Immunological Characterization of the Chemically Prepared Ghosts of Salmonella Typhimurium as a Vaccine Candidate

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Abstract

**Background:** The purpose of this study is to measure the immunogenic protective response of bacterial ghosts of Salmonella typhimurium in animals. Accordingly, the researchers will be able to do further clinical trials and confirm the efficiency of bacterial ghosts in human vaccination. The targets to be measured are humoral immune response: immunoglobulins elevation (IgG), cellular immune response: granulocytes increment; serum antibacterial activity; success the faeces and liver virulence challenge and High survival rates.

**Results:** The BG vaccine was able to protect 100% of BG subcutaneously vaccinated rats and 75% of BG-adjuvant subcutaneously vaccinated rats. The lowest survival rate was in the BG orally vaccinated group (25%). The maximum level of serum IgG titers as well as serum and faeces bactericidal activity (100% eradication) was exhibited in the subcutaneously vaccinated group with BG-adjuvant followed by the BG subcutaneously vaccinated one. Additionally, the highest granulocytes’ number was observed in the BG-adjuvant subcutaneously immunized group. The bacterial load in liver homogenate eliminated in the subcutaneously vaccinated rats by BG after virulence challenge.

**Conclusions:** The bacterial ghosts of *Salmonella enterica serovar Typhimurium* that prepared by tween 80 protocol showed an effective vaccine candidate that protected animals, eliminated the virulence in faeces and liver. These findings suggest that chemically induced bacterial ghosts of Salmonella typhimurium can be a promising vaccine.

**Background:**

The intact unaffected evacuated cellular shells of Gram-negative bacteria that are evacuated from their cellular contents are defined as bacterial ghosts (BGs) (Jaleta, Mamo, & Disassa, 2015; Kudela, Koller, & Lubitz, 2010; Langemann et al., 2010; P. Lubitz, Mayr, & Lubitz, 2009; W Lubitz, 2016; Tabrizi et al., 2004). Gram-positive bacteria are lacking the *E* lysis gene so cannot produce the lysis protein. Accordingly, Gram-positive bacterial ghosts cannot be prepared by the same principle that is used in preparation of Gram-negative ghosts(Park et al., 2016; Vinod et al., 2015).

Recently, the definition of BGs has been extended to include Gram-positive bacteria by applying a new protocol which comprises the use of some chemical agents in their minimum inhibitory concentrations (MIC) and minimum growth concentrations (MGC) and/or some physical factors(Amara, Salem-Bekhit, & Alanazi, 2013; Rabea et al., 2018; Vinod et al., 2015). By using specific chemicals in critical concentrations at critical times, the puncturing of bacterial cells and expelling of the internal contents have been achieved. Although the bacterial cells were pierced, the intactness of the cellular shell was not affected or deformed.

Bacterial ghosts can be a new biotechnology platform for vaccine production. Recently, different approaches have been used to develop human and veterinarian vaccines. Such approaches either utilize the cellular surface displaying properties (e.g. Protein A in Gram-positive bacteria and specific outer-
membrane proteins in Gram-negative bacteria) (Wernérs & Ståhl, 2004), or biotechnology based techniques (Motin & Torres, 2009). The novel biotechnology approaches involve genetically engineered vaccines, inverted pathogenicity (utilizing virulence factors to prevent or treat a disease), and bacterial ghosts system (Motin & Torres, 2009; Paukner et al., 2006). The preserved surface structures and components of the BG can induce both innate and adaptive immune response.

*Staphylococcus aureus* NaOH induced and prepared ghosts protected the whole rats’ population against virulent challenge (Vinod et al., 2015). Survival of the whole rats’ population (100%) against virulent challenge and significant antibodies titer production were achieved by *Listeria monocytogenes* ghosts. The latter ghosts were chemically produced using several reagents, such as: calcium carbonate, sodium hydroxide, sodium dodecyl sulphate, and hydrogen peroxide (Wu et al., 2017). The prepared *Escherichia coli* O157:H7 by E-lysis gene achieved 93.3% survival rate in rats after lethal challenge test (Mayr, Haller, et al., 2005).

