* isolates as the source of vaccine antigens may better mimic the immune stimulus generated by natural infections that confer protective immunity in sheep.

The vaccine was formulated with an antigen dose suitable for scalable production (i.e., 40 μg) and capable of inducing protection against chronic *T. gondii* infection in swine [20]. QuilA® was used as an adjuvant due to its well-established ability to elicit a balanced Th1/Th2 immune response, which is critical for controlling intracellular pathogens such as *T. gondii,* and it has proven safety profile in sheep [17, 29]. No significant systemic and local adverse effects were observed following vaccination and booster administration in sheep. Moreover, no effects on gestation were observed, suggesting that, unlike Toxovax®, this inactivated vaccine could be safely used in pregnant sheep.

A key component in controlling *T. gondii* infection in the host is the cellular immune response, particularly IFN-γ, which plays a role in both innate and adaptive immunity [28]. Vaccination in ewes elicited specific IgG and IFN-γ responses that persisted throughout gestation. IgG responses were observed increased at 6–8 days after primary vaccination and the booster, similarly to S48 infection [30]. IFN-γ responses determined in vitro were found 6 days after primo-vaccination and booster and remained stable until the end of gestation. These findings confirm that vaccinated ewes were able to maintain a sustained cellular response consistent with an adaptive response. Similar IFN-γ kinetics, measured in efferent lymph cells stimulated in vitro with crude *T. gondii* antigen, was observed in sheep vaccinated with the S48 live vaccine. IFN-γ production increased between days 2 and 5 post-vaccination and persisted for 6–9 days [30–32]. Focusing on immune responses after challenge, IgG levels increased earlier after the challenge in vaccinated sheep and remained higher than unvaccinated controls, providing further evidence of efficient vaccine-induced humoral responses, as was observed with S48 [12]. The challenge also elicited an increased IFN-γ response by day 9 post-infection in both vaccinated and unvaccinated ewes. Whether an earlier IFN-γ response indicative of immunological memory occurred in vaccinated ewes could not be determined in this study due to the sampling schedule. Minor efficacy of killed *T. gondii* vaccines against abortion has been attributed to weak cell-mediated immune responses [28], which apparently was not observed in this study. S48 live vaccine immunization involves the activation of CD4 + and CD8 + T cells that are required for long-term protection against *T. gondii* [28, 32]. Together, CD4 + and CD8 + lymphocytes, NK, dendritic and macrophages, monocytes and neutrophils are involved producing IFN-γ, and they are present in blood stimulation assays in this study [32]. The stimulated cell profile producing IFN-γ in vitro that could explain potential variations in efficacy was not evaluated.

The results of vaccine trial 1 showed that vaccination resulted in all gestations viable in vaccinated ewes, whereas only 50% of unvaccinated ewes delivered viable lambs at term. Foetal viability was similar in vaccinated and sentinel groups (≈ 80%) demonstrating protection against *T. gondii*-induced reproductive failure. Foetal death at 130 days of gestation was not observed in vaccinated ewes, whereas it occurred in 25% of unvaccinated but challenged sheep in vaccine trial 1. Nevertheless, vaccine trial 1 was conducted with a limited number of vaccinated ewes, and additional studies were carried out to confirm these results. Vaccine trial 2 also demonstrated an increase in viable gestations in vaccinated ewes, from 50% in unvaccinated ewes to 78% in vaccinated animals. This was associated with a decrease of 70% on early foetal losses in vaccinated ewes. Protection against late foetal losses after 130 days of gestation and stillbirths did not differ significantly between vaccinated and unvaccinated ewes. In controlled trials, the S48 vaccine also demonstrated protection against abortion occurring prior to 130 days gestation, increasing foetal survival from 35.5% (31.1%, including mummified foetuses) in unvaccinated ewes to 100% (93.3%, including mummified foetuses) following challenge with 2000 sporulated oocysts of the *T. gondii* M3 isolate at day 90 of pregnancy [12]. No differences were observed in the incidence stillborn or non-viable weak lambs between vaccinated and unvaccinated

