

Route-dependent immunogenicity and protective efficacy of a chitosan nanoparticle-based recombinant Omp31 vaccine against *Brucella abortus*

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Research Article

Keywords: *Brucella abortus*, recombinant Omp31, chitosan nanoparticles, mucosal vaccination, recombinant vaccine, protective immunity

Posted Date: October 14th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11095326/v1>

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Additional Declarations: No competing interests reported.

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Abstract

Background: Brucellosis remains an important zoonotic disease, highlighting the need for safe and effective vaccine strategies. This study evaluated recombinant Omp31 (rOmp31) formulated in chitosan nanoparticles and investigated the effects of different administration routes on immune responses and protection against *Brucella abortus* (*B. abortus*).

Methods and Materials: The *omp31* gene was characterized using immunoinformatics approaches, and rOmp31 was expressed in *Escherichia coli* BL21 (DE3) and purified by Ni-NTA chromatography. The purified protein was encapsulated in chitosan nanoparticles, and their physicochemical properties, encapsulation efficiency, antigen-release profile, and cytocompatibility were evaluated. BALB/c mice were immunized through subcutaneous, oral, or combined subcutaneous-oral routes. Antigen-specific IgG and IgA responses, cytokine production, and protective efficacy against virulent *B. abortus* strain 544 were subsequently assessed.

Results: The nanoparticles showed a mean size of 440.3 nm, PDI of 0.732, zeta potential of +16.2 mV, and approximately 90% encapsulation efficiency. Approximately 75% of the encapsulated antigen was released over 120 h, while cell viability remained approximately 90%. All rOmp31-immunized groups exhibited significantly increased antigen-specific antibody responses compared with controls. Cytokine profiles varied according to the vaccination route. Following challenge, the combined subcutaneous-oral regimen produced the greatest reduction in bacterial burden (1.55-log₁₀ CFU), followed by oral (1.12-log₁₀ CFU) and subcutaneous (0.92-log₁₀ CFU) immunization.

Conclusion: Chitosan nanoparticle-formulated rOmp31 induced systemic and mucosal immune responses and provided measurable protection against *B. abortus*. The combined administration route showed the highest protective efficacy, supporting further investigation of this formulation as a candidate brucellosis vaccine.

Keywords: *Brucella abortus*, recombinant Omp31, chitosan nanoparticles, mucosal vaccination, recombinant vaccine, protective immunity.

1. Introduction

Brucella species are the causative agents of brucellosis, a globally significant zoonosis that poses a persistent threat to both public health and livestock economies. While official reports document over 500,000 new human cases annually, the true global burden is estimated to be considerably higher [1]. The pathogen affects a diverse range of domestic and wildlife hosts, with transmission to humans occurring via direct contact with infected animals or the consumption of contaminated animal products. In humans, brucellosis manifests as a chronic, debilitating infection, whereas in livestock, it is characterized by severe reproductive complications-including abortion and infertility-leading to profound economic losses. The endemicity of brucellosis in numerous regions, despite extensive control efforts, underscores the critical necessity for more effective preventive strategies [2].

Vaccination represents a fundamental strategy for controlling brucellosis, particularly in livestock populations. Although live attenuated vaccines (e.g., *Brucella abortus* S19, RB51, and *Brucella melitensis* Rev.1) confer substantial protection, their residual virulence, safety concerns, reproductive complications, and interference with serological diagnosis limit their utility-particularly in humans, for whom no licensed vaccine exists. These limitations have therefore driven the development of next-generation platforms, including recombinant, DNA, mRNA, multi-epitope, and nanoparticle-based vaccines [3].

Given the intracellular nature of *Brucella* pathogens, effective immunization necessitates the induction of robust cellular immunity, specifically characterized by Th1-biased responses and the activation of IFN- γ -producing T cells. While humoral and mucosal immunity also play supportive roles, cellular mechanisms are paramount for controlling intracellular infection. Consequently, recombinant vaccines targeting specific immunogenic *Brucella* proteins have emerged as promising candidates, as they allow for precise antigenic targeting while circumventing the safety limitations inherent in live attenuated platforms. Recent advancements have further demonstrated that the deployment of recombinant protein combinations and multi-epitope constructs can elicit synergistic humoral and cellular responses, providing demonstrable protection in murine models of experimental brucellosis [4, 5].

Among the various candidates evaluated, outer membrane protein 31 (Omp31) stands out for its robust immunogenicity and protective capacity. As a surface-exposed antigen, Omp31 has been extensively explored across diverse delivery platforms, including recombinant proteins, DNA vaccines, and chimeric constructs. Pivotal studies have demonstrated that recombinant Omp31 (rOmp31) elicits robust antigen-specific immunity and significant protective efficacy, providing a foundation for advanced formulation-based optimizations. Moreover, the continued integration of Omp31 into contemporary multi-antigen and multi-epitope vaccine platforms underscores its indispensable role in the development of next-generation brucellosis prophylaxis [5-7].

Nevertheless, the immunogenicity of recombinant protein vaccines relies heavily on both the antigen and the chosen delivery system. Soluble recombinant proteins are often susceptible to enzymatic degradation and exhibit limited stability, necessitating appropriate adjuvants or carrier systems to promote antigen uptake and immune activation. In this context, nanoparticle-based delivery systems have emerged as powerful strategies to safeguard antigen integrity, facilitate efficient cellular transport, and amplify the immunogenicity of subunit vaccines. Among various biomaterials, chitosan stands out as an exceptionally attractive vehicle owing to its biodegradability, biocompatibility, positive surface charge, mucoadhesive properties, and intrinsic immunostimulatory activity. Chitosan nanoparticles can effectively prolong antigen retention at mucosal barriers, enhance professional Antigen-Presenting Cell (APC) uptake, protect cargo proteins from premature degradation, and provide controlled or sustained release kinetics [8, 9].

Recent experimental evidence underscores the efficacy of nanoparticle-enabled mucosal vaccination in combating *Brucella* infection. For instance, a recent 2025 investigation engineered a multi-epitope mucosal vaccine candidate, demonstrating robust systemic and mucosal immunity following oral administration. Complementary studies evaluating chitosan-based delivery platforms for *Brucella* antigens highlight their capacity to direct cargo toward specialized mucosal immune niches-such as M cells and dendritic cells-thereby orchestrating a balanced cellular, humoral, and mucosal response alongside a pronounced reduction in tissue bacterial burden [10, 11]. Collectively, these findings validate nanoparticle-mediated mucosal delivery as a compelling strategy to transcend the inherent immunogenic limitations of recombinant *Brucella* subunit antigens.

Despite these technological strides, the immunological potential of rOmp31 delivered via nanoparticle-based formulations-particularly across diverse administration routes-remains insufficiently characterized. Specifically, the extent to which the route of administration dictates systemic and mucosal antibody responses, systemic cytokine profiles, and protective efficacy against virulent *Brucella* challenge warrants comprehensive elucidation. A systematic comparative assessment of parenteral, oral, and combined administration strategies can thus yield critical insights into the capacity of rOmp31-nanoparticle formulations to elicit a harmonious and robust systemic, mucosal, and cellular immune network. Accordingly, the rational optimization of both antigen delivery modalities and administration routes remains a paramount requisite for the advancement of effective, next-generation mucosal vaccines against brucellosis [11, 12].

This study explores the potential of chitosan nanoparticles as a delivery system for a rOmp31 vaccine against *B. abortus*. After *in silico* design and prokaryotic expression, the antigen was encapsulated and characterized for its physicochemical and biological properties. Immunogenicity was subsequently assessed in BALB/c mice, focusing on humoral (IgG/IgA) and cellular immune responses. A challenge assay using virulent *B. abortus* strain 544 further confirmed the protective efficacy of the proposed vaccination strategies. Our findings highlight the influence of administration route on the induction of systemic and mucosal immunity, underscoring the efficacy of this novel nano-formulation as a promising candidate against brucellosis.

2. Methods and Materials

2.1. Immunoinformatic Design and Characterization of the Vaccine Candidate

2.1.1. Comparative Sequence Analysis and Conservation of Omp31 across *Brucella* strains

The Omp31 protein sequence of *B. abortus* was retrieved from the NCBI and UniProt databases using accession numbers WP_002971128.1 and F8WJS1, respectively. To evaluate sequence conservation among different strains and species, homologous protein sequences were retrieved from the NCBI database and aligned using ClustalW (EMBL-EBI, UK). Prior to subsequent analyses, the protein sequence was screened for the presence of signal peptides using the SignalP server. [13].

