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Evaluation of protective and immune responses following vaccination with recombinant MIP and CPAF from *Chlamydia abortus* as novel vaccines for enzootic abortion of ewes

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ABSTRACT

MIP and CPAF from *Chlamydia* have been shown to be effective in inducing immune responses important in clearing chlamydial infections. This study evaluates the protection conferred by MIP and CPAF as novel vaccines in pregnant *C. abortus* challenged ewes. Fifty *C. abortus* sero-negative sheep were randomly allocated into 5 groups of 10 according to the treatment they were to receive (1) 100 μg of MBP-MIP (2) 100 μg CPAF (3) 50 μg MBP-MIP and 50 μg CPAF (4) Tris-buffer (negative control) (5) Enzovax (positive control). Booster inoculations were administered 3 weeks after primary inoculations. Blood samples were taken pre-vaccination and weekly for 5 weeks. Five months after vaccination the ewes were mated. Pregnant ewes were then challenged on day 90 of gestation. Blood samples taken at four time-points post challenge were analysed for IFNγ levels, *TNFα* and *IL-10* expression and anti-chlamydial antibody levels. Vaginal swabs, placental and foetal tissue and bacterial shedding were analysed using qPCR to quantify levels of *C. abortus*. Enzovax was 100% effective with no abortions occurring. The MIP/CPAF combined vaccine offered the greatest protection of the novel vaccines with 67% of ewes giving birth to one or more live lambs equating to a 50% vaccine efficacy rate. MIP and CPAF administered singly did not confer protection. Enzovax and MIP/CPAF vaccinated ewes had longer gestations and lambs with higher birth weights than negative control ewes. Aborting ewes shed higher numbers of *C. abortus* than ewes that had live lambs, all vaccinated ewes demonstrated lower levels of bacterial shedding than negative control ewes with Enzovax ewes shedding significantly fewer bacteria. Ewes that went on to abort had significantly higher levels of IFNγ and *IL-10* at day 35 post challenge and significantly higher levels of anti-chlamydial antibodies at 24 h post lambing compared to ewes that had live lambs.

1. Introduction

Enzootic abortion of ewes (EAE) is a widespread and costly disease in many sheep rearing countries worldwide [1]. Ewes and lambs become infected most commonly at lambing when large numbers of infectious elementary bodies (EBs) are shed and subsequently ingested or inhaled by naïve animals [1]. Once a ewe becomes infected, *Chlamydia abortus* is believed to remain in a latent state in lymphoid tissue controlled by host cytokines such as IFNγ [2,3]. However, with the onset of pregnancy and the consequent changes in regulation of both hormone and cytokine levels, these bacteria can spread to the placenta and target trophoblast cells resulting in inflammation and placental lesions that may culminate in abortion in the last 4 weeks of pregnancy [1]. Over the last fifty years several vaccines for EAE have been trialled with various degrees of success. The first vaccines were inactivated vaccines which were widely used and considered to give good levels of protection [4,5]. However, outbreaks of abortion in vaccinated flocks led to questions regarding their efficacy and resulted in the removal of these vaccines from the market in 1992 [6,7,8]. As a result, attention turned to the live attenuated vaccine developed by Rodolakis and Souriau [9], resulting in the commercial vaccine currently available under the tradename Enzovax in Ireland (MSD Animal Health) and Ceva in France (Cevac, Animal Health

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Ltd). There is also a killed vaccine available in the UK called Mydiavac (Benchmark Vaccines Ltd. UK) under licence from Novartis (Animal Vaccines, Ltd) which consists of inactivated EBs.

Problems with the live attenuated vaccine for EAE were highlighted in a study where PCR-RFLP was used to identify SNPs associated with the 1B vaccine strain [10,11,12]. This identification of SNPs unique to the vaccine strain allowed for the discrimination of the vaccine strain from wild-type field strains and resulted in the identification of the vaccine strain in ewes from vaccinated flocks that had aborted [11]. More recently, Longbottom et al. [13] have proposed that any protection induced by *C. abortus* vaccine strain 1B was likely due to the administration of high doses of EBs that stimulate protective immunity and not due to the nitrosoguanidine (NTG)-induced temperature attenuation of the vaccine strain. These reports along with the zoonotic nature of this bacterium, the fact that current serological diagnostic tests cannot distinguish vaccinates from infected animals [10] and the fact that Enzovax does not halt the release of EBs at birth or oestrus, has given a renewed impetus to vaccine research and in particular the development of sub-unit vaccines. Many of the first subunit vaccines focused on the use of the major outer membrane protein (MOMP) due to its' surface exposure, immunogenicity and large size [14,15,16]. Tan et al. [17] found that a subcellular vaccine comprising the chlamydial outer membrane complex (COMC) which is made up of 90% MOMP resulted in significant protection from challenge compared to the placebo group. The large quantities of chlamydial EBs needed for sufficient COMC preparation however made this approach commercially non-viable [18]. Partial protection was achieved using recombinant forms of MOMP but efficacy was variable and not as successful as whole organism vaccines [19]. DNA vaccines using the *ompA* gene encoding MOMP also had limited success [20].

To date 48 immuno-reactive proteins have been identified in *C. abortus* [21] including the chlamydial protease-like activity factor (CPAF) one of the proteins already assessed in murine trials of vaccines for *C. muridarum* and *C. trachomatis* [22,23,24,25,26]. Murthy et al. [26] demonstrated the protective potential of CPAF when they reported that intra-nasal vaccination of mice induced robust $\mathrm{T_h}$1 cellular and humoral immune responses and resulted in decreased bacterial shedding accompanied by an accelerated resolution of genital *C. muridarum* infection. A second protein selected for vaccine development due to its immuno-reactive properties [27] is the macrophage infectivity potentiator (MIP). Various studies have shown MIP to be a strong immunoreactive protein [21,27,28,29]. A vaccine trial by Lu et al. [29] demonstrated that vaccination with MIP in a *C. muridarum* mouse challenge model could produce significant protection and reduce bacterial shedding. Based on these studies we sought to investigate the protective qualities of MIP and CPAF and MIP/CPAF combined in pregnant ewes subsequently challenged with *Chlamydia abortus*.

2. Materials and methods

2.1. Ethical approval

Ethical approval was granted by the Animal Research Ethics Committee (AREC) at UCD under license AREC-14-10 Markey and all procedures were performed under experimental license from the Health Products Regulatory Authority (HPRA) of Ireland under license AE 19113/P004.