sheep [12]. Similarly, vaccination with the Mic1-3KO RH mutant prior to mating protected against abortion at 105 days of gestation following challenge with 400 sporulated oocysts of the PRU strain at mid gestation, but did not provide clear protection against reproductive losses after 105 days of gestation [13]. These results are consistent with those obtained in vaccine trial 1 of this study, where viable lambs increased significantly from 31.2% in unvaccinated ewes to 83.3% in vaccinated ewes. Partial protection was observed against reproductive losses when a vaccine composed of membrane surface antigens was evaluated in pregnant sheep, from the 36.4% of viable lambs to 64.7% in vaccinated [17]. Similarly, partial protection was achieved in vaccine trial 2 when viable lambs were increased from the 33.3% in unvaccinated to 50% in vaccinated ewes.

Attempts to control infection through immune response in vaccinated ewes may have occurred during parasitaemia and parasite propagation in target tissues immediately after challenge. Peyer's patches in the medial jejunum and the medial and distal jejunal mesenteric lymph nodes are key sites for the early replication and dissemination of *T. gondii* in sheep under vaccine challenge [2]. *Toxoplasma gondii* is detected in the placenta around day 14 post-infection, where they multiplicate and reach the foetus [33]. Foetal losses in this acute phase of infection are associated with thrombosis and placental infarcts, non-associated with parasite multiplication in placenta [2, 33, 34]. A high parasite burden in placenta has been detected at 28 days after infection, associated with lesions of the maternal caruncle and foetal cotyledon –necrosis and infiltration of mononuclear inflammatory cells-, and with late abortions and stillbirths [33]. First clues of *T. gondii* control could be reflected in a shorter period of hyperthermia in vaccinated ewes and a reduced early foetal death. Thus, vaccination may delay or limit the number of parasites reaching the foetus, thereby postponing the occurrence of foetal losses in vaccinated ewes due to *T. gondii* proliferation in foetal placenta and tissues. Evidence of *T. gondii* control in vaccinated ewes was observed when parasite loads detected in cotyledon were significantly diminished in those than completed gestation with all viable lambs. Indeed, lower percentage of cotyledons exhibiting lesions of *T. gondii* infection were observed in the vaccinated ewes than in unvaccinated ewes, consistent with previous observations using the S48 live vaccine [12]. Moreover, a correlation between parasite burdens and lesion severity in the cotyledon was observed only in the unvaccinated group, supporting limited *T. gondii* proliferation in cotyledons under immune control in vaccinated sheep. Whether this effect results from infection control in the caruncles could not be assessed in this study.

Vaccine efficacy against reproductive failure varied between vaccine trials 1 and 2, likely related to the severity of clinical consequences after infection. First evidence was associated with the presentation of early foetal losses in unvaccinated and challenged sheep groups from both trials. The proportion of gestations carried to term (50%) and overall foetal/lamb mortality (66–69%) were consistent across both trials and previous studies with this model [21, 23]. However, foetal losses during the acute phase two–three weeks after challenge increased from 12.5% in unvaccinated ewes in trial 1 (18.8% involving mummified foetuses), very similar to reported in previous studies [21, 23], to 38.1% in unvaccinated in vaccine trial 2 (47.6% including mummified foetuses). Thus, late foetal losses increased to 81.3% in vaccine trial 1 versus 52.4% in vaccine trial 2. Additionally, hyperthermia persisted for two more days in unvaccinated sheep in the vaccine trial 2. We support that the increased presentation of foetal losses during acute phase in vaccine trial 2 could be related to the storage time of oocysts at 4 °C, which was limited to 3 months in vaccine trial 2 compared with 13 months in vaccine trial 1. A similar foetal loss profile to vaccine trial 1 was achieved with oocysts older than 6 months in previous studies [21, 23, Sánchez-Sánchez, personnel communication]. Significant differences in time to foetal survival were found after statistical analysis between challenged unvaccinated groups for vaccine trial 1 *versus* vaccine trial 2 ($p < 0.05$), likely due to storage time for oocysts. Oocysts can remain viable at 4 °C for up to 54 months [35], although infectivity capacities in sheep of sporozoites after 6 months at 4 °C could be limited based on the findings in this study. It has been suggested that significant variability in the viability (or infectivity) of oocysts could be the cause of different findings in experimental studies using similar doses [34]. Given that comparable variations in clinical outcomes may arise under field conditions influenced by oocyst infectivity, these findings are considered representative and appropriate for assessing vaccine efficacy against reproductive failure in two plausible field scenarios.