2.1.2. Assessment of Antigenic Potential and Immunogenicity

The antigenicity of the retrieved protein sequences was evaluated using the VaxiJen v2.0 server available at <http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) [14] with the default threshold of 0.4. VaxiJen predicts antigenicity based on the physicochemical properties of proteins using an alignment-independent approach. Protein sequences identified as antigenic were further analyzed for allergenicity using the AlgPred server [15, 16].

2.1.3. Identification of Potential B-Cell Epitopes

Linear B-cell epitopes were predicted using two complementary computational approaches, ABCpred and BepiPred. The ABCpred server (<http://crdd.osdd.net/raghava/abcpred/>) employs an artificial neural network (ANN)-based method to identify potential linear B-cell epitopes of predefined lengths within the target protein sequence [17]. In parallel, the BepiPred server (<http://www.cbs.dtu.dk/services/BepiPred/>) was used to predict linear B-cell epitopes based on amino acid sequence characteristics and residue-specific epitope propensity scores [18]. The use of both prediction tools enabled a comparative

assessment of the predicted epitopic regions. Regions identified by both approaches were considered high-confidence linear B-cell epitope candidates and were selected for subsequent analyses.

2.1.4. In Silico Prediction and Mapping of T-cell Epitopes (MHC Class I and Class II) for rOmp31

Potential T-cell epitopes restricted to MHC class I and class II molecules were predicted using the NetMHCcons 1.1 server [19] and the IEDB Analysis Resource [20], respectively. These computational tools were used to assess peptide-MHC binding interactions based on quantitative matrix-based and other predictive algorithms. Peptides with favorable predicted binding affinities were subsequently considered as potential T-cell epitopes for further analysis.

2.1.5. Multiepitope Vaccine Candidate Construction and Design

Sequences were obtained from the aforementioned databases to serve as the template for vaccine design. Candidate epitopes were subsequently selected based on their high binding affinity and predicted immunogenicity, ensuring their suitability for integration into a multi-epitope vaccine construct.

2.1.6. Physicochemical Characterization and Solubility Profiling

The physicochemical properties of the protein sequences were computed using the ExPASy ProtParam server (<http://web.expasy.org/protparam/>). This analysis yielded essential parameters, including molecular weight, amino acid composition, net charge, predicted half-life, and the instability index (PII). In addition, solubility of the rOmp31 was evaluated in silico using the Protein-Sol server (<https://protein-sol.manchester.ac.uk/>) to predict its propensity for soluble expression in an *E. coli* host system [21].

2.1.7. Structural Modeling and In Silico Validation of the Vaccine Candidate

The secondary structure of rOmp31 was predicted using the PSIPRED v3.3 and SOPMA servers [22, 23]. PSIPRED utilizes a neural network-based prediction approach, whereas SOPMA employs the Self-Optimized Prediction Method to estimate the secondary structural elements of the protein. Subsequently, the three-dimensional (3D) structure of rOmp31 was modeled using the SWISS-MODEL server. The quality of the generated model was assessed using the Ramachandran Plot Server and further validated with RAMPAGE, ProSA-web, and ERRAT to evaluate its stereochemical properties, overall structural quality, and non-bonded atomic interactions [24].

2.1.8. Codon Optimization for Heterologous Expression in *E. coli*

To enhance protein expression, the nucleotide sequence was codon-optimized for *E. coli* using Gene Optimizer server [25]. The optimized sequence was subsequently cloned *in silico* into the pET-28a (+) expression vector. To facilitate directional cloning, HindIII and XhoI restriction sites were introduced at the N- and C-termini of the construct, respectively.

2.2. Experimental analysis

2.2.1. Microbial Strains, Vectors, and Cultivation Conditions

The *B. abortus* strain 544 (ATCC 23448) was preserved at -80°C in Luria-Bertani (LB) broth supplemented with 20% (v/v) glycerol, and subsequently cultured in LB broth at 37°C. For protein expression, the recombinant construct, harboring the synthetic *omp31*, was cloned into the pET-28a (+) expression vector and transformed into competent *E. coli* BL21 (DE3) cells.

2.2.2. Recombinant Omp31 Expression and Protein Purification

Recombinant colonies were inoculated into LB broth supplemented with 50 µg/mL kanamycin and cultured overnight at 37°C. The overnight culture was then transferred to fresh LB medium and incubated until the optical density (OD₆₀₀) reached approximately 0.8. Protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG; Sigma, USA), followed by further incubation for 2 h and then overnight at 37°C. Cells were harvested by centrifugation (5,000 ×g, 10 min, 25°C), and the resulting pellets were resuspended in lysis buffer (100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea; pH 8.0).

Following sonication (six 10 s cycles at high power), the lysate was centrifuged at 10,000 ×g for 15 min at 4°C. The supernatant was loaded onto a Ni-NTA affinity chromatography column (Qiagen) for recombinant protein purification. Non-specifically bound contaminants were removed by washing with a buffer containing 100 mM NaH₂PO₄, 10 mM Tris-HCl, and 8 M urea (pH 5.9). The recombinant protein was eluted using the same buffer adjusted to pH 4.5, and the fractions were analyzed via 14% SDS-PAGE [26]. Finally, stepwise dialysis was performed against buffers with decreasing urea concentrations (pH 8.0, 5.9, and 4.5), followed by a final dialysis against PBS (pH 7.5) to remove residual urea. Protein concentration was determined using the Bradford assay [27].

2.2.3. Immunological Verification via Western Blotting

Purified rOmp31 was resolved via 14% SDS-PAGE and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane using a wet electrotransfer system with a transfer buffer consisting of 25 mM Tris base and 192 mM glycine. After blocking with 5% skim milk in TBST (20 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20, pH 7.4), the membrane was incubated with an HRP-conjugated anti-His-tag antibody (1:2000; Sigma-Aldrich). Immunoreactive bands were visualized using diaminobenzidine (DAB, 0.5 mg/mL) in PBS supplemented with H₂O₂ as the chromogenic substrate.

2.2.4. Preparation and Physicochemical Characterization of Chitosan

Chitosan nanoparticles were synthesized via ionic cross-linking between chitosan and sodium tripolyphosphate (TPP) anions. Briefly, rOmp31 (500 µg) was initially dispersed in 7.5 mL of a chitosan solution (2 mg/mL, pH 5.5) prepared in 2% acetic acid and incubated for 10 minutes. Subsequently, a sodium tripolyphosphate solution (1 mg/mL) was added dropwise under continuous magnetic stirring. The mixture was further stirred at room temperature for 1 hour to facilitate complete nanoparticle formation. The resulting suspension was then subjected to physicochemical characterization. For animal immunization, the chitosan nanoparticles were collected by centrifugation at 13,000 rpm for 20 minutes at 4°C. The resulting pellet was resuspended in phosphate-buffered saline (PBS) to create the final nanovaccine formulation.

2.2.5. Analysis of Nanoparticle Size and Zeta Potential

Dynamic light scattering (DLS) and laser Doppler electrophoresis were employed to characterize the particle size distribution and zeta potential of the rOmp31-loaded chitosan nanoparticles, respectively, using a Zeta sizer Nano-ZS (Malvern Instruments, UK). The analysis was performed at a controlled temperature of 25°C using a 633 nm laser wavelength.

2.2.6. In Vitro Cytotoxicity Assessment of Chitosan Nanoparticles

The in vitro cytotoxicity of blank chitosan nanoparticles and rOmp31-loaded chitosan nanoparticles was assessed in HEK-293 cells using the MTT assay. HEK-293 cells were initially plated at 1×10^4 cells per well and subsequently exposed to chitosan nanoparticles (2 mg/mL) or rOmp31-loaded formulations for 24 h at 37°C. Following treatment, MTT reagent (5 mg/mL) was added, and the cells were incubated for an additional 4 h to allow for formazan formation. The formazan crystals were then solubilized in DMSO, and the absorbance was recorded at 570 nm using a microplate reader. Cell viability was determined by comparing the absorbance values of treated cells with those of the untreated control group.

2.2.7. Determination of Protein Encapsulation Efficiency

The encapsulation efficiency (EE %) was determined by quantifying the amount of unencapsulated rOmp31 remaining in the supernatant after centrifugation. The protein concentration was measured using the Bradford assay, with absorbance recorded at 595 nm using a UV-Vis spectrophotometer [28]. The EE% was calculated according to the following equation [29]:

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Total rOmp31} - \text{rOmp31 in supernatant}}{\text{Total rOmp31}} \times 100$$

2.2.8. Evaluation of In Vitro Controlled Release Kinetics

The rOmp31-loaded chitosan nanoparticles were suspended in PBS and centrifuged at $13,000 \times g$ for 20 min at 4 °C. The pellet was then resuspended in PBS and incubated at 37 °C in a shaking incubator. At predetermined time intervals (0, 1, 3, 12, 24, 48, 72, 96, and 120 h), aliquots of the supernatant were collected, and the concentration of the released antigen was quantified using the Bradford assay.