2.2. Preparation of recombinant MIP and CPAF

Chlamydia abortus genomic DNA was extracted from strain C 95/27 [30] using High Pure PCR Template Preparation kit (Roche). The *CAB712* (*cpaf*) and *CAB080* (*mip*) genes were amplified from the purified genomic DNA using the high fidelity polymerase LATaq DNA polymerase (Clonetec) and the primers shown in Table 1. The PCR products were purified and subsequently cloned into the BamHI/PstI (*cpaf*) or BamHI/SalI (*mip*) sites of the vector pMALc2X. The resulting recombinant plasmids; pMAL-CPAF and pMAL-MIP encode the *C. abortus* CPAF and MIP proteins respectively, downstream of the *E. coli* maltose-binding protein (MBP), with expression under the control of an isopropyl—D-thiogalactoside (IPTG)-inducible promoter. Induction of MBP-MIP expression from pMAL-MIP was performed in *E. coli* strain MKV15 [31]. Overnight cultures were inoculated into fresh LB broth containing ampicillin (100 μg/ml) with 2 g/L glucose (Sigma-Aldrich) and incubated at 37 °C in a shaking incubator to grow to mid-log phase (OD600nm of 0.5) before induction with 0.3 mM IPTG for 18 h at 30 °C and 200 rpm (ThermoFisher Scientific Thermo Orbital Shaker). Cells were lysed by sonication on ice using the Branson Sonifier 150 (Emerson) at level 8 for 5 min in 20 s bursts with 10 s intervals and then centrifuged at 20,000*g* for 20 min. Supernatants were collected and recombinant MBP-MIP was purified from the crude extract using amylose affinity chromatography according to the NEB pMAL protocol (New England BioLabs). For CPAF purification overnight cultures of MKV15 transformed with pMAL-CPAF were inoculated into fresh LB broth containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml), incubated at 37 °C in a shaker incubator to grow to mid-log phase (OD600nm of 0.5) before induction with a final concentration of 1 mM IPTG for 16 h at 16 °C and 150 rpm. Cells were subsequently lysed by French press (ThermoFisher Scientific) at 1000 PSI, recombinant MBP-CPAF was purified from the crude extract using amylose affinity chromatography of Bio-Rad NGC system (Bio-Rad). The MBP tag ($\sim$42.5 kDa) was removed by TEV protease (Sigma-Aldrich) digestion for 16 h at 4 °C, followed by separation using a 50,000 (CPAF, $\sim$66 kDa) kDa molecular weight cut off Amicon centrifugal filter column.

2.3. Inoculum preparation

A 100 μg aliquot of each purified protein, MBP-MIP and CPAF was mixed with complete Freund's adjuvant (CFA) (Sigma-Aldrich) in a 1:1 ratio using two 3 ml luer lock syringes (Sigma-

Table 1
Primer sequences for MIP and CPAF amplification, *ompA* diagnostic PCR [34] and *TNFα* and *IL-10* cytokine analysis using qRT-PCR [This study, [35,36]].

Primer	Sequence	Reference
CPAF-F	ACGCGGATCCATGAAGCTAAAACAAATTACAGTC	This study
CPAF-R	ACGCCTGCAGTTACGACTCAGTGATTTTGTCTGCAC	This study
MIP-F	ACGCGGATCCGATCAGAGTTCTCACAATGAGAAC	This study
MIP-R	ACGCGTCGACTCATGAAGCTGTGTTTTTGTCCTC	This study
CpaOMP1-F	GCAACTGACACTAAGTCGGCTACA	34
CpaOMP1-R	ACAAGCATGTTCAATCGATAAGAGA	34
CpaOMP1-S	FAM-TAAATACCACGAATGGCAAGTTGGTTTAGCG-TAMRA	34
*IL-10*F	CCCAGTCTCTGCTGGATGAC	This study
IL-10 R	CAGAAAACGATGACAGCGCC	This study
TNFα F	TGCACTTCGGGGTAATCGG	This study
TNFα R	CGCTGATGTTGGCTACAACG	This study
H3F3A F	GAGGTCTCTATACCATGGTC	35
H3F3A R	GTACCAGGCCTGTAACGATG	35
GAPDH F	GGCGTGAACCACGAGAAGTATAA	36
GAPDH R	CCCTCCACGATGCCAAAGT	36

Aldrich) connected via a stopcock 3-way luer lock (Sigma-Aldrich). The negative control inoculum consisted of Tris-buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA) mixed with CFA in a 1:1 ratio. For the MIP/CPAF combined inoculum 50 μg of MBP-MIP and 50 μg of CPAF were mixed with CFA at a 1:1 ratio. The mixtures were passed back and forth through the luer lock system to create an emulsion of protein combined with the adjuvant. The booster inoculum was similarly prepared but using incomplete Freund's adjuvant (IFA) (Sigma-Aldrich) at a 1:1 ratio. Note: Due to difficulties in separating MIP from MBP it was decided to vaccinate~~d~~ with MIP tagged to MBP while CPAF vaccinates received CPAF alone without MBP present.

2.4. Ewe immunisation and blood sampling

Commercial cross bred yearling ewes were sourced from 3 farms with no history of EAE and animals were screened using a commercial EAE ELISA kit according to the manufacturer's guidelines (PrioCHECK™ Ruminant *Chlamydophila* spp. Ab Kit, ThermoFisher Scientific). Fifty EAE sero-negative sheep were randomly allocated into 5 groups of 10 with the following treatments administered to the groups: (1) 100 μg MBP-MIP (2) 100 μg CPAF (3) 50 μg MBP-MIP and 50 μg CPAF (4) Tris-buffer (negative control) (5) live attenuated *C. abortus* (Enzovax MSD Animal Health; positive control). The primary inoculations were administered as a 0.5 ml subcutaneous injection over the flank. The live attenuated *C. abortus* vaccine was administered according to the manufacturer's instructions; as a 2 ml dose injected over the flank. Blood samples were taken 3 days before inoculation and on day 7 and 14 post inoculation (PI). Blood samples were taken by jugular venipuncture using a 20 gauge needle (Greiner Bio-one) into a plain coagulant coated tube (Greiner Bio-one). The booster immunisation was given on day 21 PI, administered (0.5 ml) subcutaneously over the flank. The positive control ewes received Tris-buffer to mimic needle injection trauma. Blood samples were taken day 28 and day 35 post primary inoculation. The ewes were kept at pasture throughout this part of the experimental trial. The Enzovax ewes were kept segregated on a separate part of the farm. Plain coagulant tubes were spun at 1800 g for 20 min and serum stored at −20 °C until analysis.

2.5. Mating of ewes

The ewes were sponged using progestogen impregnated sponges to synchronise oestrus. The sponges were removed after 11 days and the ewes were injected with 500–700 IU of pregnant mare serum gonadotrophin (PMSG) to increase ovulation rates. The rams were introduced and removed after 4 days. Rams were then reintroduced 11 days later for a second mating to maximise the pregnancy rate. Ewes were scanned approximately 60 days after mating to estimate stage of pregnancy and number of foetuses per ewe. One ewe from the CPAF group was not pregnant and one ewe from the MIP/CPAF combined group was deemed too early in its gestation to be included in the trial. One ewe from the negative control group suffered a cardiac arrest 5 weeks after vaccination. This resulted in a final flock size of 47. Ewes were housed in pens within a large shed according to their vaccine group after pregnancy was confirmed.

2.6. Preparation of C. Abortus C 95/27 for challenge

The Irish isolate *C. abortus* C 95/27 was cultivated in the yolk sac of specific pathogen free hens' eggs using standard techniques [27,32]. Eggs were incubated at 37 °C until death at about day 7 post-infection. The yolk sacs membrane from each embryo was collected and ground using a mortar and pestle in 10 g sand and 5 ml sucrose phosphate glucose buffer [33]. The ground contents were centrifuged at 350 g for 10 min. The middle layer was collected, pooled in 50 ml tubes and aliquoted before storage at −80 °C until required. The number of IFUs present in the pooled yolk sac material was determined by titration in McCoy cells as previously described [27,32]. After 72 h the cells were washed in PBS and fixed in acetone before being stained with *Chlamydophila* FITC conjugate mouse mono-clonal antibody (Progen) for 30 min at 37 °C in 5% CO_2 in the dark. The ewes were challenged on day 90 of gestation subcutaneously over the left flank with 1 ml of 1×810^6 IFU of *C. abortus* C 95/27 passage 5 yolk sac.

