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RESEARCH PAPERS

# Xanthan gum triple emulsion adjuvant effect in an experimental vaccine with recombinant epsilon toxin (rETX) of *Clostridium perfringens*

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**Highlights**

- Developed a novel xanthan gum-based W/O/W emulsion adjuvant (Xenoil)
- Emulsion was stable, safe, and suitable for SC and IM administration
- Xenoil induced 1.5× higher IgG titers than aluminum hydroxide ($p < 0.05$)
- Upregulated IL-2, IL-15, and IL-17, suggesting Th1/Th17 immune polarization
- Promising candidate adjuvant for recombinant clostridial vaccines in cattle

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KEYWORDS
Triple emulsion technology;
Vaccine formulation;
Bovine immunization;
Immunological response;
Cytokine transcription;
Humoral response.

**Abstract:** Subunit vaccines composed of recombinant proteins are safer and more defined than traditional formulations but often require potent adjuvants to achieve strong and durable immunity. Aluminum hydroxide, the most widely used adjuvant, has limited capacity to induce robust humoral and cellular responses. This study evaluated a novel xanthan gum-based water-in-oil-in-water (W/O/W) emulsion adjuvant (Xenoil) as a delivery system for recombinant *Clostridium perfringens* epsilon toxin (rETX). Heifers (n = 15) with no prior history of clostridial vaccination were included in the study and randomly allocated into three experimental groups (n = 5 per group) as follows: 1. inoculated with rETX formulated with the triple emulsion; 2. inoculated with rETX adjuvanted with aluminum hydroxide; and 3. animals receiving the triple emulsion combined with phosphate-buffered saline (PBS), as the control formulation. An in-house indirect ELISA was used to measure specific anti-rETX IgG. Transcription levels of key cytokines (*il2*, *il15*, *il17*) were analyzed in peripheral blood mononuclear cells (PBMCs) using quantitative PCR (qPCR). The W/O/W emulsion was stable for six months under refrigeration and safe for administration in mice, horse and cattle. Heifers immunized with the emulsion exhibited a 1.5-fold increase in IgG titers compared with aluminum hydroxide ($p < 0.05$). Significant upregulation of

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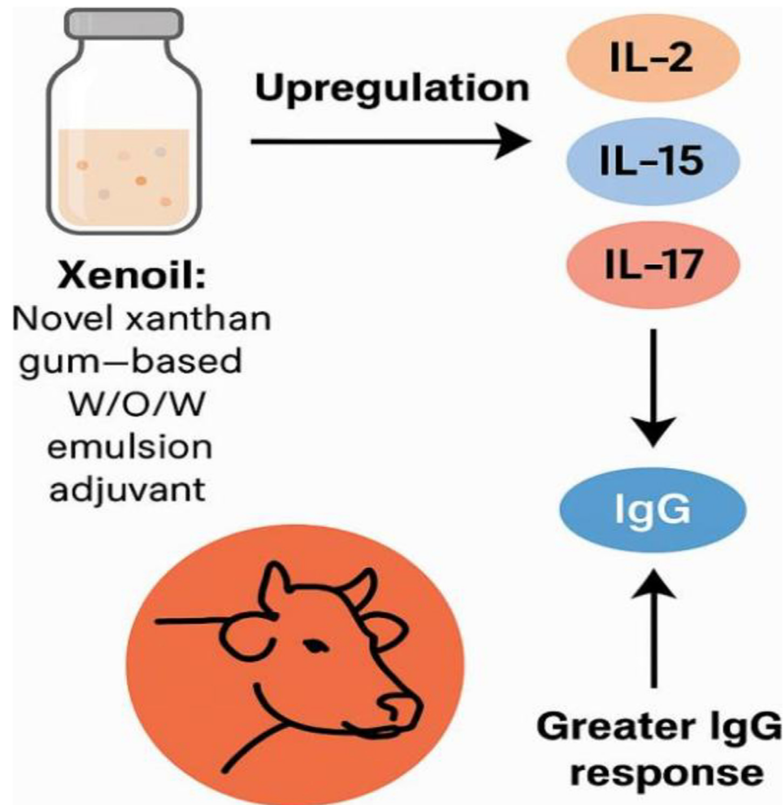
*il2*, *il15*, and *il17* transcription was observed ($p < 0.05$), suggesting a modulation towards Th1/Th17 pathways, activating T cells and B-cell differentiation. The xanthan gum-based W/O/W emulsion induced stronger humoral responses than aluminum hydroxide, demonstrating its immunomodulatory potential in cattle. Although neutralizing activity and long-term memory were not assessed, these results highlight Xenoil as a promising next-generation adjuvant candidate. Future studies should confirm antibody neutralization capacity, evaluate protective efficacy under field conditions, and determine the durability of immunity.

