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Preparation and Evaluation of a Combined Inactivated Vaccine Against *Corynebacterium pseudotuberculosis*, *Trueperella pyogenes*, and *Staphylococcus aureus*

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Abstract

Background: Caseous lymphadenitis (CLA) is a significant infectious disease in sheep and goats, traditionally associated with *Corynebacterium pseudotuberculosis*. However, recent evidence highlights the polymicrobial nature of CLA, with *Trueperella pyogenes* and *Staphylococcus aureus* frequently co-isolated from lesions. Current vaccines mainly target single pathogens, and combined inactivated vaccines against all three bacteria are lacking. Methods: We developed a trivalent inactivated vaccine using strains CpS1, TpS1, and SaS9, inactivated with 0.2% formalin and formulated with either PBS, Freund's adjuvant, or aluminum hydroxide adjuvant. Vaccine quality, safety, and immunogenicity were evaluated in experimental goats. The aluminum hydroxide-adjuvanted vaccine was further assessed for protective efficacy via challenge with individual pathogens and in field trials on goat and sheep farms. Results: All formulations induced specific antibodies against the three pathogens, with Freund's adjuvant eliciting the highest level, while aluminum hydroxide provided a favorable safety profile and induced antibody levels that were numerically lower than but not statistically distinguishable from the Freund's adjuvant group at most time points; however, the small group size (n = 6) limited the statistical power to detect differences between adjuvants. The aluminum hydroxide-adjuvanted vaccine conferred protection rates of 66.7% against *C. pseudotuberculosis*, 83.3% against *T. pyogenes*, and 83.3% against *S. aureus* in challenge trials. In field studies, vaccination was associated with a lower observed incidence of CLA, from 12.5% in unvaccinated controls to 2.5% in vaccinated goats and from 8% to 2% in vaccinated sheep within 90 days post-immunization. Conclusions: The trivalent inactivated vaccine with aluminum hydroxide adjuvant showed promising immunogenicity and preliminary protective efficacy against experimental challenge and was associated with a lower observed overall abscess incidence under the investigated field conditions.

Keywords: caseous lymphadenitis; *C. pseudotuberculosis*; *T. pyogenes*; *S. aureus*; inactivated vaccine; combined vaccine; aluminum hydroxide adjuvant; goat; sheep

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1. Introduction

With increasing awareness of the pathogenic impact of caseous lymphadenitis (CLA) in sheep and goats, greater attention has been directed toward its prevention [1–3]. In the early stages of CLA, small nodules or granulomas are often concealed by wool or are too minute to be detected [1]. As the disease progresses, typical necrotic purulent foci develop in the middle and late stages [1,4]. Once irreversible suppuration occurs, effective treatment becomes challenging, and spontaneous resolution usually requires surgical drainage or natural rupture of the abscess cavity [5]. However, even after manual intervention or spontaneous rupture, viable pathogens may persist in the host and cause relapse after a period of time [5,6]. Moreover, during both artificial and natural pus discharge, the environment and other animals can become contaminated, facilitating further transmission of the disease [1−4]. Therefore, vaccination remains the optimal strategy for the effective control of CLA.

Inactivated vaccines are widely used in veterinary practice owing to their ease of preparation and favorable safety profiles [6–9]. Considerable research has been conducted on inactivated vaccines against *Corynebacterium pseudotuberculosis* for CLA, but most of these immunization trials have been carried out in mice, with relatively limited data on immune responses in sheep and goats [10,11]. Furthermore, few studies have addressed the development and evaluation of inactivated vaccines targeting *Trueperella pyogenes* and *Staphylococcus aureus* in CLA [12]. Recent reviews have emphasized that protection against CLA remains incompletely understood and that vaccine efficacy is influenced by antigen, adjuvant, route of administration, animal model, and protection endpoint [13,14]. Current commercial vaccines are predominantly monovalent, targeting *C. pseudotuberculosis* alone, and have shown limited efficacy in fully preventing disease [13,15].

Classical CLA is primarily caused by *C. pseudotuberculosis*. However, *T. pyogenes* and *S. aureus* have also been frequently isolated from CLA-like abscesses in sheep and goats, either as primary pathogens, co-infections, or secondary invaders. Although individual abscesses often contain a single predominant pathogen, the species may vary among different animals, farms, and regions. Therefore, CLA is polymicrobial at the population level, and a trivalent vaccine containing all three major pathogens may provide broader protection than a monovalent vaccine. Thus, the present study aimed to develop a combined inactivated vaccine for the prevention of CLA in sheep and goats, and to evaluate its immunogenicity, safety, and preliminary protective efficacy under laboratory and field conditions. These results may provide useful information for the development of inactivated vaccines against CLA.

2. Methods

2.1. Preparation of Inactivated Combined Vaccine

C. pseudotuberculosis CpS1, *T. pyogenes* TpS1, and *S. aureus* SaS9 were separately inoculated into three flasks containing 100 mL of BHI broth supplemented with 5% fetal bovine serum and incubated at 36 ± 1 °C with shaking at 200 rpm for 24 h, corresponding to the mid-to-late exponential phase (OD600 ≈ 1.8–2.2 for CpS1, TpS1, and SaS9). Commercial formalin (37% *w*/*v* formaldehyde, methanol-stabilized) was then added to each culture to a final concentration of 0.2% (*v*/*v*), corresponding to approximately 0.75 g/L formaldehyde. The cultures were then incubated at 36 ± 1 °C with shaking at 200 rpm for 24 h for inactivation. To confirm complete inactivation, 100 μL of each inactivated suspension was spread onto BHI serum agar plates in triplicate and incubated at 36 ± 1 °C for 48 h. In addition, 1 mL was inoculated into 10 mL of BHI broth and incubated for 7 days. No growth was observed. The validation was repeated three times, and the final formulated vaccine was also tested. Residual formaldehyde was assessed using the acetylacetone

colorimetric method (linear range 0.25–100 μg/mL; limit of detection ≈ 0.25 μg/mL) and was below the detection limit. The final formulated vaccine contained residual formaldehyde below the detection limit (<0.25 μg/mL). The concentration of inactivated suspensions was expressed as pre-inactivation concentration (2×10^{10} CFU/mL). The bacterial cultures were transferred to sterile centrifuge tubes and centrifuged at 5000 rpm for 10 min. The supernatant was discarded, and the pellets were resuspended in an equal volume of sterile PBS. This washing step was repeated three times. After inactivation and washing, the antigens were mixed at a volume ratio of 1:1:1 based on equal pre-inactivation bacterial concentrations. Three vaccine formulations were prepared by combining the mixed antigen with sterile PBS, Freund's adjuvant, or aluminum hydroxide adjuvant.

Alhydrogel adjuvant 2% (InvivoGen, San Diego/Paris; cat. no. vac-alu-250; aluminium hydroxide gel, CAS 21645-51-2) was used as the aluminium adjuvant stock. According to the manufacturer, the preparation contains 9.0–11.0 mg/mL aluminium (expressed as elemental Al). This yielded a final adjuvant concentration of 20 mg/mL Al_2O_3 equivalent, corresponding to ~10.6 mg Al/mL in the adjuvant phase and ~15.9 mg elemental aluminium per 3 mL dose. Each bacterial suspension was adjusted to 2×10^{10} CFU-equivalents/mL (pre-inactivation concentration) before inactivation and washing. For the Freund's adjuvant group, complete Freund's adjuvant was used for the prime immunization (D0) and incomplete Freund's adjuvant for the booster immunization (D14). Emulsification of Freund's adjuvant vaccines was performed using a three-way stopcock and syringes; aluminum hydroxide adjuvant was mixed with antigen under stirring at 300 rpm for 10 min using a magnetic stirrer. The finished aluminum hydroxide-adjuvanted vaccine was adjusted to pH 7.0 ± 0.2 and stored at 2–8 °C, protected from light; freezing was avoided. All laboratory experiments were conducted using a single vaccine batch prepared from one antigen master mix.