2.7. Sampling at birth or abortion

Blood samples were taken from the ewes pre-challenge, 15 days post-challenge, 35 days post challenge and within 24 h of lambing or abortion. A final blood sample was taken approximately 3 weeks after lambing/abortions. Blood samples were collected by jugular venipuncture using a 20-gauge needle (Greiner Bio-one). Blood collected for RNA isolation was placed into Tempus blood RNA tubes (ThermoFisher Scientific) while sera was harvested from blood collected in plain coagulant tubes. At lambing/abortion the ewe identification tag and number of foetuses/lambs were recorded. Vaginal swabs, samples of placental cotyledon and a pooled sample of foetal lung and liver tissue were collected. On occasion the foetal membranes were not found, possibly consumed by the ewe. The length of gestation was recorded along with the weight and the crown to rump length for each lamb. Normal lambing care protocols were followed including aid in initiating feeding. Vaginal swabs, serum, RNA tempus tubes, placental material and foetal material were all stored at −20 °C until further analysis could be conducted.

2.8. Immunoblotting for MIP and CPAF antibody detection

The concentrations of purified recombinant CPAF and MBP-MIP were determined using the DC™ Protein assay according to the manufacturer's instructions (Bio-Rad). A 240 μl aliquot of CPAF at a concentration of 4.84 μg/μl was added to an eppendorf tube with 120 μl 1 × sample buffer (6 M urea, 75 mM Tris-HCl pH 8.8, glycerol 29.3% v/v, 2% SDS, 1% bromophenol blue). The mixture was heated at 95 °C for 5 min. A 20 μl aliquot per well was then electrophoresed on a 12% SDS-polyacrylamide gel using a Mini-PROTEAN II cell (Bio-Rad). A 120 μl aliquot of MBP-MIP (1.18 μg/μl) was incubated for 24 h at room temperature (RT) with 4 μl of factor XA (New England BioLabs) and 16 μl of factor XA buffer (New England BioLabs). After 24 h, 60 μl of 1 × sample buffer was added to the tube and the samples were heated at 95 °C for 5 min. A 20 μl aliquot was then added per well of a 12% SDS-PAGE gel and electrophoresed using a Mini-PROTEAN II cell (Bio-Rad).

The proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Millipore) using a mini trans-blot electrophoretic transfer cell (Bio-Rad). Following transfer the membranes were blocked using 5% skim milk powder (Sigma-Aldrich) overnight at 4 °C. Membranes were subsequently washed twice for 5 min in 20 ml of 1 × phosphate buffered saline (PBS) with 0.1% tween (PBST) at RT using a roller. An equal volume of serum from each ewe from the MIP inoculated group and the CPAF inoculated group from (i) 3 days pre-inoculation, (ii) day 14 post-inoculation and (iii) day 35 post-inoculation were pooled. The PVDF membranes were cut with a scalpel and probed with the pooled sera at a 1/400 dilution in PBST for 1 h at RT with rolling. Membranes were then washed 3 times with PBST for 5 min at RT with rolling and then probed with anti-sheep antibody tagged with horse raddish peroxidase (HRP) (Sigma-Aldrich) at a 1/250 dilution in 5% skimmed milk for 1 h at RT with rolling. The blots were washed as described previously and the membranes were developed using Super Signal West Dura extended duration substrate (ThermoFisher Scientific) before being visualised using the G Box chemiluminescent imager (Syngene).

2.9. *Chlamydia abortus quantitative PCR*

The *ompA* gene of *Chlamydia abortus* was amplified using the protocol described by Pantchev et al. [33] and using the primers listed in Table 1. PCR assays were conducted on placental material, vaginal swabs and foetal tissue.

Swabs: The cotton bud portion of each swab was added to a micro-centrifuge tube containing 700 µl of sterile 0.5 M PBS. The samples were then vortexed for 60 s twice and 250 µl of each sample was added to a plate for DNA extraction using the MagNa pure LC DNA isolation kit and the MagNA pure 96 instrument (Roche Diagnostics Ltd., Ireland) according to the manufacturer's guidelines. The master mix for PCR was made as detailed for a 1× reaction and multiplied up for the amount of reactions to run; 12.5 µl of Quanti Tect probe master mix plus 0.5 µl of Cpa OMP1 probe plus 0.5 µl of the forward and reverse primers and 6 µl of water. 20 µl of master mix was added to each well and 5 µl of extracted DNA from each sample was added to each well. The QuantiTect probe PCR kit (Qiagen USA, cat no. 204345) was used with the following thermocycler profile: 95 °C for 15 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. The fluorescence data was collected during the 60 °C annealing step using ROX and FAM filters.

Foetal tissue: Approximately 0.25 g of each foetal tissue sample was chopped finely and added to a micro-centrifuge tube containing 700 µl of sterile 0.5 M PBS. The DNA extraction was then conducted as described previously for the swab samples.

Placental material: Approximately 0.25 g of placental tissue was added to a 2 ml micro-centrifuge tube containing 0.25 g of sterile sand and 250 µl of MagNa Pure Total Nucleic Acid Isolation Kit lysis buffer (Roche Diagnostics Ltd., Ireland). The tube was then vortexed for 10 s. 20 µl of proteinase K was then added to the tube and vortexed for 10 s. Samples were then incubated for 1 h at 56 °C and then spun at 9000 g for 2 min. 450 µl from each sample tube was added to the extraction plate for DNA extraction using the MagNa pure LC DNA isolation kit and the MagNA pure 96 instrument (Roche diagnostics, Ireland) according to the manufacturer's guidelines and as described previously for swab samples.

Serial dilutions of the neat inoculum of C95/27 (10^6 IFU/ml) used to challenge the ewes were made down to 10^{-10} and the DNA in each diluted sample was extracted using the MagNa pure LC DNA isolation kit and the MagNA pure 96 instrument to generate a standard curve to estimate the number of genome copies of *C. abortus* in each sample.

2.10. *Chlamydial antibody detection*

The commercial diagnostic PrioCHECK™ Ruminant *Chlamydophila spp*. Ab Kit (ThermoFisher Scientific) was used to measure circulating antibodies to chlamydiae as per manufacturers' instructions.

2.11. *Interferon gamma detection in sera*

Serum samples were analysed for levels of IFNγ using a commercial bovine IFNγ ELISA kit which cross-reacts with ovine samples (Mabtech) according to the manufacturer's instructions.