## Graphical abstract




## Introduction

Veterinary vaccines have been widely used for decades and are essential for protecting animal health, enhancing welfare, and supporting sustainable food production (Garg et al., 2017). A key part of most vaccines is the adjuvant, which boosts the immune response by making it faster, stronger, and longer lasting (Coffman et al., 2010). Adjuvants also help save doses by reducing the amount of antigen needed and decreasing the number of booster shots required. However, no single adjuvant can meet all the criteria of an ideal vaccine. When used alone, adjuvants might produce weak, short-lived, or unbalanced immune responses. In contrast, combining adjuvants can create synergistic effects, activating a wider range of immune cells such as dendritic cells, macrophages, and lymphocytes, which improves vaccine effectiveness (Levast et al., 2014). These combination adjuvant platforms are especially promising for recombinant and subunit vaccines, which often have low inherent immunogenicity (Garg et al., 2017).

Several combinations of adjuvants, including Montanide, ISCOMs, and liposomes, either alone or in association with other molecules, have demonstrated encouraging results in veterinary models (Levast et al., 2014). Montanide, a mineral oil widely used as an adjuvant carrier, exerts its effect by enhancing both antibody and cytotoxic T-lymphocyte (CTL) responses when mixed with antigens (Awate et al., 2012). Its mechanism is linked to depot formation at the injection site, which slows antigen release, induces local inflammation, and recruits immune cells such as macrophages, neutrophils, and dendritic cells (Mutwiri et al., 2007). This cascade promotes lymphocyte accumulation in draining lymph nodes, sustaining antigen exposure and immune activation (Dar et al., 2012).

Among polysaccharide-based adjuvant candidates, Xanthan gum has attracted attention as a natural immunostimulant. Xanthan is a negatively charged extracellular polysaccharide,

consisting of a (1,4)-β-D-glucan cellulose backbone produced by *Xanthomonas campestris* (Becker et al., 1998; Borges & Vendruscolo, 2008). It exhibits intrinsic adjuvant activity (Ishizaka et al., 1983), partly mediated by Toll-like receptor 4 (TLR4) recognition, which activates the NF-κB pathway and promotes the production of pro-inflammatory cytokines (Takeuchi et al., 2009). Through TLR signaling, xanthan gum enhances antigen presentation and bridges innate and adaptive immunity (Athman & Philpott, 2004), making it a valuable candidate for inclusion in vaccine formulations.

Within veterinary medicine, *Clostridium perfringens* is of particular concern. Although it is a commensal of the gastrointestinal tract in humans and animals certain toxinotypes act as highly pathogenic agents (Ferreira et al., 2016). Types B and D produce epsilon toxin (ETX), a potent prototoxin that becomes biologically active after proteolytic cleavage of its C-terminal region (C-23). Once activated in the intestine, ETX induces rapid and often fatal enterotoxemia, particularly in cattle and small ruminants, leading to severe production losses (Rocha et al., 2008; Yao et al., 2016).

Vaccination remains the most effective strategy for controlling clostridioses. Currently available vaccines to prevent enterotoxemia in cattle are mainly based on inactivated *C. perfringens* toxins or toxoids formulated with aluminum hydroxide as an adjuvant (Oliveira et al., 2021; Songer, 1996). Although effective under field conditions, protection is often variable and influenced by factors such as antigenic composition, vaccination schedules, herd management, and individual immune responsiveness (Freitas et al., 2021; Songer, 1996). In addition, alum-adjuvanted toxoid vaccines frequently require repeated booster doses, which may limit the duration and consistency of protection (Reed et al., 2013). Recombinant epsilon toxin (rETX) has been successfully produced (Moreira et al., 2016); however, as with most recombinant subunit vaccines, potent adjuvants are required to induce robust and long-lasting immunity, driving continued efforts to improve adjuvant vaccine formulations systems (Di Pasquale et al., 2015).