2.2. Vaccine Quality Control

2.2.1. Emulsion Stability Test

A 100-μL aliquot of each emulsified vaccine was dropped onto the surface of clean water in a Petri dish. The vaccine was considered well-emulsified if it remained as a round droplet without spreading into a cloud-like pattern within a few minutes.

2.2.2. Viscosity Test

Using a 1-mL pipette with internal diameters of 1.2 mm (upper) and 2.7 mm (lower), 1.0 mL of each Freund's adjuvant vaccine was aspirated at room temperature. The pipette was held vertically, and the time required for 0.4 mL of vaccine to flow out was recorded; less than 8 s was considered acceptable.

2.2.3. Accelerated Physical Stability and Emulsion Stability Assessment

Each oil-adjuvanted vaccine was centrifuged at 3000 rpm for 15 min and examined for phase separation.

2.2.4. Safety Test

Six experimental goats per group were subcutaneously injected with twice the immunizing dose of each vaccine formulation; the control group ($n = 6$) received an equal volume of sterile PBS. The health status of all goats was monitored continuously for 2 weeks post-injection.

2.3. Animal Screening and Health Criteria

All animals used in this study were clinically examined by a veterinarian before enrollment and confirmed to be healthy, with normal body temperature, no visible abscesses or lesions, and normal appetite and behavior. Serum samples were collected and tested for antibodies against *C. pseudotuberculosis*, *T. pyogenes*, and *S. aureus* using the ELISA kits described below, and only seronegative animals were included. Nasal swabs and any suspicious skin lesions were cultured on BHI serum agar to confirm the absence of the three target pathogens. Animals with positive serology or positive bacterial culture were excluded.

2.4. Immunogenicity Evaluation of Different Vaccines

Twenty-four healthy male dairy goats of similar body weight were randomly divided into four groups ($n = 6$ per group). Randomisation was performed using a computer-generated random number table. Three groups received vaccines and one group served as the control. Each animal was vaccinated subcutaneously in the neck at Day 0 (D0) and Day 14 (D14) with 3 mL of vaccine or sterile PBS. Blood samples were collected from the jugular vein at D0, D7, D14, D28, and D60, and sera were separated. All laboratory personnel performing the ELISA assays were blinded to the group allocation until the completion of all measurements. Grouping and vaccination schemes are shown in Table 1. Morbidity was defined as the presence of a palpable abscess. Protection was defined as the absence of abscess formation. The protection rate was calculated as (number of protected animals/total number of vaccinated animals) × 100%. Humane endpoints were defined as body weight loss > 20%, inability to stand, or severe respiratory distress.

Table 1. Grouping and vaccination protocol of experimental goats.

Group	Number	Injection
NAV	6	Inactivated trivalent vaccine with PBS (no adjuvant)
FA	6	Inactivated trivalent vaccine with CFA (prime) and IFA (booster)
ATH	6	Inactivated trivalent vaccine with aluminum hydroxide adjuvant
NC	6	Sterile PBS

Specific antibodies against *C. pseudotuberculosis* and *S. aureus* were detected using commercial qualitative ELISA kits (Goat *Corynebacterium pseudotuberculosis* Antibody ELISA Detection Kit, Jiangsu Jingmei Biotechnology Co., Ltd., Yancheng city, Jiangsu province, China; Goat *Staphylococcus aureus* Antibody ELISA Detection Kit, Shanghai Tongwei Industrial Co., Ltd., Jinshan District, Shanghai, China) according to the manufacturer's instructions. For both kits, 10 µL of serum was added to 40 µL of sample diluent, and the plates were incubated at 37 °C. After washing, HRP-conjugated reagent was added, followed by substrates A and B, and the reaction was stopped with stop solution. The OD450 value was measured. Each plate included two negative control wells, two positive control wells, and one blank well. Assay validity required a mean positive control OD450 > 1.00 and a mean negative control OD450 < 0.20 for the *C. pseudotuberculosis* kit, and a mean positive control OD450 ≥ 1.00 and a mean negative control OD450 ≤ 0.15 for the *S. aureus* kit. The cutoff was calculated as the mean negative control OD450 + 0.15. Samples with OD450 below the cutoff were considered negative, and those at or above the cutoff were considered positive. Antibody levels are reported as OD450 values.

Anti-*T. pyogenes* antibodies were measured using a laboratory-developed indirect ELISA. The coating antigen was prepared by ultrasonic disruption of washed bacterial cells (Sonics & Materials, VCX-750, 20 kHz, 40% amplitude, 10 min on ice) followed by centrifugation at 12,000× *g* for 30 min at 4 °C and filtration through a 0.22-µm membrane. The protein concentration of the supernatant was determined by BCA assay (Thermo Fisher Scientific, Waltham, MA, USA) and was 13.28 µg/mL. The optimal coating

concentration was determined by checkerboard titration to be 33.2 ng/mL (1:400 dilution of the stock). Briefly, 96-well plates were coated with antigen at the optimal concentration of 33.2 ng/mL (50 μL per well) at 37 °C for 2 h and then at 4 °C overnight. After washing five times with PBST, the plates were blocked with 50 μL of 10% BSA at 37 °C for 1 h. Serum samples were diluted 1:200 in PBS, added at 50 μL per well, and incubated at 37 °C for 1 h. After washing, HRP-conjugated rabbit anti-goat IgG (diluted 1:10,000 in PBST) was added at 50 μL per well and incubated at 37 °C for 1 h. After washing, 50 μL of TMB substrate was added, and the plates were incubated at 37 °C in the dark for 15 min. The reaction was stopped with 50 μL of 20% HCl, and OD450 was measured immediately.

The cutoff was determined using 32 serum samples from neonatal lamb cord blood collected before colostrum intake (mean OD450 = 0.264, SD = 0.034). These samples were confirmed negative for antibodies against *C. pseudotuberculosis* and *S. aureus* using the commercial ELISA kits described above. The cutoff was defined as the mean + 3 SD = 0.366; samples with OD450 ≥ 0.366 were considered positive. The use of ovine cord-blood sera for cutoff determination was based on the following rationale: (i) neonatal cord blood collected before colostrum intake contains no maternal antibodies, providing a true seronegative reference; (ii) sheep and goat IgG share high structural homology, and the HRP-conjugated anti-goat IgG used in this assay cross-reacts strongly with sheep IgG; and (iii) the cutoff was defined as mean + 3SD, a conservative threshold. To confirm the applicability of this cutoff to goat samples, 20 serum samples from clinically healthy goats that tested negative by the commercial *C. pseudotuberculosis* and *S. aureus* ELISA kits were evaluated in the *T. pyogenes* ELISA. The mean OD450 of these goat sera was 0.271 ± 0.039, and all 20 samples fell below the cutoff of 0.366, confirming that the ovine-derived cutoff is applicable to goat samples.