2.12. *TNFα and IL-10 cytokine RNA expression in blood.*

Tempus blood RNA tubes (ThermoFisher Scientific) stored at −20 °C were thawed and the RNA extracted from whole blood using the Tempus Spin RNA Isolation kit (ThermoFisher Scientific) according to the manufacturer's guidelines. The quality of RNA extracted was assessed using the Agilent RNA 6000 Nano assay run on a 2100 Bioanalyser and total RNA quantified in ng/µl using the NanoDrop ND-100 spectrophotometer (ThermoFisher Scientific). All RNA samples extracted were also run on a 1% agarose gel to visualize the 18 s and 28 s bands and ensure that the RNA was intact. Extracted RNA (500 ng) was converted to cDNA using the High Capacity cDNA reverse transcription kit (ThermoFisher Scientific). Real time PCR was then conducted with the cDNA using the Fast SYBR Green Master Mix kit (Applied Biosystems, Ireland). Primers for *TNFα* and *IL-10* (Table 1) were designed using the NCBI database and it was ensured that the primers spanned an exon-exon boundary. *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* and *H3 histone family member 3A (H3F3A)* were used as internal controls. The 20 µl mix for each reaction consisted of 10 µl Fast SYBR Green Master Mix kit, with 0.4 µl of the relevant forward and 0.4 µl of the relevant reverse primer, 8.2 µl of dH_2O and 1 µl of cDNA. The melt curve profiles were examined and the mean CT value for each sample was recorded. Results were expressed as fold changes using the $2^{-\Delta\Delta CT}$ method [37] and by relating day 35 post challenge and day 24 h post lambing to pre-infection levels.

2.13. *Statistical analysis*

For comparison between vaccine groups log transformation ($\log_{10}(x + 1)$) was applied to *TNFα*, *IL-10* and IFNγ to stabilize the variance. To account for repeated measures, data were analysed using Proc MIXED (SAS v9.4). The model used had fixed effects for treatment, day and their interaction. The differences among treatments were evaluated on a within-day basis using Dunnett's test with the negative control as the control group. Indicators of the success of pregnancy, such as gestation length, lamb birth weight and foetal length were analysed using independent T-tests to compare vaccine groups to the negative control group. For comparisons between ewes that aborted and ewes that had live pregnancies it was decided that one or more live lambs born was constituted as a live birth, while pregnancies that ended in all dead lambs counted as an abortion. Ewes that aborted and ewes that had live lambs were then grouped separately and Mann-Whitney T-tests were conducted using GraphPad Prism (V 5.03) for each variable.

3. Results

3.1. *Humoral response to MIP and CPAF*

CPAF and MIP induced a specific humoral immune response with anti-CPAF and anti-MIP antibodies detected at day 14 and 35 post-inoculation in the groups treated with CPAF and MIP respectively. A strong antibody response was induced by day 14 post-inoculation which subsequently increased substantially by day 35 (Fig. 1).

3.2. *Challenge with Chlamydia abortus.*

The challenge trial provided evidence of protection for some treatment groups (Table 2). Enzovax was 100% protective with no abortions occurring in this group, while the negative control

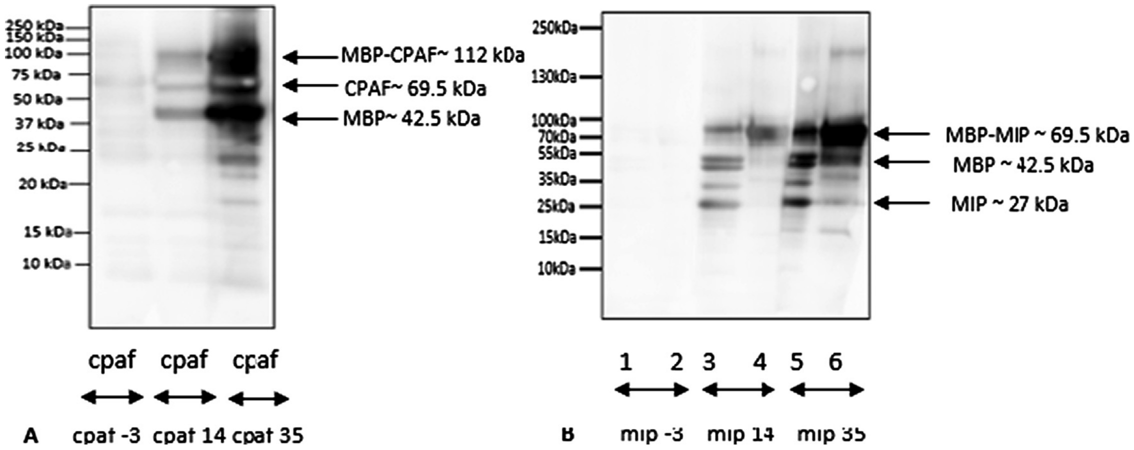

Fig. 1. (A) Immunoblot of recombinant CPAF probed with pooled serum from CPAF vaccinated ewes from day −3, day 14 and day 35 post inoculation. The arrows indicate sero-reactive bands to MBP, CPAF and MBP-CPAF fusion protein. (B) Immunoblot of recombinant MBP-MIP and MIP cleaved from MBP using factor XA probed with pooled serum from MIP vaccinated ewes from days −3 (Lanes 1 & 2), day 14 (Lanes 3 & 4) and day 35 (Lanes 5 & 6) post-inoculation. Lanes 1, 3, 5 = MIP cleaved with factor XA; Lanes 2, 4, 6 = MBP-MIP. The arrows indicate sero-reactive bands to MIP, MBP and MBP-MIP fusion protein.

group had an abortion rate of 67%. Vaccine efficacy was calculated according to the equation [38]:

$$\text{Efficacy} = \frac{\text{Abortion rate}_{\text{novel test vaccine}} - \text{Abortion rate}_{\text{positive control}}}{\text{Abortion rate}_{\text{negative control}}} \times 100$$

MIP/CPAF combined was the most effective of the novel vaccines explored and resulted in 50% efficacy and 67% of one or more live births (Table 2). Both MIP/CPAF combined and Enzovax vaccinated ewes had lambs of significantly higher birth weight than the negative control group ($P < 0.05$). Both MIP/CPAF and Enzovax vaccinated ewes also had longer gestation length than negative control ewes with Enzovax ewes being significantly longer ($P < 0.05$) while for MIP/CPAF vaccinated ewes the difference approached significance ($P = 0.06$) as shown in Table 2.

3.3. *Chlamydia abortus* quantitative PCR

Placental material and vaginal swabs from the Enzovax group had significantly lower numbers of genome copies of *C. abortus* than negative control ewes ($P < 0.05$) (Fig. 2A). All novel vaccine groups had lower numbers of copies of *C. abortus* present in vaginal swabs than the negative control group, though this difference was not statistically significant (Fig. 2A). Ewes which had live lambs had significantly lower numbers of genome copies in vaginal swab samples compared to ewes which aborted (Fig. 2B) ($P = 0.01$).

3.4. *Anti-chlamydial antibodies*

The antibody levels in the protected groups, Enzovax and MIP/CPAF vaccinated ewes, were lower than in the other vaccine groups but only significantly so for the Enzovax group (Fig. 3A). Enzovax ewes were the only group to have significantly lower levels of antibodies than negative control group at 24 h post lambing and at 3 weeks post lambing ($P < 0.05$). Anti-chlamydial antibodies were consistently higher in ewes that went on to abort compared to ewes that went to term and had live lamb births (Fig. 3B) and significantly so at 24 h post-lambing ($P = 0.01$) (Fig. 3B).

