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Comparative Immunogenicity of Bacterial Ghost and Whole Killed Vaccines Against Avian Pathogenic *Escherichia coli* in Broiler Chickens

Article Info.

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Abstract

The objective was to compare the immunological efficacy of bacterial ghosts (BG) and whole killed bacteria (WKV) vaccines in poultry. Both vaccine formulations significantly increased phagocytic activity and cytokine production compared to the negative control. However, BG vaccination induced a strong Th1-biased immune response, as evidenced by elevated levels of IL-12 and IFN-γ, whereas WKV vaccination favored a Th2-associated response, characterized by higher IL-4 production. Additionally, both vaccines increased IL-10 levels, an increase in IgY in BG and WKV compared to N.C is a good indication that immunization was effective in inducing the production of antibodies. suggesting an immunoregulatory component that may contribute to immune homeostasis. Both BG and WKV vaccines raise IL-1b levels in a significantly higher manner than negative controls do, which is indicative of a strong activation of the innate immune system. There was a significant increase in phagocytic activity in both vaccination groups (BG: 59.5 ± 8.61; WKV: 61.00 ± 4.81) compared to the negative control (26.67 ± 7.91; $P < 0.001$), with no significant difference between the BG and WKV groups. Collectively, these findings indicate that BG vaccination elicits a more balanced immune response with a Th1 bias, whereas WKV vaccination predominantly promotes humoral immunity.

Keywords: Vaccine, bacterial ghost, IL-10 , IL-4, , IL-12, IFN-γ , IgY

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Introduction

Upon exposure to a specific disease, the vaccine will provide strong, effective acquired immunity against the disease (1). The vaccine normally has an agent that is like the disease-causing microorganism, and it may be a weakened or killed form of the microbe or one of its surface proteins or toxins (2). The bacterial ghost system is a novel vaccine delivery system endowed with intrinsic adjuvant properties. Bacterial ghosts are nonliving Gram-negative bacterial cell envelopes devoid of cytoplasmic contents while maintaining their cellular morphology and native surface antigenic structures (3).

BGs can target epithelial cell TLR4 and TLR5 to stimulate local and systemic immune responses (4) Chemically produced bacterial ghosts are usually empty, envelopes generated through the controlled removal of cytoplasmic contents while preserving the structural and antigenic integrity of the cell wall demonstrated strong immunogenicity and safety in preclinical studies. Their ability to present native surface antigens while lacking genetic material makes them promising candidates for vaccine delivery, particularly against enteric and intracellular pathogens. Additionally, their hollow interior allows loading of drugs, proteins, and nucleic acids. The bacteria E.coli are expanding their role in biomedical and nanotechnological applications (5).(APEC) causes economic loss in chicken through a combination of (a) direct mortality and culling, (b) slaughter condemnation and yield loss, (c) reduced growth and feed efficiency, (d) declines in egg production and hatchability in layers and breeders, and (e) increased spending on treatment, prevention and diagnosis costs that are increasingly affected by antibiotic resistance. (6) Also, salpingitis and peritonitis syndromes associated with Escherichia coli bacteria that are pathogenic to poultry may lead to the culling of chickens, increased veterinary costs, and poultry disposal expenses, and may also lead to a decrease in egg quality (7).

Moreover, APEC demonstrates a high resistance to penicillin, tetracyclines, cephalosporins, fluoroquinolones and colistin-based classes of antibiotics because of such mechanisms as ESBLs and plasmid genes (8). A systematic review and meta-analysis of vaccination against colibacillosis reported an overall trend supporting certain vaccine approaches for reducing mortality. Bacterial ghost (BG) vaccines are a new vaccine system in vaccine material development, which takes advantage of the empty cell envelope of Gram-negative bacteria as an antigen delivery system. The production of these ghosts is achieved by the controlled expression of the E gene belonging to bacteriophage PhiX174 that creates a lysis tunnel structure in the bacterial membrane, and the cytoplasmic content is expelled, retaining the outer membrane and surface antigens (9). The other protocol, including chemical procedure, is the “sponge-like” method. Most importantly, the BG they do not pose any risks linked with attenuated forms of vaccines; they elicit robust cell-mediated and humoral immune responses (10). Some of the oldest

and most common vaccine platforms include whole bacterial vaccines, which comprise either live attenuated or killed (killed) cells of bacteria. These vaccines introduce the host immune system to the entire repertoire of bacterial antigens, such as surface proteins (LPS), flagella, and other (PAMPs), which induce systemic and long-lasting immune response (11). Genetic diversity APEC strains are generally known to carry a variety of virulence-related genes that aid adhesion, invasion, acquisition of iron, production of toxins & evasion. of the immune response. Relevant virulence factors are adhesins (fimbria, curli), iron-binding systems (siderophores like aerobactin and yersiniabactin), protection (capsules, enhanced serum survival genes like iss), and toxins (hemolysin and cytotoxic necrotizing factors). (12).