The assay showed good sensitivity (a positive serum remained positive at 1:1280 dilution), specificity (no cross-reactivity with positive sera against Streptococcus ovis, orf virus, sheep pox virus, *C. pseudotuberculosis*, *S. aureus*, or peste des petits ruminants virus), and repeatability (intra-assay CV < 7.5%, inter-assay CV < 9.1%).

2.5. Bacterial Isolation, Strain Selection, and Challenge Protection Test

The isolation and identification of bacteria from field abscess samples were performed as described in our previous study [16]. Briefly, abscess contents were cultured on 5% sheep blood agar under both aerobic and anaerobic conditions at 36 ± 1 °C for 24–48 h. Individual colonies with different morphologies were purified and identified by Gram staining and 16S rRNA gene PCR amplification using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), followed by sequencing and BLAST (v2.17.0) comparison. Isolates with ≥99% sequence homology were identified to species level. At least 5 individual colonies were picked per sample to detect mixed infections.

Based on this identification, three representative strains were selected for the present study: *C. pseudotuberculosis* CpS1, *T. pyogenes* TpS1, and *S. aureus* SaS9. All three strains were isolated from mandibular lymph node abscesses of goats in Shaanxi Province, China, in 2021. The strains were stored at −80 °C in BHI broth with 20% glycerol. The virulence of each strain was confirmed by experimental infection in goats, in which all three strains induced local abscesses at the inoculation site [16].

Thirty-six healthy male dairy goats of similar weight were randomly divided into six groups ($n = 6$) using a computer-generated random number table: three vaccinated groups and three control groups. The veterinarian responsible for daily clinical observation and abscess assessment was blinded to the vaccination status of the animals. At D0 and D14, the vaccinated groups received 3 mL of the aluminum hydroxide-adjuvanted trivalent inactivated vaccine subcutaneously in the neck; the control groups received sterile PBS.

At D28, the vaccinated groups were challenged subcutaneously in the neck with 1 mL of bacterial suspension (10^8 CFU/mL) containing *C. pseudotuberculosis* CpS1, *T. pyogenes* TpS1, or *S. aureus* SaS9, respectively. Similarly, the three control groups were challenged with the corresponding strains. Animals were observed for 30 days for local abscess formation and mortality. At necropsy, euthanasia, or unexpected death, lesion/abscess material from each challenged animal was aseptically collected for bacterial re-isolation. Samples were cultured on 5% sheep blood agar under aerobic and anaerobic conditions at 36 ± 1 °C for 24–48 h, identified by Gram staining and 16S rRNA gene PCR using primers 27F/1492R, and compared with the reference challenge strains CpS1, TpS1, and SaS9. A strain was considered confirmed if the sequence showed ≥99% homology with the corresponding challenge strain. Animals meeting pre-defined humane endpoints (body weight loss >20%, inability to stand, or severe respiratory distress) were euthanized by intravenous injection of pentobarbital sodium; if spontaneous death occurred before euthanasia, the carcass was subjected to immediate post-mortem examination. The grouping and challenge strains are shown in Table 2.

Table 2. Grouping and challenge strains for the protection test.

Group	Number	Vaccination	Challenge Strain
ATH-1	6	Trivalent $Al(OH)_3$ vaccine	CpS1
ATH-2	6	Trivalent $Al(OH)_3$ vaccine	TpS1
ATH-3	6	Trivalent $Al(OH)_3$ vaccine	SaS9
NC-1	6	PBS	CpS1
NC-2	6	PBS	TpS1
NC-3	6	PBS	SaS9

2.6. Field Immunization Trials

A field plate agglutination test (PAT) was developed as a rapid on-farm screening method to exclude animals with pre-existing antibodies before enrolment. Formalin-inactivated whole-cell antigens of *C. pseudotuberculosis*, *T. pyogenes*, and *S. aureus* were prepared as described in Section 2.1 and adjusted to OD600 = 1.880, 1.980, and 1.816, respectively. Serum samples were twofold diluted and mixed with the corresponding antigen at the optimized dilution: *C. pseudotuberculosis* at 1:4, *T. pyogenes* at 1:2, and *S. aureus* at 1:4. The antigen–serum mixtures were incubated at 37 °C for 30 min and read by naked eye.

A sample was considered positive for a given pathogen if visible agglutination or floccule formation was observed at or above the specified serum dilution; samples in which the mixture remained uniformly cloudy/smooth were recorded as negative. For every plate, three controls were included: (i) antigen control (antigen + PBS, no serum) to check auto-aggregation; (ii) positive control (hyperimmune or known positive caprine/ovine serum) to confirm antigen reactivity; and (iii) negative control (neonatal lamb cord serum or confirmed seronegative goat/sheep serum) to monitor nonspecific agglutination. A run was accepted only when the antigen control showed no agglutination, the negative control remained smooth, and the positive control showed clear agglutination.

The PAT was used for pre-enrolment screening only. A formal diagnostic validation against a reference standard (validated ELISA or bacterial culture) was not performed; therefore, diagnostic sensitivity, diagnostic specificity, and their 95% confidence intervals were not calculated. This limitation is acknowledged in the Discussion Section. Repeatability was monitored by including the same positive and negative controls on every plate and by retesting 10% of samples within the same run; no discrepant results were observed across the duplicated samples.

In total, 80 goat sera and 100 sheep sera were screened. Animals showing a positive PAT reaction to any of the three antigens were excluded from the corresponding field trial; only seronegative animals were enrolled. A confirmed CLA case was defined as an animal with a palpable abscess from which *C. pseudotuberculosis* was isolated. Abscesses from which only *T. pyogenes* or *S. aureus* was isolated were recorded separately as abscesses associated with other bacterial species and were not classified as confirmed CLA cases.

All animals were housed indoors under farm-simulated husbandry conditions with free access to feed and water.

Booster vaccination was performed on day 14 (D14). Groups were co-mingled on day 15 (D15) following a 24 h post-vaccination observation for adverse reactions. Co-mingling guaranteed identical housing conditions to assess authentic disease outcomes and vaccine practicality in vaccinated and control animals.

2.6.1. Field Trial in Goats

In a goat fattening farm in Baoji City, Shaanxi Province, which was positive for CLA, 80 animals aged 2.5 to 4 months were screened using the plate agglutination test, and only those negative for antibodies against *C. pseudotuberculosis*, *T. pyogenes*, and *S. aureus* were selected. The 80 animals were randomly divided into two groups (n = 40) using a computer-generated random number table and identified. The farm personnel and the veterinarian who recorded CLA incidence and collected abscess samples were blinded to the group allocation until the end of the 90-day observation period. The vaccinated group received 3 mL of the trivalent aluminum hydroxide-adjuvanted inactivated vaccine subcutaneously in the neck at D0 and D14; the control group received 3 mL of sterile PBS at the same time points. At D15, the two groups were merged and raised together under identical management. The incidence of CLA was recorded over 90 days under natural conditions.

2.6.2. Field Trial in Sheep

In a sheep fattening cooperative in Qingyang City, Gansu Province, which was positive for CLA, 100 animals aged 2 to 4 months were screened by plate agglutination as above, and only seronegative animals were enrolled. The animals were randomly divided into two groups (n = 50) using a computer-generated random number table. The farm personnel and the veterinarian who recorded CLA incidence and collected abscess samples were blinded to the group allocation until the end of the 90-day observation period. The vaccinated group received 3 mL of the trivalent aluminum hydroxide-adjuvanted vaccine at D0 and D14; the control group received sterile PBS. At D15, the two groups were merged and raised together. CLA incidence was recorded over 90 days.