3.5. *Interferon gamma ELISA*

IFNγ levels showed an increase in all vaccine groups post-challenge (Fig. 4A). IFNγ levels were significantly higher in ewes that aborted compared to ewes that had live lambs at 35 days post challenge ($P = 0.01$) (Fig. 4B).

3.6. *PCR analysis of TNFα and IL-10 expression in sera*

Expression of *IL-10* was significantly higher in ewes that went on to abort than in ewes that had live lambs at 35 days post challenge (Fig. 5A and B). There were no statistically signifcant differences in *TNFα* or *IL-10* expression between any vaccine groups and the negative control ewes (Fig. 5C and D).

Table 2
Results from each vaccine group of % live lambs, births with 1/+ live lambs (births with all live lambs), abortions, vaccine efficacy, mean gestation length, mean birth weight and mean crown to rump length per vaccine group.

	MIP	CPAF	MIPCPAF	Enzovax	Negative control
No of ewes	10	9	9	10	9
%live lambs	20%	29%	41%	100%	27%
	4/20	5/17	7/17	16/16	4/15
Births 1/+ live lamb (births with all live lambs)	3 (2)	3 (2)	6 (2)	10 (10)	3 (2)
Abortions	7	6	3	0	6/9
Vaccine efficacy	0%	0%	50%	100%	0%
Mean gestation length	136 days	135 days	138 days	145 days	134 days
Mean birth weight	2.9 kg	3.3 kg	3.5 kg	4.5 kg	2.8 kg
Mean crown to rump length	42.4 cm	45.6 cm	43.9 cm	46.6 cm	42.2 cm

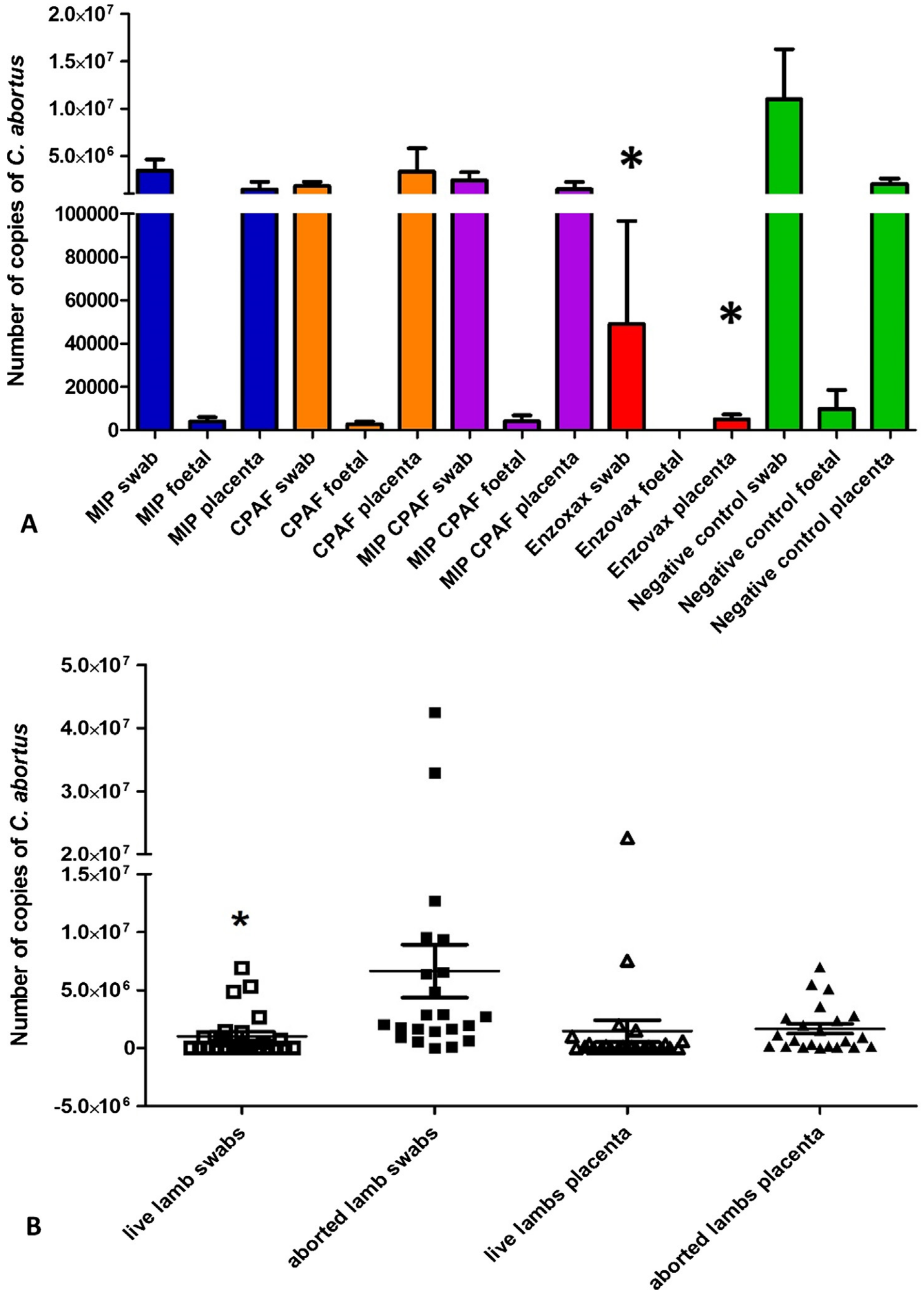

Fig. 2. (A) Mean (+SEM) number of copies of *C. abortus* from vaginal swabs, foetal tissues and placental material collected at lambing/abortion from ewes vaccinated with MIP (blue), CPAF (orange), MIP + CPAF (purple), Enzovax (red) or PBS (negative control, green) and subsequently challenged with 10^6 IFU of *C. abortus* C 95/27. No foetal material was obtained from Enzovax vaccinated ewes as no abortions occurred. *Placental material and vaginal swabs from Enzovax ewes had significantly lower number of copies of *C. abortus* values than negative control ewes ($P < 0.05$). (B) Number of copies of *C. abortus* (±SD) present in vaginal swabs and placental material collected at lambing/abortion from ewes that gave birth to live lambs compared to those that aborted. *Number of copies of *C. abortus* in vaginal swabs were significantly lower in ewes that had live lambs ($P < 0.05$).

