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Immunopathologic Evaluation of *E. Coli* Adjuvant Proteins Conjugated with ZnO Nanoparticles in Young Lambs

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ABSTRACT

This study evaluated the immunogenic potential of *Escherichia coli* adjuvant proteins conjugated with zinc oxide nanoparticles as a novel immunization strategy in young lambs (3–3.5 months of age). Twenty lambs were randomly divided into four groups (n=5) after adaptation. Group 1 served as a negative control, while Group 2 was a positive control challenged subcutaneously with virulent *E. coli* (1×10^7 CFU/ml) on day 28. Groups 3 and 4 received two subcutaneous doses of WCECK and ECCFPs proteins, respectively, at 14-day intervals, and were similarly challenged. Blood samples were collected on days 0, 7, 14, 28, and 35 to measure antibody levels (IgG-3, IgM) and proinflammatory cytokines (CXCL-2, IL-1β) via ELISA. Liver, spleen, and intestinal tissues were collected post-euthanasia for histopathological examination. The results demonstrated that both vaccinated and infected groups showed a time-dependent increase in IgG-3, IgM, CXCL-2, and IL-1β levels, reaching peak concentrations on day 35. Histopathological findings revealed necrotic and degenerative changes, inflammatory infiltration, congestion, and edema in target tissues. Conclusion, *E. coli* protein vaccines formulated with zinc oxide nanoparticles induced robust humoral and inflammatory responses in young lambs, suggesting their potential as candidate formulations for early protection against *E. coli* infections.

التقييم المناعي المرضي لبروتينات المعزز الخاصة بالإشريكية القولونية المرتبطة بجزيئات الزنك النانوية في الحملان حديثة الولادة

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الخلاصة

هدفت هذه الدراسة إلى تقييم الكفاءة المناعية لبروتينات المساعدة المناعية (Adjuvant Proteins) لبكتيريا الإشريكية القولونية المقترنة بجسيمات أكسيد الزنك النانوية كاستراتيجية تلقيح مبتكرة في الحملان اليافعة بعمر (3–3.5 أشهر). تم توزيع عشرين حملاً عشوائياً إلى أربع مجموعات متساوية (n = 5) بعد فترة التكيف. المجموعة الأولى سيطرة سالبة، في حين مثّلت المجموعة الثانية سيطرة موجبة تم تحديها تحت الجلد ببكتيريا الإشريكية القولونية الضارية بتركيز (1×10^7 CFU/ml) في اليوم 28 من التجربة. أما المجموعتان الثالثة والرابعة فقد حقنت تحت الجلد ببروتينات WCECK وECCFPs على التوالي، بفاصل زمني 14 يوماً بين الجرعتين، ثم تم تعريضهما للتحدي البكتيري بنفس الطريقة. تم جمع عينات الدم في الأيام 0 و7 و14 و28 و35 لقياس مستويات الأجسام المضادة (IgG-3, IgM) والسيتوكينات الالتهابية (CXCL-2, IL-1β) باستخدام ELISA. كما جُمعت عينات من الكبد والطحال والأمعاء بعد القتل الرحيم لغرض الفحص النسجي المرضي. أظهرت النتائج أن المجموعات الملقحة والمصابة سجلت زيادة تدريجية معتمدة على الزمن في مستويات IgG-3 وIgM وCXCL-2 وIL-1β، حيث بلغت أعلى قيمها في اليوم الخامس والثلاثين. وكشفت الفحوصات النسيجية المرضية عن وجود تغيرات تنكسية ونخرية، وارتشاحات التهابية، واحتقان، ووذمة في الأنسجة المستهدفة. وتخلص الدراسة إلى أن لقاحات بروتينات الإشريكية القولونية باستخدام جسيمات أكسيد الزنك النانوية قادرة على تحفيز استجابات مناعية خلطية والتهابية قوية في الحملان اليافعة، مما يشير إلى إمكانية اعتمادها كصيغ لقاحية واعدة لتوفير حماية مبكرة ضد إصابات الإشريكية القولونية.

الكلمات المفتاحية: الاجسام المضادة، الاستجابة المناعية، الالتهاب

INTRODUCTION

Nanotechnology has increasingly been applied in biomedical and veterinary research to improve vaccine delivery and immunogenicity through enhanced antigen stability, targeted delivery, and controlled immune activation. Nanoparticle-based adjuvants have demonstrated the capacity to enhance antigen presentation, prolong antigen persistence, and strengthen immune responses compared with conventional vaccine formulations (Farokhzad and Langer, 2009; Zhao *et al.,* 2014; Ali and Al-Juthery, 2017; Muna *et al.,* 2023; Ahmed and Yaaqoob, 2025). Using concepts from physics, chemistry, material science, biology, engineering, and medicine, nanotechnology is an interdisciplinary approach. A nanoparticle is any particle that is between 1 and 100 nm in size (Farokhzad and Langer, 2009; Zúñiga *et al.,* 2025). Clinical therapies have recently benefited greatly from nanotechnology and nanomedicine's use of biocompatible nanoscale drug carriers and vaccines, including liposomes, micelle nanoparticles, dendrimer nanoparticles, polymeric nanoparticles, and metal nanoparticles, for more effective and safer administration of several anticancer medications. Drug administration or vaccine carried with nanoparticles offers enhanced pharmacokinetics, longer circulation half-lives, and less undesirable side effects (Zhang *et al.,* 2008; Hu and Zhang, 2010).

Zinc oxide nanoparticles (ZnO-NPs) have attracted increasing interest as vaccine adjuvants due to their favorable immunomodulatory properties, biocompatibility, and cost-effectiveness compared with noble metal nanoparticles such as silver or gold. ZnO-NPs have been reported to enhance antigen uptake, support innate immune activation, and promote balanced inflammatory responses without excessive cytotoxicity at appropriate doses (Roselli *et al.,* 2003; Xu *et al.,* 2021). In food-producing animals, zinc compounds are widely used as nutritional supplements and exhibit an established safety profile when

properly formulated, making ZnO-NPs a practical and scalable option for veterinary vaccine applications. *Escherichia coli (E. coli)* is a unique bacterium that is thought to be responsible for a wide range of illnesses, including gastroenteritis, external gastrointestinal tract infections, bloodstream infections, and disorders of the central nervous system (Croxen and Finlay, 2010; Hameed and Mahmood, 2025). Colibacillosis is one of multiple disease states that result from infection by the bacterium *Escherichia coli*. This bacterium falls under the category of gram-negative organisms and is part of a normal microbiota that inhabits the intestines of ruminants as well as some other animal species. *E. coli* is not monotypic, as there are multiple known variations that have been categorized on their virulence mechanisms to include enterotoxigenic, enteropathogenic, and enteroinvasive varieties. Colibacillosis most commonly manifests in diarrhea, with particular regard to its high incidence in animals that are neonatal in status, as this is a population group that is particularly susceptible to the harmful effects of this disease (Bertani and Ruiz, 2018; El-Nady *et al.,* 2023). *E. coli* is excreted in the feces of infected animals and is transmitted between animals primarily via the fecal-oral route.