The present study aimed to develop and evaluate a novel triple W/O/W emulsion adjuvant combining Xanthan gum and Montanide. We hypothesize that the water portion containing Xanthan and the antigen will be liberated and sensitize the host immune system, then the oil portion (Montanide) will slowly liberate the antigen, eliciting a highly effective vaccine response. This innovative platform, referred to as Xenoil, proved to be highly effective in cattle vaccination against *C. perfringens* epsilon toxin, offering a promising strategy for improving clostridial vaccine efficacy.

## Material and methods

### Ethics declaration

All protocols were reviewed and approved by the Ethical Committee on the Use of Animals at UFPel (CEUA No. 9339). The CEUA/UFPel is accredited by the Brazilian National Council for the Control of Animal Experimentation (CONCEA).

### Vaccine antigen

For recombinant ETX, the codon-optimized synthetic gene for *Escherichia coli* expression of ETX (Epoch Life Science), which lacks the signal sequence (the first 45 amino acid residues of the N-terminal portion of the protein), was extracted from the pAE plasmid and subcloned into a pET28a expression vector. Expression, purification, and safety tests of rETX were conducted according to the procedure described by Moreira et al. (2016). rETX was purified using affinity chromatography with a high-performance HisTrap™ Ni-Sepharose™ column (GE Healthcare). The protein concentration was measured using the BCA protein assay kit (Pierce, USA).

### Xanthan gum

Xanthan gum powder was commercially obtained (Deosen Biochemical) and used as the bacterial polysaccharide (BPS) component for adjuvant emulsion preparation. Purity of xanthan gum was evaluated by an iodine test to detect possible starch contamination, in which 2 mL of a 1% polysaccharide solution was transferred to a test tube followed by the addition of two drops of 5% Lugol's solution (Ultrafarma) (Iodine test, 2017). Microbiological purity was assessed by inoculating the BPS solution onto Petri dishes containing blood agar (Newprov) and agar Sabouraud dextrose (Sigma-Aldrich) to verify the presence of microorganisms and potential contamination.

### Vaccine preparation

Briefly, the experimental vaccine emulsion was prepared in three stages: the first stage involved a water-in-oil (W/O) emulsion (0.5 ml containing xanthan and 80 µg of rETX antigen); the second stage consisted of an oil phase (0.5 ml of Montanide ISA 50 V2 and Span® 80); and the third stage included an aqueous phase (1 ml containing 120 µg of rETX antigen, xanthan, Tween® 80, and propylene glycol). The first stage was heated to 40 °C and poured over the second stage. The W/O emulsion from the second stage was then mixed under agitation until a W/O/W emulsion was formed (Figure 1).

### Emulsion stability and safety assessment

The emulsion was assessed for organoleptic characteristics (color, odor, and appearance) and physical instability parameters, including phase separation, precipitation, and turbidity (Reed et al., 2013). Stability was evaluated under different storage conditions: room temperature (± 2 °C), elevated temperature (37 °C ± 2 °C), refrigeration (4 °C), freezing (−20 °C), light exposure, and freeze–thaw cycles (Aucouturier et al., 2001; Coffman et al., 2010; Pulendran et al., 2021). Temperature conditions were continuously monitored throughout the study.

Xanthan emulsions (without protein) were analyzed for viscosity using a Haake Rheostress RS 150 rheometer coupled to a Haake DC 50 temperature controller. Measurements were performed at 25 °C under shear rates ranging from 0.01 to 500 $s^{-1}$ (Ishizaka et al., 1983). Formulations included 0.3% and 0.5% xanthan aqueous solutions and xanthan emulsions

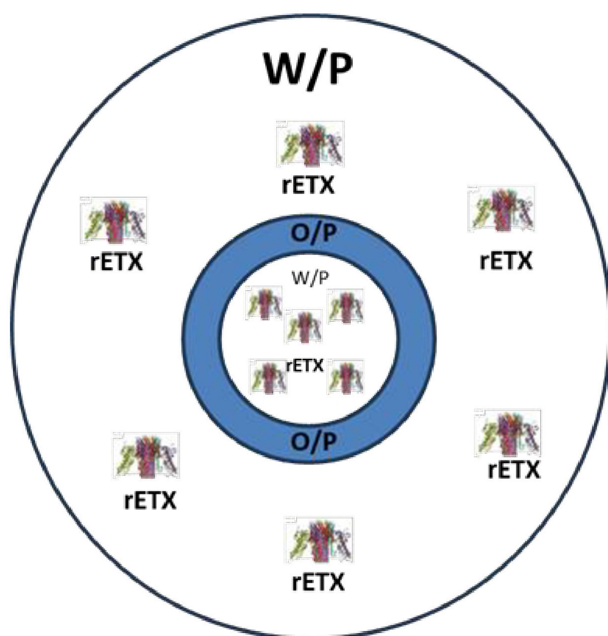



**Figure 1.** Representation of the Recombinant epsilon toxin (rETX) triple emulsion. Water phase (W/P) was formulated by mixing the Xanthan gum with rETX, and oil phase (O/P) was formulated using Montanide ISA 50 V2 with rETX, giving the W/O/W emulsion.

subjected to thermal treatments (121 °C for 20 min or 90 °C for 1 h).