Efficacy against congenital transmission was also assessed in foetuses/lambs using serology, histopathology and the highly sensitive PCR detection in target foetal tissues, revealing complete lack of protection. Live vaccines also do not prevent vertical transmission in sheep [12]. Accordingly, 65% of lambs born from S48-vaccinated ewes were seropositive for *T. gondii*, similar to the findings of this study. In addition, placental cotyledon lesions consistent with *Toxoplasma*-induced abortion were observed in 46.8% of cases from vaccinated, indicating

foetal infection [12]. All these results suggest that the *T. gondii* live vaccine does not protect against vertical transmission, although PCR was not applied for the detection of *T. gondii* in foetal tissues.

In conclusion, the potential efficacy of this vaccine prototype lies in increasing the number of full-term gestations resulting in the birth of viable lambs in vaccinated ewes. Preserving viable gestations and reducing early foetal losses, as demonstrated in this study, could help mitigate the economic impact associated with *T. gondii* infection in the flocks. Recently, the direct economic cost associated with a *T. gondii* outbreak in a meat flock was estimated at €63.6 per abortion, with the main income lost due to unsold lambs. These economic losses were higher in a dairy flock, estimated at €171.8 per abortion, with the main income loss resulting from reduced milk production [9]. Thus, the use of the inactivated vaccine evaluated in the present study that, at least, could provide up to 50% protection against loss of gestation, would ensure viable pregnancies, thereby resulting in increased economic benefits in dairy production. Further studies on the efficacy against *T. gondii* reproductive failure of this vaccine prototype under field conditions will be necessary to assess its potential for a broad use and its commercialization.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13567-026-01720-2.

Additional file 1. Scoring of local reactions following vaccination of the ewes.

Additional file 2. Apgar score for the evaluation of lamb viability at birth.

Additional file 3. Characterization of the *T. gondii* parasite cultures and antigen extract used for vaccine production. A Photomicrograph showing a *T. gondii* culture analysed for cyst formation. Cyst wall was labelled with FITC-conjugated Dolichos biflorus lectin (labelled green) and parasites were labelled using mouse anti-T. gondii antibody (labelled red). The white scale bar represents 100 µm (40X). **B** Immunoblot analysis of different production batches of the parasite antigen extract used in the vaccine (passage 28 [p28], p29, and four replicates of p30 as a reference). The blot was probed with an anti-TgBAG1 monoclonal antibody to confirm the presence of bradyzoite-specific antigens. White arrows indicate the molecular weight of the Precision Plus Protein Standards KaleidoscopeTM marker and black arrow points to the 28 kDa band corresponding to the expected molecular weight of the TgBAG1 protein.

Additional file 4. Detailed data on gestation outcomes and parasite DNA detection in placentas and foetal tissues from vaccine trial 1.

