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Comparative Serum Immune Responses to Bacterial Ghost and Liposomal APEC Vaccine Formulations in Broiler Chickens

Ahmid. Hamid Dawood Alatbee[1*], Ali. A. Al-iedani[1]

1. Department of Microbiology, College of Veterinary Medicine, University of Basrah, Iraq

*** Corresponding author email address:** ahmed.hamed@uobasrah.edu.iq

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A B S T R A C T

This study compared the preliminary serum immune-marker responses induced by two vaccine preparations against avian pathogenic Escherichia coli (APEC) in broiler chickens: a bacterial ghost preparation (BGs) and a liposome-based preparation (LIPO). Serum IL-4, IL-10, IL-12, IL-1β, IFN-γ, and total IgY were measured by ELISA to evaluate humoral, cellular, and inflammatory responses relative to a negative control group. Overall differences among the three groups were statistically significant for all measured markers (p<0.0001). Direct comparison of the vaccinated groups showed significantly higher IL-10, IL-1β, and total IgY concentrations in the BGs group, whereas IL-4 was significantly higher in the LIPO group. IL-12 and IFN-γ did not differ significantly between the two vaccinated groups. These findings indicate that the two preparations generated distinct preliminary serum immune-marker profiles. Because protective efficacy was not evaluated in an APEC challenge model, further studies are required before either preparation can be considered an effective vaccine against colibacillosis.

Keywords: *APEC; Bacterial ghosts; Broiler chickens; Cytokines; Immunogenicity; Liposomal vaccine; Total IgY*

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1 Introduction

Avian pathogenic *Escherichia coli* (APEC) comprises a diverse group of extraintestinal pathogenic *E. coli* strains that cause colibacillosis, one of the most economically important bacterial diseases affecting poultry production worldwide (Adams et al., 2025; Kathayat et al., 2021; Nawaz et al., 2024). Colibacillosis may present as respiratory disease, airsacculitis, pericarditis, perihepatitis, cellulitis, or septicemia, particularly when birds are exposed to inadequate ventilation, overcrowding, environmental stress, or concurrent infections (Dziva & Stevens, 2008; Nolan et al., 2020). The disease may lead to mortality, impaired growth and productivity, carcass condemnation, and increased treatment costs.

Control of APEC infections requires an integrated approach involving biosecurity, hygienic husbandry, appropriate nutrition, environmental management, vaccination, and prudent antimicrobial use. The emergence of antimicrobial-resistant *E. coli* in poultry and livestock further underscores the need for effective preventive strategies to reduce reliance on antimicrobial treatment (Kathayat et al., 2021; Yassin et al., 2017).

The avian immune response to APEC involves both innate and adaptive mechanisms. Heterophils, macrophages, antimicrobial peptides, and other innate components provide an early response to bacterial invasion, whereas cellular and humoral immune mechanisms contribute to bacterial clearance and longer-term immunity. Cytokines coordinate these responses, but excessive or poorly regulated inflammation may also contribute to tissue injury (Abed et al., 2022; Nawaz et al., 2024). Immune competence can additionally be influenced by age, nutrition, environmental stress, and concurrent disease (Nolan et al., 2020).

Bacterial ghosts (BGs) are non-living bacterial envelopes from which the cytoplasmic contents have been removed while the native three-dimensional structure and many surface components are preserved (Kudela et al., 2010; Langemann et al., 2010). Retained pathogen-associated molecular patterns and surface antigens may provide intrinsic immunostimulatory activity, facilitate uptake by antigen-presenting cells, and support delivery of vaccine antigens (Chen et al., 2021; Hajam et al., 2017).

Liposomal vaccines represent another adaptable approach for antigen delivery. Liposomes are phospholipid-bilayer vesicles capable of carrying antigens, adjuvants, or immunostimulatory molecules. Their size, surface charge, lipid composition, antigen localization, and route of administration can influence antigen stability, uptake by antigen-presenting cells, and the resulting immune response (Krasnopolsky & Pylypenko, 2022; Perrie et al., 2016; Schwendener, 2014; Watson et al., 2012).