2.7. Ethics Statement

All animal experiments were approved by the Animal Ethics Committee of Northwest A&F University (Approval No. DY2022-05-016). The study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Northwest A&F University and with international standards for animal welfare. Animals were housed under standard conditions with free access to water and feed. Humane endpoints were defined as body weight loss >20%, inability to stand, or severe respiratory distress; animals meeting these criteria were euthanized by intravenous injection of pentobarbital sodium. For field trials, informed consent was obtained from the farm owners. All efforts were made to minimize animal suffering in accordance with the 3Rs principles.

2.8. Statistical Analysis

Statistical analyses were performed using SPSS 26.0 software (IBM Corp., Armonk, NY, USA; MIXED procedure). Antibody OD450 values were analysed using a linear mixed-effects model with treatment, time, and their interaction (treatment × time) as fixed effects, and animal (nested within treatment) as a random intercept to account for the repeated measures. The covariance structure for the repeated measures was first-order autoregressive [AR(1)], selected on the basis of the Akaike information criterion (AIC) and residual diagnostics. Parameters were estimated by restricted maximum likelihood (REML), and denominator degrees of freedom were calculated using the Satterthwaite approximation. Post-hoc multiple comparisons of estimated marginal means were performed with Tukey's adjustment. For dichotomous outcomes (challenge protection and field incidence), groups were compared using Fisher's exact test (two-tailed). Relative risks (RR) and their 95% confidence intervals (CI) were reported as descriptive effect sizes: RR was calculated as the risk in the vaccinated group divided by the risk in the control group, and 95% CIs were obtained on the log scale by the Katz–Wald method [$\ln(RR) \pm 1.96 \times SE$, where $SE = \sqrt{(1/a + 1/c - 1/(a + b) - 1/(c + d))}$], with the Haldane–Anscombe 0.5 correction applied to each cell when a table contained a zero count. Because these intervals are large-sample approximations, they are not derived by inverting Fisher's exact test and may therefore not agree with it in sparse 2 × 2 tables; primary inference was accordingly based on Fisher's exact test. A p value < 0.05 was considered statistically significant. Graphs were generated using GraphPad Prism 8 and Microsoft Excel 2010.

3. Results

3.1. Inactivation Verification

After 24 h of culture on BHI serum agar, no bacterial growth was observed from any of the inactivated suspensions, confirming that 0.2% formalin for 24 h completely inactivated *C. pseudotuberculosis*, *T. pyogenes*, and *S. aureus*.

3.2. Vaccine Quality Control Results

Both complete and incomplete Freund's adjuvant vaccines formed stable droplets without spreading on water, indicating good emulsification. The time required for 0.4 mL of Freund's adjuvant vaccine to flow out was 6–7 s, within the acceptable limit of 8 s. No significant stratification was observed after centrifugation, indicating uniform emulsification and good physical stability.

Goats receiving PBS-adjuvanted vaccine, aluminum hydroxide-adjuvanted vaccine, or sterile PBS showed no fever, local reactions, or mortality within 2 weeks. In the complete and incomplete Freund's adjuvant groups, one goat in each group showed a transient temperature rise to 40.7–41 °C within 8 h, which returned to normal after 14 h. Injection sites in Freund's adjuvant groups showed slow absorption, with local swelling persisting for 3 days, but no deaths occurred. These results indicated that all three vaccine types were safe at twice the immunizing dose, although Freund's adjuvants caused local adverse reactions.

3.3. Immunogenicity of Different Vaccines

Antibody levels are expressed as OD450 values. All vaccinated groups showed significantly higher anti-*C. pseudotuberculosis* OD450 values than the control group from D7 to D60 ($p < 0.001$). Similar trends were observed for anti-*T. pyogenes* and anti-*S. aureus* OD450 values. It should be noted that OD450 values from different ELISAs are not directly comparable; therefore, comparisons among the three pathogens are based on dynamic trends within each assay.

3.3.1. Anti-*C. pseudotuberculosis* Antibody Levels and Dynamics (Figure 1A,B)

Anti-*C. pseudotuberculosis* OD450 values were analyzed using a linear mixed-effects model with treatment, time, and treatment × time interaction as fixed effects and animal as a random effect. Significant main effects of time ($F(1.482, 37.04) = 211.5$, $p < 0.0001$) and treatment ($F(3, 100) = 313.3$, $p < 0.0001$) were observed, along with a significant treatment × time interaction ($F(12, 100) = 23.95$, $p < 0.0001$), indicating that antibody dynamics differed among groups. Post-hoc analysis using Tukey's correction showed that all vaccinated groups had significantly higher OD450 values than the control group at D7, D14, D28, and D60 ($p < 0.001$). Among the vaccinated groups, the Freund's adjuvant group showed the highest OD450 values, followed by the aluminum hydroxide group, while the non-adjuvanted group showed the lowest. Within each group, OD450 values were highest at D28 and decreased by D60, but the magnitude of decline differed among groups, consistent with the significant interaction.