Materials and Methods

Laboratory animals

Broiler chickens were used in this study to investigate immune system responses. The chickens were divided into three groups of 10 birds each: Group 1 received a vaccine containing bacterial ghost antigens administered subcutaneously at a dose of 0.5 ml (1.5×10^8 CFU/ml); Group 2 received a vaccine containing whole killed bacteria at the same dose; and Group 3 served as the control group. The vaccine was administered on days 10, 20, and 30, and blood samples were collected on day 35. Cytokine levels were measured to compare the effects of the two vaccines.

Bacterial Isolate

Local (avian pathogenic *E. coli*) isolate was obtained from Basrah governorate and donated by the Department of Microbiology at the University of Basrah, College of Veterinary Medicine. The isolate was diagnosed using different microbiological and molecular PCR Techniques (13).

Preparation of killed bacteria (whole cell vaccine). Brain-heart infusion broth was used to cultivate stock E. Coli at 37° C for 24 hours. While being shaken. Cells were treated with 3.7% formalin and were incubated for a night. Following four PBS washes, the inactivated bacteria were adjusted to 1.5×108 CFU/ml by comparison with 0.5 McFarland Standard Solution. Until they were used, these preparations were kept at 4°C. A loopful of the dead isolate was streaked onto blood agar and MacConkey agar plates, and the plates were incubated at 37°C for 24 to 48 hours to verify sterility (14).

Preparation of Bacterial ghost vaccine

Chemical methods for BG production rely on carefully controlled exposure to near their Minimal inhibitory concentrations(MICs).The chemical protocol is the “sponge-like” method, in which

bacteria are sequentially treated with low concentrations of agents such as 1.15 mg/ml sodium dodecyl sulfate (SDS), 3.125 mg/ml sodium hydroxide (NaOH), and 8.79 µl/ml hydrogen peroxide (H_2O_2). Experimental design tools (e.g., Plackett–Burman designs) are often used to identify combinations that ensure complete killing, DNA removal, and maintenance of envelope integrity. Which is confirmed by Electron microscopy. (15)

Preparation of Whole bacterial vaccine

A loopful of the E. coli isolate was added to the brain heart infusion 5ml of broth media, and then incubated for 24 hours at 37°C to enrich it. B-Treatment of bacteria with formalin. The Bacteria were treated with formalin to form whole-cell vaccines (WKV). After incubation, the culture broth was treated with 3.7% formalin for 18 hours. The inactivated bacteria were washed four times with phosphate buffer saline (PBS) and used as an antigen (16). By using standard McFarland, the immunogenic concentration of E. coli reported by (17) was adjusted to 1.5 × 108 CFU/ml before killing the bacteria. Until they were used, these preparations were kept at 4°C for four weeks. Whole killed bacteria suspension 1.5×108 CFU/ml in PBS mixed with alum as an adjuvant. Aluminium hydroxide gel was prepared by mixing 1.0 L of 10% (w/v) potassium aluminium sulfate with NaOH solution under stirring, maintaining pH 6.0–7.0, and allowing the precipitate to mature with the ratio of 1:1 (volume/volume) .

Vaccine dose and route of injection

In the present study,.the.total subcutaneous dose. Was 1.5 × 108 CFU/ml of the vaccine through injection dose .(18).Vaccination: Samples were collected from three main groups of 10 chicks each, under standard hygiene conditions. (Sterile. needles after disinfecting the skin of the animal. with 70% ethanol). (19). The (G1) of chicks was injected subcutaneously with normal saline solution as a control group.The second group (G2) was immunized with a bacterial ghost vaccine, while the third group (G3) was immunized with a whole bacterial vaccine.

Blood Samples

The blood samples were. collected on day 35 after the last booster, dose using tubes with EDTA and six different parameters. Cytokines (IL-4, IFN-γ, IL-10, IL-1β and IL-12) were measured by using an ELISA Kit from (Sun Long Biotech,Ltd).The concentration of Igy was Measured by an ELISA Kit from Sun Long Biotech Ltd by ELISA Techniques . To determine the immune response induced by the two vaccine types against APEC.