Although both BGs and liposomes have been proposed as vaccine-delivery platforms, direct comparative data on their serum immune marker profiles in broiler chickens remain limited. Therefore, this study compared the preliminary immunogenic responses induced by bacterial ghost and liposomal APEC preparations by measuring serum IL-4, IL-10, IL-12, IL-1β, IFN-γ, and total IgY. Protective efficacy was not assessed through an experimental APEC challenge.

2 Material and Methods

2.1 Molecular Confirmation of the APEC Isolate

The avian pathogenic *Escherichia coli* isolate used in this study was obtained from the bacterial collection previously described by Mohammed and Al-Iedani (2024) (Mohammed & Al-Iedani, 2024). The isolate was phenotypically evaluated on eosin methylene blue agar and molecularly examined by PCR. The *uidA* marker was used to support identification as *Escherichia coli*. Additional PCR assays targeted virulence-associated genes documented in the available laboratory documentation, including *iss*, *ompT*, *iutA*, *hly*, and an iron-acquisition-associated target. PCR products were separated by agarose-gel electrophoresis and interpreted by comparison with their recorded expected amplicon sizes: 203 bp for *uidA*, 227 bp for *iss*, 196 bp for *ompT*, 230 bp for *iutA*, 228 bp for *hly*, and 146 bp for the iron-acquisition-associated target.

2.2 Bacterial Ghost Vaccine Preparation

Bacterial ghosts were prepared using a chemical "sponge-like" method based on the sequential application of surfactant, alkaline, and oxidative agents. The purpose of the procedure was to remove bacterial cytoplasmic contents while preserving the structural integrity of the cell envelope.

1. Surfactant treatment: The bacterial suspension was exposed to 1.15 mg/mL sodium dodecyl sulfate to permeabilize the bacterial membrane.
2. Alkaline treatment: The suspension was subsequently treated with 3.125 mg/mL sodium hydroxide.
3. Oxidative treatment: The bacterial suspension was treated with 8.79 µL/mL hydrogen peroxide and 1.05 µL/mL of the calcium carbonate preparation.

Preservation of bacterial-envelope morphology and formation of membrane pores were evaluated by electron microscopy according to the chemical bacterial-ghost preparation approach described by Thanh and Hanh (2020) (Thanh & Hanh, 2020). The final preparation was standardized to an antigenic equivalent of 1.5×10^8 CFU/mL. A separate post-treatment culture-based assessment of residual bacterial viability was not documented in the available study records.

2.3 Preparation of the Liposomal Vaccine

The liposomal preparation was produced using the thin-film hydration method. Phospholipids at 0.08 mg/mL and cholesterol at 0.02 mg/mL were dissolved in a methanol-chloroform mixture at a ratio of 1:2 (v/v). The organic solvents were removed by rotary evaporation, leaving a thin lipid film on the flask wall.

The APEC antigen was incorporated into the liposomal preparation, and the total protein concentration was measured using the bicinchoninic acid assay. The final formulation contained 0.5 µg/mL of encapsulated antigen and had an encapsulation efficiency of 90%. Because each bird received 0.5 mL, the administered amount was equivalent to 0.25 µg of encapsulated antigen per bird per injection.

Particle size and distribution were assessed by dynamic light scattering, and liposome morphology was evaluated by transmission electron microscopy, following the general thin-film preparation approach described by Al-Amin et al. (2020) (Al-Amin et al., 2020).

2.4 Measurement of Immune Markers

The systemic immune response was evaluated by measuring serum interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-12 (IL-12), interleukin-1β (IL-1β), interferon-gamma (IFN-γ), and total immunoglobulin Y (IgY). Measurements were performed using chicken ELISA kits supplied by Sun Long Biotech Co., Ltd., according to the manufacturer's instructions.

2.5 Animal Model and Vaccination

Thirty-one-day-old male and female Ross 308 broiler chickens were randomly assigned to three groups of ten birds each: G1, negative control; G2, bacterial ghost preparation; and G3, liposomal preparation. The birds were maintained under controlled husbandry conditions with appropriate temperature, humidity, photoperiod, nutrition, and water availability. Animal caretakers, laboratory technicians, and the statistician were blinded to the coded treatment groups.