Additional file 5. Rectal temperature kinetics (A and C) and local lesion scores (B and D) after vaccination and booster. Data from the two vaccinated groups G2a (vaccinated/challenged) and G2c (vaccinated/unchallenged) are pooled (G2a + G2c) for all comparisons against the unvaccinated control group (G2b). Panels **A** and **C** show the mean rectal temperature (± S.E.M.) of ewes after the vaccination (**A**) and the booster dose (**C**). The left Y-axis displays the mean rectal temperature. The discontinuous horizontal line indicates the threshold for hyperthermia (≥40 ∘C). Columns over the X-axis (read against the right Y-axis) represent the percentage of ewes that experienced hyperthermia daily. Green asterisks indicate a statistically significant increase in temperature in the pooled vaccinated group (G2a + G2c) compared to the unvaccinated group (G2b) (****$p < 0.0001$, Two-way ANOVA test). Panels **B** and **D** display the individual lesion scores recorded at the inoculation site after the vaccination (**B**) and the booster dose (**D**), respectively. Scores (Additional File 1) correspond to local adverse reactions observed in ewes from the pooled vaccinated group (G2a + G2c). No statistical differences in the lesion scoring were found when comparing the vaccinated group (G2a + G2c) to the unvaccinated group (G2b) (data not shown).

Additional file 6. Detailed data on gestation outcomes, parasite DNA detection, histopathological analyses in placentas and foetal tissues and IgG foetal response in vaccine trial 2.

Acknowledgements
We thank Silvia Jara, Elena Neves, Michela Re and Patricia Fariñas for their technical assistance in conducting this study. The authors gratefully acknowledge Dr. Julio Benavides and Dr. Daniel Gutiérrez-Expósito from the Instituto de Ganadería de Montaña del CSIC-Universidad de León (Grulleros, Spain), for performing the histopathological analyses of this study. We thank Alfonso Martin Fontecha from SIVA for his revision, comments and suggestions to the manuscript.

Authors' contributions
JRC, Conceptualization; Data curation; Formal analysis and Writing-original draft; ALT, Investigation, Methodology, Writing-review & editing; RSS, Investigation, Methodology, Writing-review & editing; IF, Conceptualization, Writing-review & editing; JMG, Investigation, Methodology; LMO, Conceptualization; Supervision, Writing-review & editing & Funding acquisition. All authors read and approved the final manuscript

Funding
This work was partially supported by Laboratorios SYVA and SALUVET-Innova S.L. The authors also acknowledge the support received from the General Directorate of Research and Innovation of the Regional Government of Madrid (TEC-2024/BIO-66/SALAINDEC-CM).

Data availability
All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate
Protocols involving animals for vaccine trials were approved by the Animal Welfare Committee of the Community of Madrid, Spain (PROEX 128.1/21 and 36.7/24), following Spanish and European Union legislation (Law 32/2007, R.D. 53/2013), and Council Directive 2010/63/EU). Oocyst production, described below, was also approved by the same Committee (238.3/22). Dr. Roberto Sánchez Sánchez consented to participate in this publication and shared oocyst time of preservation at 4 °C for previous studies [21, 23].

Competing interests
The authors declare no competing interests.

Received: 18 November 2025 Accepted: 9 February 2026
Published online: 19 March 2026

References

1. Dubey JP (2022) Toxoplasmosis of animals and humans, 3rd edn. CRC Press, Boca Raton
2. Sánchez-Sánchez R, Horcajo P, Fernández M, Arranz-Solís D, Velasco-Jiménez N, Re M, Gutiérrez-Expósito D, Valdivia G, Alonso-Diez A, Benavides J, Ortega-Mora LM (2025) Early dynamics of *Toxoplasma gondii* infection in sheep inoculated at mid-gestation with archetypal type II oocysts. Vet Res 56:134. https://doi.org/10.1186/s13567-025-01557-1