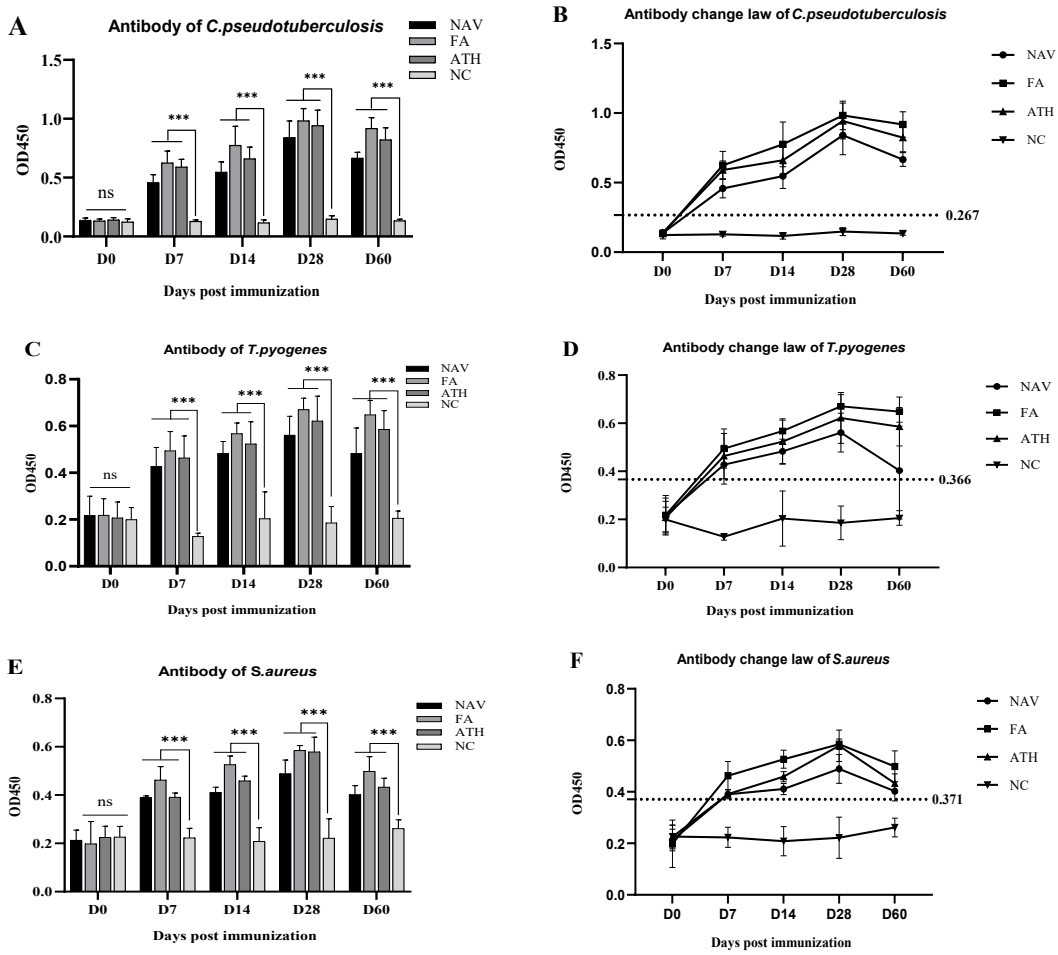


Figure 1. Antibody responses induced by different trivalent inactivated vaccines against *C. pseudotuberculosis*, *T. pyogenes*, and *S. aureus*. Data are presented as mean ± SD ($n = 6$ per group). Statistical comparisons were performed using a linear mixed-effects model with Tukey's post-hoc test. *** $p <$ 0.001 vs. control group. (**A**,**B**) Anti-C. *pseudotuberculosis* OD450 values at days 0, 7, 14, 28, and 60 post-vaccination. All vaccinated groups showed significantly higher OD450 values than the control group ($p < 0.001$). The Freund's adjuvant group exhibited the highest antibody levels, followed by the aluminum hydroxide group, while the non-adjuvanted group showed the lowest. OD450 values were highest at day 28 and decreased by day 60 but remained above day 14 levels. (**C**,**D**) Anti-*T. pyogenes* OD450 values. All vaccinated groups produced significantly higher OD450 values than controls from day 7 to day 60 ($p < 0.001$). The highest values occurred at day 28. By day 60, the non-

adjuvanted group showed a marked decrease approaching the cutoff, whereas the Freund's and aluminum hydroxide groups maintained relatively high levels. (**E**,**F**) Anti-*S. aureus* OD450 values. Vaccinated groups had significantly higher OD450 values than controls from day 7 onward ($p < 0.001$). At all time points, the Freund's adjuvant group showed the highest levels, followed by the aluminum hydroxide and non-adjuvanted groups. By day 60, OD450 values in all vaccinated groups had decreased markedly, approaching day 7 levels. OD450 values from different ELISAs are not directly comparable.

3.3.2. Anti-*T. pyogenes* Antibody Levels and Dynamics (Figure 1C,D)

Anti-*T. pyogenes* OD450 values were analyzed using the same mixed-effects model. Significant main effects of time ($F(2.975, 59.50) = 65.98$, $p < 0.0001$) and treatment ($F(3, 20) = 59.17$, $p < 0.0001$) were observed, along with a significant treatment × time interaction ($F(12, 80) = 9.077$, $p < 0.0001$). Post-hoc analysis using Tukey's correction showed that all vaccinated groups had significantly higher OD450 values than the control group from D7 to D60 ($p < 0.001$). Among the vaccinated groups, the Freund's adjuvant and aluminum hydroxide groups maintained relatively high levels at D60, whereas the non-adjuvanted group showed a marked decline approaching the cutoff.

3.3.3. Anti-*S. aureus* Antibody Levels and Dynamics (Figure 1E,F)

Anti-*S. aureus* OD450 values were analyzed using the same mixed-effects model. Significant main effects of time ($F(3.144, 62.88) = 116.7$, $p < 0.0001$) and treatment ($F(3, 20) = 78.26$, $p < 0.0001$) were observed, along with a significant treatment × time interaction ($F(12, 80) = 17.15$, $p < 0.0001$). Post-hoc analysis using Tukey's correction showed that all vaccinated groups had significantly higher OD450 values than the control group from D7 onward ($p < 0.001$). Among the vaccinated groups, the Freund's adjuvant group showed the highest levels at all time points, followed by the aluminum hydroxide and non-adjuvanted groups. By D60, OD450 values in all vaccinated groups had decreased markedly, approaching D7 levels.

3.3.4. Summary of Antibody Responses

All three formulations induced significant specific antibody responses against the three pathogens. Within each assay, the Freund's adjuvant group showed the highest OD450 values, while the aluminum hydroxide group showed intermediate levels and the non-adjuvanted group the lowest. However, because OD450 values from different ELISAs are not directly comparable, no conclusion can be drawn regarding the relative strength of antibody responses among the three pathogens. The significant treatment × time interactions observed for all three pathogens indicate that the adjuvants influenced not only the magnitude but also the persistence of antibody responses. The aluminum hydroxide-adjuvanted vaccine induced antibody levels that were intermediate between the Freund's and non-adjuvanted groups. At most time points, the aluminum hydroxide group showed numerically lower antibody levels than the Freund's adjuvant group, although the differences were not statistically significant ($p > 0.05$). It should be noted that this study was not designed or powered to formally test equivalence between the two adjuvants, and the absence of a statistically significant difference does not imply equivalence. However, given the small group size ($n = 6$ per group), this absence of statistical significance should not be interpreted as evidence of equivalent immunogenicity. The Freund's adjuvant group generally showed the highest numerical antibody levels.

3.4. Challenge Protection Test Results

In the CpS1 challenge group, the vaccinated group showed an observed protection rate of 66.7% (4/6; 95% CI: 22.3–95.7%), compared with 0% (0/6) in the control group. The

difference was not statistically significant (p = 0.0606, Fisher's exact test; descriptive RR = 0.33). In the TpS1 challenge group, the vaccinated group showed an observed protection rate of 83.3% (5/6; 95% CI: 35.9–99.6%), compared with 0% (0/6) in the control group, and the difference was statistically significant (p = 0.0152, Fisher's exact test; RR = 0.17). In the SaS9 challenge group, the vaccinated group also showed an observed protection rate of 83.3% (5/6; 95% CI: 35.9–99.6%), compared with 0% (0/6) in the control group, and the difference was statistically significant (p = 0.0152, Fisher's exact test; RR = 0.17). No mortality was observed in any vaccinated group (Table 3).

Table 3. Challenge protection test results.

Group	Number	Challenge Strain	Morbidity Rate	Mortality Rate	Protection Rate	Observed Protection Rate (95% CI)	p Value	RR (95% CI)
ATH-1	6	CpS1	33.3% (2/6)	0	66.7% (4/6)	66.7% (22.3–95.7%)	0.0606	0.33 (0.15–0.76)
ATH-2	6	TpS1	16.7% (1/6)	0	83.3% (5/6)	83.3% (35.9–99.6%)	0.0152	0.17 (0.03–0.94)
ATH-3	6	SaS9	16.7% (1/6)	0	83.3% (5/6)	83.3% (35.9–99.6%)	0.0152	0.17 (0.03–0.94)
NC-1	6	CpS1	100% (6/6)	16.7% (1/6)	/	/	/	/
NC-2	6	TpS1	100% (6/6)	16.7% (1/6)	/	/	/	/
NC-3	6	SaS9	100% (6/6)	0	/	/	/	/

RR = risk in the vaccinated group/risk in the control group; 95% CIs were calculated on the log scale by the Katz–Wald method with the Haldane–Anscombe 0.5 correction for zero cells and are reported as descriptive effect sizes (they are not derived by inverting Fisher's exact test). Primary inference was based on Fisher's exact test.