The negative control group received 0.5 mL of sterile saline by subcutaneous injection. Birds in the BGs group received 0.5 mL of the bacterial ghost preparation, corresponding to 7.5×10^7 CFU-equivalent per injection. Birds in the LIPO group received 0.5 mL of the liposomal preparation containing 0.25 µg of encapsulated antigen per injection. Vaccinations were administered subcutaneously on days 10, 20, and 30 after hatching. Blood samples were collected on day 35 for measurement of serum cytokines and total IgY. Birds showing clinical illness or signs suggestive of APEC infection before vaccination were excluded.

2.6 Safety and Reactogenicity

Birds were monitored for mortality, clinical signs, and local injection-site reactions, including swelling and redness. Three birds initially assigned to the negative control group died during the acclimation period before vaccination, reportedly because of transport-related stress. They were replaced with healthy birds of the same age and source before the vaccination programme began, maintaining ten birds in each group. No treatment-related mortality was reported during the vaccination period.

2.7 Statistical Analysis

Immune-marker concentrations were summarized as Mean±Standard Deviation (SD). Because assumptions of normality or homogeneity of variance were not satisfied for all variables, differences among the three groups were evaluated using the Kruskal-Wallis test followed by Dunn's post hoc multiple-comparison test. Pairwise comparisons between the BGs and LIPO groups were reported using adjusted *p*-values. Statistical significance was defined as an adjusted *p*-value below 0.05. Individual bird measurements were used in the analysis, with 10 birds per group.

3 Results

3.1 Microbiological and Molecular Confirmation of the APEC Isolate

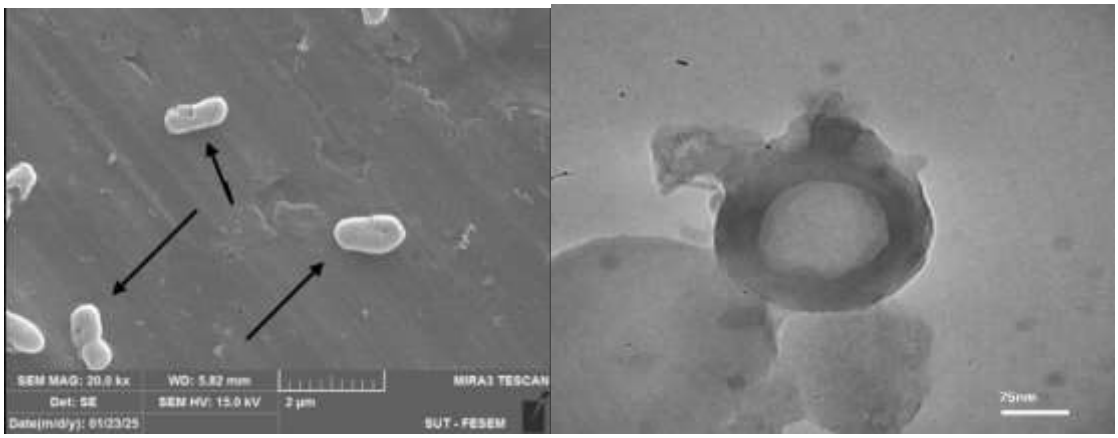
The *Escherichia coli* isolate produced characteristic colonies with a green metallic sheen on eosin methylene blue agar. Molecular identification by PCR confirmed amplification of the expected 203-bp *uidA* product.

PCR analysis also detected five virulence-associated targets: *iss* (227 bp), *ompT* (196 bp), *iutA* (230 bp), *hly* (228 bp), and an iron-acquisition-associated target (146 bp). The corresponding amplification products are shown in Figure 3.

3.2 Characterization of the Vaccine Preparations

Scanning electron microscopy revealed BG preparations with preserved bacterial envelope morphology and visible surface pores (Figure 1A). Transmission electron microscopy showed approximately spherical liposomal vesicles with a diameter of approximately 75 nm (Figure 1B).

Dynamic light-scattering analysis showed a main hydrodynamic-diameter peak at approximately 150 nm. The liposomal preparation had a polydispersity index of 0.18±0.02 and a zeta potential of −25.4±3.1 mV (Figure 2).



A **B**

Figure 1. Electron micrographs of the bacterial ghost and liposomal preparations. (A) Scanning electron micrograph showing preserved bacterial-envelope morphology and visible pores. (B) Transmission electron micrograph showing a liposomal vesicle with an approximate diameter of 75 nm.