Syringeability was evaluated to determine injectability and plunger force requirements (Boyman & Sprent, 2012). For horses and cattle, a 5 ml syringe (2 ml test volume) fitted with a 25 × 8 mm needle was used. For mice, a 1 ml syringe (150 µl test volume) fitted with a 13 × 0.45 mm needle was employed.

For safety assessment, 150 µl of the adjuvant emulsion was administered subcutaneously to five mice, and 2 ml was administered intramuscularly to five horses and five cattle. Animals were monitored daily for seven days for local and systemic adverse reactions, particularly at the injection site, according to MAPA guidelines (Takeuchi & Akira, 2010).

## Heifers vaccination

The study involved 15 Aberdeen Angus heifers (≈1.5 years old) from a commercial farm in southern Brazil (32°00'29" S, 53°30'42" W), with no history of clostridial vaccination. . The animals were maintained under extensive grazing conditions on native pasture, with free access to water and mineral supplementation, and without confinement or concentrated feeding. The animals were divided into three experimental groups (five per group) and inoculated intramuscularly (IM) on days 0 and 21. Group 1 - Control group, received 2ml of the emulsion without antigen (PBS and adjuvant emulsion); Group 2 - vaccinated with the adjuvant emulsion of 200 µg rETX, 2 ml dose; Group 3 - vaccinated with 10% aluminum hydroxide Alhydrogel® (Brenntag Nordic, Haslev, Denmark) containing 200 µg rETX, 2 ml dose. Serum samples were collected on days 0, 14, 21, and 32, and stored at -20 °C until use.

## Serum IgG dynamics

Serum IgG dynamics against rETX were studied. Antibody responses with modifications were monitored using an in-house indirect ELISA with rETX as the antigen, as described by Moreira et al. (2016). Plates were coated with rETX at 100 ng/well; sera were diluted 1:800 in PBS-T and applied in duplicates; the anti-bovine peroxidase-conjugated antibody (Sigma-Aldrich) was diluted 1:10,000. Optical density (OD) was measured at 492 nm using a microplate reader (Multiskan MCC / 340 MKII), and an intra-plate control ELISA was performed.

## Cytokine transcription

For cytokine transcription analysis, 10 ml of blood was collected from each heifer by jugular venipuncture using BD Vacutainer® needles (Becton Dickinson) into tubes containing 0.38% sodium citrate. The samples were centrifuged at 500 × *g* for 30 minutes using a Sorvall RC-6 Plus® centrifuge (Langenselbold) to isolate peripheral blood mononuclear cells (PBMCs), following the protocol described (Leite et al., 2004). Subsequently, PBMCs from three cattle were combined to create one pool, resulting in three independent pools used as biological replicates for statistical analysis.

Subsequently, PBMCs were cultured (2 x $10^6$ cells/ml) in 24-well plates (Kasvi) with RPMI 1640 medium (ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Sigma®) and antibiotics/antifungals (Penicillin 10,000 IU/ml, Streptomycin 10 mg/ml, Amphotericin B 25 mg/ml, Sigma) in 24-well plates (TPP®), and incubated for 24 h at 37 °C with 5% $CO_2$. The cells were then stimulated with concanavalin A (Sigma) at 2.5 µg/ml, rETX at 10 µg/ml, or adjuvant emulsion + rETX (10 µg/ml) for 24 h at 37 °C with 5% $CO_2$. On day three, the cells were harvested in TRIzol® (Invitrogen), and RNA extraction was performed using the TRIzol protocol. For cDNA synthesis and subsequent quantitative PCR (qPCR), 400 ng of total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA), according to the manufacturer's protocol.

qPCR assays were conducted with 1 µl of cDNA, 5 µl of GoTaq® qPCR Master Mix, 0.25 µl of each primer (Table 1), and 3.5 µl of RNase-free water, on a CFX96/BioRad® qPCR system. The mRNA transcription levels of Interleukin 2 (*il2*), Interleukin 15 (*il15*), and Interleukin 17 (*il17*) were measured, using β-actin as the reference gene. Each sample was analyzed in triplicate. The relative mRNA transcription of the target genes was determined by comparing the threshold cycle (Ct) values with the β-actin reference gene, and relative transcription was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Fold changes in gene transcription were calculated by comparing the Ct values of the target genes to those obtained from cells incubated with culture medium alone (controls).