2.2.9. Experimental Animal Models, Immunization, and Sample Collection

Female BALB/c mice (5 weeks old, $n = 49$; Razi Vaccine and Serum Research Institute) were randomly allocated into three treatment (T1-T3) and corresponding control groups. The immunization regimens were established as follows: T1 received four weekly subcutaneous (s.c.) injection of 15 µg rOmp31-loaded chitosan nanoparticles. T2 was primed with a single s.c. dose of 15 µg and subsequently boosted with four oral gavage administrations of 60 µg of the formulation. T3 underwent five consecutive oral immunizations (60 µg per dose). Control animals were mock-immunized with PBS or blank chitosan nanoparticles following identical administration routes and dosing intervals Table 1. Blood was sampled retro-orbitally post-third and post-fifth immunizations. For mucosal antibody analysis, fecal pellets were homogenized in PBS (1:1 w/v; 1 g/mL) and centrifuged ($4,000 \times g$, 10 min, 4 °C); the resulting supernatants, alongside serum samples, were stored at -70 °C until evaluation. All procedures adhered strictly to institutional guidelines, received approval from the Animal Ethics Committee (approval code: IR.SHAHED.AEC.1405.003), and were reported in compliance with the ARRIVE 2.0 guidelines.

2.2.10. Quantification of Humoral Immune Responses (IgG/IgA)

Antigen-specific humoral and mucosal antibody responses were assessed by indirect ELISA. Briefly, 96-well microplates were coated overnight at 4°C with 1 µg of purified rOmp31 diluted in carbonate-bicarbonate buffer (pH 9.8). After blocking nonspecific binding sites with 5% skim milk, serial dilutions of serum samples were added for the determination of IgG (1:100-1:6400) and IgA (1:5-1:640) antibodies. Fecal samples were also analyzed for antigen-specific IgA responses. Following incubation at 37°C for 1 h, HRP-conjugated rabbit anti-mouse IgG or IgA antibodies (1:2000; Sigma) were added as secondary antibodies. The enzymatic reaction was developed using OPD (0.5 mg/mL) containing H_2O_2 , terminated with 2.5 M H_2SO_4 , and the absorbance was recorded at 492 nm using a microplate reader.

2.2.11. Analysis of Pro-inflammatory and Regulatory Cytokines

One month after the final immunization, seven mice from each group were sacrificed, and their spleens were aseptically removed. Splenocytes were isolated by mechanical homogenization and suspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were seeded in duplicate wells at a concentration of 4×10^6 cells/mL and cultured either without stimulation as a negative control or in the presence of 10 µg of purified rOmp31 or 0.25 µg of concanavalin A (ConA). Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 . After 48 h of antigen stimulation, culture supernatants were collected, and the concentrations of IFN- γ , IL-4, IL-12, and IL-17 were quantified using mouse-specific ELISA kits according to the manufacturer's instructions. All measurements were performed in triplicate.

2.2.12. Evaluation of Protective Efficacy against *Brucella* Challenge

Four weeks post-final immunization, mice were challenged intraperitoneally (i.p.) with 2×10^7 CFU of *B. abortus* strain 544 (ATCC 23448). Four weeks post-infection, animals were euthanized via carbon dioxide (CO_2) inhalation. Spleens were aseptically harvested, homogenized, and serially diluted on *Brucella* agar plates to quantify the bacterial burden. Results are expressed as mean $\log_{10}$ CFU $\pm$ SD per group. The level of protection was determined by calculating the difference in the mean $\log_{10}$ CFU between the negative control group and the respective experimental groups.

2.2.13. Statistical analysis

All experimental measurements were performed independently at least three times, where applicable, and the results are presented as mean $\pm$ standard deviation (SD). Statistical differences in antibody responses, cytokine production, and splenic bacterial burdens among the experimental groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. Bacterial burdens were expressed as log₁₀ CFU per spleen. A *p*-value of < 0.05 was considered statistically significant for all analyses.

3. Results

3.1. Sequence Conservation Suggests Functional Importance of Omp31

The retrieved Omp31 consisted of 141 amino acid residues. Multiple sequence alignment of Omp31 sequences from different strains demonstrated a high degree of sequence conservation among the analyzed isolates. Amino acid substitutions were mainly confined to variable and non-conserved regions, whereas the conserved regions remained highly preserved across all sequences. Signal peptide analysis identified an N-terminal signal peptide, which was removed prior to subsequent analyses. The resulting mature protein sequence was used for downstream epitope prediction and vaccine construct design.

3.2. Antigenic Profiling and Induction of Immune Responses by rOmp31

The antigenicity of the target protein was assessed using the VaxiJen v2.0 server, which revealed a strong antigenic potential. The predicted antigenicity score about rOmp31 was 0.65, significantly exceeding the default threshold of 0.4, thereby confirming its suitability as a vaccine candidate. Subsequently, the allergenic potential of the rOmp31 was evaluated via the AlgPred web server. The results indicated that the construct is a probable non-allergenic candidate, suggesting its safety for further immunoinformatic development.

3.3. Prediction and Mapping of B-Cell Epitopes

To improve the reliability of linear B-cell epitope prediction, two complementary immunoinformatics tools, ABCpred and BepiPred, were employed. ABCpred, based on an artificial neural network algorithm, identified several potential linear B-cell epitopes with prediction scores ranging from 0.80 to 0.89 Table 2. Consistently, BepiPred predicted these regions as potential epitopes, with scores exceeding the established threshold of 0.1512; most were located in surface-accessible regions of the protein. To enhance confidence in the predictions, only the overlapping epitopes identified by both servers were considered reliable candidates and selected for incorporation into the multi-epitope vaccine construct.

3.4. Prediction and Selection of T-cell Epitopes (MHC Class I and II)

To predict potential T-cell epitopes, the IEDB analysis resource was utilized, employing NetMHCpan for MHC-I and the recommended consensus method for MHC-II binding predictions. Epitopes were evaluated based on their percentile rank scores, and only those with a percentile rank of 0.01 to 0.15 for MHC I Table 3 and 3 to 7.70 for MHC II Table 4, respectively, were selected as candidate binders. This filtering approach was implemented to ensure that candidate peptides with favorable predicted binding ranks were selected for further analysis were prioritized for the subsequent design of the multi-epitope vaccine construct.

3.5. Physicochemical Properties and Solubility Prediction

Analysis of the 141-amino acid rOmp31 antigen using ProtParam confirmed favorable physicochemical characteristics, including stability, Theoretical pI and Aliphatic Index. These parameters indicate that the antigen is a suitable candidate for efficient recombinant expression Table 5. Moreover, the predicted solubility of rOmp31 was evaluated to estimate its potential for soluble expression in *E. coli*. The analysis yielded a solubility score of 0.686, indicating a high likelihood of soluble protein expression. These findings suggest that the recombinant construct possesses favorable properties for subsequent experimental expression and production.

3.6. Secondary and Tertiary Structure Characterization and Validation

The secondary structure of rOmp31 was analyzed using multiple *in silico* prediction algorithms. The PSIPRED results indicated that the predicted structure was predominantly composed of α -helical elements, suggesting a well-organized secondary structural arrangement. The substantial contribution of α -helical regions may support the overall structural stability of rOmp31 and could contribute to the preservation of conformational features relevant to its antigenic properties (Fig. 1). In addition, the three-dimensional (3D) structure of rOmp31 was predicted via homology modeling using the SWISS-MODEL server, based on the template A0A7L7MPK2.1.A, which shared 94.33% sequence identity with the target protein (Fig. 2a). The low RMSD value further validates the structural accuracy of the predicted fold, indicating that the model accurately represents the protein's native conformation. Furthermore, Ramachandran plot analysis confirmed the stereochemical quality of the model, with the vast majority of residues located in energetically favored regions. The minimal occurrence of outliers demonstrates high structural fidelity with no significant steric clashes (Fig. 2b).