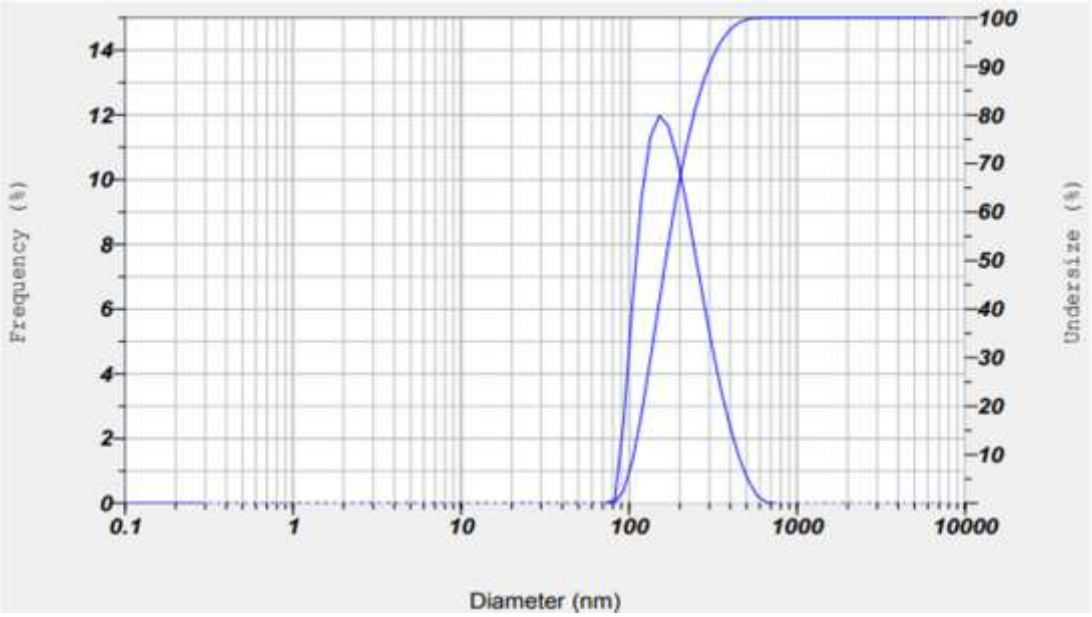


Figure 2. Dynamic light-scattering analysis of the liposomal preparation. The main hydrodynamic diameter peak was centred at approximately 150 nm; the polydispersity index was 0.18±0.02, and the zeta potential was −25.4±3.1 mV.

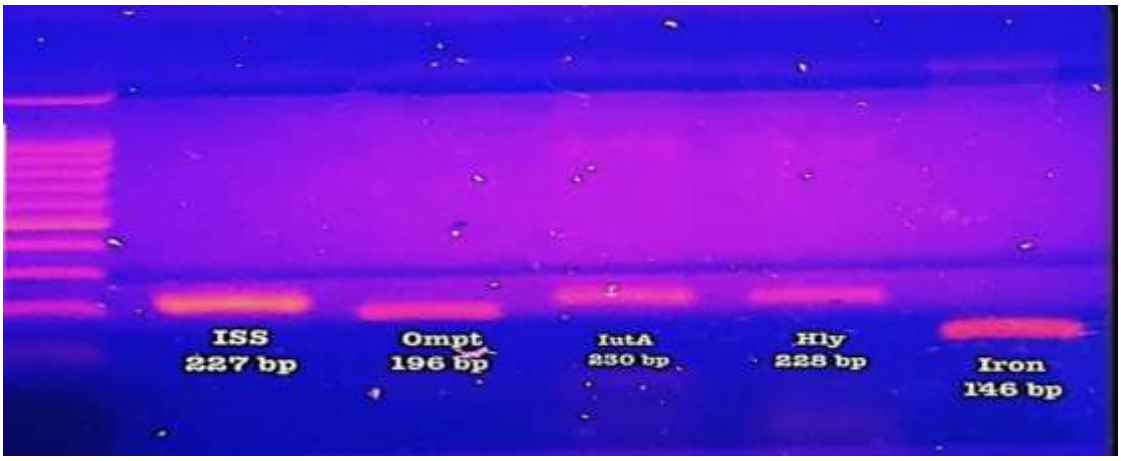


Figure 3. Agarose-gel electrophoresis showing amplification products for the APEC-associated virulence targets iss (227 bp), ompT (196 bp), iutA (230 bp), hly (228 bp), and the iron-acquisition-associated target (146 bp).