Several trials of animal vaccination by BGs of different Gram-positive and Gram-negative bacteria were highly effective. The dead cellular shells as vaccine candidates were prepared by either pathway; lysis protein induction or chemical agents (Francis O Eko et al., 1994; Talebkhan et al., 2010; Sheweita, 2014; Vinod et al., 2015).

**Materials And Methods:**

**BGs Preparation:**

Bacterial ghosts were produced from *Salmonella enterica serovar typhimurium* ATCC 11331 using the previously described protocol (Rabea et al., 2018). Briefly, the cells were incubated in Muller-Hinton broth containing 7% v/v tween 80 for 24 hours at 37 C°. The grown cells were posed to lactic acid (pH=3.6). By centrifugation, ghosts’ pellets were separated then washed three times by a sterile solution of half normal saline. Finally, the obtained ghosts were lyophilized and stored.

**Experimental animals:**

The ethical approval for this study was given (No. MI 1506) on 28/10/2015 by the research ethical committee, college of pharmacy, Cairo university. Twenty four normal young adult male Sprague–Dawley rats (Average weight 150 gm) obtained from the animal house of the faculty of veterinary medicine, Cairo University, were used in this study.

**Vaccination of animals:**

After 2 weeks of acclimatization and housing in animal house of the holding company of the vaccines and sera, Giza, Egypt, the rats were divided equally into six groups (4 rats in each group). According to previous studies (Sheweita, 2014; Y. Yang et al., 2013), the groups were assigned as the following: group 1: Control group: was subcutaneously (S.C.) injected by 1 ml PBS; group 2: was S.C. injected by 1 ml adjuvant (Alum); group 3: was orally vaccinated by 1 ml suspension of (300 μg/ml) of
Salmonella enterica serovar typhimurium ATCC 13311 ghosts (BGs); group 4: was orally vaccinated by equal volumes of BGs + Alum (0.5 ml STG + 0.5 alum); group 5: was S.C. vaccinated by 1 ml of (300 μg/ml) of BGs suspension and group 6: was S.C. vaccinated by equal volumes of BGs + alum (0.5 ml STG + 0.5 alum). Vaccination was repeated every 14 days for two cycles.

**Withdrawing blood samples procedure:**

The blood samples (2 ml) were withdrawn via retro-orbital sinus puncture (Yardeni, Eckhaus, & Morris, 2011) from each animal under anesthesia.

**Determination of antibodies response:**

Serum was separated by centrifugation at 1400 g for 20 minutes and stored at -20°C until analysis. The serum samples were collected every 14 days and were challenged against anti-rat IgG (whole molecule) – Horseradish Peroxidase (HRP) conjugate antibodies followed by ELISA analysis. As described before (Francis O Eko, Hensel, Bunka, & Lubitz, 1994), the live Salmonella cells were added to each well of the 96- microwell ELISA plates as 50 μl (10 x 10^9) and left to dry. Plates were blocked with 10% BSA for 1 hour. Sera samples were 2 fold serially diluted and dispensed to the plates for 1 hour, then were incubated and washed with wash buffer as 200 μl. The anti-rat IgG HRP conjugate was added to the whole plates as 1/1000 final dilution as 50 μl. The plates were incubated for 1 hour then washed as previous. Substrate buffer was added (50 μl). The developed color was stopped using 2N H_2SO_4. The readings were taken using the microplate reader at 450 nm filter. The mean optical density was plotted against the time post vaccination.

**Antibacterial activity in serum:**

After 42 days and finishing of the whole vaccination program, serum (25 μl) were collected from each group and challenged by addition of 100 μl (1.5 X 10^8 CFU/ml) live Salmonella enterica serovar typhimurium ATCC 13311 suspension. This mixture was incubated for 1 hour at 37°C. Then it was mixed uniformly with Shigella-Salmonella (S-S) media to count viable cells (Yang et al., 2013).

**The granulocytes’ percentage:**

Forty two days later, 2 ml of withdrawn blood (from each group) were examined for CBC in order to calculate the granulocytes’ percentage.