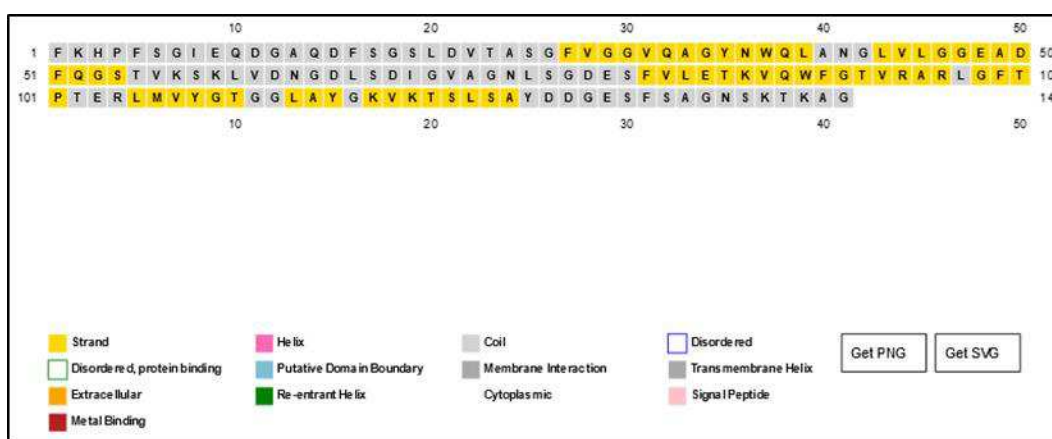


Fig. 1 Predicted secondary structure composition of rOmp31. PSIPRED analysis reveals a predominant α -helical content

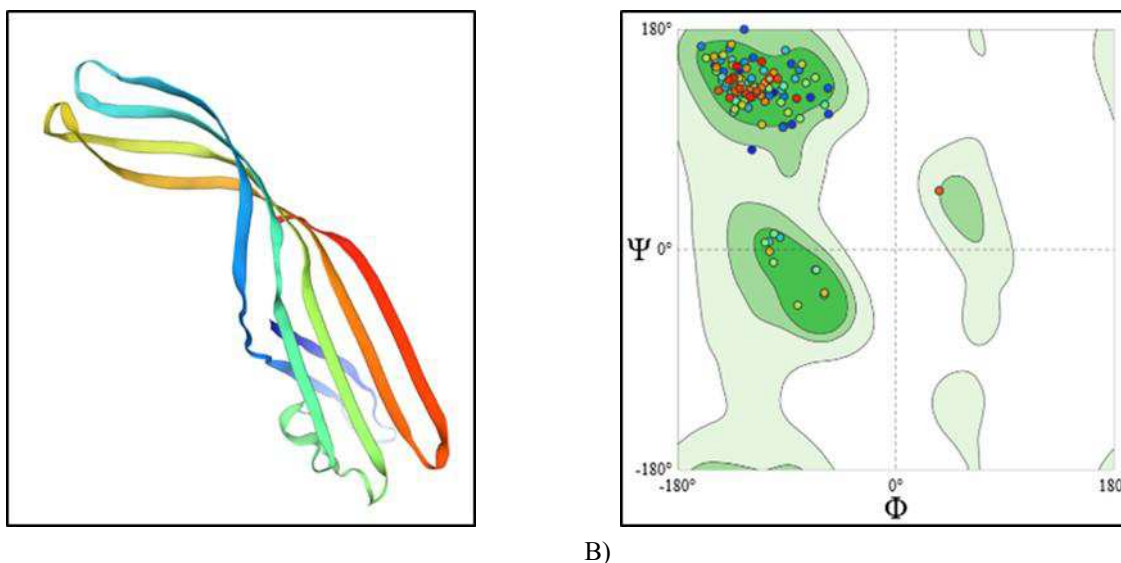


Fig. 2 Three-dimensional structure prediction and stereochemical validation of the rOmp31 construct. **a** Predicted 3D model of the designed recombinant protein generated via homology modeling, depicted as a ribbon diagram. The color gradient

transitions from the N-terminus (blue) to the C-terminus (red). **b** Ramachandran plot illustrating the distribution of ϕ (ϕ) and ψ (ψ) dihedral angles. The clustering of most residues within the energetically favorable regions confirms the high stereochemical quality and structural reliability of the predicted model

3.7. Codon Optimization of the rOmp31 Construct

The codon-optimized sequence, exhibiting a GC content of 58.22% and a Codon Adaptation Index (CAI) of 0.93, was cloned into the pET-28a (+) expression vector using *Hind*III and *Xho*I restriction sites. The high CAI value indicates an optimal codon usage bias, which is highly conducive to robust protein expression in the *E. coli* host.

3.8. Expression and Purification of rOmp31

The synthetic *omp31*, fused with an N-terminal His-tag, was expressed in *E. coli* BL21 (DE3). The rOmp31 was purified under denaturing conditions (8 M urea) via Ni-NTA affinity chromatography. SDS-PAGE analysis (14%) revealed a distinct band at approximately 20.5 kDa (Fig. 3a, b), consistent with the predicted molecular weight of rOmp31. Western blot analysis using an anti-His-tag antibody confirmed the successful expression and identity of the protein (Fig. 3c). The concentration of the purified rOmp31 was determined to be 1.505 mg/mL using the Bradford assay (Fig. 3d).

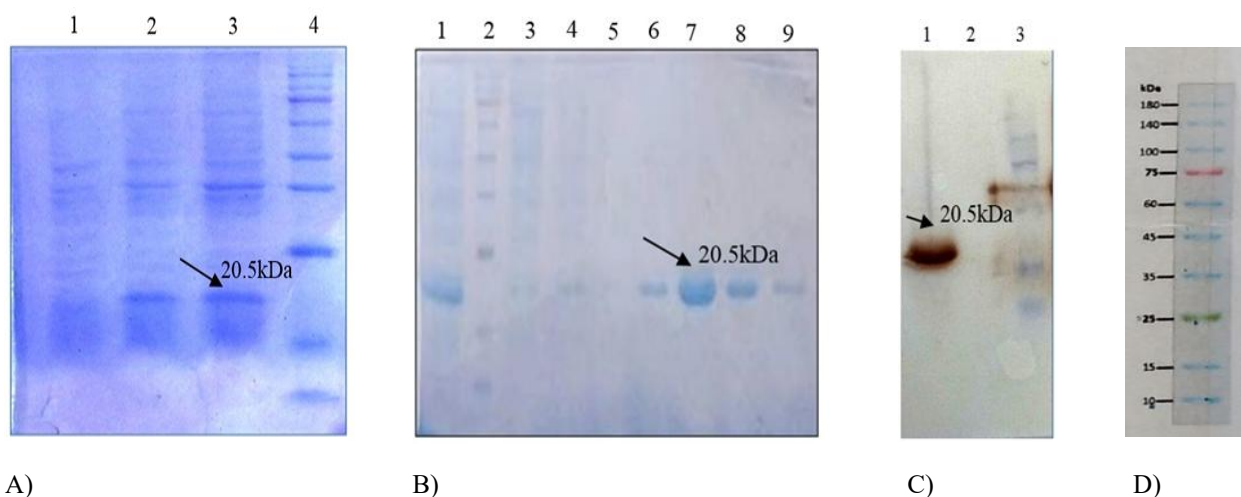
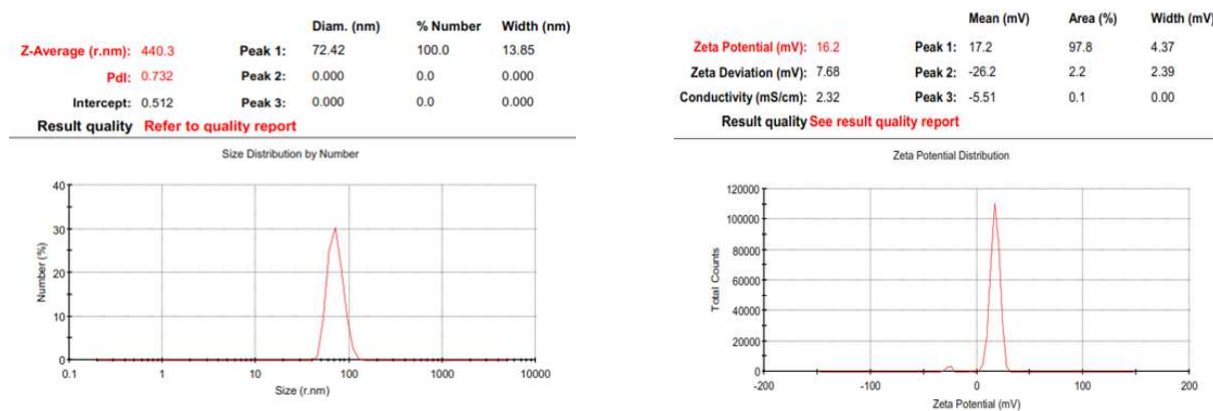