4. Discussion

This is the first time, to the best of our knowledge, that the abilities of MIP and CPAF to protect against *C. abortus* challenge in sheep have been investigated. It was found that of the novel vaccine combinations examined, the MIP/CPAF combined vaccine offered the greatest protection with 67% of ewes giving birth to one or more live lambs equating to a 50% vaccine efficacy rate. Similar to the postive control vaccinated ewes, MIP/CPAF vaccinated ewes had longer gestations and lambs with significantly higher birth weights than negative control ewes. In this trial the live commercial vaccine was 100% effective with no abortions recorded in this vaccine group. As expected, aborting ewes shed higher numbers of *C. abortus* than ewes that had live lambs. IFNγ levels and the relative expression of *TNFα* and *IL-10* were all analysed to determine if there were any differences between ewes that aborted compared to those that had live lambs. Interestingly ewes that went on to abort had significantly higher levels of IFNγ and *IL-10*

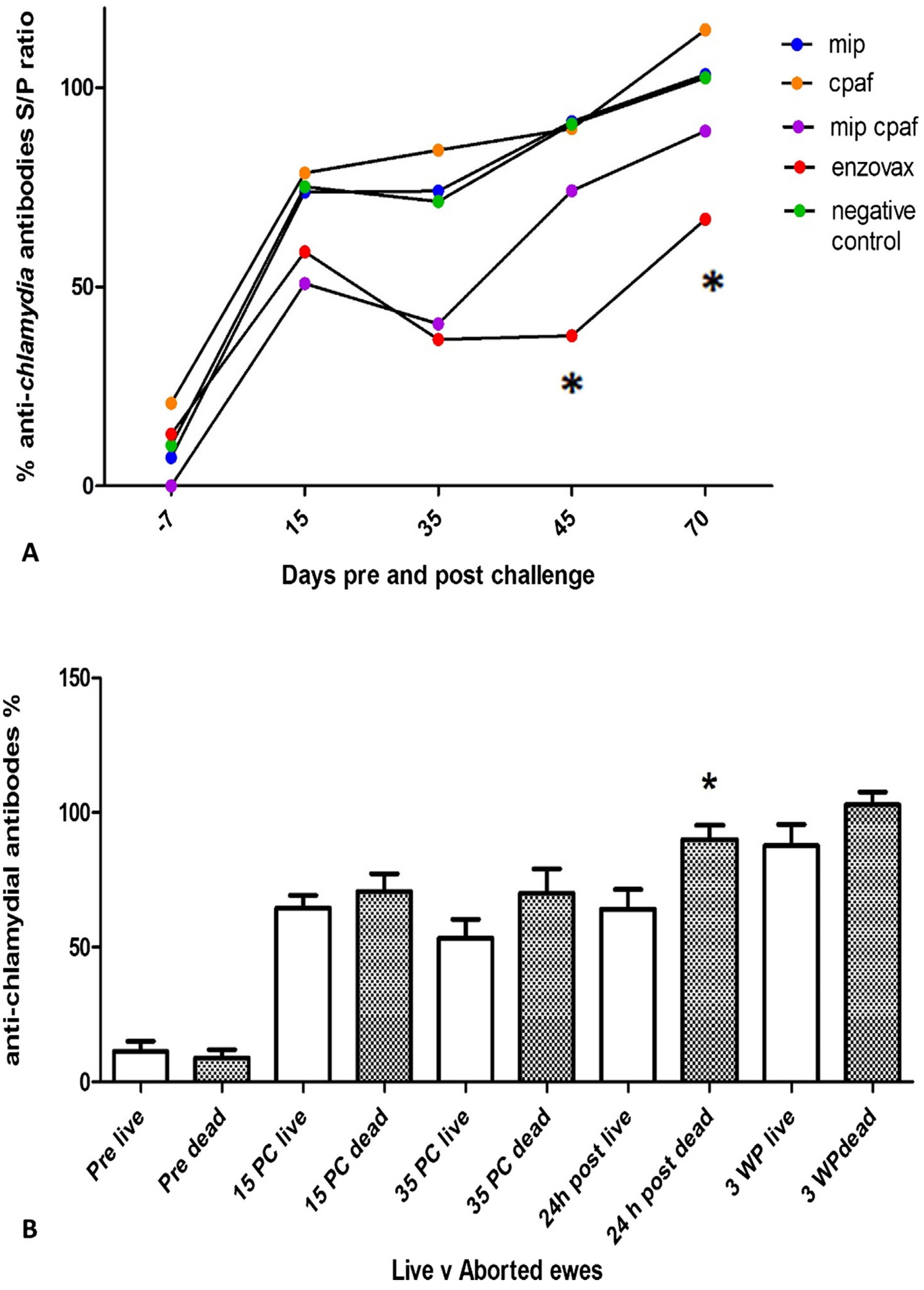

Fig. 3. A Least squares mean anti-chlamydial antibodies (sample to positive (S/P) ratio) in sera from ewes vaccinated with MIP (blue), CPAF (orange), MIP + CPAF (purple), Enzovax (red) or PBS (negative control, green) and subsequently challenged with 10^6 IFU of *C. abortus* C 95/27. Challenge on day 0 corresponded to ~ 3 months gestation with final blood samples taken 3 weeks after lambing/abortion (~day 70 post challenge). *Significantly lower levels of anti-chlamydial antibodies in Enzovax ewes compared to negative control ewes at 24 h post lambing and 3 weeks post lambing ($P < 0.05$) B) Mean anti-chlamydial antibodies (S/P ratio) in sera from pregnant *C. abortus* challenged ewes comparing those that had live lambs (white bars) to those that aborted (shaded bars) at each time point. * Significantly higher levels in ewes that aborted compared to ewes that had live lambs ($P < 0.05$) at 24 h post lambing. PC = post challenge, WP = weeks post lambing, live = ewes that had at least one live lamb, dead = ewes that aborted and had all dead lambs.

expression at day 35 post challenge compared to ewes that had live lambs. Ewes that aborted also had significantly higher levels of anti-chlamydial antibodies at 24 h post lambing.

All novel vaccinations administered were well tolerated by the ewes with the combination of both MIP and CPAF together offering the greatest protection demonstrated by higher birth weight of lambs, longer gestation length and more live lambs compared to ewes that received either of the proteins singly. It is important to note that if one simply recorded protection based on the gestations in which all ewes had live lambs then the protection provided by the MIP/CPAF combination would have been missed. Neither single subunit vaccination offered protection greater than the negative control, indicating that a complex response is needed for complete protection against abortion. Similar concentrations of recombinant proteins were administered in trials involving vaccination with sheep against fasciolosis [39]. However further work examining and optimising vaccine dose in terms of protein quantity along with details on the number of booster vaccinations will be vital to the creation of a new vaccine. Future work on multi-subunit protein vaccines for this bacterium could pave the way forward for novel chlamydial vaccine development and eliminate the need for live attenuated vaccines and the associated risks.