3.3 Serum Humoral Immune Markers

Serum IL-4, IL-10, and total IgY concentrations differed significantly among the negative control, BGs, and LIPO groups ($p<0.001$; Table 1). The highest mean IL-4 concentration was observed in the LIPO group. In contrast, IL-10 and total IgY concentrations were highest in the BGs group. Direct comparison of the two vaccinated groups showed that IL-4 was significantly higher in the LIPO group ($p=0.03$), whereas IL-10 ($p=0.03$) and total IgY ($p=0.03$) were significantly higher in the BGs group.

Table 1. Serum concentrations of humoral immune markers in broiler chickens following vaccination

IL-4 (pg/mL)	11.56±0.41	13.64±0.63	18.10±0.53	<0.001
IL-10 (pg/mL)	24.99±0.49	74.70±1.19	45.71±0.97	<0.001
Total IgY	3.66±0.44	44.65±0.67	40.73±0.46	<0.001

Values are presented as Mean±SD. Overall differences among groups were evaluated using the Kruskal-Wallis test.

3.4 Serum Cellular and Inflammatory Immune Markers

Serum IL-12, IL-1β, and IFN-γ concentrations also differed significantly among the three experimental groups ($p<0.001$; Table 2). Mean concentrations of all three markers were higher in the vaccinated groups than in the negative control group. The BGs group had the highest mean concentrations of IL-12, IL-1β, and IFN-γ. However, direct comparison between the vaccinated groups showed no statistically significant differences in IL-12 ($p=0.13$) or IFN-γ ($p=0.27$). IL-1β was significantly higher in the BGs group than in the LIPO group ($p=0.03$).

Table 2. Serum concentrations of cellular and inflammatory immune markers in broiler chickens following vaccination

IL-12 (pg/mL)	8.52±0.43	32.51±0.42	27.80±9.66	<0.001
IL-1β (pg/mL)	20.15±0.43	80.83±0.52	62.06±0.44	<0.001
IFN-γ (pg/mL)	10.35±0.42	30.53±0.51	29.56±0.55	<0.001

Values are presented as Mean±SD. Overall differences among groups were evaluated using the Kruskal-Wallis test.

Figures 4-6 illustrate the observed differences in the measured immune markers among the three experimental groups.

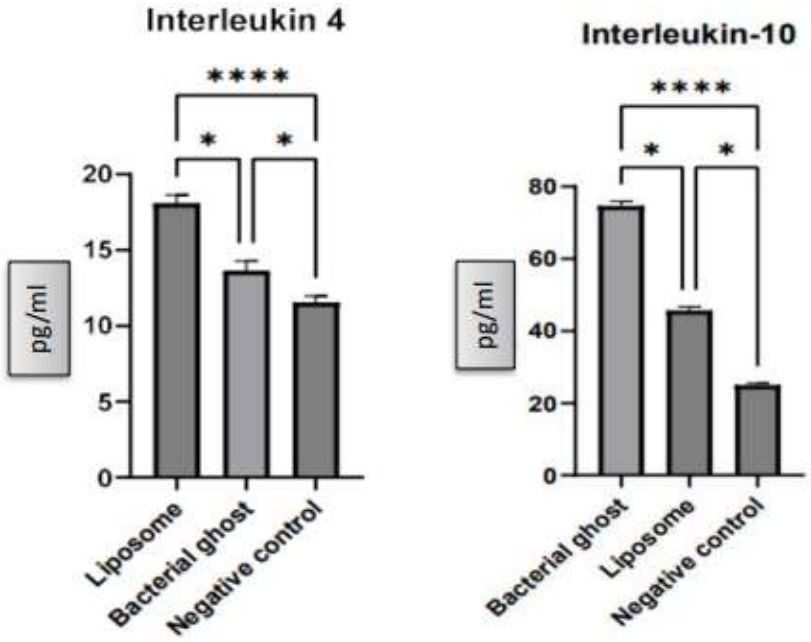


Figure 4. Serum concentrations of (A) IL-4 and (B) IL-10 in the BGs, LIPO, and negative control groups. Bars show mean values with error bars as supplied by the authors. *p<0.05; ****p<0.001.