Fig. 3 Expression, purification, and immunological validation of rOmp31. **a** Expression analysis via 14% SDS-PAGE. Lane 1: *E. coli* BL21 harboring pET-28a-omp31 before IPTG induction; Lane 2: *E. coli* BL21 harboring pET-28a-omp31 after 2 h of IPTG induction; Lane 3: *E. coli* BL21 harboring pET-28a-omp31 following overnight IPTG induction; Lane 4: Protein molecular weight marker (Sinaclon, SL7013 and SL7014). The arrow indicates the rOmp31 band. **b** Purification profile of rOmp31 via 14% SDS-PAGE. Lane 1: Cell lysate prior to column loading; Lane 2: Molecular weight marker; Lane 3: Column flow-through; Lane 4: Wash fraction (pH 5.9); Lanes 5-9: Elution fractions (pH 4.5). **c** Western blot analysis of rOmp31. Detection was performed using an anti-His tag antibody. Lane 1: Purified rOmp31; Lane 2: Negative control; Lane 3: Molecular weight marker. **d** Protein ladder marker

3.9. Physicochemical Characterization of Chitosan Nanoparticles

The physicochemical characterization of rOmp31-loaded chitosan nanoparticles showed an average particle size of approximately 440.3 nm and a polydispersity index (PDI) of 0.732 (Fig. 4a). Zeta potential measurements revealed a positive surface charge of +16.2 mV, suggesting the positively charged nature of the rOmp31-loaded chitosan nanoparticles (Fig. 4b).



A)

B)

Fig. 4 Physicochemical characterization of rOmp31-loaded chitosan nanoparticles. **a** Particle size distribution (DLS analysis) showing the hydrodynamic diameter of the rOmp31-loaded chitosan nanoparticles. **b** Zeta potential analysis illustrating the surface charge of the rOmp31-encapsulated chitosan nanoparticles

3.10. In Vitro Cytotoxicity Assessment

The cytotoxicity of the chitosan nanoparticles was evaluated using an MTT assay. HEK-293 cells treated with 2 mg/mL of the nanoparticles for 24 h exhibited approximately 90% cell viability. This high viability indicates minimal cytotoxic effects and excellent biocompatibility of the chitosan nanoparticles under the tested conditions, suggesting low cytotoxicity under the tested conditions.

3.11. Encapsulation Efficiency and In Vitro Release Profile

The encapsulation efficiency (EE) of rOmp31 within the chitosan nanoparticles was determined to be 90%, demonstrating effective protein incorporation into the nanoparticle matrix. The *in vitro* release profile exhibited a gradual and sustained release pattern, with approximately 75% of the encapsulated rOmp31 released after 120 h in PBS (Fig. 5). This sustained-release behavior suggests that the chitosan nanoparticles function as an effective delivery system, potentially facilitating prolonged antigen availability following administration.

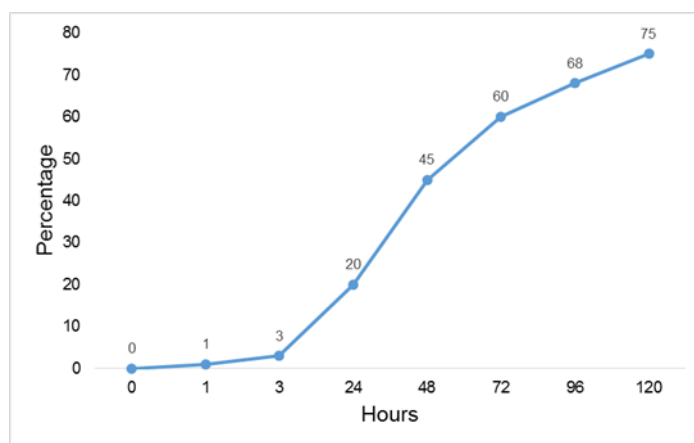


Fig. 5 Release of nanoparticled rOmp31. In vitro release profile of rOmp31 from chitosan nanoparticles

3.12. Evaluation of Humoral Immune Responses (Antibody Titers)

The immunized groups (T1-T3) exhibited significantly elevated levels of rOmp31-specific antibodies compared to their respective control groups. A robust humoral response was evidenced by serum anti-rOmp31 IgG titers, which remained detectable at dilutions as high as 1:6400 (Fig. 6a). Furthermore, the comparative evaluation of systemic and mucosal IgA

profiles revealed distinct immune response patterns across the different administration regimens (Fig. 6b, c). Consistent with these findings, a marked increase in serum anti-rOmp31 IgG titers was observed in all immunized groups following the final booster dose, underscoring the efficacy of the formulated nanoparticle-based vaccine.

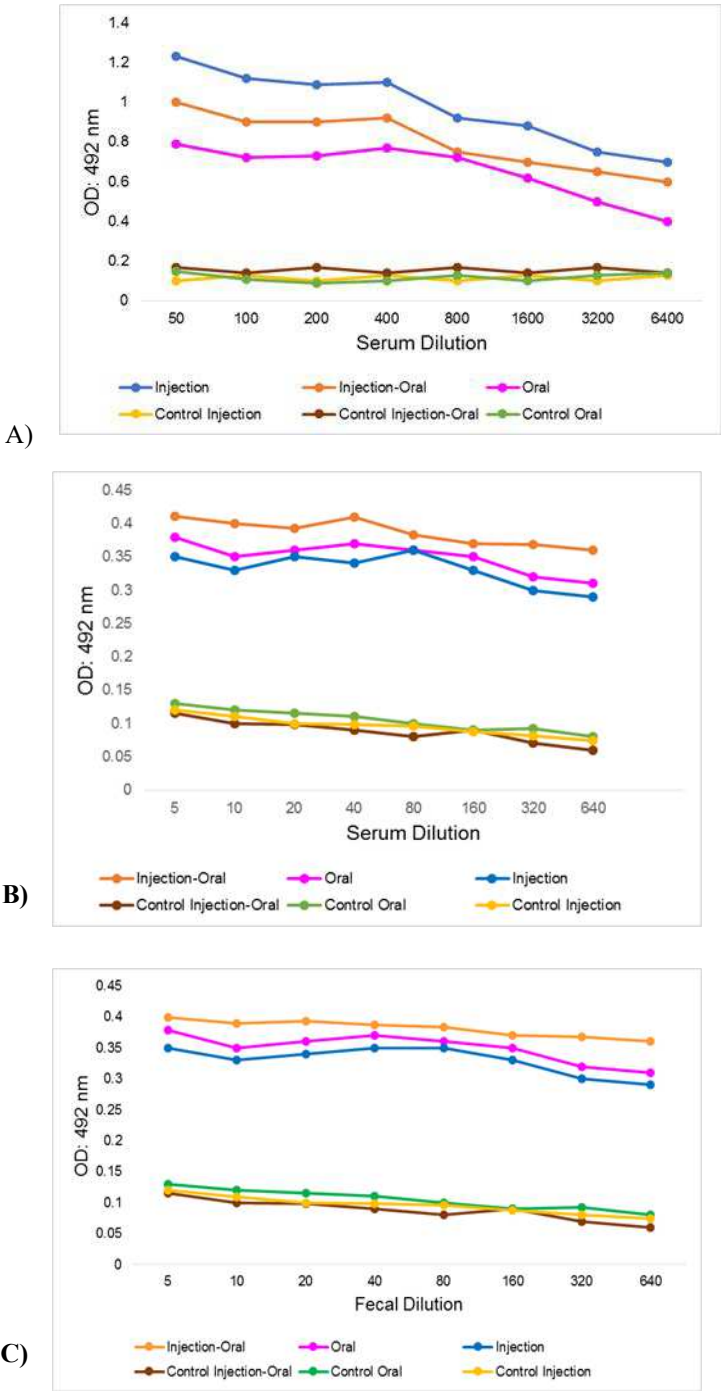
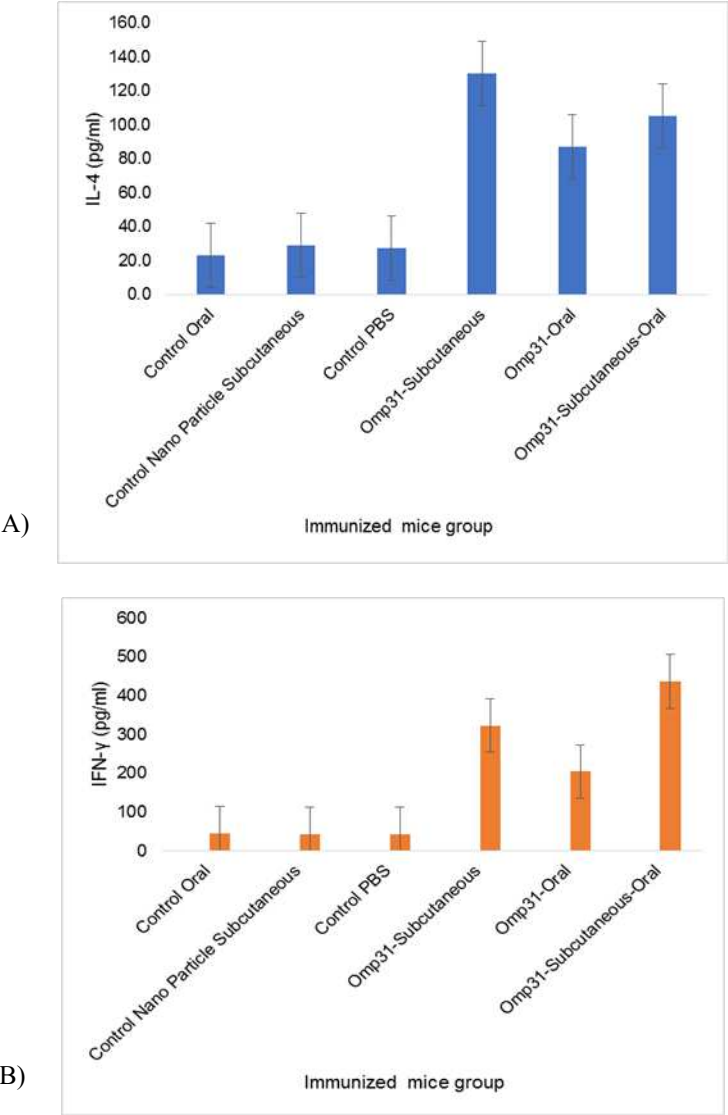