3. Dubey JP, Murata FHA, Cerqueira-Cézar CK, Kwok OCH, Villena I (2021) Congenital toxoplasmosis in humans: an update of worldwide rate of congenital infections. Parasitology 148:1406–1416. https://doi.org/10.1017/S0031182021001013
4. Buxton D, Maley SW, Wright SE, Rodger S, Bartley P, Innes EA (2007) *Toxoplasma gondii* and ovine toxoplasmosis: new aspects of an old story. Vet Parasitol 149:25–28. https://doi.org/10.1016/j.vetpar.2007.07.0035
5. Innes EA, Bartley PM, Buxton D, Katzer F (2009) Ovine toxoplasmosis. Parasitology 136:1887–1894. https://doi.org/10.1017/S0031182009991636
6. Castaño P, Fuertes M, Regidor-Cerrillo J, Ferre I, Fernández M, Ferreras MC, Moreno-Gonzalo J, González-Lanza C, Pereira-Bueno J, Katzer F, Ortega-Mora LM, Pérez V, Benavides J (2016) Experimental ovine toxoplasmosis: influence of the gestational stage on the clinical course, lesion development and parasite distribution. Vet Res 47:43. https://doi.org/10.1186/s13567-016-0327-z
7. Dubey JP, Murata FHA, Cerqueira-Cézar CK, Kwok OCH, Su C (2020) Economic and public health importance of *Toxoplasma gondii* infections in sheep: 2009-2020. Vet Parasitol 286:109195. https://doi.org/10.1016/j.vetpar.2020.109195
8. Stelzer S, Basso W, Benavides Silván J, Ortega-Mora LM, Maksimov P, Gethmann J, Conraths FJ, Schares G (2019) *Toxoplasma gondii* infection and toxoplasmosis in farm animals: risk factors and economic impact. Food Waterborne Parasitol 15:e00037. https://doi.org/10.1016/j.fawpar.2019.e00037
9. Gutiérrez-Expósito D, Tejerina F, Gutiérrez J, Fernández-Escobar M, Ortega-Mora LM, Mantecón AR, Dagleish MP, Pérez V, Benavides J (2021) Direct economic losses of *Toxoplasma gondii* abortion outbreaks in two Spanish sheep flocks. Vet Parasitol Reg Stud Reports 26:100623. https://doi.org/10.1016/j.vprsr.2021.100623
10. Kijlstra A, Jongert E (2008) Control of the risk of human toxoplasmosis transmitted by meat. Int J Parasitol 38:1359–1370. https://doi.org/10.1016/j.ijpara.2008.06.002
11. Zhu S, Shapiro K, VanWormer E (2022) Dynamics and epidemiology of *Toxoplasma gondii* oocyst shedding in domestic and wild felids. Transbound Emerg Dis 69:2412–2423. https://doi.org/10.1111/tbed.14197
12. Buxton D, Thomson K, Maley S, Wright S, Bos HJ (1991) Vaccination of sheep with a live incomplete strain (S48) of *Toxoplasma gondii* and their immunity to challenge when pregnant. Vet Rec 129:89–93. https://doi.org/10.1136/vr.129.5.89
13. Mévélec MN, Ducournau C, Bassuny Ismael A, Olivier M, Sèche E, Lebrun M, Bout D, Dimier-Poisson I (2010) Mic1-3 knockout *Toxoplasma gondii* is a good candidate for a vaccine against *T. gondii*-induced abortion in sheep. Vet Res 41:49. https://doi.org/10.1051/vetres/2010021
14. Hasan T, Nishikawa Y (2022) Advances in vaccine development and the immune response against toxoplasmosis in sheep and goats. Front Vet Sci 9:951584. https://doi.org/10.3389/fvets.2022.951584
15. Beverley JK, Archer JF, Watson WA, Fawcett AR (1971) Trial of a killed vaccine in the prevention of ovine abortion due to toxoplasmosis. Br Vet J 127:529–535. https://doi.org/10.1016/s0007-1935(17)37286-x