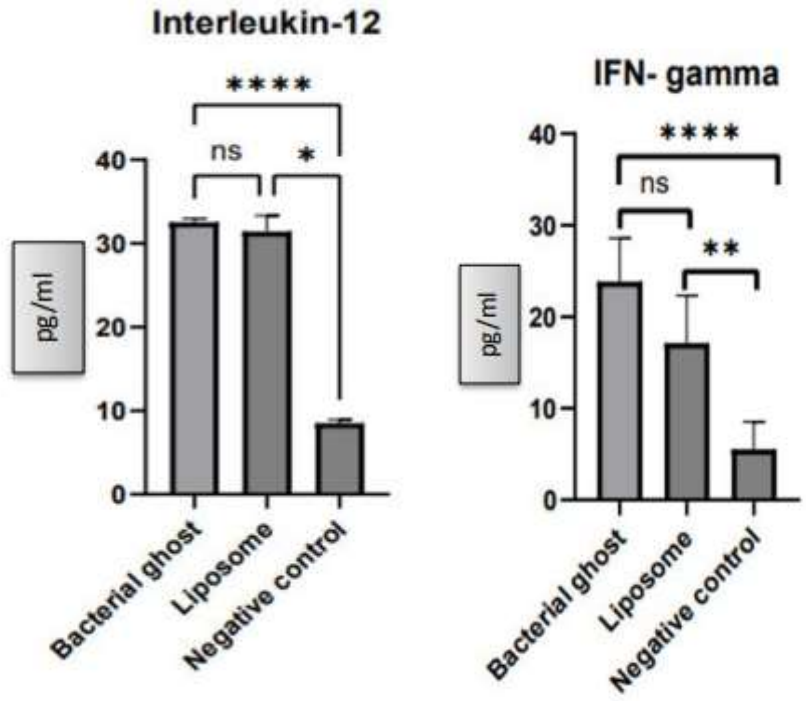


Figure 5. Serum concentrations of (A) IL-12 and (B) IFN-γ in the BGs, LIPO, and negative control groups. Bars show mean values with error bars as supplied by the authors. *$p<0.05$; **$p<0.01$; ****$p<0.001$; NS, not significant.

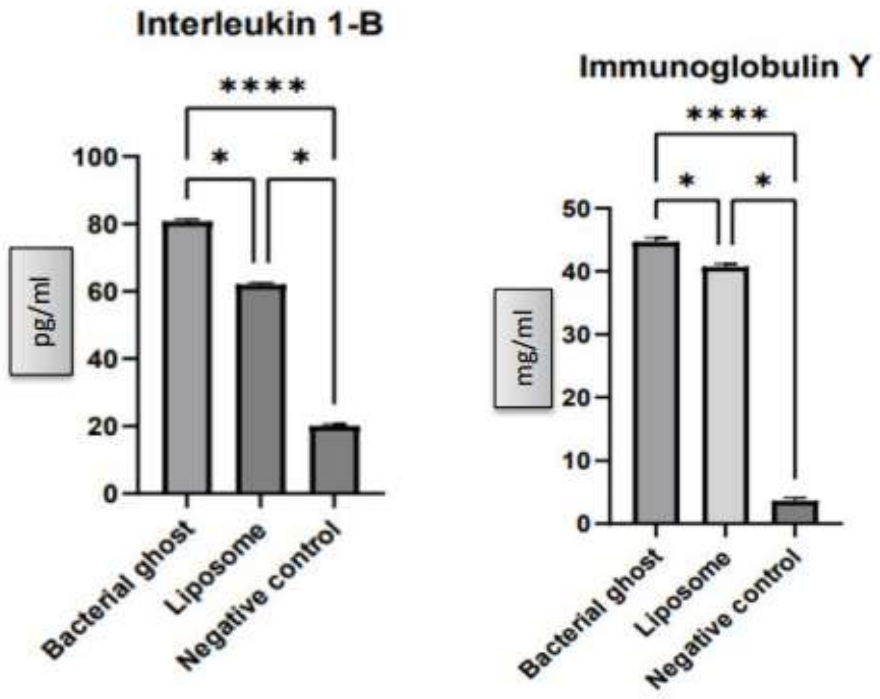


Figure 6. Serum concentrations of (A) IL-1β and (B) total IgY in the BGs, LIPO, and negative control groups. Bars show mean values with error bars as supplied by the authors. **p<0.05; ****p<0.001.*