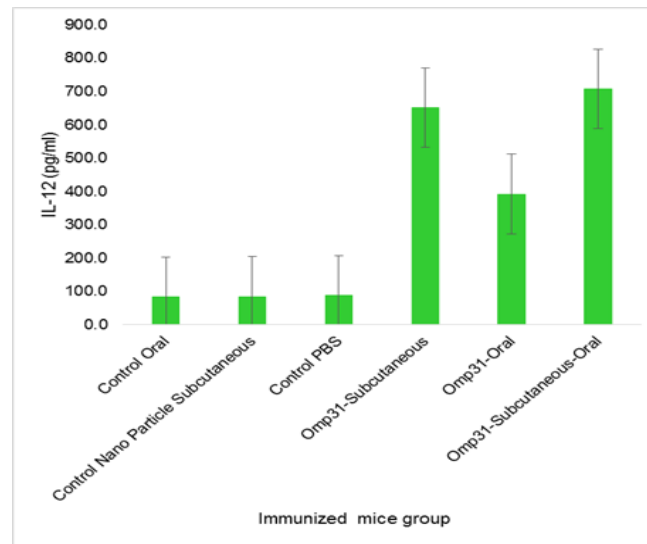
Fig. 6 Evaluation of humoral immune responses in mice immunized with rOmp31. **a** Serum IgG titers, **b** Serum IgA titers, and **c** Fecal IgA titers

3.13. Analysis of Cytokine Production Profiles

The culture supernatants obtained from splenocytes of immunized mice were analyzed for cytokine production by ELISA. IL-4 levels were significantly increased in mice immunized subcutaneously with rOmp31, whereas no significant increase was observed in the other immunized groups ($p < 0.0002$) (Fig. 7a). In contrast, significantly elevated production of IFN- γ and IL-12 was observed in splenocytes from mice immunized through the combined subcutaneous-oral gavage route with rOmp31, as well as in orally immunized mice receiving rOmp31-loaded nanoparticles (Fig. 7b, c). Moreover, a significant increase in IL-17 production was detected in the orally immunized mice (Fig. 7d).



C)



D)

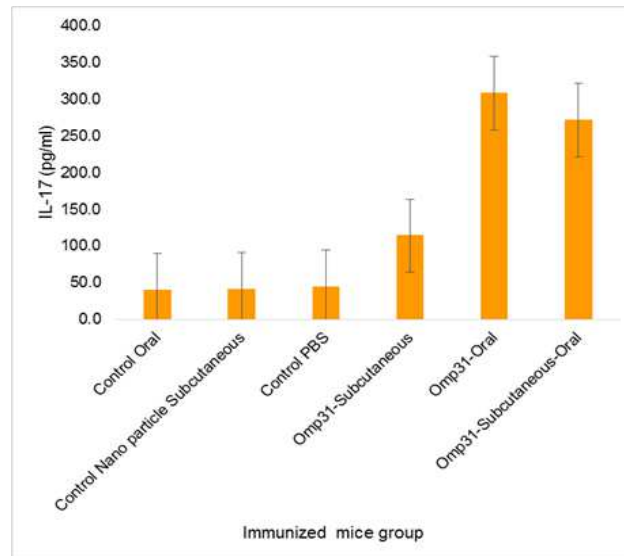


Fig.7 Cytokine production by splenocytes from mice immunized with rOmp31 formulated in chitosan nanoparticles. Levels of **a** IL-4, **b** IFN- γ , **c** IL-12, and **d** IL-17 were measured in culture supernatants following antigen stimulation. Data are presented as mean $\pm$ SD

3.14. Protective Efficacy against *B. abortus* Challenge

The protective efficacy of rOmp31 against *B. abortus* strain 544 was assessed in immunized BALB/c mice by determining splenic bacterial loads four weeks after challenge. Mice receiving rOmp31 through the combined subcutaneous-oral route exhibited the highest level of protection, with a reduction of 1.55 log units compared with the control groups ($p < 0.0001$). Oral and subcutaneous immunization with rOmp31-loaded nanoparticles resulted in 1.12 and 0.92 log units of protection, respectively (Fig. 8a). Following i.p. challenge with 2×10^7 CFU of *B. abortus* strain 544, immunized mice showed significantly reduced bacterial colonization and shedding compared with control groups ($p < 0.05$). Although all immunized groups demonstrated a protective effect, no significant differences were observed among them regarding the duration of bacterial shedding (Fig. 8b) and (Table 6).

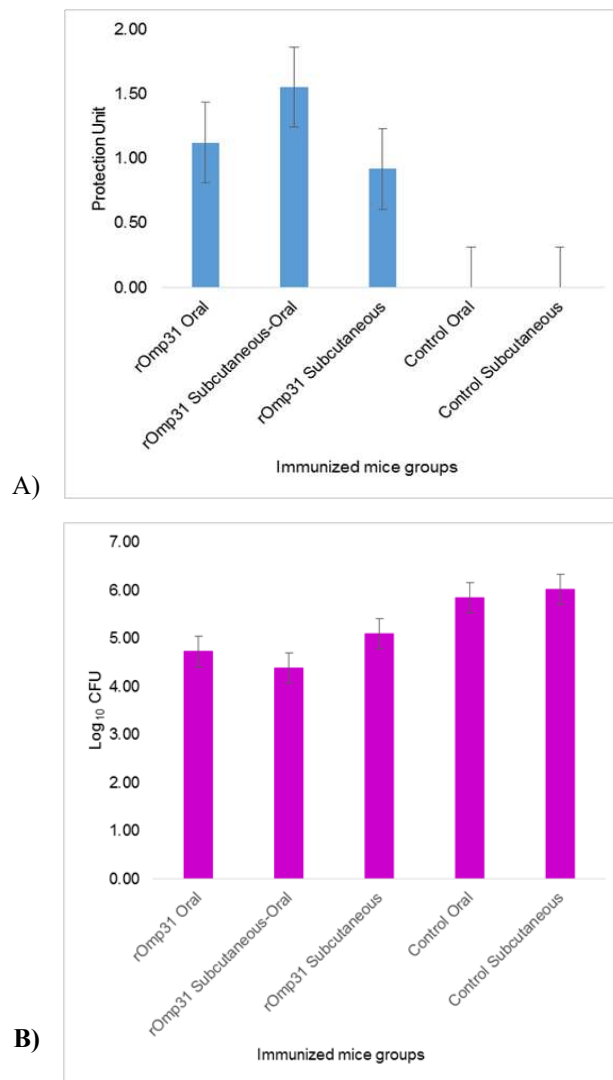


Fig.8 Evaluation of protection and reduction in bacterial shedding in immunized mice following infectious challenge. Data are presented as mean $\pm$ standard deviation (SD)

Discussion

The present study evaluated the immunogenicity and protective potential of rOmp31 formulated in chitosan nanoparticles using different immunization routes in BALB/c mice. The main findings were that rOmp31 was successfully expressed and purified, efficiently incorporated into chitosan nanoparticles, and capable of inducing both humoral and cellular immune responses. Most importantly, all rOmp31-immunized groups showed significant reductions in bacterial burden following challenge with *B. abortus* strain 544, while the combined subcutaneous-oral regimen produced the greatest reduction in splenic bacterial load. These findings support rOmp31 as a promising subunit vaccine antigen and further suggest that formulation and immunization route may influence the magnitude and profile of the resulting immune response.