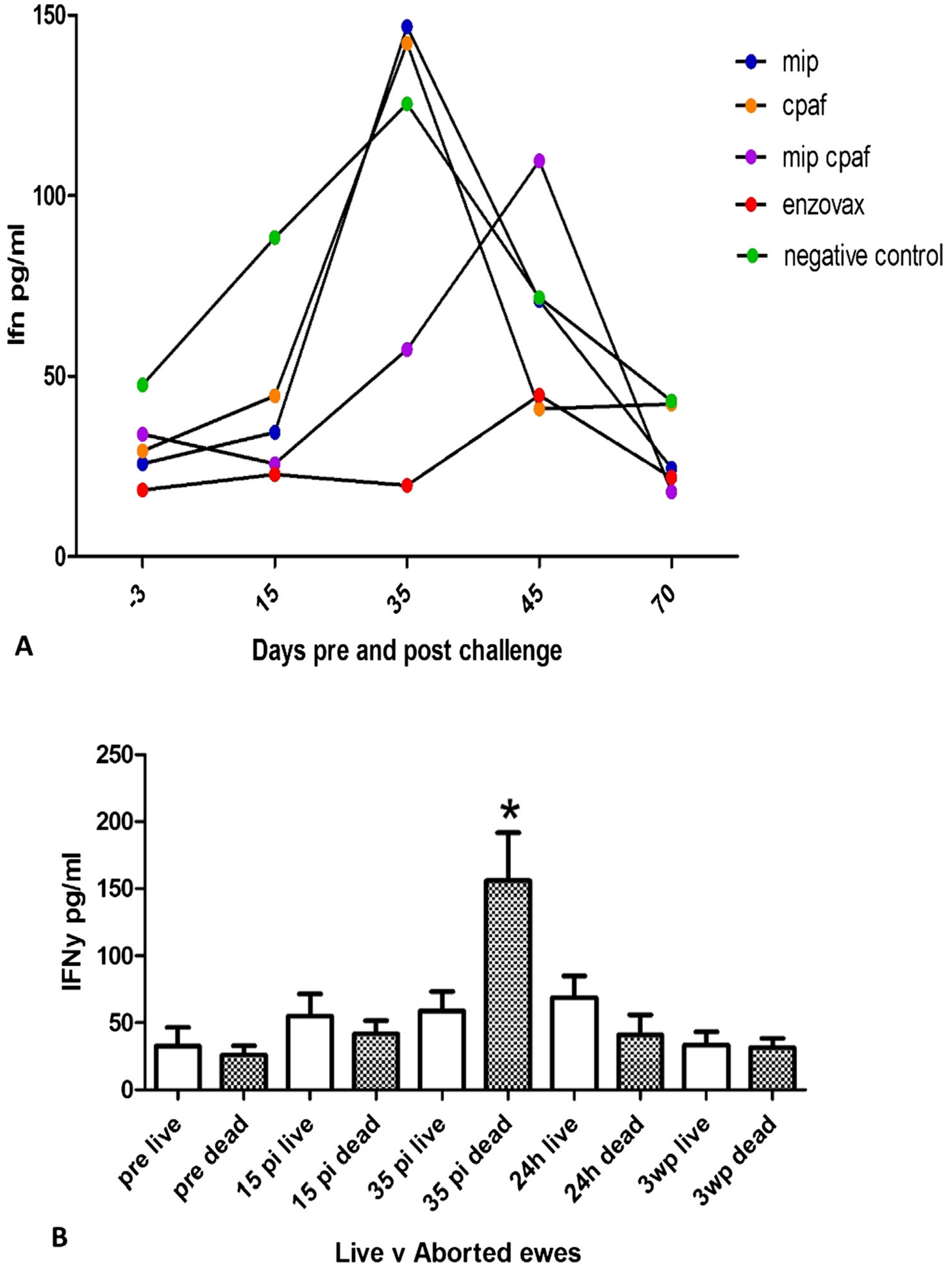

Fig. 4. (A) Least squares mean IFNγ (ρg/ml) in sera from ewes vaccinated with MIP (blue), CPAF (orange), MIP + CPAF (purple), Enzovax (red) or PBS (negative control, green) and subsequently challenged with 10^6 IFU of *C. abortus* C 95/27. Challenge on day 0 corresponded to ∼ 3 months gestation with final blood samples taken 3 weeks after lambing/abortion (∼day 70 post challenge). (B) Mean IFNγ (ρg/ml) in sera from pregnant *C. abortus* challenged ewes comparing those that had live lambs (white bars) to those that aborted (shaded bars) at each time point. * Significantly higher levels in ewes that aborted (shaded bars) compared to ewes that had live lambs (white bars) ($P < 0.05$) at 35 days post lambing PC = post challenge, WP = weeks post lambing, live = ewes that had at least one live lamb, dead = ewes that aborted and had all dead lambs.

Cytokines are considered to play an important role in the pathogenesis of *C. abortus* infection [40]. The production of IFNγ has been reported as a major player in an effective immune response to *C. abortus* both *in vitro* and *in vivo* [2,3]. IFNγ stands out as the strongest correlate of protection against chlamydial infection in many species including EAE [41]. However, it is also associated with persistence of infection [41,42]. Cytokines in serum were evaluated by Del Rio et al. [43] in a mouse model of *C. abortus* infection. They found that IFNγ expression in the placenta can be useful in controlling the level of infection locally. Interestingly low levels of *IFNγ* expression were found in the placentas of *C. abortus* affected ewes in the study carried out by Buxton et al. [40]. This low *IFNγ* expression could mean the animal was unable or was failing to control chlamydial replication in the placenta. Buxton et al. [40] on the other hand noted that *TNFα* mRNA was readily found in the cytoplasm of mononuclear cells in affected placentas. It was postulated that in *C. abortus* infection the production of TNFα associated with macrophages expressing MHC II damages the ovine placenta and contributes to the pathogenesis of abortion or pre-mature birth. Studies have found that elevated levels of TNFα have been associated with spontaneous embryo loss in pregnant mice [44,45]. High levels of TNFα in neutrophil depleted *C. abortus* infected mice were also associated with death while surviving mice had lower levels [46]. These studies demonstrate the opposing roles that cytokines can play in EAE. TNFα can contribute to pathology and abortion while increases in *IFNγ*