However, despite significant advancements in vaccination, have been made, new effective vaccinations are still needed because none have yet been developed to prevent diseases like tuberculosis, malaria, AIDS, or *E. coli* infection (Bertani and Ruiz, 2018). Diarrhea, which is linked to complicated microbial illnesses, is one of the most often reported disorders in young farm animals (lambs under two weeks old, calves under ten days old, and newborn or recently weaned piglets) (El-Gbily *et al.,* 2025) And, research has indicated that the primary pathogenic bacterium that causes diarrhea is diarrheagenic *E. coli* (Kaper *et al.,* 2004). A vast population of bacteria makes up the intestinal microbiota, which is frequently regarded as a novel "organ" in various biological advancements. Inflammatory bowel disease is one of the many illnesses that are intimately associated with changes in the composition of the intestinal microbiota (Zhang *et al.,* 2022). Recent research on diarrhea caused by *E. coli* has shown that variations in the composition of the intestinal microbiota have distinct impacts on the susceptibility of the intestinal tract to enterotoxigenic *Escherichia coli* (ETEC) infection, such as an increase in pigs and lambs infected with *E. coli* F4. A level of *Lactobacillus rhamnosus* can raise serum IL-17 (Siuce *et al.,* 2020). Despite the growing interest in nanoparticle-based vaccine adjuvants, most existing studies have focused on small laboratory animals or in vitro models, with limited data available in food-producing ruminants. Moreover, the immunological and tissue-level responses induced by zinc oxide nanoparticle–formulated bacterial antigens remain poorly characterized under in vivo challenge conditions. In young lambs, particularly, there is insufficient information regarding how ZnO–antigen formulations influence humoral immunity, inflammatory mediators, and intestinal histopathology following exposure to pathogenic *Escherichia coli*. This knowledge gap limits rational optimization of nanoparticle-assisted vaccines for veterinary field applications. This study aims to evaluate the immunogenic potential of Escherichia coli antigens formulated with zinc oxide (ZnO) nanoparticles in young lambs and to explore their associated immune and histopathological responses.

MATERIALS AND METHODS

Animal Ethics Approval

The animals for this research were housed at a very clean location at Tikrit University's College of Veterinary Medicine, adhering to the No. Tu. Vet. 161 regulations and ethics (https://2u.pw/W6FOJTsQ).

Field experiment and animals used in research

A field experiment was conducted in September and October 2023. The neonatal lambs were kept in the animal house at Tikrit University, College of Veterinary Medicine. Twenty young lambs aged 3–3.5 months with an average body weight of 10–12 kg was used in this study.

E. coli strain

The *Escherichia coli* O157:H7 isolate used in this study was obtained from the Central Laboratory, University of Tikrit, and originated from a clinical human sample. Identification was performed using routine bacteriological and biochemical tests consistent with *E. coli* O157:H7. Molecular serotyping and virulence gene characterization (stx1, stx2, eae) were not performed, and the strain was used as a reference pathogenic isolate provided by the diagnostic laboratory.

Preparation *E. coli* O157:H7 antigens

Whole cells of *E. coli* killed with formalin (WCECK)

Escherichia coli was cultured on brain heart infusion agar (Himedia) at 37 °C for 24 h. Bacterial cells were harvested at late exponential growth phase, centrifuged, and washed three times with phosphate-buffered saline (PBS). Cells were inactivated using 1% formalin for 24 h under refrigerated conditions, followed by repeated washing with sterile PBS to remove residual formalin. Complete inactivation was confirmed by absence of growth after plating on nutrient agar. The bacterial concentration was expressed as CFU equivalents/mL, which was estimated based on viable counts obtained prior to inactivation and adjusted spectrophotometrically to a final concentration equivalent to 2×10^8 CFU/mL (Kumru *et al.,* 2014).

Preparation of *E. coli* culture filtrated proteins (ECCFPs)

Shake culture overnight at 37°C was carried out on brain-heart infusion broth. The bacterial cells were then harvested and centrifuged to recover the culture filtrate proteins (CFPs) which are primarily exoproteins. The filtered resultant supernatant used filters with holes measuring 0.2 µM. After incubation for 24 hours, concentrated ECCFPs carry out sterility check in nutrient agar at 37°C, whereas Bradford's procedure was used to calculate total protein concentration, 15 mg/ml and decrease concentration to 1 mg/ml (Tucci *et al.,* 2020).

Mixed Whole cells of *E. coli* were killed, and culture filtrated proteins with zinc oxide nanoparticles (ZnO-NPs) and dose determination for young lambs

Use commercially available ZnO-NPs (typically 20–50 nm; ZnO-NPs: 25 µg /kg body weight) and mixing each protein alone via sonication (use probe sonicator 23 MHz / 10 min, sonicate on ice to avoid heat damage, Amplitude: 30–40%, Time: 5–10 min, Pulse: 30s on/30s off) with each bacterial protein alone (Whole cells of *E. coli* killed 1 ml (2×10^8) Zn + WCECK + Culture filtrated proteins 1ml (1 mg/ml) Zn + ECCFPs.

Challenge dose with *E. coli*

Animals were challenged 14 days after the last immunization S/C with $1x10^7$ CFU of virulent *E. coli* (Ponce-Gordo and García-Rodríguez, 2021).

Blood sampling schedule and rationale

Blood samples were collected from the jugular vein, allowed to clot at room temperature in gel tubes, centrifuged at 2,500 rpm for 10 min, and serum was stored at −20 °C until analysis. Blood sampling was performed at defined time points to capture sequential phases of the immune response following immunization and challenge. Day 0 samples were collected to establish baseline immunological levels prior to any immunization or challenge. Day 7 sampling was designed to detect the early primary immune response, when IgM and early pro-inflammatory cytokines (IL-1β) typically begin to rise within the first week after antigen exposure (Zinkernagel, 2000; Papp *et al.,* 2019). Day 14 corresponded to the booster immunization and early class-switching phase of humoral immunity (IgG-3) (Papp *et al.,* 2019). Day 21 sampling reflected the developing adaptive and inflammatory response (CXCL2), allowing assessment of chemokine activity associated with leukocyte recruitment (De Filippo et al., 2008; Kumar et al., 2024). Day 28 sampling was performed to evaluate antibody maturation and persistence following the booster immunization (Zhang *et al.,* 2023). Day 35 sampling represented the late phase of the immune response (IgM and CXCL2), enabling evaluation of whether antibody and inflammatory activity persisted or resolved over time (Zinkernagel, 2000; Chandrashekar *et al.,* 2020). This structured schedule permits temporal correlation between vaccination events and immune response dynamics.

ELISA Procedure

The concentrations of IgM, IgG-3, IL-1β, and CXCL2 in serum samples were determined using commercially available ELISA kits (Manufacturer: SUNLONG BIOTECH CO., China), following the supplier's protocol. In summary, 96-well plates pre-coated with specific capture antibodies were loaded with 100 µL of diluted serum samples and standard solutions, then incubated at 37°C for 1–2 hours. After washing to remove unbound material, HRP-linked detection antibodies were added and incubated for 1 hour. A second washing step was performed before adding the TMB substrate, which was incubated in the dark for 10–15 minutes. The enzymatic reaction was terminated by adding 50 µL of stop solution, and optical density was measured at 450 nm using a microplate reader. Each sample was analyzed in duplicate to ensure accuracy and reproducibility.