3.5 Pairwise Comparison of the BGs and LIPO Groups

Dunn's post hoc comparisons showed significant differences between the BGs and LIPO groups for IL-4, IL-10, total IgY, and IL-1β. No significant differences were detected for IL-12 or IFN-γ.

Table 3. Pairwise comparison of serum immune markers between the BGs and LIPO groups

IL-12	8.00	0.13	Not significant
IL-10	10.00	0.03	Higher in BGs
IL-4	10.20	0.03	Higher in LIPO
Total IgY	10.00	0.03	Higher in BGs
IL-1β	10.00	0.03	Higher in BGs
IFN-γ	6.70	0.27	Not significant

Pairwise comparisons were performed using Dunn's post hoc test. Adjusted p-values below 0.05 were considered statistically significant.

4 Discussion

This preliminary study compared the physicochemical characteristics and serum immune-marker responses associated with bacterial ghost and liposomal APEC preparations in broiler chickens. Both preparations produced measurable changes in humoral, cellular, and inflammatory markers, but the response patterns differed between the platforms.

Electron microscopy showed preservation of bacterial-envelope morphology in the BG preparation, whereas the liposomal formulation consisted of approximately spherical vesicles. The liposomes measured approximately 75 nm by transmission electron microscopy, while dynamic light scattering indicated a hydrodynamic diameter of approximately 150 nm. This difference is expected because transmission electron microscopy evaluates dehydrated particles, whereas dynamic light scattering measures particles in suspension together with their associated

hydration layer. The polydispersity index of 0.18±0.02 indicated a relatively narrow size distribution, and the zeta potential of −25.4±3.1 mV suggested moderate colloidal stability. The biological behavior of liposomal preparations can be influenced by particle size, charge, lipid composition, antigen loading, and administration route (Al-Amin et al., 2020; Alavi et al., 2017; Krasnopolsky & Pylypenko, 2022; Pati et al., 2018; Perrie et al., 2016; Schwendener, 2014; Watson et al., 2012).

Phenotypic growth on eosin methylene blue agar and amplification of the *uidA* gene supported identification of the isolate as *Escherichia coli*. Detection of *iss*, *ompT*, *iutA*, *hly*, and an iron-acquisition-associated target supported an APEC-associated virulence profile. Such virulence-associated genes contribute to the pathogenic potential and diversity of APEC strains; however, their presence alone does not establish the degree of virulence or vaccine protection (Adams et al., 2025; Kathayat et al., 2021; Mohammed & Al-Iedani, 2024; Nawaz et al., 2024). Protective efficacy would require confirmation in a controlled challenge model.

The two preparations produced distinct humoral immune marker profiles. IL-4 was significantly higher in the LIPO group than in the BGs group. IL-4 is associated with Th2-type responses and supports B-cell activation and antibody production. The stronger IL-4 response observed with the liposomal preparation may reflect improved antigen delivery and presentation, consistent with liposomal systems' ability to modulate humoral immune responses based on their formulation characteristics (Jumaah & Al-Iedani, 2024; Perrie et al., 2016; Zhou et al., 2023).

In contrast, the BGs group had significantly higher IL-10 and total IgY concentrations than the LIPO group. IL-10 is an immunoregulatory cytokine that can limit excessive inflammation while supporting aspects of B-cell function and antibody production (Jumaah & Al-Iedani, 2024; Saraiva & O'Garra, 2010). Its elevation in the BGs group may therefore indicate a stronger regulatory component; however, increased IL-10 should not be interpreted as evidence of superior protection.