## Statistical analysis

Data was analyzed using the software GraphPad Prism 7 (GraphPad®). IgG values, obtained by indirect ELISA, were submitted to analysis of variance (Two-way ANOVA), and post-hoc comparisons were made by the Tukey test. The relative

**Table 1.** Target genes and primers (5′ to 3′) used to determine gene transcription levels in PBMCs of vaccinated and control heifers.

| Gene | Forward (5´-3´) | Reverse (5´-3´) |
|---|---|---|
| *il2* | CCTCGAGTCCTGCCACAATG | CCGTAGAGCTTGAAGTAGGTGC |
| *il15* | CATATTTGAGAAGTACTTCCATCCAG | GAAGTGTTGATGAACATTTGCAC |
| *il17* | GGACTCTCCACCGCAATGAG | GATACAGCCTGAGTGGCTGC |
| *B - actin* | TGTCCACCTTCCAGCAGATG | CTAGAAGCATTTGCGGTGGA |

transcription levels in PBMCs were analyzed by one-way ANOVA followed by Dunnett's test. Significance was set at $p<0.05$. Asterisks (*) indicate a significant difference ($p<0.05$) between the supplemented and control groups.

## Results

### Quality, stability, and safety assessment of the xanthan-based adjuvant emulsion

The xanthan gum exhibited no evidence of starch contamination, as demonstrated by the absence of color change in the iodine test following the addition of Lugol's solution. Furthermore, microbiological evaluation revealed no visible microbial growth on blood agar or YPD agar plates after incubation, confirming the microbiological purity of the polysaccharide and the absence of detectable bacterial or fungal contamination under the experimental conditions.

The emulsions exhibited stable organoleptic properties throughout the evaluation period, maintaining uniform color, characteristic odor, and homogeneous appearance, with no evidence of phase separation, precipitation, or turbidity under any storage condition tested. Stability assessments performed at room temperature, elevated temperature (37 °C), refrigeration (4 °C), freezing (−20 °C), light exposure, and during freeze–thaw cycles demonstrated preservation of physicochemical integrity, confirming formulation robustness across environmental stresses.

Rheological analyses revealed non-Newtonian shear-thinning behavior for xanthan solutions and emulsions, with viscosity decreasing as shear rate increased from 0.01 to 500 $s^{-1}$ at 25 °C. Xanthan aqueous solutions at 0.5% displayed higher viscosity compared to 0.3% formulations, while thermal treatments (121 °C for 20 min or 90 °C for 1 h) did not compromise the structural consistency of xanthan emulsions. Syringeability tests indicated satisfactory injectability for all formulations, with smooth plunger displacement and absence of needle obstruction in both syringe systems, supporting their suitability for administration in large animals and mice.

The safety evaluation of the adjuvant emulsion was performed through daily clinical monitoring during the seven-day post-inoculation period, encompassing both local and systemic parameters. Local reactogenicity was assessed at the injection site by inspection and palpation for the presence of erythema, edema, nodules, induration, ulceration, increased local temperature, and pain response upon handling. Systemic safety was evaluated through observation of general behavior, posture, locomotion, grooming, feed and water intake, body weight variation, and the presence of clinical signs such as lethargy, piloerection, respiratory alterations, diarrhea, or fever. Adverse effects were defined as any persistent or progressive deviation from baseline clinical status temporally associated with inoculation, including measurable inflammatory reactions at the injection site or systemic clinical abnormalities. The absence of adverse reactions was determined by the lack of detectable local inflammatory changes and by the maintenance of normal physiological and behavioral parameters throughout the observation period, indicating no evidence of acute local or systemic toxicity attributable to the adjuvant emulsion.