Histopathological findings

Tissue specimens (liver, spleen, and small intestine) (approximately 1 × 1 × 0.5 cm per animal) were collected immediately after necropsy and rinsed briefly in phosphate-buffered saline to remove excess blood. Samples were fixed in 10% formalin at a tissue-to-fixative ratio of at least 1:10 for 24–48 h at room temperature. Following fixation, tissues were processed routinely through graded ethanol dehydration, xylene clearing, and paraffin embedding. Paraffin blocks were sectioned at 4–5 µm thickness using a rotary microtome, mounted on glass slides, and stained with hematoxylin and eosin (H&E) for microscopic examination. Slides were examined using a light microscope and representative images were captured at ×100 and ×400 magnifications. The histological evaluation was performed descriptively based on standard pathological criteria, and blinded scoring was not performed. Lesions were documented qualitatively and supported by representative photomicrographs.

Research methodology

This study aimed to evaluate the immunological impact of vaccination and explore strategies to reduce or neutralize its adverse effects. Twenty young lambs were purposively and randomly selected and assigned to four equal groups (n=5). All procedures followed ethical standards approved by the Animal Use Committee at the University of Tikrit, College of Veterinary Medicine. The immunization dose was accurately calculated based on prior studies and appropriate conversions from earlier experimental data (Rocha-Melogno *et al.,* 2022): (**Group 1**: Control without immunization); (**Group 2**: Positive control was injection with 1 ml SC of virulent *E. coli* 1×10^7 CFU/ml on day 28. (**Group 3**: Injected with two subcutaneous doses of 1-ml Whole cells of *E. coli* killed with formalin (WCECK) in 1 day and repeated at 14 days, young lambs were injected S/C on day 28 with 1-ml of virulent *E. coli* containing 1×10^7 CFU/ml. (**Group 4**: Injected with 1-ml *E. coli* culture filtrated proteins (ECCFPs), two doses, S/C with day 14 intervals for each neonatal lamb and at day 28, were injection S/C with 1 ml with a challenged effective dose (1×10^7 CFU/ml) of virulent *E. coli*. On the day, 35 animals were euthanized for post-mortem to collect autopsies from the organs (liver, spleen and small intestine) and then preserved in 10% formalin for the preparation of tissue sections for histopathological examination by using hematoxylin and eosin stain (Al-Bayati *et al.,* 2024).

Statistical analysis

Statistical analyses were performed using GraphPad Prism. Due to differences in sampling schedules among immune markers, each biomarker was analyzed independently. For each sampling day, group differences were evaluated using one-way analysis of variance (ANOVA). When a significant overall effect was detected, pairwise comparisons among groups were performed using Tukey's multiple comparisons test. Results are expressed as mean ± standard error (SE). Differences were considered statistically significant at $p < 0.05$. Different lowercase letters within the same column indicate statistically significant differences among groups.

RESULT AND DISCUSSION

At Day 0, no significant differences in IgM levels were observed among all experimental groups, confirming comparable baseline immune status prior to immunization or challenge. At Day 7, both ZnO-conjugated vaccine groups showed significantly higher IgM concentrations compared with the negative and positive control groups ($p < 0.05$–0.01), indicating early humoral immune activation following immunization. No significant difference was detected between the two ZnO formulations. At Day 35, IgM levels were significantly elevated in the positive control (challenged only) and both immunization groups relative to the negative control ($p < 0.01$–0.001), reflecting strong antibody responses following bacterial challenge by live *E. coli* infection and sustained immunization -induced immunity. Overall, ZnO-based immunization effectively enhanced early and late IgM responses compared with non- immunization controls as (Table 1 and figure 2). Consequently, the present data indicate enhanced antibody production following vaccination, while nanoparticle-based formulations such as ZnO–antigen complexes are reported to improve antigen delivery and immunogenicity, which

may contribute to the observed humoral responses, in line with Golubovskaya and Wu (2016). Nanoparticle-based adjuvants have been reported to enhance antigen delivery and immunogenicity, thereby supporting improved protective immune responses in vaccine formulations (Zhao *et al.,* 2014), which may help explain the elevated antibody levels observed in the present study, Furthermore, previous studies have demonstrated that nanoparticle-based adjuvants can enhance humoral immune responses and vaccine immunogenicity, supporting their application in vaccine formulation (Xu *et al.,* 2013). Moreover, Higher levels of IgM, the primary immunoglobulin isotype reactive to LPS O157, were found in the mothers' sera. The fact that lipopolysaccharide is a type-I thymus-independent antigen and that mice respond to it with type IgM, which has a poor affinity and a limited isotype switch to certain IgG subclasses, may help to explain this observation (Palmeira *et al.,* 2007).

Table 1: Effect of vaccination and non-vaccination groups at (0, 7, 35) days on IgM (μg/mL) level in serum.

Group	Day 0	Day 7	Day 35
Negative Control	1344.313 ± 63.607 **a**	1329.927 ± 73.786 **b**	1343.335 ± 63.023 **b**
Positive Control	1332.632 ± 20.032 **a**	1377.439 ± 37.412 **b**	4878.393 ± 216.202 **a***
ZnO + ECCFPs	1159.113 ± 109.010 **a**	2859.000 ± 385.924 **a** **	3714.000 ± 484.213 **a** **
ZnO + WCECK	1150.407 ± 50.843 **a**	3309.000 ± 303.521 **a****	3854.000 ± 680.781 **a** **

Values are Mean ± SE (n = 5). Different letters within the same column indicate significant differences (one-way ANOVA + Tukey, $p < 0.05$). Stars indicate differences vs negative control within the same day (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

At Day 0, IgG-3 concentrations did not differ significantly among groups, indicating comparable baseline antibody levels. At Day 14, the positive control and both ZnO-vaccinated groups showed significantly higher IgG-3 levels than the negative control ($p < 0.01$–0.001), demonstrating vaccine-induced humoral maturation. By Day 28, IgG-3 remained significantly elevated in all stimulated groups compared with the negative control, with the positive control exhibiting the highest response. These findings confirm sustained antibody class switching following vaccination and bacterial challenge. The present study demonstrated in (Table 2 and Figure 1) demonstrates that both vaccination and infection promote class switching and maturation of the humoral response. The sustained increase in IgG-3, particularly in the ZnO+WCECK group, suggests that whole-cell protein formulations may provide a broader antigenic stimulus. IgG-3 is known for its strong opsonizing and complement-fixing capabilities, which are essential in the clearance of extracellular pathogens like *E. coli* (Behrouz *et al.,* 2017; Vidarsson *et al.,* 2014). Likely due to natural antigenic stimulation from the *E. coli* infection, lipopolysaccharide can activate humoral immunity and protein antigens, which stimulate cell-mediated immune responses. As an adjuvant vaccine, it may also raise the blood's vaccine-specific serum IgG level and improve Th1-mediated bacterial-clearing responses to balance Th2 re-epithelialization in the intestinal and urinary tract mucosal sites (Xing *et al.,* 2023). Studies have shown that LPS can induce IgG3 production as part of a Th1-type immune response,

which is associated with the activation of macrophages and the enhancement of cell-mediated immunity (Van der Bruggen *et al.,* 1999).