Two control animals died: one in NC-1 (CpS1) and one in NC-2 (TpS1). Both had severe local abscessation and systemic decline and met pre-defined humane endpoints before death; they died spontaneously before scheduled euthanasia. Available lesion material was cultured, and the corresponding challenge strains CpS1 and TpS1 were re-isolated and confirmed by 16S rRNA sequencing. Detailed systematic post-mortem records for these two animals were not preserved; therefore, only the re-isolation results and gross observations at the time of death are reported. This limitation is acknowledged in the Discussion Section.

3.5. Field Immunization Results

All animals were observed daily; animals showing typical CLA lesions were recorded as positive, and abscess contents were subjected to bacterial isolation and identification.

In the goat field trial, the vaccinated group had 0 confirmed *C. pseudotuberculosis* CLA cases (0/40, 0%) and 1 abscess associated with *S. aureus* (1/40, 2.5%). The control group had 1 confirmed *C. pseudotuberculosis* CLA case (1/40, 2.5%) and 4 abscesses associated with *S. aureus* (4/40, 10.0%). The overall abscess incidence (including both confirmed CLA and abscesses associated with other bacteria) was 2.5% (1/40) in the vaccinated group and 12.5% (5/40) in the control group, but the difference was not statistically significant (p = 0.2007; Fisher's exact test; descriptive RR = 0.20, 95% CI: 0.02–1.72).

In the sheep field trial, the vaccinated group had 0 confirmed *C. pseudotuberculosis* CLA cases (0/50, 0%) and 1 abscess associated with *S. aureus* (1/50, 2.0%). The control group had 0 confirmed *C. pseudotuberculosis* CLA cases (0/50, 0%), 2 abscesses associated with *T. pyogenes* (2/50, 4.0%), and 2 abscesses associated with *S. aureus* (2/50, 4.0%). The overall abscess incidence was 2.0% (1/50) in the vaccinated group and 8.0% (4/50) in the control group, but the difference was not statistically significant (p = 0.3622; Fisher's exact test; descriptive RR = 0.25, 95% CI: 0.03–2.18). The isolate from the vaccinated group was identified as *S. aureus*. In the control group, 2 isolates were *T. pyogenes* and 2 were *S. aureus*.

Notably, *T. pyogenes* was isolated only from control sheep, and no *T. pyogenes* was isolated from vaccinated sheep.

These field results should be interpreted as preliminary evidence due to the small number of events, the single farm per species, and the mixing of vaccinated and control animals after D15 (Figure 2).

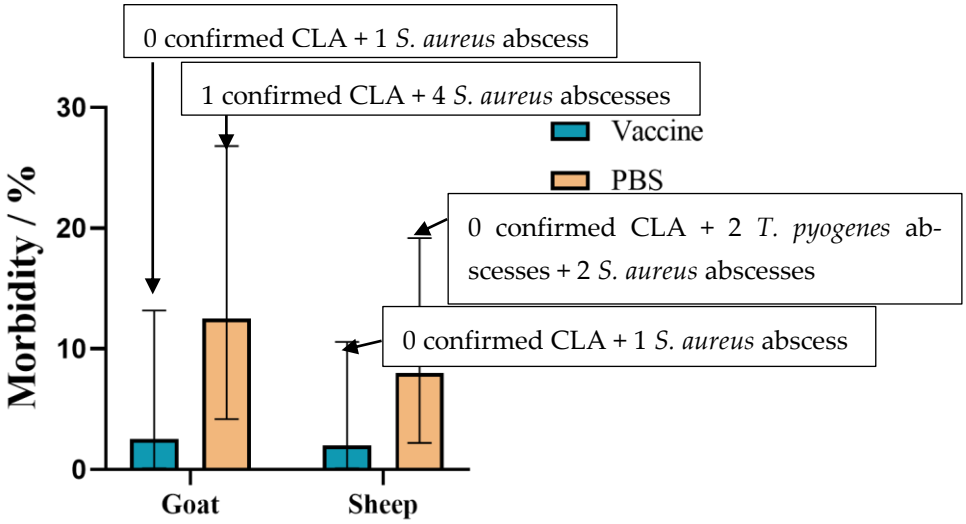


Figure 2. Field trial results in goats nd sheep. Confirmed CLA cases (*C. pseudotuberculosis* isolated from abscess material) and abscesses associated with other bacterial species (*S. aureus* or *T. pyogenes* isolated) are shown separately. Absolute case numbers are indicated above each bar. Vaccinated group: $n = 40$ (goats) or $n = 50$ (sheep); control group: $n = 40$ (goats) or $n = 50$ (sheep).

4. Discussion

In recent years, combined inactivated vaccines containing multiple antigens have become increasingly common in veterinary immunization [17], and the choice of adjuvant significantly affects the immunogenicity of inactivated vaccines [18]. To our knowledge, this is among the first studies to develop a trivalent inactivated vaccine against *C. pseudotuberculosis*, *T. pyogenes*, and *S. aureus* for the prevention of CLA, and we performed preliminary laboratory and field evaluations. Freund's adjuvant was used in this study only as an experimental comparator to provide a strong immunological benchmark. It is not a realistic formulation for veterinary commercial vaccines due to its strong local reactogenicity. Aluminum hydroxide was selected as the practical adjuvant because of its favorable safety profile, low cost, and suitability for veterinary use.

Freund's adjuvants are widely used for producing hyperimmune sera because they stimulate high levels of specific antibodies. In experimental immunogenicity evaluations, comparing test vaccines with Freund's adjuvant-adjuvanted formulations is a common approach [19,20]. Our immunogenicity results showed that the Freund's adjuvant vaccine consistently induced the highest antibody levels against all three pathogens at all time points. The aluminum hydroxide-adjuvanted vaccine induced antibody levels that were numerically lower than those of the Freund's adjuvant group at most time points, and the differences were not statistically significant. However, formal equivalence testing was not performed, and the small group size ($n = 6$ per group) limits the statistical power to detect true differences between the two adjuvants. Therefore, equivalence between the two adjuvants cannot be concluded from this study. Given that aluminum hydroxide is safe, inexpensive, and easy to prepare, it was selected for further protection and field trials. Within each assay, the dynamic trends differed. This may be attributable to inherent differences in the immunogenicity of surface antigens among these species.