**Virulence Challenge-antibacterial response in faeces:**

After finishing of the whole vaccination program (42 days), every rat in all groups was infected by S.C. injection of 1 ml (1.5 X 10^8 CFU/ml) live Salmonella enterica serovar typhimurium ATCC 13311 cells. After one week, faeces samples (1 gm) were collected from each group and were mixed uniformly with S-S media to investigate bacterial counts (MM, A, Ghazy A, S, & Fattah Amara, 2017).
**Virulence Challenge-antibacterial response in liver homogenate:**

One week after infection, all rats were sacrificed and liver tissues (1 gm) were homogenized and mixed uniformly with S-S media to investigate viable bacterial counts (MM et al., 2017).

**Termination of animals:**

After finishing all required procedures, all animals were terminated by decapitation under anesthesia. Their cadavers and tissues were frozen until incinerated according to the standard procedure (Stokes, 2002).

**Results:**

**Production of BGs:**

The obtained ghosts were intact bacterial shells showing several intra-membranous tunnels (Fig. 1). The optimizing physical and chemical conditions of the incubation led to production of high-quality *S. Typhimurium* ghosts.

**Determination of antibodies response:**

At the end of the first cycle, 14 days, vaccination of 24 rats which were divided into six groups (4 rats in each group), the elicited serum IgG’s quantities were close to each other among the six groups (Control/PBS, control/alum, Oral BG, Oral BG + alum, s.c. BG, and s.c. BG + alum). Although there was no significant difference among vaccinated groups, BG+ alum group showed the highest serum IgG titers, Figure 2. The orally vaccinated group by BG + alum, and subcutaneously vaccinated groups by BG and BG+ alum showed significant difference *P* value $0.0001$ (one-way ANOVA) after the second cycle (28 days). At the end of this cycle, the highest IgG titers was shown in the subcutaneously vaccinated group by BG+A, Figure 2.

At the end of the last cycle of vaccination, 42 days, both BG-sc and BG + alum-sc vaccinated groups showed a significant difference *P* value $0.0001$ (one-way ANOVA) in the produced serum IgG antibodies. The subcutaneously vaccinated group by BG + alum showed the ultimate highest serum IgG during whole 42-day vaccination period which reached 3.125 OD, which also followed by the subcutaneously vaccinated group by BG only that were hitting 2.686 OD at 450 nm, Figure 2.

**Antibacterial activity in serum:**

At the end of the whole vaccination period, the collected sera from all groups were challenged against live Salmonella cells. The virulence challenge test showed failure of all vaccinated groups to be protected from bacteremia except the subcutaneously vaccinated group by BG + alum which showed full protection. The sera of this group showed absence of any viable salmonella cells, Figure 3. The alum control group and orally vaccinated group by BG combined with alum showed significant increase in
viable cells in their sera, 4 \times 10^5 \text{CFU/ml} and 1.6 \times 10^5 \text{CFU/ml} respectively, \( P \) value 0.0001 (One-way ANOVA analysis), Figure 3. On the other hand, the other groups; PBS control, orally vaccinated by BG, and subcutaneously vaccinated by BG was showing the same viable counts, 0.8 \times 10^5 \text{CFU/ml} in serum, Figure 3.

**The of granulocytes’ percentage:**

The percentage of granulocytes (24.8\%) was significantly increased \( P \)-value 0.0001 (one-way ANOVA) among the subcutaneously vaccinated group by BG combined with alum. The granulocytes were present in almost the same percentage (18\%) among both control groups, PBS and alum, in addition to the orally vaccinated group by BG + alum, Figur 4. The least percentage (6.2\%) of granulocytes were showed among the orally vaccinated group by BG. The subcutaneously vaccinated group by BG showed only granulocytes’ percentage of 12\%, Figure 4.

**Virulence Challenge-antibacterial response in faeces:**

All rats’ groups were infected subcutaneously by Salmonella after finishing the whole cycles of vaccination, then a week later, fecal bacterial count had been done. The maximum antibacterial activity response was shown among the subcutaneously vaccinated groups by BG and BG + alum as well as the alum control group. All of these groups showed disappearance of viable salmonella cells in their faeces, Figure 5.

Both orally vaccinated groups by BG and BG combined with alum in addition to the PBS control group failed to be protected against the virulent bacteria in faeces, as they showed variable viable counts. The highest viable counts (2.4 \times 10^6 \text{CFU/gm}) was shown among the PBS control group, while the orally vaccinated groups by BG and BG + alum showed 1.5 \times 10^5 and 1.16 \times 10^5 \text{CFU/gm} respectively \( P \) value 0.0001 (One-way ANOVA), Figure 5.