Table 2: Serum levels of IgG-3 in immunized and nonimmunized animals at day (0, 14 and 28) days post immunization (µg/mL).

Group	Day 0	Day 14	Day 28
Negative Control	1.536 ± 0.188 **a**	1.112 ± 0.186 **b**	1.060 ± 0.169 **b**
Positive Control	1.648 ± 0.464 **a**	2.092 ± 0.716 **b**	12.528 ± 0.562 **a***
ZnO + ECCFPs	1.509 ± 0.117 **a**	5.804 ± 1.032 **a** **	6.380 ± 0.696 **b***
ZnO + WCECK	1.026 ± 0.010 **a**	6.694 ± 1.415 **a** **	6.500 ± 0.661 **b***

Values are Mean ± SE (n = 5). Different letters within the same column indicate significant differences (one-way ANOVA + Tukey, p < 0.05). Stars indicate differences vs negative control within the same day (* p < 0.05, ** p < 0.01, *** p < 0.001).

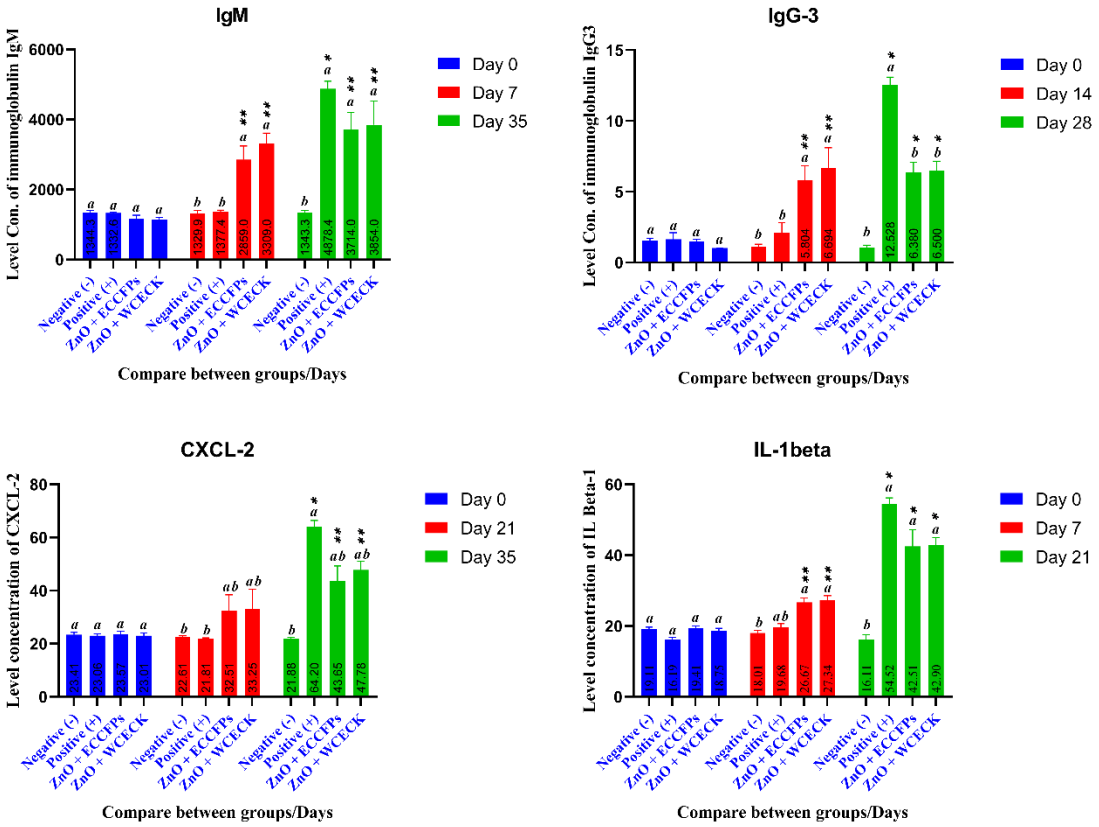

Figure 1. Serum immunological responses in lambs following immunization and challenge with Escherichia coli antigens formulated with zinc oxide nanoparticles. Changes in IgM, IgG-3, CXCL-2, and IL-1β concentrations were measured at the indicated time points in negative control, positive control, ZnO + ECCFPs, and ZnO + WCECK groups. Data are presented as mean ± SE (n = 5). Different lowercase letters indicate significant differences among groups within the same time point (one-way ANOVA followed by Tukey's multiple comparisons test, p < 0.05). Asterisks indicate significant differences compared with the negative control within the same day (*p < 0.05, **p < 0.01, ***p < 0.001).

Baseline CXCL2 levels were comparable among all groups at Day 0. At Day 21, CXCL2 concentrations tended to increase in both ZnO-vaccinated groups, although partial overlap with control groups was observed), consistent with heightened inflammatory activity following infection. At Day 35, CXCL2 levels were significantly higher in the positive control and ZnO-vaccinated groups compared with the negative control ($p < 0.01$–0.001), indicating enhanced chemokine-mediated inflammatory recruitment during the late immune phase see (Table 3 and figure 1). The CXCL-2 (also known as MIP-2α) is a neutrophil chemoattractant involved in early innate immune responses. Its elevation in response to vaccination indicates that ZnO-conjugated antigens can effectively activate local inflammatory signaling and neutrophil mobilization. This is consistent with findings that nanoparticle adjuvants such as ZnO enhance antigen uptake and stimulate chemokine expression via dendritic cell activation (Xu *et al.,* 2021). Through the current results, the present data indicate that vaccination with ZnO–antigen formulations is associated with elevated inflammatory signaling and chemokine production compared with non-immunized controls and perpetuating inflammation, thus increasing the production and raising the level of inflammatory chemical mediators that work to activate cells for longer periods and attract inflammatory cells to the site of infection, thus reducing the percentage of damage resulting from bacterial infection, The elevated levels in vaccinated groups suggest the vaccines triggered appropriate inflammatory signaling pathways that enhance pathogen clearance through CXCL-2 (Kamei *et al.,* 2006), Proinflammatory cytokines and chemokines, including IL-1β, IL-6, TNF-α, CCL-2, and IL-8, are produced in response to LPS generated from *E. coli* and Pseudomonas. others result also demonstrate how LPS causes monocytic THP-1 cells to express CCL2 both mRNA and protein (Cho *et al.,* 2014). Moreover, the TLR4 was required for the generation of CCL2 triggered by LPS. LPS-induced TLR4 stimulation results in the production of important proinflammatory cytokines that contribute to the activation of several immunological responses (Lu *et al.,* 2008).

Table 3: Serum levels of CXCL-2 in immunized and nonimmunized animals at day (0, 21 and 35) days post immunization (pg/mL).