Within each assay, the dynamic trends of antibody responses differed among the three pathogens. However, OD450 values from different ELISAs are not directly comparable, and no conclusion can be drawn regarding which antigen induced the strongest antibody response. The protection rate against CpS1 challenge was the lowest (66.7%), which raises the possibility that humoral immunity may not be the sole mechanism of protection against *C. pseudotuberculosis*, given its intracellular nature. However, this interpretation should be regarded as a hypothesis rather than a demonstrated mechanism. Several alternative explanations cannot be excluded, including the small challenge group size (n = 6 per group), differences in antigen quality among the three vaccine components, differences in challenge dose and virulence among the three strains, antigenic differences among the pathogens, assay characteristics, the possible contribution of cellular immunity (which was not assessed), and biological variability among individual animals. Further studies incorporating cellular immunity assays and larger group sizes are needed to test this hypothesis. In the sheep field trial, *T. pyogenes* was isolated only from control animals (2/50), and no *T. pyogenes* was isolated from vaccinated animals. However, due to the small number of field cases, no conclusion can be drawn regarding the field efficacy of the vaccine against *T. pyogenes*. For *S. aureus,* isolates were obtained from both vaccinated and control animals, suggesting that the vaccine did not completely prevent natural *S. aureus* infection. It should be noted that only one confirmed *C. pseudotuberculosis* case was identified across both field trials (in the goat control group), and the remaining field abscesses were associated with *S. aureus* or *T. pyogenes*. Therefore, the observed reduction in overall abscess incidence in vaccinated animals was driven primarily by a reduction in abscesses associated with *S. aureus* and *T. pyogenes*, rather than by a measurable reduction in confirmed *C. pseudotuberculosis* CLA cases within the 90-day observation period. Possible reasons include suboptimal antigen dosage or waning antibody levels over time, as by D60 the antibody levels for these two pathogens were approaching the cutoff. Regarding challenge strains, our earlier findings indicated that CLA lesions typically involve single-pathogen infections rather than mixed infections; therefore, we used single-strain challenges in this study.

Previous studies on CLA vaccines have largely focused on *C. pseudotuberculosis* alone, and most evaluations were performed in mice. The limited ovine and caprine trials indicated that monovalent *C. pseudotuberculosis* vaccines could not fully prevent natural CLA [21,22]. Based on our results and bacterial isolation from field-vaccinated animals that still developed CLA, we hypothesize that the residual incidence may be partly due to the absence of protection against *T. pyogenes* and *S. aureus* in monovalent vaccines. Although individual inactivated vaccines against *T. pyogenes* and *S. aureus* have been evaluated in mice [23–25], no direct comparison with monovalent vaccines was performed in this study, and the protection rates observed here are within the range reported for monovalent vaccines. Further comparative trials are needed to confirm the potential of the trivalent formulation for practical application.

It should be acknowledged that the single-pathogen challenge model does not fully reproduce the complexity of natural CLA, in which mixed infections or sequential exposure to different pathogens may occur. Single-pathogen challenges were used to allow standardized assessment and independent attribution of protection against each vaccine component. However, protection against mixed infection was not evaluated in this study. Future studies using mixed or heterologous challenge models are needed to confirm the field efficacy of the trivalent vaccine.

A limitation of this study is that the antigen concentration per dose was not experimentally determined after inactivation. The 1:1:1 ratio was based on equal pre-inactivation bacterial concentrations, and antigenic equivalence among the three components was not established. Equal CFU before formalin treatment does not necessarily represent

equivalent quantities of immunologically relevant antigen after inactivation and formulation. Future studies should include total protein quantification and antigenic mass determination at the time of formulation to optimize the antigen ratio and should move toward more analytical, antigen-specific, and fit-for-purpose approaches for veterinary vaccine quality assessment, integrating molecular, immunochemical, and physicochemical methods within lifecycle-based regulatory frameworks [26]. The on-farm plate agglutination test was used as a serological screening tool rather than a validated diagnostic assay; its diagnostic sensitivity and specificity were not established against a reference standard, and field results should be interpreted with this caveat.

It should be emphasized that the present study evaluated only humoral immune responses. Given the intracellular nature of *C. pseudotuberculosis*, cellular immunity is likely to play a critical role in protection against CLA. However, IFN-γ production, T-cell proliferation, and other cellular immune parameters were not assessed in this study. Therefore, the cellular mechanisms underlying the observed protection remain undetermined. Future studies should incorporate cellular immunity assays to better elucidate the protective mechanisms of the trivalent vaccine.

A major limitation of this study is the short interval between vaccination and challenge. Animals were challenged at D28, only 14 days after the booster immunization. Therefore, the results primarily reflect early post-vaccination protection and do not establish the duration of immunity. Given that antibody levels had already declined by D60, the durability of protection beyond D28 remains unknown. Future studies should include later challenge time points (e.g., D60 or D90) to evaluate how long the vaccine-induced protection persists.

The field trials have several important design limitations. Only one farm per species was included, and the farm is effectively the experimental unit, making farm-level confounding difficult to exclude. In addition, vaccinated and control animals were merged after D15, so the two groups were no longer physically separated. Cross-transmission between groups may have occurred, and clustering within farms limits statistical inference. Environmental exposure and management effects cannot be excluded. Therefore, the field data should be regarded as preliminary field evidence rather than definitive proof of field efficacy.

Overall, the trivalent aluminum hydroxide-adjuvanted inactivated vaccine showed promising immunogenicity and partial protective effects against homologous experimental challenge and was associated with a lower observed incidence of CLA under the investigated field conditions. Larger, blinded, adequately powered multi-farm studies using heterologous strains are required to confirm safety and field effectiveness.

5. Conclusions

In this study, we developed a trivalent inactivated vaccine against *C. pseudotuberculosis*, *T. pyogenes*, and *S. aureus* for the prevention of CLA. The aluminum hydroxide-adjuvanted formulation showed promising immunogenicity, with antibody levels that were numerically lower than but not statistically distinguishable from the Freund's adjuvant group at most time points, and partial protection against homologous experimental challenge and was associated with a lower observed overall abscess incidence under the investigated field conditions. However, several limitations should be acknowledged: the challenge study had a small sample size (n = 6 per group); the challenge was an artificial homologous challenge; cellular immune responses were not assessed; the follow-up period was short; the duration of protection was not evaluated; field cases were few; only one farm per species was included; and efficacy against mixed or heterologous field strains remains to be determined. Larger, blinded, adequately powered multi-farm

studies using heterologous strains are required to confirm the safety and field effectiveness of this vaccine.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vaccines14110930/s1. Table S1: Individual antibody OD450 values; Table S2: Individual challenge outcomes; Table S3: Individual field trial outcomes.

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References

1. de Oliveira Silva, M.T.; Barbosa, T.N.; de Pinho, R.B.; Moron, L.D.; Scholl, N.R.; Borsuk, S. Multi-epitope recombinant chimera and inactivated *Escherichia coli* expressing chimeric gene of *Corynebacterium pseudotuberculosis* as vaccines for caseous lymphadenitis. *Res. Vet. Sci.* **2025**, *197*, 105956.
2. Bastos, B.L.; Loureiro, D.; Raynal, J.T.; Guedes, M.T.; Vale, V.L.; Moura-Costa, L.F.; Guimarães, J.E.; Azevedo, V.; Portela, R.W.; Meyer, R. Association between haptoglobin and IgM levels and the clinical progression of caseous lymphadenitis in sheep. *BMC Vet. Res.* **2013**, *9*, 254.
3. Dorella, F.A.; Pacheco, L.G.; Oliveira, S.C.; Miyoshi, A.; Azevedo, V. *Corynebacterium pseudotuberculosis*: Microbiology, biochemical properties, pathogenesis and molecular studies of virulence. *Vet. Res.* **2006**, *37*, 201–218.
4. Abebe, D.; Sisay Tessema, T. Determination of Corynebacterium pseudotuberculosis prevalence and antimicrobial susceptibility pattern of isolates from lymph nodes of sheep and goats at an organic export abattoir, Modjo, Ethiopia. *Lett. Appl. Microbiol.* **2015**, *61*, 469–476.
5. Ashrafi Tamai, I.; Mohammadzadeh, A.; Zahraei Salehi, T.; Mahmoodi, P. Genomic characterisation, detection of genes encoding virulence factors and evaluation of antibiotic resistance of Trueperella pyogenes isolated from cattle with clinical metritis. *Antonie Leeuwenhoek* **2018**, *111*, 2441–2453.
6. Burakova, Y.; Madera, R.; McVey, S.; Schlup, J.R.; Shi, J. Adjuvants for Animal Vaccines. *Viral Immunol.* **2018**, *31*, 11–22.
7. Coppens, J.; Van Heirstraeten, L.; Ruzin, A.; Yu, L.; Timbermont, L.; Lammens, C.; Matheeussen, V.; McCarthy, M.; Jorens, P.; Ieven, M.; et al. Comparison of genexpert mrsa/sa eta assay with semi-quantitative and quantitative cultures and nuc gene-based qpcr for detection of staphylococcus aureus in endotracheal aspirate samples. *Antimicrob. Resist. Infect. Control* **2019**, *8*, 4.