Measuring the immune response

1-Measurement of phagocytic activity: This test was conducted by the procedure provided by Shanmugam et al. (2015) by using Candida albicans (20).

2-Measurement of the concentration of humoral immunity (IL-4, IL-10, and IgY) for two types of vaccine compared with N.C& and Measurement of the concentration of Cellular immunity parameters (IL-12, IFN-γ, IL-1β) for two types of vaccine compared with Negative control.

Statistical analysis

The statistical analysis of results to determine whether differences exist among the means of three groups measured with their means (M) and standard deviations (SD) is presented along with significance testing (P-values). statistical analysis by one-way ANOVA.

Result

The two vaccines were prepared and injected subcutaneously at doses of 0.5 ml (1.5×10^8 CFU/ml). Cellular and humoral immunity were then measured to determine the immune response. The innate immune responses were measured using phagocytic percentage; the results indicate that WKV and BG were statistically significant compared with the N.C. group, and the WKV group was the highest, Table (1).

Table (1): Comparison of phagocytic activity between negative control& bacterial ghosts and Whole killed bacteria after the third dose of the vaccine.

Group	Vaccine	Mean±SD	Significance
G1	N.C (Negative Control)	26.67± 7.91	Ns
G2	BG(Bacterial Ghosts)	59.5 ± 8.61	****
G3	WKV(Whole killed bacteria)	61.00± 4.81	****

Note: the groups show as the following: G1 Negative Controal and G2 Bacterial ghost vaccine& G3 Whole bacterial vaccine (Significance Codes: **** =P < 0.0001, * =P < 0.0 1, ns = not significant).

Table (2): Comparison of Humoral immune (IL-4, IL10, IgY) between NC &bacterial ghosts and whole killed bacteria after the third dose of the vaccine.

Type of cytokine	N.C	BG	WKV	Significance
IL-4 M ± SD	11.56 ± 0.4127	13.64 ± 0.6348	33.78 ± 0.4900	P < 0.0001
IL-10 M± SD	24.99 ± 0.4952	74.70 ± 1.194	71.99 ± 0.8469	P < 0.0001
IgY M ± SD	3.664 ± 0.4370	44.65 ± 0.6701	37.23±1.006	P < 0.0001

The Humoral immune were measured using cytokine concentration of vaccinated chickens. Results indicate that WKV and BG were statistically significant compared with the N.C. BG group stimulation immune response has a better in Th1& WKV has better in Th2, Table (2) & Figure (2,4-B).

Table (2) presents the mean (M) and standard deviation (SD) values for humoral immune markers IL-4, IL-10, and IgY across negative control (N.C.), bacterial ghost (BG), and (WKV)vaccine groups, revealing distinct. immunomodulatory profiles in poultry models.

Table (3): Comparison of cellular immunity indicators (IL12, IL-1B, IFN-γ) between N.C &bacterial ghosts and whole killed bacteria group after the third dose of the vaccine.

Type of cytokine	N.C	BG	WKV	Significance
IL12 M ± SD	8.522 ± 0.4260	32.51± 0.4230	11.89 ±0.5535	P < 0.0001
IL-1B M ± SD	20.15 ± 0.4352	80.83 ± 0.5164	59.69 ± 0.8440	P < 0.0001
IFN-γ M ± SD	10.35 ± 0.4157	30.53 ± 0.5063	20.00 ± 0.4033	P < 0.0001

The Cellular immune response was measured using the cytokine concentration of vaccinated chickens. Results indicate that WKV and BG were statistically significant compared with the N.C. Th2 Table (3) & Figure (3,4-A).Table (3) displays mean (M) and standard deviation (SD) values for cellular immune markers IL-12, IL-1β, and IFN-γ across negative control (N.C.), bacterial ghost (BG), and (WKV)vaccine groups, highlighting divergent Th1 activation profiles in poultry vaccination. Serological Tests by the ELISA technique were explained in figures (1) ,(2),and(3).