**Virulence Challenge-antibacterial response in liver homogenate:**

None of the vaccinated group showed any liver protection against the virulence challenge by salmonella except the subcutaneously vaccinated group by BG + alum. All the vaccinated groups showed variable viable counts, the subcutaneously vaccinated group by BG showed significant decrease in the virulent viable count \( P \)-value 0.0001 (One-way ANOVA) that was 7000 \text{CFU/gm}. Both control groups, PBS and alum, showed uncountable number of viable counts, Table 1.

**The survival animals after virulence Challenge test:**

After seven days of virulence challenge test and intentional salmonella infection of all vaccinated groups, full survival was achieved among both groups of subcutaneously vaccinated by BG and orally vaccinated by BG combined with alum. Only one rat (25\%) died among the group of subcutaneously vaccinated by BG + alum. The survival percentage was the same (50\%) in both: control groups (PBS and alum). The lowest survival percentage (25\%) was among the orally vaccinated group by BG, Figure 6.
Discussion:

Recently, different approaches have been used to develop human and veterinarian vaccines. Such approaches either utilize the cellular surface displaying properties (e.g., Protein A in Gram-positive bacteria and specific outer-membrane proteins in Gram-negative bacteria) (Wernérus & Ståhl, 2004), or biotechnology-based techniques (Motin & Torres, 2009). The novel biotechnology approaches involve genetically engineered vaccines, inverted pathogenicity (utilizing virulence factors to prevent or treat a disease), and bacterial ghosts system (Motin & Torres, 2009; Paukner et al., 2006). The preserved surface structures and components of the BG can induce both innate and adaptive immune response.

In the current study, the highest serum bactericidal activity (100% eradication) was achieved in the BG-adjuvant subcutaneously immunized animals. Salmonella cells disappeared totally in the faeces of the immunized rats by subcutaneous injection of both BG and BG-adjuvant vaccine after virulence challenge test. In previous studies, the sodium hydroxide (MIC) induced BG of S. aureus showed significant lowering of the total bacterial load within the internal organs (liver, spleen, lungs, and kidneys) of all vaccinated rats groups (orally, subcutaneously, and intravenously) (Park et al., 2016).

In a related study, *Salmonella enteritidis* ghosts (SEG) that were also prepared by the same agent (MIC of NaOH) showed a comparable immune response. The highest serum bactericidal effect was shown in the intramuscular (SEG) with complete Freund's adjuvant followed by intramuscular then finally orally vaccinated rats group at the sixth week. Similarly, the intramuscular (SEG) vaccine with adjuvant gave the highest IgG titers and showed the least bacterial load in the internal organs' homogenates, followed by intramuscular, then orally vaccinated group in week 8 and week 10. All vaccinated groups exhibited significant humoral and cellular immune responses in comparison to the non-vaccinated rat group (Vinod et al., 2014).

In this study, the highest IgG titers were at the maximum level in the subcutaneously vaccinated group with BG-adjuvant followed by the BG subcutaneously vaccinated group at the last day (day 42) in the last period of immunization program. The bacterial load in liver homogenate significantly reduced in the subcutaneously vaccinated rats by BG only after virulence challenge and disappeared in the vaccinated group by BG-alum. In a previous study, the highest IgG antibody activity and the serum bactericidal activity was elicited in subcutaneously vaccinated group at week 9 followed by intravenously then finally orally vaccinated group. Likewise, CD4 + and CD8 + T-cell populations were produced in the largest percentage in the subcutaneously intravenously then orally vaccinated group. The survival rate was 100% in the intravenously immunized, while about 60% in non-immunized group (Vinod et al., 2015).