### IgG immune response

Both groups, 2 (W/O/W adjuvant) and 3 $Al(OH)_3$, responded to vaccination by exhibiting significantly higher specific total IgG anti-rETX levels than Group 1 (control) at all assessed time points. No increase in IgG levels against rETX was noted in the control group, indicating that the animals had no exposure to *C. perfringens* during the experimental period. Group 2, W/O/W adjuvant, demonstrated a significant difference ($p < 0.01$) in serum IgG levels against rETX on days 14, 21, and 32, being 2.5-, 4.4-, and 3.6-fold increases, respectively, compared to the control group. Also, it demonstrated a significant difference ($p < 0.05$) in serum IgG levels against rETX on days 21 and 32 when compared to Group 3, which received aluminum hydroxide as an adjuvant (Figure 2).

### Cytokine transcription

Cytokine transcription levels in the PBMCs of vaccinated animals were evaluated by qPCR on day 32 of the experiment, which was 11 days following the booster. PBMCs from cattle vaccinated with the adjuvant emulsion and aluminum hydroxide, when stimulated with rETX, exhibited increased gene transcription levels of *il2*, *il15*, and *il17* compared to the control group (unvaccinated). In comparison to the control group, the adjuvant emulsion and aluminum hydroxide groups showed increases in *il2* transcription of 5.3-fold and 1.4-fold, respectively. *il15* transcription rose by 7.6-fold in the adjuvant emulsion group and by 2-fold in the aluminum hydroxide group. For *il17*, transcription levels were 13.6-fold and 7.6-fold higher in the adjuvant emulsion and aluminum hydroxide groups, respectively (Figure 3).

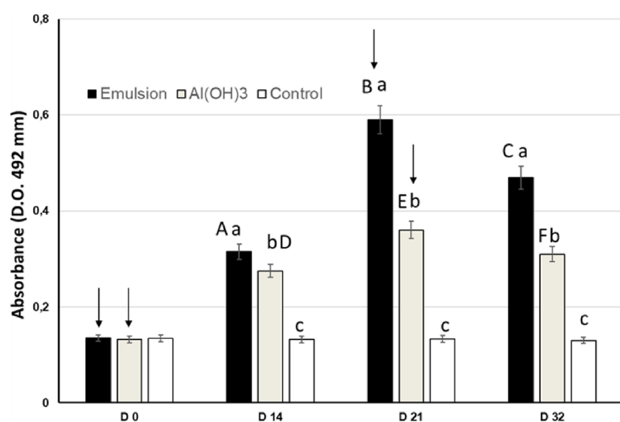



**Figure 2.** Dynamics of total serum IgG-specific rETX. The data represent the mean ± standard error of the mean (SEM) absorbance values for individual sera for anti-rETX-specific IgG in experimental groups. Arrows indicate vaccination days. Capital letters indicate statistical (p≤0.05) difference among the same group in different days, lowercase letters indicate statistical (p≤0.05) difference between groups among experimental days, evaluated by two-way analysis of variance (ANOVA) followed by Sidak for multiple comparison tests.

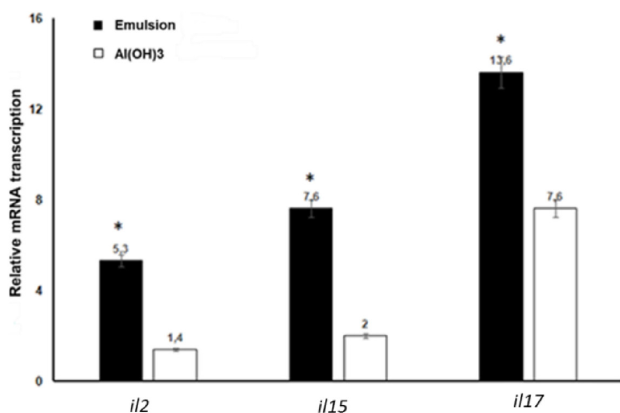



**Figure 3.** Relative cytokine transcription in PBMCs. Data are presented as the mean (± standard error) of *il2*, *il15*, and *il17* mRNA expression in PBMCs from heifers vaccinated with rETX on experimental day 32. Asterisk (*) indicates statistical difference (p < 0.05).

## Discussion

In recent years, several innovative combination adjuvants have been developed, typically incorporating two or three distinct immunostimulatory components to achieve synergistic effects (Ananya et al., 2023; Di Pasquale et al., 2015; Zarandi et al., 2025). In this context, we developed and evaluated Xenoil as a proof of concept for a next-generation adjuvant platform for recombinant protein–based vaccines. Throughout the experimental period, clinical monitoring revealed no adverse effects attributable to either the evaluated adjuvant or the vaccination procedure, confirming the formulation's safety under experimental conditions.