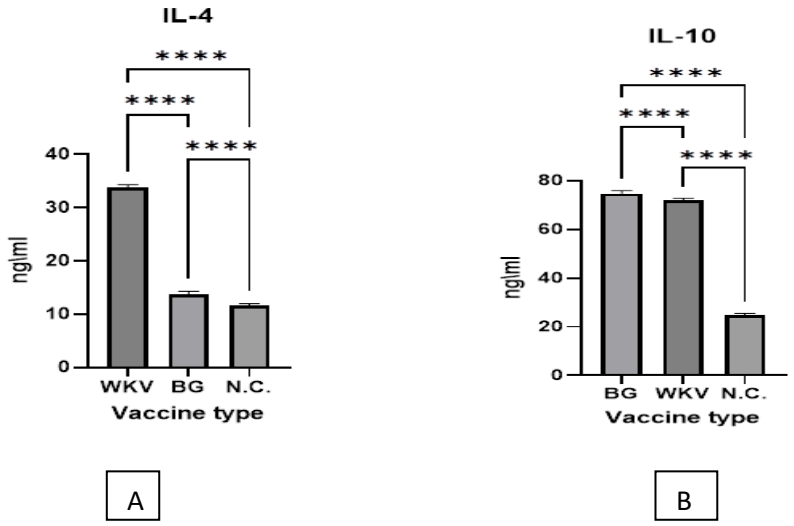

Figure (1)-A-Concentration of IL-4 in all groups (BG. wkv) compared with the. N.C group, B- Concentration of IL-10 in all groups (BG. wkv) compared. with the N.C. groups

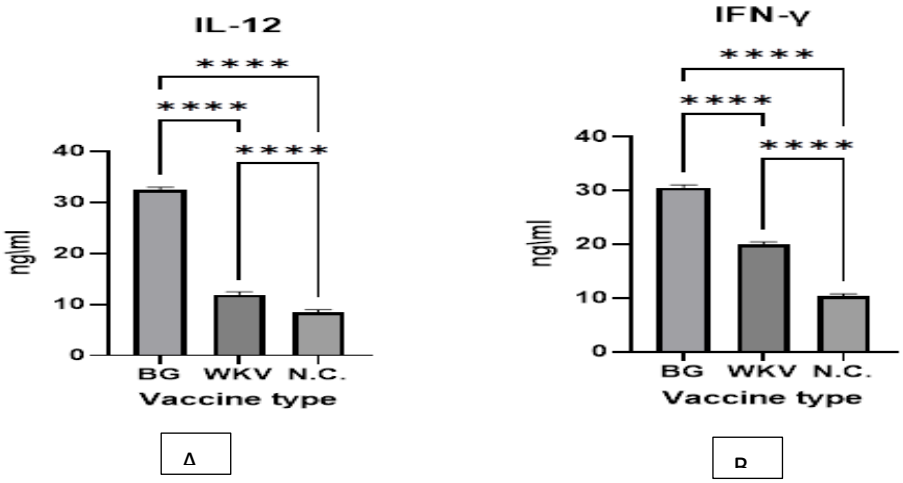

Figure (2)-A-Concentration of IL-12 in all groups (BG.Wkv) compared. with the N.C .group, B- Concentration of IFN-gamma in all groups (BG.Wkv) compared with the N.C group

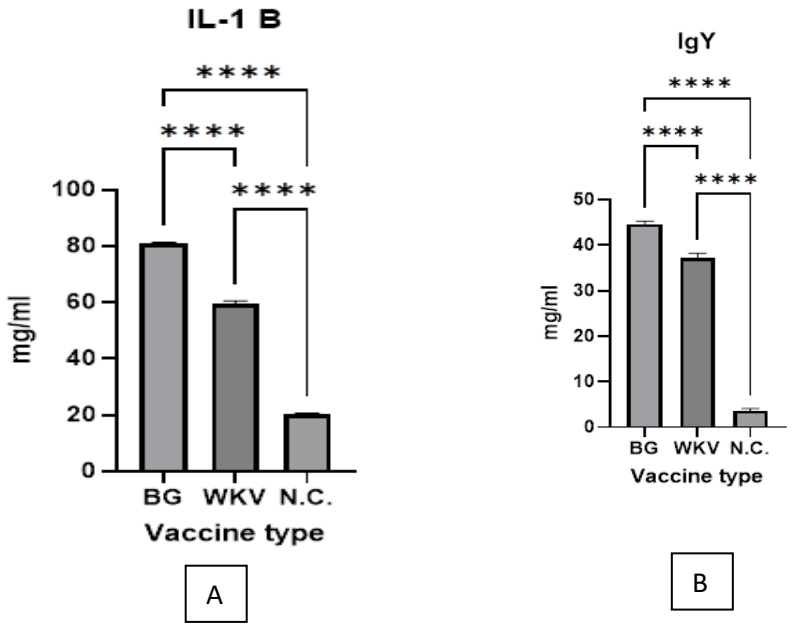

Figure (3)-A-Concentration of IL-1B in all groups (BG.wkv) compared with the N.C group, B- Concentration of IgY in all groups (BG.wkv) compared with the N.C group