The initial in silico analyses provided a rational basis for selecting Omp31 as a vaccine antigen. Omp31 showed a high degree of sequence conservation among the analyzed *B. abortus* strains, and its predicted antigenicity score was above the commonly used VaxiJen threshold. In addition, overlapping B-cell epitopes were identified by ABCpred and BepiPred, while candidate MHC class I- and class II-binding peptides were identified using the IEDB platform. Although these computational findings

cannot establish protective immunogenicity by themselves, they support the experimental evaluation of Omp31 and are consistent with previous studies identifying Omp31 as an immunogenic and protective *Brucella* antigen [6, 30].

The recombinant protein was successfully expressed in *E. coli* BL21 (DE3) and purified under denaturing conditions using Ni-NTA affinity chromatography. The approximately 20.5-kDa band observed by SDS-PAGE and confirmed by anti-His Western blotting was consistent with the expected recombinant product. Previous studies have likewise demonstrated the feasibility of using rOmp31 or Omp31-containing fusion constructs as vaccine antigens. Cassataro et al. reported that rOmp31 induced protective immunity in BALB/c mice, while Golshani et al. showed that a recombinant L7/L12-truncated Omp31 fusion protein generated significant protection against both *B. melitensis* and *B. abortus* [6, 30].

An important feature of the present study was the incorporation of rOmp31 into chitosan nanoparticles. The formulation had a mean particle size of approximately 440.3 nm, a positive zeta potential of +16.2 mV, and a PDI of 0.732. The positive surface charge is compatible with the cationic nature of chitosan and may facilitate interaction with negatively charged mucosal surfaces. Chitosan has been extensively investigated as a vaccine delivery material because of its biocompatibility, biodegradability, mucoadhesive properties, and potential to support sustained antigen delivery and mucosal immune responses [8, 31].

Despite this limitation, the formulation showed a high encapsulation efficiency of approximately 90% and a gradual release profile, with approximately 75% of the encapsulated rOmp31 released over 120 h in PBS. This sustained release behavior is potentially advantageous because prolonged antigen availability may facilitate continued antigen exposure and uptake. Previous literature has described controlled or sustained antigen release as one of the potential advantages of chitosan-based particulate vaccine systems, particularly for mucosal delivery [8, 31]. However, because release was evaluated only in vitro, the present results cannot establish the duration or pattern of antigen release in vivo.

The MTT assay showed approximately 90% viability of HEK-293 cells after exposure to the tested nanoparticle concentration for 24 h, suggesting limited cytotoxicity under the experimental conditions. This observation is compatible with the generally favorable biocompatibility reported for chitosan-based delivery systems [32]. Nevertheless, MTT measures cellular metabolic activity and represents only one aspect of nanomaterial safety. Additional assessments of inflammatory responses, oxidative stress, membrane integrity, tissue compatibility, and repeated-dose toxicity would provide a more comprehensive evaluation. The immunization experiments demonstrated clear antigen-specific humoral responses. All rOmp31-immunized groups showed significantly higher anti-rOmp31 antibody responses than their respective controls, and serum IgG remained detectable at a dilution of 1:6400. These findings are consistent with previous studies demonstrating that Omp31-containing vaccine formulations can induce substantial antibody responses. Cassataro et al. reported strong antibody responses following rOmp31 vaccination, whereas Golshani et al. observed a vigorous IgG response, including increased IgG2a, after vaccination with an L7/L12-truncated Omp31 fusion protein [6, 30].

The differences in serum and fecal IgA responses among the immunization regimens are particularly relevant to the present formulation. The distinct antibody patterns observed after oral and combined administration suggest that the route of antigen delivery influenced the quality of the immune response. This is biologically plausible because chitosan is a mucoadhesive polymer and has been widely investigated for oral and other mucosal vaccine applications. Chitosan-based particles can increase antigen retention at mucosal surfaces and facilitate interaction with antigen-presenting cells, thereby supporting both mucosal and systemic immune responses [32].

The cytokine results further indicated that the immunization routes generated different cellular immune profiles. Subcutaneous administration significantly increased IL-4, whereas the combined subcutaneous-oral regimen and oral administration of

rOmp31-loaded nanoparticles significantly increased IFN- γ and IL-12. Oral immunization also significantly increased IL-17. The IFN- γ and IL-12 responses are particularly relevant to *Brucella* infection because effective control of this intracellular pathogen depends substantially on cell-mediated immunity. These findings are consistent with previous Omp31 studies. Cassataro et al. demonstrated a CD4⁺ Th1-oriented response following rOmp31 vaccination, while Golshani et al. reported increased IFN- γ and IL-2 together with T-cell proliferation after vaccination with an L7/L12-truncated Omp31 construct [6, 30].

The increased IL-17 observed after oral immunization is also noteworthy, particularly in the context of mucosal vaccination. Th17-associated responses may contribute to mucosal host defense; however, the present study did not directly establish a functional role for IL-17 in protection. Therefore, the increased IL-17 should be interpreted as an immunological association rather than evidence of a causal mechanism. Future studies incorporating cellular phenotyping and functional assays would be necessary to determine whether Th17 responses contribute to the protection induced by the oral formulation.

The challenge experiment provided the strongest evidence for the biological relevance of the immune responses. Four weeks after i.p. challenge with *B. abortus* strain 544, all rOmp31-immunized groups showed significantly reduced bacterial burdens compared with controls. The combined subcutaneous-oral regimen produced the greatest reduction, corresponding to 1.55 log₁₀ units, followed by oral and subcutaneous immunization with reductions of 1.12 and 0.92 log₁₀ units, respectively. These findings are consistent with previous evidence supporting Omp31 as a protective *Brucella* antigen. Cassataro et al. reported approximately 1.16-log protection against *B. melitensis* following rOmp31 vaccination, while Golshani et al. demonstrated significant protection against both *B. melitensis* and *B. abortus* using an L7/L12-truncated Omp31 fusion protein [6, 30].

The magnitude of protection observed in the present study should, however, be compared cautiously with previous reports because the studies differed in antigen format, adjuvant or delivery system, immunization schedule, challenge strain, and experimental endpoint. The present results are nevertheless important because they extend previous Omp31 findings to a chitosan nanoparticle formulation and directly compare different administration routes. The combined subcutaneous-oral regimen produced the highest reduction in splenic bacterial burden, suggesting that simultaneous induction of systemic and mucosal immune responses may be beneficial. This interpretation is consistent with the recognized potential of chitosan-based systems to support mucosal as well as systemic immunity [32].

Previous Omp31 studies also indicate that protection is not necessarily dependent on antibody responses alone. An Omp31 DNA vaccine protected BALB/c mice against *B. melitensis* and *B. ovis* and induced predominantly CD8⁺ T-cell-mediated cytotoxic activity, while also involving CD4⁺ T cells [6]. In another study, a chimeric BLS-Omp31 vaccine induced humoral, Th1, and cytotoxic responses and provided protection against *B. ovis* and, to a lesser extent, *B. melitensis* [33]. These observations support the concept that Omp31-mediated protection can involve multiple arms of cellular immunity. In the present study, the association between increased IFN- γ /IL-12 responses and reduced bacterial burden is compatible with this concept, although the absence of direct T-cell functional assays prevents definitive identification of the cellular mechanisms responsible for protection.

The present study therefore adds to the existing Omp31 literature in two important respects. First, it evaluates full-length rOmp31 in a chitosan nanoparticle delivery platform rather than relying solely on free recombinant protein, DNA vaccination, or Omp31-containing fusion constructs. Second, it demonstrates that administration route can influence both the immune profile and the magnitude of protection, with the combined subcutaneous-oral regimen producing the strongest reduction in splenic bacterial burden. These findings suggest that optimization of the delivery system and vaccination schedule may be as important as antigen selection when developing Omp31-based subunit vaccines.