In another previous study, fish that were immunized intraperitoneally by genetically produced *Edwardsiella tarda* showed stronger serum agglutination titer than those immunized by traditional (formalin-killed pathogen) vaccine (Kwon, Yoon, Sung, & Kim, 2006). The immunized cattle by genetically prepared BGs of *Brucella suis* S2 showed the same titers of IgG, interleukin 4, INF-γ and T-cells as that showed in the conventional formalin-killed Brucella) immunized cattle (Liu et al., 2015).
In extraordinary route of immunization which was through aerosol of the genetically induced BG of *Actinobacillus pleuropneumoniae* elicited higher antigen-specific IgM and IgA in the bronchoalveolar lavage and plasma cells influx in comparison to the traditionally (irradiated-killed bacteria) immunized pigs. Additionally the survival rate was 100% in the BG vaccinated animals (Hensel et al., 2000; Katinger et al., 1999). The antibody titer reached to the maximum in the immunized rats at the third week with neglected titers in the immunized ones (Wu et al., 2017). In the current trial, the survival rate in the BG subcutaneously vaccinated rats’ group was 100%. This was followed by the BG-adjuvant subcutaneously vaccinated group (75%), while the lowest survival rate was in the orally vaccinated group. In a corresponding results in another study, the immunization of rats by subcutaneous injection of BGs of *Listeria monocytogenes* that were prepared by sponge-like protocol (a chemically induced method) (Amro, Salem-Bekhit, & Alanazi, 2014) protected the immunized rats by 100% in the opposite of 0% percent survival in non-immunized animals. By using the same protocol, sponge-like protocol, *Salmonella typhimurium* ATCC 14028 was turned into BGs then orally administered to a rat group. The serum of the orally vaccinated group showed agglutination reactions between antigen O and H against antibodies that prove the correct Salmonella envelop structure that may protect against live Salmonella infection (Sheweita, 2014).

In a previous study, the prepared *Klebsiella pneumonia* by the same protocol (Sponge-like chemical protocol) gave both cellular and humoral immune responses in form of subcutaneous (the highest activity), inhalation, intraperitoneal, and intramuscular routes. The produced phagocytic activity, INF-γ levels, and bactericidal actions were much higher in the vaccinated rats (MM et al., 2017). Different strains of Vibrio cholerae were evacuated using the E lýsis gene –genetic approach- and exploited in vaccination of the experimental animals via different routes of administration. *Vibrio cholerae* El Tor, Ogawa strains ghosts intraperitoneal vaccination of rats were compared to the conventional vibrio vaccination and produced more IgG titers and vibriocidal activity (Francis O Eko et al., 1994).

In contrast to the result of the current study, the oral vaccines of salmonella’s BGs failed to protect rats, another study showed that orally vaccinated animals by BGs of *E. coli* O157:H7 (Mayr et al., 2005b) and *H. pylori* (Talebkhan et al., 2010) were survived by 93% and 100% respectively. Additionally, the bacterial colonization in intestine (*E. coli* BG vaccine) and stomach (*H. pylori* BG vaccine) was reduced. Also, there was significant existence of anti *H. pylori* and Omp-specific antibodies in *H. pylori* BG vaccinated animal group (Talebkhan et al., 2010). Furthermore, the oral vaccine of *V. cholerae* O1 or O139 strains protected about 50% of the immunized infant mice cholera models. It also produced anti-LPS and antitoxin coregulated pilli (F. O. Eko, Mayr, Attridge, & Lubitz, 2000) and vibriocidal antibodies (Francis O Eko et al., 2003) in the vaccinated group of rabbits. Further, it could reduce- dose correlated- the duodenal mucosal colonization by vibrio after vibrio challenge local injection (Jalava, Eko, Riedmann, & Lubitz, 2003).

In this study, the highest percentage of granulocytes was raised in the BG-adjuvant subcutaneously immunized group. Significant induction of dendritic cells antigen presentation and release of different interleukins and anaphlytoxins were followed the subcutaneous immunization of rabbits by BG of *V. cholerae* H1 strains (Francis O Eko et al., 2015). Significant increase in antigen-specific T-cells
proliferation (Jawale, Chaudhari, & Lee, 2014), plasma antigen-specific IgA and IgG, INF-γ, interleukin 2, T-cells as well as bacterial colonization reduction in the immunized chicken internal organs (Jawale & Lee, 2016). It was compatible with the results of the current study that 100% protection was accomplished by the immunization of both rabbits and mice by genetically induced *Pasteurella multocida* and *Pasteurella haemolytica* BGs by subcutaneous route (Marchart et al., 2003). In another report, both species of salmonella, *S. gallinarum* and *S. typhimurium* BGs that were genetically synthesized, significantly reduced the mortality rates in immunized chicken when they were administered intramuscularly (Jawale & Lee, 2016) or even orally (Jawale et al., 2014).