Group	Day 0	Day 21	Day 35
Negative Control	23.412 ± 0.868 **a**	22.612 ± 0.355 **b**	21.884 ± 0.461 **b**
Positive Control	23.059 ± 0.650 **a**	21.812 ± 0.390 **b**	64.197 ± 2.221 **a***
ZnO + ECCFPs	23.565 ± 1.114 **a**	32.513 ± 5.921 **ab**	43.647 ± 5.677 **ab** **
ZnO + WCECK	23.012 ± 0.948 **a**	33.249 ± 7.242 **ab**	47.776 ± 3.272 **ab** **

Values are Mean ± SE (n = 5). Different letters within the same column indicate significant differences (one-way ANOVA + Tukey, $p < 0.05$). Stars indicate differences vs negative control within the same day (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

No significant differences in IL-1β concentrations were observed among groups at baseline. At Day 7, both ZnO-vaccinated groups exhibited significantly elevated IL-1β levels compared with the negative control and positive control groups ($p < 0.01$) following immunization, reflecting early innate immune activation. By Day 21, IL-1β levels were significantly increased in the positive control and immunization groups relative to the negative control ($p < 0.001$), demonstrating sustained pro-inflammatory immune stimulation following antigen exposure as (Table 4 and Figure 1), it is clear to us that the difference in these proportions is due to the possession of *E. coli* of necessary factors that enable it to stimulate the immune system, both cellular and humoral, to form a focus of

necrosis and sepsis, due to the fact that bacteria contain endotoxin in their walls that are considered among their most important virulence factors, such as lipopolysaccharides and this fact represented in level of IL-1β in control positive group, this study is consistent with many articles that indicate that the *E. coli* stimulates the production of IL-1β and IL-6 in the infected animals (Jang *et al.,* 2018), all *E. coli* pathotype has distinct virulence factors expressed by distinct gene clusters, as well as unique pathogenicity mechanisms, numerous virulence factors linked to *E. coli* pathotypes implicated in intestinal and extraintestinal illnesses have been discovered (Yang *et al.,* 2017), many of the virulence factors associated with enteric *E. coli* pathotypes implicated in intestinal and extraintestinal disorders have been identified in the last years (Gomes *et al.,* 2016). On the other hand, the noticeable increase in IL-1β levels in the conjugated groups (43.900±1.306, 45.9±2.474) likely reflects combined effects of antigenic stimulation and nanoparticle-based delivery, as supported by previous literature. Through what was mentioned, we agree with studies, ZnO increases the expression of the TGFb gene (Roselli *et al.,* 2003) and the kind of pro-inflammatory cytokine activation negatively impacts immune system proteins, including TNFa, IL1b, and IFNg (Capaldo and Nusrat, 2009). The purpose of an adjuvant is to increase the immunogenicity of highly purified antigens in vaccines (Sun *et al.,* 2018). Additionally, in animals that are not responding well to vaccination, adjuvants can increase the immune system's reaction to antigens and lower the dosage and manufacturing costs of vaccines, zinc oxide is a good inducer of the mucosal immune system and improves a cell-mediated type-1 helper T cell (Th1) or cytotoxic T lymphocyte (CTL) response.

Table 4: Effect of vaccination and non-vaccination groups at (0, 7, 21) days on IL-1beta (pg/mL) level in serum

Group	Day 0	Day 7	Day 21
Negative Control	19.111 ± 0.581 **a**	18.012 ± 0.803 **b**	16.110 ± 1.390 **b**
Positive Control	16.194 ± 0.646 **a**	19.678 ± 0.980 **ab**	54.519 ± 1.689 **a***
ZnO + ECCFPs	19.413 ± 0.686 **a**	26.669 ± 1.249 **a** **	42.509 ± 4.700 **a***
ZnO + WCECK	18.747 ± 0.637 **a**	27.335 ± 1.233 **a** **	42.904 ± 2.052 **a***

Values are Mean ± SE (n = 5). Different letters within the same column indicate significant differences (one-way ANOVA + Tukey, $p < 0.05$). Stars indicate differences vs negative control within the same day (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Under specific circumstances, *E. coli*, a common conditional pathogen, can infect humans and a variety of animals (Ponce-Gordo and García-Rodríguez, 2021). The main pathological lesion in intestine of control positive groups (Figure 2 A1, A2, A3) represented by hemorrhagic and hypertrophy of mesenteric lymph node with detachment of intestinal mucosa a necrosis in the intestinal villi with edematous fluid with aggregation of inflammatory cell lesion in liver characterized by necrotic area in liver parenchyma with congestion of central vein, the pathological changes in spleen of the infected lambs' organs that were noted in this study featured by depilation and necrosis in medulla, Lambs usually infected with colibacillosis diarrhea via contaminated by bacteria (bacteremia). The pathohistological findings that were typical of diarrheal histopathology were in line with the findings of Rocha-Melogno *et al.* (2022). These inflammatory lesions were comparable to earlier research on *E. coli* infections or other *E. coli* toxins that alter gut shape and cause severe intestinal damage (Touchette *et al.,* 2002), However, in order to destroy tissues, the bacteria produce inflammatory chemical mediators and toxins, which cause organ cell necrosis and degenerative cell alterations (Madzingira, 2016). The opposite situation was

found in tissue sections of animals immunized to groups 3 and 4 of the study (Figure 2 B1, B2, B3), where it was observed hyperplasia of the mesenteric lymph node with intact villi, while in the spleen, the lymphoid follicles with hyperplasia due to stimulation and the finding in agree with this. In an intraperitoneal sepsis of *E. coli*, immunizing animals with either of the conjugated vaccine proteins produced robust humoral and cellular immune responses and shielded against challenge colibacillosis (Wieser *et al.,* 2010). Two doses of the identical bacterial vaccinations containing O157 or O157:H7; K99 stimulate immunity to serum antibodies (Yousif *et al.,* 2013), (Vulcano *et al.,* 2014) examined the immunological and histopathological changes in *E. coli*-infected triggers immune response and intestinal histological alterations. However, more research is required to examine the part that cellular immunity and antibodies play in the *E. coli* infection. Additionally, strengthening immune responses can be suggested as a way to manage the effects of E. coli infection. In addition, the relation between the IgG-3, IgM, CXCL-2, IL-1β and histopathology tissue of liver, spleen, small intestine of positive control group challenged with the effective dose (1×10^7 CFU/ml) of virulent *E. coli* leading to hyperplasia white pulp of spleen during infection with elevated IgM and IgG-3, processing immune complexes due to liver infection, furthermore, small intestine infected directly, where local immune cells activate systemic antibody response via the mesenteric lymph nodes as in (Figure 2). A group receiving only ZnO-NPs was not included, so it is not possible to distinguish independent immune system modulation by ZnO-NPs from antigen-specific immune system modulation. Rather, variations in IgM, IgG-3, CXCL-2, and IL-1β concentrations are indicative of immune system modulation to ZnO-antigen vaccines. Future studies should include a group treated only with ZnO-NPs to quantitate immune system modulation specifically due to nanoparticle administration. In addition, a sample size of n=5 is relatively small. Immunomodulatory analysis was limited to certain humoral markers of inflammation. Cellular immunity, mucosal antibodies, and functional immunity were not evaluated. Furthermore, challenge was done with only one *E. coli* strain. This strain might not reflect typical *E. coli*. Notwithstanding the limitations, the increase in humoral factors (IgM, IgG-3) and pro-inflammatory mediators (CXCL-2, IL-1β) is in line with the histology results indicating the presence of intact tissue, lymphoid hyperplasia, and minimal necrosis in the inoculated animals. The result is indicative of successful priming with moderate inflammation to facilitate clearance and minimize damage. The presence of intact villi and lymphoid follicles indicates ongoing mucosal and systemic immunity in young lambs inoculated with ZnO-antigen formulations, which are biologically relevant as vaccine delivery systems in young lambs.