16. Wilkins MF, O'Connell E, Te Punga WA (1987) Toxoplasmosis in sheep I Effect of a killed vaccine on lambing losses caused by experimental challenge with Toxoplasma gondii. N Z Vet J 35:31–34. https://doi.org/10.1080/00480169.1987.35371
17. Buxton D, Uggla A, Lövgren K, Thomson K, Lundén A, Morein B, Blewett DA (1989) Trial of a novel experimental *Toxoplasma* iscom vaccine in pregnant sheep. Br Vet J 145:451–457. https://doi.org/10.1016/0007-1935(89)90053-5
18. Colos-Arango A, de la Largo- Torre A, Calero-Bernal R, Ortega-Mora LM, Regidor-Cerrillo J (2023) Short-term culture adaptation of *Toxoplasma gondii* archetypal II and III field isolates affects cystogenic capabilities and modifies virulence in mice. Int J Parasitol 53:491–504. https://doi.org/10.1016/j.ijpara.2023.03.004
19. Müller J, Regidor-Cerrillo J, Arranz-Solís D, Braga-Lagache S, Uldry AC, Heller M, Calero-Bernal R, Hemphill A, Ortega-Mora LM (2025) Proteome changes during *in vitro* culture adaptation of *Toxoplasma gondii* archetypal II and III field isolates. Front Cell Infect Microbiol 15:1633384. https://doi.org/10.3389/fcimb.2025.1633384
20. de la Largo- Torre A, Sánchez-Sánchez R, Diezma-Díaz C, Ferre I, Regidor-Cerrillo J, Ortega-Mora LM (2025) An inactivated vaccine against acquired *Toxoplasma gondii* infection in pigs as a tool to minimize the zoonotic transmission risk. Vet Res 56:206. https://doi.org/10.1186/s13567-025-01645-2
21. Sánchez-Sánchez R, Ferre I, Regidor-Cerrillo J, Gutiérrez-Expósito D, Ferrer LM, Arteche-Villasol N, Moreno-Gonzalo J, Müller J, Aguado-Martínez A, Pérez V, Hemphill A, Ortega-Mora LM, Benavides J (2019) Virulence in mice of a *Toxoplasma gondii* type II isolate does not correlate with the outcome of experimental infection in pregnant sheep. Front Cell Infect Microbiol 8:436. https://doi.org/10.3389/fcimb.2018.00436
22. Fernández-Escobar M, Calero-Bernal R, Regidor-Cerrillo J, Vallejo R, Benavides J, Collantes-Fernández E, Ortega-Mora LM (2020) Isolation, genotyping, and mouse virulence characterization of *Toxoplasma gondii* from free ranging iberian pigs. Front Vet Sci 7:604782. https://doi.org/10.3389/fvets.2020.604782
23. Sánchez-Sánchez R, Ferre I, Re M, Ramos JJ, Regidor-Cerrillo J, Pizarro Díaz M, González-Huecas M, Tabanera E, Benavides J, Hemphill A, Hulverson MA, Barrett LK, Choi R, Whitman GR, Ojo KK, Van Voorhis WC, Ortega-Mora LM (2019) Treatment with bumped kinase inhibitor 1294 is safe and leads to significant protection against abortion and vertical transmission in sheep experimentally infected with *Toxoplasma gondii* during pregnancy. Antimicrob Agents Chemother 63:e02527-18. https://doi.org/10.1128/AAC.02527-18
24. Wallace JM, Shepherd PO, Milne JS, Aitken RP (2021) Perinatal complications and maximising lamb survival in an adolescent paradigm characterised by premature delivery and low birthweight. PLoS One 16:e0259890. https://doi.org/10.1371/journal.pone.0259890
25. López-Ureña NM, Calero-Bernal R, Vázquez-Calvo Á, Sánchez-Sánchez R, Ortega-Mora LM, Álvarez-García G (2023) A comparative study of serological tests used in the diagnosis of *Toxoplasma gondii* infection in small ruminants evidenced the importance of cross-reactions for harmonizing diagnostic performance. Res Vet Sci 165:105052. https://doi.org/10.1016/j.rvsc.2023.105052