8. Dowling, D.J.; Sanders, H.; Cheng, W.K.; Joshi, S.; Brightman, S.; Bergelson, I. A Meningococcal Outer Membrane Vesicle Vaccine Incorporating Genetically Attenuated Endotoxin Dissociates Inflflammation from Immunogenicity. *Front. Immunol.* **2016**, *7*, 562.
9. Huang, T.; Song, X.; Jing, J.; Zhao, K.; Shen, Y.; Zhang, X.; Yue, B. Chitosan-DNA nanoparticles enhanced the immunogenicity of multivalent DNA vaccination on mice against Trueperella pyogenes infection. *J. Nanobiotechnology* **2018**, *16*, 8.
10. Brogden, K.A.; Cutlip, R.C.; Lehmkuhl, H.D. Immunogenicity of Corynebacterium pseudotuberculosis and the effect of adjuvants in mice. *J. Comp. Pathol.* **1985**, *95*, 167–173.
11. Zhang, W.; Wang, P.; Wang, B.; Ma, B.; Wang, J. A combined Clostridium perfringens/Trueperella pyogenes inactivated vaccine induces complete immunoprotection in a mouse model. *Biologicals* **2017**, *47*, 1–10.
12. Beikzadeh, B. Immunoinformatics design of novel multi-epitope vaccine against Trueperella Pyogenes using collagen adhesion protein, fimbriae, and pyolysin. *Arch. Microbiol.* **2024**, *206*, 90.
13. de Pinho, R.B.; de Oliveira Silva, M.T.; Bezerra, F.S.B.; Borsuk, S. Vaccines for caseous lymphadenitis: Up-to-date and forward-looking strategies. *Appl. Microbiol. Biotechnol.* **2021**, *105*, 2287–2296.
14. Windsor, P.A. Control of caseous lymphadenitis. *Vet. Clin. N. Am. Food Anim. Pract.* **2011**, *27*, 193–202.
15. Dorella, F.A.; Pacheco, L.G.; Seyffert, N. Antigens of Corynebacterium pseudotuberculosis and prospects for vaccine development. *Expert Rev. Vaccines* **2009**, *8*, 205–213.
16. Wang, B.; Wei, Y.C.; Bai, X.D.; Wang, C.X.; Feng, H.; Yang, Z.Q. Investigation and analysis of bacterial species in caseous lymphadenitis-like abscesses of goats and sheep in Shaanxi and Gansu provinces. *Chin. J. Vet. Sci.* **2022**, *42*, 2427–2433.
17. Moreira, L.S.; Lopes, N.D.R.; Pereira, V.C.; Andrade, C.L.B.; Torres, A.J.L.; Ribeiro, M.B.; Freire, S.M.; Santos, R.M.D.; D'ávila, M.; Nascimento, R.M. The Association of Bacterin and Recombinant Proteins Induces a Humoral Response in Sheep against Caseous Lymphadenitis. *Vaccines* **2022**, *10*, 1406.
18. Machado, V.S.; Bicalho, M.L.; Meira Junior, E.B.; Rossi, R.; Ribeiro, B.L.; Lima, S.; Santos, T.; Kussler, A.; Foditsch, C.; Ganda, E.K. Subcutaneous immunization with inactivated bacterial components and purified protein of *Escherichia coli*, Fusobacterium necrophorum and *Trueperella pyogenes* prevents puerperal metritis in Holstein dairy cows. *PLoS ONE* **2014**, *9*, e91734.
19. Yang, L.; Liang, H.; Wang, B.; Ma, B.; Wang, J.; Zhang, W. Evaluation of the Potency of Two Pyolysin-Derived Recombinant Proteins as Vaccine Candidates of Trueperella Pyogenes in a Mouse Model: Pyolysin Oligomerization and Structural Change Affect the Efficacy of Pyolysin-Based Vaccines. *Vaccines* **2020**, *8*, 79.
20. Garcia-Romo, G.S.; Gonzalez-Ibarra, M.; Donis-Hernandez, F.R.; Zendejas Buitron, V.M.; Pedroza-Gonzalez, A. Immunization with Heat-Inactivated *Staphylococcus aureus* Induced an Antibody Response Mediated by Igg1 and Igg2 in Patients with Recurrent Tonsillitis. *Microbiol. Immunol.* **2015**, *59*, 193–200.
21. De Oliveira, L.P.O.; Cerqueira, R.B.; de Oliveira Lima, Â.C.; da Silva Cavalcante, A.K.; Barreto, K.A.; Vieira, V.P.; da Fonseca, L.A.; Ayres, M.C.C. Humoral and innate response in goats immunized with vaccine against Corynebacterium pseudotuberculosis. *Vet. Ital.* **2022**, *58*, 391–398. https://doi.org/10.12834/VetIt.2440.14841.1.
22. Rodríguez-Domínguez, M.C.; Montes-de-Oca-Jiménez, R.; Vázquez-Chagoyán, J.C.; Rivadeneira-Barreiro, P.E.; Zambrano-Rodríguez, P.C.; Ruiz-Riva-Palacio, M.E.; Gutiérrez-Castillo, A.D.C.; de-Castro-Soares, S.; Vieyra-Reyes, P.; Arteaga-Troncoso, G. Bioinformatic Approach of B and T Cell Epitopes of PLD and CP40 Proteins of *Corynebacterium pseudotuberculosis ovis* Mexican Isolate 2J-L towards a Peptide-Based Vaccine. *Int. J. Mol. Sci.* **2023**, *25*, 270.
23. Beikzadeh, B.; Ashrafi Tamai, I.; Zahraei Salehi, T. Construction of live-attenuated Trueperella pyogenes by antibiotic treatment and sequential passage: Methods for vaccine development. *Arch. Microbiol.* **2023**, *205*, 147.
24. Chand, U.; Priyambada, P.; Kushawaha, P.K. Staphylococcus aureus vaccine strategy: Promise and challenges. *Microbiol. Res.* **2023**, *271*, 127362.
25. Shinwari, K.; Vieira, B.; Ciaparin, I.; Hakansson, A.P.; Darrieux, M.; Converso, T.R. Overcoming Immune Evasion in *Staphylococcus aureus*: Strategies for Rational Vaccine Design. *ACS Infect. Dis.* **2025**, *11*, 2692–2705.
26. Abousenna, M.S. Reframing veterinary vaccine quality control: Integrating alternative assays within lifecycle-based regulatory frameworks. *Vet. Immunol. Immunopathol.* **2026**, *296*, 111109.

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