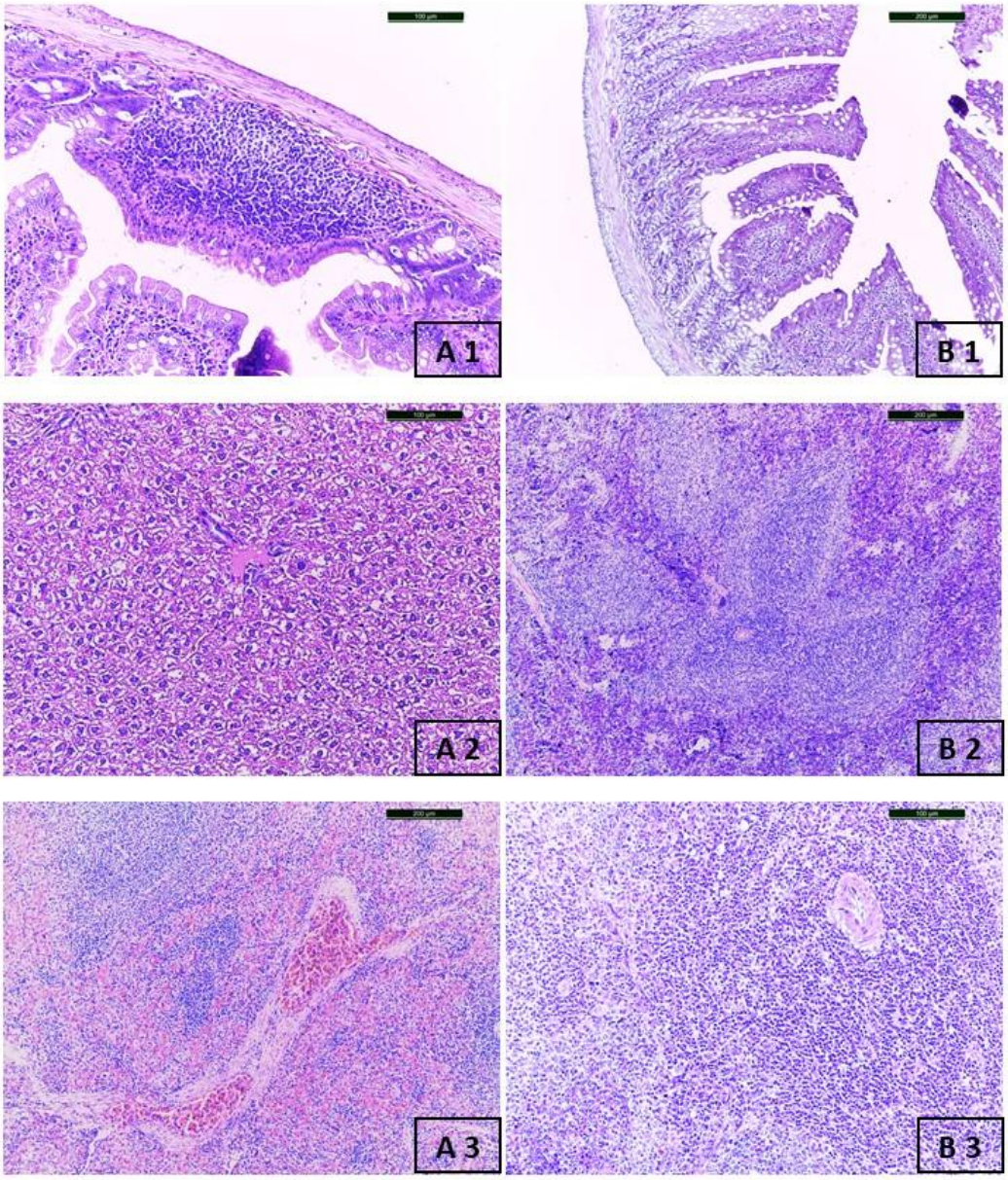

Figure 2. Histopathological sections of the intestine of (Cont. +, G2) (H.E. stain 100×). A1. Necrosis of intestinal villi (black arrow), congestion of the capillary in the mucosal layer (yellow arrow), with hyperplasia of the mesenteric lymph node and sloughing in the mucosal layer (green arrow) and infiltration of inflammatory cells (red arrow). A2. Histopathological sections of the liver (Cont. +, G2) show a necrotic lesion in a hepatocyte (black arrow), with inflammatory edema (yellow arrow) (100×). A3. Spleen shows depletion in lymphoid follicles (yellow arrow), congestion of the splenic artery (red arrow) (100×). Histopathological sections of the intestine of ZnO+WCECK (G3), and ZnO+ECCFPs (G4) (H.E. stain100×). B1. Show normal section with intact intestinal villi without lesion (black arrow). B2 and B3. Spleen section showing hyperplastic lesion in lymphoid follicles (hyperplasia) (yellow arrow), with amyloid deposition (red arrow) (100×).

CONCLUSION

ZnO-conjugated *Escherichia coli* vaccines stimulated robust early-life immune responses in young lambs, as demonstrated by increased IgM, IgG-3, CXCL-2, and IL-1β levels, indicating enhanced immunogenicity through nanoparticle-based antigen delivery without excessive inflammation. Unimmunized lambs exhibited marked intestinal, hepatic, and splenic lesions characteristic of colibacillosis, whereas vaccinated animals showed preserved tissue integrity and lymphoid hyperplasia, supporting a beneficial association between immune activation and tissue protection. However, clinical disease severity, mortality, bacterial load reduction, and direct protective efficacy were not evaluated, and no formal statistical correlation analysis was performed between immunological markers and histopathological outcomes; therefore, these associations remain descriptive rather than quantitatively validated. Future studies should incorporate clinical scoring systems, bacteriological quantification, quantitative lesion scoring, and immune–pathology correlation analyses to confirm protective performance and mechanistic relationships.

CONFLICT OF INTEREST

The authors confirm that there are no financial or personal conflicts of interest that could have influenced the outcomes of this research.