Evaluation of the vaccine-induced immune response demonstrated that animals vaccinated with the Xenoil emulsion exhibited significantly higher anti-rETX IgG levels compared with those receiving aluminum hydroxide. Although neutralizing antibody titers were not directly measured, these results are encouraging, as the emulsion-vaccinated group showed significantly higher antibody levels than both the aluminum hydroxide and control groups. Similar ELISA titers were reported by Moreira et al. (2016), who demonstrated effective neutralization of epsilon toxin in a murine model at comparable antibody levels. Although interspecies differences must be considered, the comparable titers observed here suggest that Xenoil may elicit functionally relevant humoral responses.

Water-in-oil emulsions are well known to activate the innate immune system, providing essential signals that support the development of adaptive immune responses (Aucouturier et al., 2002; Coffman et al., 2010). Their depot effect promotes antigen retention at the injection site, prolongs antigen availability, and enhances recruitment of antigen-presenting cells. Xanthan gum, one of the components of the emulsion tested in this study, is a polysaccharide produced by *Xanthomonas* spp. (Borges & Vendruscolo, 2008; Sutherland, 2001). Its immunostimulatory properties were first described in the 1980s (Ishizaka et al., 1983), and later studies demonstrated its recognition by Toll-like receptor 4 (Takeuchi et al., 2009). The combined presence of xanthan gum and oil suggests a synergistic adjuvant effect, resulting in a more robust antibody response against rETX. Emulsion-based adjuvants have been associated with mixed immune profiles, including Th1- and Th2-related responses, depending on formulation and antigen context (O'Hagan & Valiante, 2003; Reed et al., 2013). Worth noting that our group and others have reported a mixed Th1/Th2 immune response using oil-based adjuvants (Ciabattini et al., 2016; Cui et al., 2024; Dummer et al., 2014; Habjanec et al., 2008). However, using Xenoil, we observed a vaccinal immune response towards Th1/Th17. Thus, one might speculate that xanthan might be playing a role in the Th1/Th17 response.

By contrast, aluminum hydroxide, one of the most widely used adjuvants, primarily acts by forming antigen depots and facilitating uptake by antigen-presenting cells. Its mechanism involves phagocytosis of aluminum salts, activation of the NALP3 inflammasome, and caspase-1–mediated production of proinflammatory cytokines (Eisenbarth et al., 2008; Reed et al., 2009). However, aluminum-based adjuvants are generally considered weak inducers of cellular immunity and may be suboptimal for recombinant protein antigens (Di Pasquale et al., 2015).

Cytokine profiling demonstrated that vaccination with the Xenoil triple emulsion induced a coordinated immune activation pattern characterized by upregulation of *il2*, *il15*, and *il17*. IL-2 plays a central role in T-cell proliferation, differentiation, and memory formation (Bachmann & Oxenius, 2007; Gaffen & Liu, 2004; Li & Pauza, 2015), while IL-15 supports memory T-cell survival, NK-cell activation, and B-cell differentiation (Poon et al., 2009; Saikh et al., 2008). IL-17, predominantly produced by Th17 cells, contributes to antigen-presenting cell recruitment, germinal center maintenance, and high-affinity antibody production

(Bystrom et al., 2013; Mitsdoerffer et al., 2010). Th17-associated cytokines have been linked to improved vaccine efficacy (Stoppelenburg et al., 2014).

Taken together, these findings highlight Xenoil as a promising next-generation adjuvant for recombinant clostridial vaccines. The enhanced immunogenicity observed suggests potential applicability both for improving existing vaccine formulations and for the development of new recombinant vaccines, as well as for evaluation in other relevant animal models.

This study has limitations, including the lack of direct toxin neutralization assays and the relatively short experimental period that precluded assessment of long-term immune memory, as well as the limited experimental sample design. Nevertheless, the results clearly demonstrate the immunomodulatory potential of the xanthan gum–based W/O/W emulsion in cattle. Future studies should focus on evaluating neutralizing activity, protective efficacy under field conditions, and durability of immune responses.

## Conclusions

These results highlight the xanthan gum–based W/O/W emulsion as a promising adjuvant candidate, warranting further studies to confirm its protective efficacy and long-term immunological benefits.

## Conflict of interests

The authors declare that they have no conflict of interest.

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