Total IgY was higher in both vaccinated groups than in the negative control group and was significantly higher in the BGs group than in the LIPO group. This finding suggests stimulation of the systemic humoral response. However, the assay measured total IgY rather than APEC-specific IgY, so the results do not confirm an antigen-specific antibody response. The greater total IgY response in the BGs group may be related to the preservation of bacterial surface antigens and pathogen-associated molecular patterns that provide intrinsic immunostimulatory activity (Chen et al., 2021; Hajam et al., 2017; Kudela et al., 2010; Langemann et al., 2010).

The vaccinated groups also showed higher mean concentrations of IL-12, IL-1β, and IFN-γ than the negative control group. IL-12 contributes to Th1 differentiation and promotes IFN-γ production, thereby linking innate antigen recognition with cellular immune activation (Abed et al., 2022; Zundler & Neurath, 2015). However, IL-12 did not differ significantly between the BGs and LIPO groups, so the results do not demonstrate superiority of either platform for this marker.

IL-1β was significantly higher in the BGs group than in the LIPO group. IL-1β is an important mediator of innate inflammation and can enhance recruitment and activation of immune cells (Dinarello, 2018; Jumaah & Al-Iedani, 2024). The greater IL-1β response in the BGs group may reflect stronger recognition of preserved bacterial-envelope components. Nevertheless, an increased inflammatory response is not necessarily indicative of improved protection, as excessive inflammation may contribute to tissue injury.

IFN-γ concentrations were elevated in both vaccinated groups relative to the negative control mean. IFN-γ is associated with macrophage activation and cell-mediated responses against bacterial pathogens (Abed et al., 2022; Bagheri et al., 2023). However, the difference between the BGs and LIPO groups was not statistically significant, indicating comparable IFN-γ-associated responses rather than a significant advantage for either preparation.

Collectively, the liposomal formulation was associated with a stronger IL-4 response, whereas the BGs preparation produced higher IL-10, IL-1β, and total IgY concentrations. No significant differences were observed between the vaccinated groups for IL-12 or IFN-γ. The two preparations should therefore be described as inducing distinct preliminary immune-marker profiles rather than one platform producing a universally stronger or more balanced immune response.

4.1 Study Limitations

This study evaluated serum cytokines and total IgY as preliminary indicators of immunogenicity. Protective efficacy was not assessed using an APEC challenge, survival analysis, lesion scoring, bacterial shedding, or organ colonization measurements. APEC-specific antibody

responses, mucosal IgA, and respiratory immune responses were not measured. The relatively small sample size, single experimental setting, and absence of long-term immune monitoring may also limit generalizability. In addition, the marked variability observed in some measurements, particularly IL-12 in the LIPO group, should be taken into account. Future studies should include larger sample sizes, antigen-specific antibody assays, mucosal immune assessments, longer follow-up, and controlled challenge experiments using a virulent APEC strain.

5 Conclusion

Both bacterial ghost and liposomal APEC preparations induced measurable serum immune-marker responses in broiler chickens. The liposomal preparation produced a significantly higher IL-4 response, whereas the bacterial ghost preparation was associated with significantly higher IL-10, IL-1β, and total IgY concentrations. IL-12 and IFN-γ did not differ significantly between the two vaccinated groups.

These findings indicate that the two platforms generated distinct preliminary immune-response profiles. Because APEC-specific antibodies and protective efficacy were not evaluated, the results do not establish protection against colibacillosis. Controlled challenge studies and assessment of antigen-specific systemic and mucosal immune responses are required before either preparation can be considered an effective APEC vaccine.

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Use of Artificial Intelligence

Generative artificial intelligence was used solely for language editing and grammatical improvement. All scientific content was reviewed and approved by the authors, who take full responsibility for the final manuscript.

Conflict of Interest

We declare that no conflict of interest.

Author Contributions

A.A. and A.H. contributed to conceptualization and study design. A.H. performed sampling and laboratory analyses. M.A. conducted the statistical analysis and contributed to data interpretation. A.H. and A.A. drafted the manuscript. All authors reviewed and approved the final manuscript.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethical Considerations

The study protocol was reviewed and approved by the Ethics Committee of the College of Veterinary Medicine, University of Basrah, Department of Microbiology. All methods were carried out in accordance with the approved ethical guidelines and protocols (Ethical Approval Code: 92/37/2025).

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