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REFERENCES

Ahmed, H. O., & Yaaqoob, L. A. (2025). Evaluation of antibacterial activity of nickel oxide nanoparticles against *Escherichia coli*. *Iraqi Journal of Agricultural Sciences*, 56(1), 502–511. https://doi.org/10.36103/aws0zt84

Al-Bayati, H. H. K., Abdullah, S. A., Shihab, T. J., Sultan, M., & Jumaa, Q. S. (2024). Immunogenicity of culture filtrated proteins and whole-cell formalin killed of *Listeria monocytogenes* to induced cellular immune response in vivo. *Open Veterinary Journal*, 14(12), 3581–3598. https://doi.org/10.5455/OVJ.2024.v14.i12.40

Ali, N. S., & Al-Juthery, H. W. A. (2017). The application of nanotechnology for micronutrient in agricultural production. *Iraqi Journal of Agricultural Sciences*, 48(4), 984. https://doi.org/10.36103/ijas.v48i4.355

Behrouz, B., Hashemi, F. B., Fatemi, M. J., Naghavi, S., Irajian, G., Halabian, R., & Imani Fooladi, A. A. (2017). Immunization with bivalent flagellin protects mice against fatal *Pseudomonas aeruginosa* pneumonia. *Journal of Immunology Research*, 2017, 5689709. https://doi.org/10.1155/2017/5689709

Bertani, B., & Ruiz, N. (2018). Function and biogenesis of lipopolysaccharides. *EcoSal Plus*, 8(1), eESP-0001-2018. https://doi.org/10.1128/ecosalplus.ESP-0001-2018

Capaldo, C. T., & Nusrat, A. (2009). Cytokine regulation of tight junctions. *Biochimica et Biophysica Acta*, 1788(4), 864–871. https://doi.org/10.1016/j.bbamem.2008.08.027

Cho, J. S., Kang, J. H., Um, J. Y., Han, I. H., Park, I. H., & Lee, H. M. (2014). Lipopolysaccharide induces pro-inflammatory cytokines and MMP production via TLR4 in nasal polyp-derived fibroblast and organ culture. *PLoS One*, 9(11), e90683. https://doi.org/10.1371/journal.pone.0090683

Croxen, M. A., & Finlay, B. B. (2010). Molecular mechanisms of *Escherichia coli* pathogenicity. *Nature Reviews Microbiology*, 8(1), 26–38. https://doi.org/10.1038/nrmicro2265

De Filippo, K., Henderson, R. B., Laschinger, M., & Hogg, N. (2008). Neutrophil chemokines KC and macrophage-inflammatory protein-2 are newly synthesized by tissue macrophages using distinct TLR signaling pathways. *Journal of Immunology*, 180(6), 4308–4315. https://doi.org/10.4049/jimmunol.180.6.4308

El-Gbily, S., Eldokmak, M. M., Diabb, R., Abas, O. M., Ata, E. B., Kamal, S., & Hassan, S. M. H. (2025). Severe lamb diarrhea outbreak: Clinical features, identification of the causative agent, and a prophylactic approach. *Comparative Immunology, Microbiology and Infectious Diseases*, 118, 102318. https://doi.org/10.1016/j.cimid.2025.102318

El-Nady, H. H., Eissa, M. I., Abou-Zeid, N. Z., Abd-Elfatah, E. B., Shehata, A. A., & Fawzi, E. M. (2023). Colibacillosis in lambs and children in Egypt: Prevalence, serogroups, antibiogram profile, virulence genes distribution and antimicrobial resistance genes. *Open Veterinary Journal*, 13(9), 1106–1115. https://doi.org/10.5455/OVJ.2023.v13.i9.6

Farokhzad, O. C., & Langer, R. (2009). Impact of nanotechnology on drug delivery. *ACS Nano*, 3(1), 16–20. https://doi.org/10.1021/nn900002m

Golubovskaya, V., & Wu, L. (2016). Different subsets of T cells, memory, effector functions, and CAR-T immunotherapy. *Cancers*, 8(3), 36. https://doi.org/10.3390/cancers8030036

Gomes, T. A., Elias, W. P., Scaletsky, I. C., Guth, B. E., Rodrigues, J. F., Piazza, R. M., Ferreira, L. C., & Martinez, M. B. (2016). Diarrheagenic *Escherichia coli*. *Brazilian Journal of Microbiology*, 47(Suppl 1), 3–30. https://doi.org/10.1016/j.bjm.2016.10.015

Hameed, H. N., & Mahmood, S. S. (2025). A role of some *Escherichia coli*–virulence factors in causing vaginosis. *Iraqi Journal of Agricultural Sciences*, 56(1), 495–501. https://doi.org/10.36103/vvb3zr35

Hu, S., & Zhang, Y. (2010). Endostar-loaded PEG-PLGA nanoparticles: In vitro and in vivo evaluation. *International Journal of Nanomedicine*, 5, 1039–1048. https://doi.org/10.2147/IJN.S14753

Jang, H. M., Lee, K. E., Lee, H. J., & Kim, D. H. (2018). Immobilization stress-induced *Escherichia coli* causes anxiety by inducing NF-κB activation through gut microbiota disturbance. *Scientific Reports*, 8(1), 13897. https://doi.org/10.1038/s41598-018-31764-0

Kamei, N., Tobe, K., Suzuki, R., Ohsugi, M., Watanabe, T., Kubota, N., et al. (2006). Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *Journal of Biological Chemistry*, 281(36), 26602–26614. https://doi.org/10.1074/jbc.M601284200

Kaper, J. B., Nataro, J. P., & Mobley, H. L. (2004). Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*, 2(2), 123–140. https://doi.org/10.1038/nrmicro818

Kumru, O. S., Joshi, S. B., Smith, D. E., Middaugh, C. R., Prusik, T., & Volkin, D. B. (2014). Vaccine instability in the cold chain: Mechanisms, analysis and formulation strategies. *Biologicals*, 42(5), 237–259. https://doi.org/10.1016/j.biologicals.2014.05.007

Lu, Y. C., Yeh, W. C., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. *Cytokine*, 42(2), 145–151. https://doi.org/10.1016/j.cyto.2008.01.006

Madzingira, O. (2016). Shiga toxin-producing *E. coli* isolated from sheep in Namibia. *Journal of Infection in Developing Countries*, 10(4), 400–403. https://doi.org/10.3855/jidc.6903

Muna, H. M., Alshaikhly, N. S., & Al-khafaji, M. M. (2023). Synergistic effect of antibiotic with green synthesized silver nanoparticles against uropathogenic *E. coli* biofilm. *Iraqi Journal of Agricultural Sciences*, 54(6), 1622–1635. https://doi.org/10.36103/ijas.v54i6.1862

Palmeira, P., Ito, L. Y., Arslanian, C., & Carneiro-Sampaio, M. M. (2007). Passive immunity acquisition of maternal anti-enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 IgG antibodies by the newborn. *European Journal of Pediatrics*, 166(5), 413–419. https://doi.org/10.1007/s00431-006-0250-9

Ponce-Gordo, F., & García-Rodríguez, J. J. (2021). *Balantioides coli*. *Research in Veterinary Science*, 135, 424–431. https://doi.org/10.1016/j.rvsc.2020.10.028

Rocha-Melogno, L., Crank, K. C., Ginn, O., Bergin, M. H., Brown, J., Gray, G. C., et al. (2022). Quantitative microbial risk assessment of outdoor aerosolized pathogens in cities with poor sanitation. *Science of the Total Environment*, 827, 154233. https://doi.org/10.1016/j.scitotenv.2022.154233

Roselli, M., Finamore, A., Garaguso, I., Britti, M. S., & Mengheri, E. (2003). Zinc oxide protects cultured enterocytes from the damage induced by *Escherichia coli*. *Journal of Nutrition*, 133(12), 4077–4082. https://doi.org/10.1093/jn/133.12.4077

Siuce, J., Maturrano, L., Heeler, J. C., & Rosadio, R. (2020). Diarrheagenic *Escherichia coli* isolates from neonatal alpacas mainly display F17 fimbriae adhesion gene. *Tropical Animal Health and Production*, 52(6), 3917–3921. https://doi.org/10.1007/s11250-020-02415-2