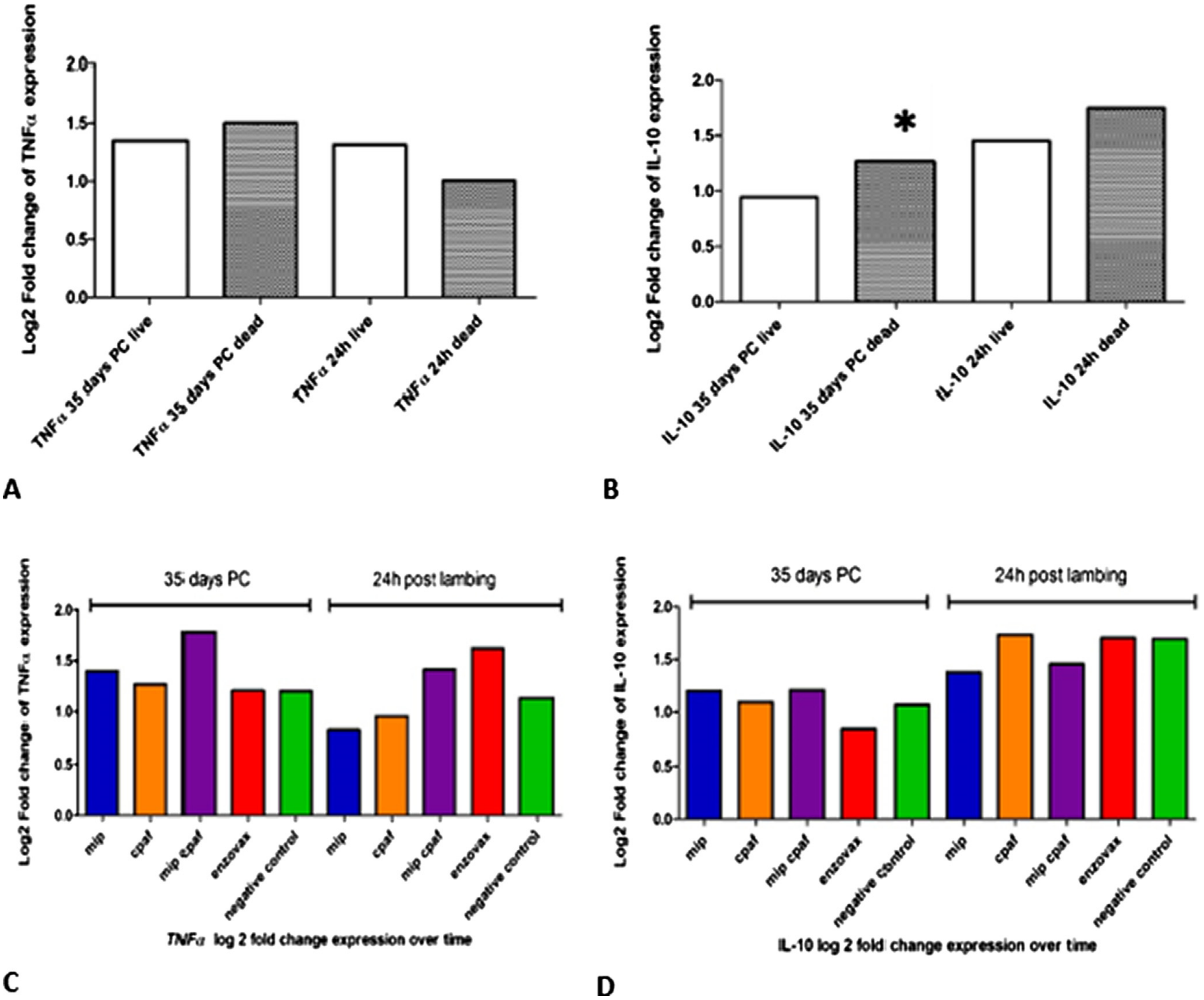

Fig. 5. Fold change in *TNFα* expression (A) and *IL-10* expression (B) at 35 days post-challenge and 24 h post-lambing/abortion in sera from pregnant *C. abortus* challenged ewes that gave birth to live lambs (white bars) compared to those that aborted (shaded bars). Gene expression is relative to pre-infection levels. *Significantly different ($P < 0.05$). Fold change in *TNFα* expression (C) and *IL-10* expression (D) at 35 days post-challenge and 24 h post-lambing/abortion in sera from ewes vaccinated with MIP (blue), CPAF (orange), MIP + CPAF (purple), Enzovax (red) or PBS (negative control, green) and subsequently challenged with 10^6 IFU of *C. abortus* C 95/27.

expression can control the infection locally. However, alterations of cytokine levels at the maternofoetal interface can also disturb pregnancy. Interestingly, although significant differences in *TNFα* expression were not observed in this study, significantly higher levels of IFNγ and *IL 10* expresson were detected in aborting ewes 35 days post challenge. A peak in the level of IFNγ at the time of abortion in infected ewes has been observed previously by Rocchi et al. [42]. It was hypothesised that this peak corresponded to extensive placental infection in these ewes resulting from strong antigenic challenge stimulating IFNγ production [42]. Our study also found that the expression levels of *IL-10* were significantly higher at 35 days post challenge in ewes that that went on to abort. This is in contrast to Rocchi et al. [42] where signifcant differences in *IL-10* production between groups were not observed. It could be hypothesised that the presence of elevated levels of IL-10 should have an anti-inflammatory effect and reduce IFNγ levels and *TNFα* expression. The significant regulatory role that IL-10 can play in controlling the levels of pro-inflammatory cytokines has been shown in human placental tissue [47]. In our study however, an increase in *IL-10* expression levels did not in turn lead to a reduction in IFNγ levels or *TNFα* expression. It may be that the IL-10 response was belated or insufficient to control the inflammatory response evoked by chlamydial placentitis. The results obtained using blood samples for these analyses demonstrates the localised nature of this infection and it could be hypothesised that using more localised techniques to examine the placenta would provide more statistically significant differences.

The antibody response associated with chlamydial abortion in sheep is believed to play a less significant role than the cellular responses in terms of immunological protection and prevention of abortion [41]. In a vaccine study exploring the use of live-attenuated vaccines, antibody levels from ewes vaccinated prior to challenge did not differ between ewes that went on to abort and ewes that had live lambs [48]. This suggests that antibodies do not play a major role in protection against *C. abortus*-induced abortion. Interestingly in our trial anti-chlamydial antibody levels were found to be consistently higher at all time points after challenge in ewes that aborted compared to ewes that had live lambs with ewes that aborted displaying significantly higher levels of antibodies at 24 h post lambing than ewes that had live lambs.

Enzovax vaccinated ewes also demonstrated significantly lower levels of antibodies compared to negative control ewes at 24 h post lambing and 3 weeks post lambing. Studies have shown increases in antibody levels associated with *C. abortus* reproductive failure [49,50,51]. Gutierrez *et al* [52] and García-Saco *et al* [53] have also noted that antibody titres were lower in animals that did not go on to abort. This current study and others clearly show how elevated antibody levels could be useful in identifying pregnant ewes that will go on to abort. It is possible that the antibody increase detected in all groups at 3 weeks post lambing could be associated with the development of protective immunity against subsequent abortions [1,53] or could simply be a diagnostic marker of exposure to high numbers of *C. abortus*.

Even though all the placentas from aborting ewes showed necrotic lesions and a high burden of *C. abortus,* the timing of abortion varied. In our trial the degree of infection and colonisation of placenta differed from ewe to ewe and between vaccine groups with the negative control ewes being the first group to abort. Buxton et al. [40] similarly found that the placentas of all infected ewes displayed signs of infection but the most severe signs of infection were present in the ewes that aborted. Clearly, the degree of infection and extent of resulting placental lesion are significant determinants of abortion and foetal loss. In the present study sampling of the foetal lung and liver failed to demonstrate high levels of infection using PCR. Vaginal swab samples demonstrated much higher infectious loads in ewes that aborted compared to those that gave birth to at least one live lamb. Interestingly higher levels of infection were noted in vaginal swabs than in placental material, this may be due to the controlled conditions of this trial and the promptness of sampling after birth. Gutierrez et al. [52] reported that *C. abortus* was readily detected in the vaginal discharge after birth. These findings suggest that a vaginal swab may be the best sample followed by placenta and foetal tissues for confirmation of EAE.

In conclusion, it is clear from this study that MIP and CPAF in combination offered some protection and could contribute to a subunit vaccine. This investigation of the systemic response to *C. abortus* infection does complement the many localized studies of this disease [1,40,51,54]. This trial demonstrated that a high titre of anti-chlamydial antibodies within 24 h of lambing along with higher levels of IFNγ and higher expression of *IL-10* 35 days post challenge are all markers that correlate with an infected ewe in train to abort. This research has clearly demonstrated the potential of subunit vaccines for EAE prevention. Besides safety assurance, such vaccines also offer the possibility of permitting discrimination of vaccinated from naturally infected ewes (DIVA). Future studies of MIP and CPAF in combination with other immunogenic chlamydial proteins and effective adjuvants could pave the way for the creation of a new, safe and highly effective vaccine for *Chlamydia abortus.*

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Declarations of Competing Interest

None.

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