Discussion

The results revealed that evaluation of phagocytic activity alongside interleukin profiling showed marked immunological responses. Table (1) Phagocytic activity indicates strongly positive levels in groups vaccinated with bacterial ghosts and whole killed bacteria relative to the negative control at the end of the third dose of the vaccines, with no significant difference between the two vaccine groups. Bacterial ghosts stimulate innate immune responses by serving as effective adjuvants, which stimulate the phagocytosis of cell remnants using preserved cell envelopes full of pathogen-associated molecular pattern (PAMPs), which targets antigen-presenting cells such as macrophages and dendritic cells Entire killed bacteria vaccines also enhance phagocytic uptake, but can cause less specific cellular response (unintentionally damaged structures, but recent studies show similar efficacy in cytokine induction, e.g. TNF- a, IL-10) following repeated immunizations. (21).

Table (2) presents considerably high means and standard deviation values of the humoral immune markers IL-4, IL-10 and IgY in bacterial ghost (BG) and WKV vaccine groups above the negative control (N.C.) in poultry, and all the differences are statistically significant (P=0.0001). Both vaccines cause a strong humoral response, with the wkv vaccine demonstrating the best IL-4 response, indicating more skewing of Th2. (22). Table (3) demonstrates that bacterial ghosts (BG) stimulate detectably larger concentrations of Th1 cytokines-IL-12,IL-1 b, IFN-γ in the poultry vaccination in contrast to whole killed bacteria (wkv) and negative control (N.C). This deviation points to the fact that BG has a better Th1.activation, which facilitates cell-mediated immunity by promoting dendritic cell differentiation and T cell polarization. WKV registers moderate increases yet falls short of BG, which is in line with the PAMP delivery intact by ghosts. (23). Figure (1-A) was used to determine the level of interleukin-4 (IL-4) in all the experimental groups, the wkv vaccine, BG vaccine and the negative control (N.C) group. The outcomes show that the increase in IL-4 of the two vaccinated groups was statistically significant over the negative control ($p < 0.0001$), and the wkv vaccine group had the highest mean IL-4 concentration. (24).High IL-4 levels after vaccination indicate that adaptive immune activation, with the focus on the formation of antibodies, has been induced successfully. The much greater IL-4 level in the WKV group suggests that this vaccine will stimulate a stronger Th2-biased immune response than the BG vaccine. (25).

The data presented in Figure (1-B) illustrate the concentration of interleukin-10. Both vaccinated groups exhibited a marked and statistically significant increase in IL-10 levels relative to the N.C group ($p < 0.0001$). The elevated IL-10 levels that are the result of vaccination could be evidence of a balanced response of the immune system, where prevention of immunopathology is accompanied by the production of protective signals. These reactions are especially beneficial in vaccines that trigger long-term immunity without a high level of inflammatory side effects (26). The lack of a large difference between the BG and wkv groups suggests that both vaccine formulations have the same ability of triggering IL-10 mediated regulatory responses, which could be triggered by APCS: macrophages and Dendritic cells that are known sources of IL-10 after immunization(27). Figure (3)-B The great increase in IgY in BG and wkv compared to N.C is an evidence that immunization was effective in inducing the production of antibodies, and that the IgY was then detected in large amounts in the sampled material (usually serum and/or extracts of egg yolk, depending on the design of the study). This is compatible with the fundamental biology of IgY: the hens produce IgY following stimulation with antigens, and IgY is moved into the egg yolk, where it is deposited. indicating successful induction of a strong humoral response following immunization. This pattern is consistent with Eablished IgY biology, whereby antigen stimulation In hens increases IgY production and promotes transfer of antibodies from the circulation into the egg yolk for accumulation and harvest. (28). Figure (2)-A) The highest level

of IL-12 concentration was in the BG group, amounting to about 3032 ng/ml, which was much higher than the wkv and NC groups.