The chemically prepared (tween 80 protocol) *Salmonella enterica serovar typhimurium* BGs vaccines were able to protect all the immunized rats (survival rate 100%) without adjuvant. It also offered both humoral and cellular immune responses in case of subcutaneous route of administration. The minor immune response of the oral BG vaccine may refer to the gastric intestinal digestion as well as possible intentional reflux by the animal itself.

**Conclusions:**

The immunological characters of salmonellas’ BG that have been revealed in this study suggest that BGs can be a promising platform for effective vaccine production to immunize against a variety of bacterial infections in animals. Further preclinical trials are required to assure the ghosts’ vaccines safety and applicability, then shifting to the clinical trials phases can be approached.

**Abbreviations**

ANOVA: Analysis of variance; ATCC: The American type culture collection; APC: Antigen presenting cells; BG: Bacterial ghosts; CBC: Complete blood count; CD4+: Cluster of differentiation 4+; CD8+: Cluster of differentiation 8+; CFU: Colony forming unit; DC: Dendritic cells; ELISA: Enzyme linked immunosorbent assays; HPBG: Helicobacter pylori ghost; HPLC: High-performance liquid chromatography; IFN-γ: Interferon-γ; IL-: Interleukin-; IM: Inner membrane; KPG: Klebsiella pneumoniae ghost; LMG: Listeria monocytogenes ghost; LPS: Lipopolysaccharide; MGC: Minimum growth concentrations; MHC: Major histocompatibility complex; MIC: Minimum inhibitory concentration; MOMP: Major outer membrane protein; OM: Outer membrane; Omp: Outer membrane protein; PAMP: Pathogen associated molecular patterns; PBS: Phosphate buffered saline; SEG: Salmonella enteritidis ghost; SLRP: Sponge-like reduced protocol; TA: Target antigen; Th:Thymocyte-helper cells; TLR: Toll-like receptor; VCG: Vibrio cholerae ghost; VPG: Vibrio parahaemolyticus ghost.

**Declarations**

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**Authors’ contributions:**

Conceptualization, S.R.; methodology, S.R.; A.F., and E.A.; formal analysis, S.R.; A.F., and E.A; investigation, A.M. and A.S.; resources, F.A. and M.M.; data curation, A.F. and S.R.; writing—original draft preparation, S.R.; writing—review and editing, S.R. and A.S.; visualization, A.S. and M.M.; supervision, A.M., A.S., and M.M.; project administration, M.M.; funding acquisition, F.A. All authors have read and agreed to the published version of the manuscript.

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**Availability of data and materials:**

Not applicable

**Ethical approval:**

The ethical approval for this study was given by the research ethical committee, college of pharmacy, Cairo University and given (No. MI 1506) /28/10/2015. All procedures and methods that have been used in this study were performed in the compliance with the guidelines and regulations of the research ethical committee in the college of pharmacy, Cairo University as well as with the ARRIVE guidelines. All authors have no conflicts of interest to disclose in all aspects of this work.

**Consent for publication:**

Not applicable

**Competing interests:**

The authors declare they have no competing interests.

**References**

1. Amara, A. A., Salem-Bekhit, M. M., & Alanazi, F. K. (2013). Sponge-like: A new protocol for preparing bacterial ghosts. *The Scientific World Journal, 2013*. https://doi.org/10.1155/2013/545741

2. Amro, A. A., Salem-Bekhit, M. M., & Alanazi, F. K. (2014). Plackett-Burman randomization method for Bacterial Ghosts preparation form E. coli JM109. *Saudi Pharmaceutical Journal, 22*(3), 273–279. https://doi.org/10.1016/j.jsps.2013.06.002
3. Eko, F. O., Mayr, U. B., Attridge, S. R., & Lubitz, W. (2000). Characterization and immunogenicity of Vibrio cholerae ghosts expressing toxin-coregulated pili. *Journal of Biotechnology, 83*(1–2), 115–123. https://doi.org/10.1016/S0168-1656(00)00315-1

4. Eko, Francis O, Hensel, A., Bunka, S., & Lubitz, W. (1994). Immunogenicity of Vibrio cholerae ghosts following intraperitoneal immunization of mice. *Vaccine, 12*(14), 1330–1334.