26. Sánchez-Sánchez R, Huertas-López A, de la Largo- Torre A, Ferre I, Dini FM, Re M, Moreno-Gonzalo J, Choi R, Hulverson MA, Ojo KK, Arnold SLM, Hemphill A, Van Voorhis WC, Ortega-Mora LM (2025) Treatment with BKI-1748 after *Toxoplasma gondii* systemic dissemination in experimentally infected pregnant sheep improves fetal and lamb mortality and morbidity and prevents congenital infection. Antimicrob Agents Chemother 69:e0144824. https://doi.org/10.1128/aac.01448-24
27. Innes EA, Bartley PM, Rocchi M, Benavidas-Silvan J, Burrells A, Hotchkiss E, Chianini F, Canton G, Katzer F (2011) Developing vaccines to control protozoan parasites in ruminants: dead or alive? Vet Parasitol 180:155–163. https://doi.org/10.1016/j.vetpar.2011.05.036
28. Innes EA, Hamilton C, Garcia JL, Chryssafidis A, Smith D (2019) A one health approach to vaccines against *Toxoplasma gondii*. Food Waterborne Parasitol 15:e00053. https://doi.org/10.1016/j.fawpar.2019.e00053
29. Livingstone M, Aitchison K, Palarea-Albaladejo J, Caspe SG, Underwood C, Hill H, Cunnea C, Stronach K, Chianini F, Entrican G, Wattegedera SR, Longbottom D (2025) Effects of different adjuvants on the protective efficacy of a subcellular vaccine against *Chlamydia abortus* infection in sheep. Vaccines 13:609. https://doi.org/10.3390/vaccines13060609
30. Wastling JM, Harkins D, Maley S, Innes E, Panton W, Thomson K, Buxton D (1995) Kinetics of the local and systemic antibody response to primary and secondary infection with S48 *Toxoplasma gondii* in sheep. J Comp Pathol 112:53–62. https://doi.org/10.1016/s0021-9975(05)80089-1
31. Innes EA, Panton WR, Thomson KM, Maley S, Buxton D (1995) Kinetics of interferon gamma production *in vivo* during infection with the S48 vaccine strain of *Toxoplasma gondii*. J Comp Pathol 113:89–94. https://doi.org/10.1016/s0021-9975(05)80074-x
32. Innes EA, Panton WR, Sanderson A, Thomson KM, Wastling JM, Maley S, Buxton D (1995) Induction of CD4+ and CD8+ T cell responses in efferent lymph responding to *Toxoplasma gondii* infection: analysis of phenotype and function. Parasite Immunol 17:151–160. https://doi.org/10.1111/j.1365-3024.1995.tb01017.x
33. Vallejo R, Benavides J, Arteche-Villasol N, Sánchez-Sánchez R, Calero-Bernal R, Ferreras MC, Criado M, Pérez V, Ortega-Mora LM, Gutiérrez-Expósito D (2023) Experimental infection of sheep at mid-pregnancy with archetypal type II and type III *Toxoplasma gondii* isolates exhibited

different phenotypic traits. Vet Parasitol 315:109889. https://doi.org/10.1016/j.vetpar.2023.109889
34. Castaño P, Fuertes M, Ferre I, Fernández M, Ferreras MC, Moreno-Gonzalo J, González-Lanza C, Katzer F, Regidor-Cerrillo J, Ortega-Mora LM, Pérez V, Benavides J (2014) Placental thrombosis in acute phase abortions during experimental *Toxoplasma gondii* infection in sheep. Vet Res 45:9. https://doi.org/10.1186/1297-9716-45-9
35. Marciano MAM, Silva RA, Barbosa ML, Ferreira ARS, Pereira-Chioccola VL (2020) Determination of the viability of *Toxoplasma gondii* oocysts by PCR real-time after treatment with propidium monoazide. Rev Inst Med Trop Sao Paulo 62:e84. https://doi.org/10.1590/S1678-9946202062084

Publisher's Note