This rise is an indication that the BG vaccine is a potent activator of Th1-bias immunological response because IL-12 is an important cytokine that promotes the differentiation of naïve T cells to become IFN-gamma producing Th1-cells. Throughout the production of IL-12, enhanced IL-12 is usually linked to improved cell-mediated immunity and activation of natural killer (NK) cells and cytotoxic T lymphocytes. (29).Comparatively, it was observed that IL-12 levels in the WKV group rose moderately (between 11and 12 ng/ ml)compared to NC group, which showed no response (exhibited no change). Though much more so than the NC group, the IL-12 concentration of WKV group was still very low when compared to that of the BG group. This indicates that although the formulation of the WKV vaccines can induce the production of IL-12, the immunostimulatory ability of the vaccine might not be as effective as BG to induce a Th1 response (30). Figure (2)- B shows that the two vaccine regimens (BG and wkv) both enhance the production of IFN- γ significantly, compared to the negative control, which indicated the induction of strong Th 1-type cellular immunity. The BG formulation has been shown to result in the most robust response of the tested groups, which, perhaps, indicates the highest immunogenicity in terms of T-cell activation. These consistent with recent immunological findings in which IFN-γ response caused by vaccines was associated with increased adaptive immunity.(31) The figure 3-A indicates the average interleukin-1 beta (IL-1b) concentrations in three groups, namely: N.C (negative control), BG, and Wkv. BG and WKV vaccine groups are significantly more elevated in IL-1b than the N.C group, with the highest concentration of BG (~80 ng/ml) and WKV (~60 ng/ml) in the middle range. These results point to the idea that both BG and WKV vaccines raise IL-1b levels in a significantly higher manner than negative controls do, which is indicative of a strong activation of the innate immune (32, 33).

Conflicts of interest

The authors declare that there is no conflict of interest.

Ethical Clearance

This work is approved by the Research Ethical Committee Approval number 92\37\2025.

Conclusion

BG vaccine continuously elicited a greater Th1-biased immune response with significantly elevated concentrations of IL-12, IFN-γ, and IL-1β, the key participants of the cell-mediated immunity. This implies that BG vaccines are highly effective in the activation of antigen-presenting cells and the polarization of T-cells to a protective profile of Th1. These types of responses are necessary to counteract intracellular pathogens as well as to form long-term

immune memory. Conversely, the Wkv vaccine exhibited a relatively superior Th2-related response, including the elevation of IL- 4 levels, combined with the considerable levels of IL-10 and IgY that measure the strength of humoral immunity.

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مقارنة الاستجابة المناعية للقاحات الشبحية البكتيرية واللقاحات الكاملة المقتولة ضد بكتيريا الإشريكية القولونية الممرضة للطيور في دجاج التسمين

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الخلاصة

هدفت هذه الدراسة إلى مقارنة الفعالية المناعية للقاحات الأشباح البكتيرية (BG) والبكتيريا الكاملة المقتولة (WKV) في الدواجن. أدى كلا اللقاحين إلى زيادة ملحوظة في النشاط البلعمي وإنتاج السيتوكينات مقارنةً بالمجموعة الضابطة السالبة. حثّ لقاح الأشباح البكتيرية استجابة مناعية قوية منحازة نحو الخلايا التائية المساعدة من النوع الأول (Th1)، كما يتضح من ارتفاع مستويات الإنترلوكين-12 (IL-12) والإنترفيرون غاما (IFN-γ)، بينما فضّل لقاح البكتيريا الكاملة المقتولة استجابة مناعية من النوع الثاني (Th2)، تميزت بزيادة إنتاج الإنترلوكين-4 (IL-4). بالإضافة إلى ذلك، زاد كلا اللقاحين من مستويات الإنترلوكين-10 (IL-10)، مما يشير إلى وجود عنصر تنظيمي مناعي قد يساهم في استتباب المناعة. كما ان يؤدي كل من لقاحي BG و WKV إلى رفع مستويات IL-1b بشكل أعلى بكثير من الضوابط السلبية، مما يدل على تنشيط قوي للمناعة الفطرية.لوحظت زيادة ملحوظة في النشاط البلعمي في كلتا مجموعتي اللقاح (الأشباح البكتيرية: 59.5 ± 8.61؛ البكتيريا الكاملة المقتولة: 61.00 ± 4.81) مقارنةً بالمجموعة الضابطة السالبة (26.67 ± 7.91؛ $P < 0.001$)، دون وجود فرق معنوي بين مجموعتي الأشباح البكتيرية والبكتيريا الكاملة المقتولة. تشير هذه النتائج مجتمعة إلى أن التلقيح بـ BG يثير استجابة مناعية أكثر توازناً مع تحيز باتجاه ال Th1، في حين أن التطعيم بـ WKB يعزز بشكل أساسي المناعة الخلطية.

الكلمات المفتاحية: لقاح، البكتريا الشبحية ، IFN-γ، IL-4, IL-10 , IL-12 , IgY