A major strength of the study is the integration of antigen characterization, recombinant protein production, nanoparticle formulation, humoral and cytokine analyses, and an in vivo challenge model within a single experimental framework. The use of *B. abortus* strain 544 challenge also provides direct evidence of biological protection against the target pathogen. In addition, comparison of oral, subcutaneous, and combined administration provides useful information regarding the influence of vaccination route.

Conclusion:

In conclusion, the present study demonstrates that rOmp31 formulated in chitosan nanoparticles can induce substantial antigen-specific humoral and cellular immune responses and significantly reduce *B. abortus* colonization following experimental challenge. The combined subcutaneous-oral regimen produced the greatest reduction in splenic bacterial burden, suggesting that vaccination route and delivery platform can influence the protective outcome. Together with previous studies demonstrating the protective potential of Omp31-based vaccines, these findings support further investigation of chitosan-encapsulated rOmp31 as a candidate subunit vaccine platform. Further optimization of nanoparticle homogeneity and more comprehensive characterization of cellular and mucosal immunity will be required to define the mechanisms and durability of the observed protection.

Acknowledgments: The authors gratefully acknowledge the financial support for this work that was provided by Faculty of Medicine, Shahed University.

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Figure legends

Fig. 1 Predicted secondary structure composition of rOmp31. PSIPRED analysis reveals a predominant α -helical content

Fig. 2 Three-dimensional structure prediction and stereochemical validation of the rOmp31 construct. **a** Predicted 3D model of the designed recombinant protein generated via homology modeling, depicted as a ribbon diagram. The color gradient transitions from the N-terminus (blue) to the C-terminus (red). **b** Ramachandran plot illustrating the distribution of ϕ (ϕ) and ψ (ψ) dihedral angles. The clustering of most residues within the energetically favorable regions confirms the high stereochemical quality and structural reliability of the predicted model

Fig. 3 Expression, purification, and immunological validation of rOmp31. **a** Expression analysis via 14% SDS-PAGE. Lane 1: *E. coli* BL21 harboring pET-28a-omp31 before IPTG induction; Lane 2: *E. coli* BL21 harboring pET-28a-omp31 after 2 h of IPTG induction; Lane 3: *E. coli* BL21 harboring pET-28a-omp31 following overnight IPTG induction; Lane 4: Protein molecular weight marker (Sinaclon, SL7013 and SL7014). The arrow indicates the rOmp31 band. **b** Purification profile of rOmp31 via 14% SDS-PAGE. Lane 1: Cell lysate prior to column loading; Lane 2: Molecular weight marker; Lane 3: Column flow-through; Lane 4: Wash fraction (pH 5.9); Lanes 5-9: Elution fractions (pH 4.5). **c** Western blot analysis of rOmp31. Detection was performed using an anti-His tag antibody. Lane 1: Purified rOmp31; Lane 2: Negative control; Lane 3: Molecular weight marker. **d** Protein ladder marker

Fig. 4 Physicochemical characterization of rOmp31-loaded chitosan nanoparticles. **a** Particle size distribution (DLS analysis) showing the hydrodynamic diameter of the rOmp31-loaded chitosan nanoparticles. **b** Zeta potential analysis illustrating the surface charge of the rOmp31-encapsulated chitosan nanoparticles

Fig. 5 Release of nanoparticled rOmp31. In vitro release profile of rOmp31 from chitosan nanoparticles

Fig. 6 Evaluation of humoral immune responses in mice immunized with rOmp31. **a** Serum IgG titers, **b** Serum IgA titers, and **c** Fecal IgA titers

Fig. 7 Cytokine production by splenocytes from mice immunized with rOmp31 formulated in chitosan nanoparticles. Levels of **a** IL-4, **b** IFN- γ , **c** IL-12, and **d** IL-17 were measured in culture supernatants following antigen stimulation. Data are presented as mean $\pm$ SD

Fig. 8 Evaluation of protection and reduction in bacterial shedding in immunized mice following infectious challenge. Data are presented as mean $\pm$ standard deviation (SD)

Tables

Table 1 Experimental immunization schedule for BALB/c mice

Animal Experimental Groups	Administration Route	Type of Administered Formulation	Experimental Immunization Schedule		Administered Antigen dose
			Phase	Day	
T1 (n=7)	Subcutaneous	Chitosan nanoparticled rOmp31	1st	0	15µg/mouse
			2nd	7	15µg/mouse
			3rd	14	15µg/mouse
			4th	21	15µg/mouse
T2 (n=7)	Subcutaneous Oral gavage	Chitosan nanoparticled rOmp31	1st	0	15µg/mouse
			2nd	7	60µg/mouse
			3rd	14	60µg/mouse
			4th	21	60µg/mouse
			5th	28	60µg/mouse
T3 (n=7)	Oral gavage	Chitosan nanoparticled rOmp31	1st	0	60µg/mouse
			2nd	7	60µg/mouse
			3rd	14	60µg/mouse
			4th	21	60µg/mouse
C1 (n=7)	Oral gavage	Chitosan nanoparticled	1st	0	60µg/mouse
			2nd	7	60µg/mouse
			3rd	14	60µg/mouse
			4th	21	60µg/mouse
C2 (n=7)	Subcutaneous	PBS	1st	0	15µl/mouse
			2nd	7	15µl/mouse
			3rd	14	15µl/mouse
			4th	21	15µl/mouse
C3 (n=7)	Subcutaneous	Chitosan nanoparticled	1st	0	15µg/mouse
			2nd	7	15µg/mouse
			3rd	14	15µg/mouse
			4th	21	15µg/mouse

Table 2 The predicted B-cell epitopes by ABCpred

Rank	Sequence	Start Position	Score
1	GIEQDGAQDFSGSLDV	7	0.89
1	LSAYDDGESFSAGNSK	122	0.89
2	GSTVKSKLVDNGDLSD	53	0.87
3	ETKVQWFGTVRARLGF	84	0.86
4	MVYGTGGLAYGKVKTS	106	0.84
5	ARLGFTPTERLMVYGT	95	0.83
6	FKHPFSGIEQDGAQDF	1	0.80

Table 3 Predicted binding affinities of candidate peptides to BALB/c MHC class I molecules via IEDB

Allele	#	Start	End	Length	Peptide	Core	Icore	Score	Percentile Rank
H-2-Kd	1	114	122	9	AYGKVKTSL	AYGKVKTSL	AYGKVKTSL	0.892471	0.01
H-2-Ld	1	123	131	9	SAYDDGESF	SAYDDGESF	SAYDDGESF	0.164751	0.47
H-2-Ld	1	100	108	9	TPTERLMVY	TPTERLMVY	TPTERLMVY	0.117224	0.67
H-2-Kd	1	89	97	9	WFGTVRARL	WFGTVRARL	WFGTVRARL	0.09778	0.79
H-2-Dd	1	97	105	9	LGFTPTERL	LGFTPTERL	LGFTPTERL	0.093885	0.15

Table 4 Predicted binding affinities of candidate peptides to BALB/c MHC class II molecules via IEDB

Allele	#	Start	End	Length	Core Sequence	Peptide Sequence	Score	Percentile Rank
H2-IAd	1	65	79	15	DIGVAGNLS	DLSDIGVAGN LSGDE	0.4950	3
H2-IAd	1	64	78	15	DIGVAGNLS	GDLSDIGVAG NLSGD	0.4337	4.20
H2-IAd	1	63	77	15	LSDIGVAGN	NGDLSDIGVA GNLSG	0.3935	5.10
H2-IAd	1	87	101	15	FGTVRARLG	VQWFQTVRA RLGFTP	0.3821	5.40
H2-IAd	1	110	124	15	LAYGKVKTS	TGGLAYGKVK TSLSA	0.3397	6.40
H2-IAd	1	24	38	15	VGGVQAGYN	ASGFVGGVQA GYNWQ	0.3097	7.40
H2-IAd	1	115	129	15	VKTSLSAYD	YGKVKTSLSA YDDGE	0.3022	7.70

Table 5 Assessment of Physicochemical properties of rOmp31

Number of amino acids	141
Theoretical pI	4.70
Instability Index (II)	8.39
Aliphatic index	71.21

Table 6 Protection against *B. abortus* in BALB/c mice immunized with rOmp31 compared with control groups

Experimental Groups	Splenic bacterial burden (log ₁₀ CFU)	Protection (log ₁₀ reduction)
rOmp31 Oral	4.72±0.23	1.12±0.0959
rOmp31 Subcutaneous-Oral	4.38±0.14	1.55±0.0121
rOmp31 Subcutaneous	5.10±0.16	0.92±0.1770
Control Oral	5.85±0.19	0
Control Subcutaneous	6.02±0.13	0