Sun, B., Yu, S., Zhao, D., Guo, S., Wang, X., & Zhao, K. (2018). Polysaccharides as vaccine adjuvants. *Vaccine*, 36(35), 5226–5234. https://doi.org/10.1016/j.vaccine.2018.07.040

Touchette, K. J., Carroll, J. A., Allee, G. L., Matteri, R. L., Dyer, C. J., Beausang, L. A., & Zannelli, M. E. (2002). Effect of spray-dried plasma and lipopolysaccharide exposure on weaned pigs: I. Effects on the immune axis of weaned pigs. *Journal of Animal Science*, 80(2), 494–501. https://doi.org/10.2527/2002.802494x

Tucci, P., Portela, M., Chetto, C. R., González-Sapienza, G., & Marín, M. (2020). Integrative proteomic and glycoproteomic profiling of *Mycobacterium tuberculosis* culture filtrate. *PLoS One*, 15(3), e0221837. https://doi.org/10.1371/journal.pone.0221837

Van der Bruggen, T., Nijenhuis, S., van Raaij, E., Verhoef, J., & van Asbeck, B. S. (1999). Lipopolysaccharide-induced tumor necrosis factor alpha production by human monocytes involves the raf-1/MEK1-MEK2/ERK1-ERK2 pathway. *Infection and Immunity*, 67(8), 3824–3829. https://doi.org/10.1128/IAI.67.8.3824-3829.1999

Vidarsson, G., Dekkers, G., & Rispens, T. (2014). IgG subclasses and allotypes: From structure to effector functions. *Frontiers in Immunology*, 5, 520. https://doi.org/10.3389/fimmu.2014.00520

Vulcano, A. B., Tino-De-Franco, M., Amaral, J. A., Ribeiro, O. G., Cabrera, W. H., et al. (2014). Oral infection with enteropathogenic *Escherichia coli* triggers immune response and intestinal histological alterations in mice selected for their minimal acute inflammatory responses. *Microbiology and Immunology*, 58(6), 352–359. https://doi.org/10.1111/1348-0421.12153

Wieser, A., Romann, E., Magistro, G., Hoffmann, C., Nörenberg, D., Weinert, K., & Schubert, S. (2010). A multiepitope subunit vaccine conveys protection against extraintestinal pathogenic *Escherichia coli* in mice. *Infection and Immunity*, 78(8), 3432–3442. https://doi.org/10.1128/IAI.00174-10

Xing, Y., Clark, J. R., Chang, J. D., Chirman, D. M., Green, S., et al. (2023). Broad protective vaccination against systemic *Escherichia coli* with autotransporter antigens. *PLoS Pathogens*, 19(2), e1011082. https://doi.org/10.1371/journal.ppat.1011082

Xu, M. N., Li, L., Pan, W., Zheng, H. X., Wang, M. L., Peng, X. M., et al. (2021). Zinc oxide nanoparticles prime a protective immune response in *Galleria mellonella* to defend against *Candida albicans*. *Frontiers in Microbiology*, 12, 766138. https://doi.org/10.3389/fmicb.2021.766138

Xu, Y., Tang, H., Liu, J. H., Wang, H., & Liu, Y. (2013). Evaluation of the adjuvant effect of silver nanoparticles both in vitro and in vivo. *Toxicology Letters*, 219(1), 42–48. https://doi.org/10.1016/j.toxlet.2013.02.010

Yang, S. C., Lin, C. H., Ljuffali, I. A., & Fang, J. Y. (2017). Current pathogenic *Escherichia coli* foodborne outbreak cases and therapy development. *Archives of Microbiology*, 199(6), 811–825. https://doi.org/10.1007/s00203-017-1393-y

Yousif, A. A., Al-Taai, N. A., & Mahmood, N. M. (2013). Humoral and cellular immune response induced by *E. coli* [O157:H7 and O157:H7:K99] vaccines in mice. *International Journal of Immunology Research*, 3(1), 17.

Zhang, L., Liu, W., Lin, L., Chen, D., & Stenzel, M. H. (2008). Degradable disulfide core-cross-linked micelles as a drug delivery system prepared from vinyl functionalized nucleosides via the RAFT process. *Biomacromolecules*, 9(11), 3321–3331. https://doi.org/10.1021/bm800867n

Zhang, Y., Si, X., Yang, L., Wang, H., Sun, Y., & Liu, N. (2022). Association between intestinal microbiota and inflammatory bowel disease. *Animal Models and Experimental Medicine*, 5(4), 311–322. https://doi.org/10.1002/ame2.12255

Zhao, L., Seth, A., Wibowo, N., Zhao, C. X., Mitter, N., Yu, C., & Middelberg, A. P. (2014). Nanoparticle vaccines. *Vaccine*, 32(3), 327–337. https://doi.org/10.1016/j.vaccine.2013.11.069

Zinkernagel, R. M. (2000). Localization dose and time of antigens determine immune reactivity. *Seminars in Immunology*, 12(3), 163–344. https://doi.org/10.1006/smim.2000.0253

Zúñiga, E., Contreras-Trigo, B., Buchert, J., Sáez-Ahumada, F., Hernández, L., et al. (2025). Gold nanoparticles as a platform for delivery of immunogenic peptides to THP-1 derived macrophages: Insights into nanotoxicity. *Vaccines*, 13(2), 119. https://doi.org/10.3390/vaccines13020119

Papp, K. A., Haraoui, B., Kumar, D., Marshall, J. K., Bissonnette, R., Bitton, A., & Wade, J. (2019). Vaccination guidelines for patients with immune-mediated disorders on immunosuppressive therapies. *Journal of cutaneous medicine and surgery*, *23*(1), 50-74. https://doi.org/10.1177/1203475418811335

Kumar, A., Tripathi, P., Kumar, P., Shekhar, R., & Pathak, R. (2024). From Detection to Protection: Antibodies and Their Crucial Role in Diagnosing and Combatting SARS-CoV-2. *Vaccines*, *12*(5), 459. https://doi.org/10.3390/vaccines12050459

Zhang, H., Hua, Q., Nani Xu, N., Zhang, X., Chen, B., Ma, X., Hu, J., Chen, Z., Yu, P., Lei, H., Wang, S., Ding, L., Fu, J., Liao, Y., Yang, J., Jiang, J., & Lv, H. (2023). Evaluation of antibody kinetics and durability in healthy individuals vaccinated with inactivated COVID-19 vaccine (CoronaVac): A cross-sectional and cohort study in Zhejiang, China. *eLife*, *12*, e84056. https://doi.org/10.7554/eLife.84056

Chandrashekar, A., Liu, J., Martinot, A. J., McMahan, K., Mercado, N. B., Peter, L., Tostanoski, L. H., Yu, J., Maliga, Z., Nekorchuk, M., Busman-Sahay, K., Terry, M., Wrijil, L. M., Ducat, S., Martinez, D. R., Atyeo, C., Fischinger, S., Burke, J. S., Slein, M. D., Pessaint, L., & Barouch, D. H. (2020). SARS-CoV-2 infection protects against rechallenge in rhesus macaques. Science (New York, N.Y.), 369(6505), 812–817. https://doi.org/10.1126/science.abc4776