5. Eko, Francis O, Mania-Pramanik, J., Pais, R., Pan, Q., Okenu, D. M. N., Johnson, A., ... Igietseme, J. U. (2015). Vibrio cholerae ghosts (VCG) exert immunomodulatory effect on dendritic cells for enhanced antigen presentation and induction of protective immunity. *BMC Immunology, 15:*584, 1–12. https://doi.org/10.1186/s12865-014-0056-x

6. Eko, Francis O, Schukovskaya, T., Lotzmanova, E. Y., Firstova, V. V, Kutyrev, V. V, Igietseme, J. U., & Lubitz, W. (2003). Evaluation of the protective efficacy of Vibrio cholerae ghost (VCG) candidate vaccines in rabbits. *Vaccine, 21*, 3663–3674. https://doi.org/10.1016/S0264-410X(03)00388-8

7. Hensel, A., Huter, V., Katinger, A., Raza, P., Strnistschie, C., Roesler, U., ... Lubitz, W. (2000). Intramuscular immunization with genetically inactivated (ghosts) Actinobacillus pleuropneumoniae serotype 9 protects pigs against homologous aerosol challenge and prevents carrier state. *Vaccine, 18*, 2945–2955.

8. Jalava, K., Eko, F. O., Riedmann, E., & Lubitz, W. (2003). Bacterial ghosts as carrier and targeting systems for mucosal antigen delivery. *Expert Review of Vaccines, 2*(1), 45–51. https://doi.org/10.1586/14760584.2.1.45

9. Jawale, C. V., Chaudhari, A. A., & Lee, J. H. (2014). Generation of a safety enhanced Salmonella Gallinarum ghost using antibiotic resistance free plasmid and its potential as an effective inactivated vaccine candidate against fowl typhoid. *Vaccine, 32*(9), 1093–1099. https://doi.org/10.1016/j.vaccine.2013.12.053

10. Jawale, C. V., & Lee, J. H. (2016). Evaluation of immunogenicity and protective efficacy of adjuvanted Salmonella Typhimurium ghost vaccine against salmonellosis in chickens. *Veterinary Quarterly, 36*(3), 130–136. https://doi.org/10.1080/01652176.2016.1138248

11. Katinger, A., Lubitz, W., Szostak, M. P., Stadler, M., Klein, R., Indra, A., ... Hensel, A. (1999). Pigs aerogenously immunized with genetically inactivated (ghosts) or irradiated Actinobacillus pleuropneumoniae are protected against a homologous aerosol challenge despite differing in pulmonary cellular and antibody responses. *Journal of Biotechnology, 73*, 251–260.

12. Kwon, S. R., Yoon, K. N., Sung, K. K., & Kim, K. H. (2006). Protection of tilapia (Oreochromis mosambicus) from Edwardsiellosis by vaccination with Edwardsiella tarda ghosts. *Fish and Shellfish Immunology, 20*(4), 621–626. https://doi.org/10.1016/j.fsi.2005.08.005

13. Liu, J., Li, Y., Sun, Y., Ji, X., Zhu, L., Guo, X., ... Feng, S. (2015). Immune responses and protection induced by Brucella suis S2 bacterial ghosts in mice. *Veterinary Immunology and Immunopathology, 166*(3–4), 138–144. https://doi.org/10.1016/j.vetimm.2015.04.008

14. Marchart, J., Rehagen, M., Dropmann, G., Szostak, M. P., Alldinger, S., Lechleitner, S., ... Lubitz, W. (2003). Protective immunity against pasteurellosis in cattle, induced by Pasteurella haemolytica
ghosts. *Vaccine*, 27(13–14), 1415–1422. https://doi.org/10.1016/S0264-410X(02)00635-7

15. Mayr, U. B., Haller, C., Haidinger, W., Atrasheuskaya, A., Bukin, E., Lubitz, W., & Ignatyev, G. (2005a). Bacterial Ghosts as an Oral Vaccine: a Single Dose of *Escherichia coli* O157: H7 Bacterial Ghosts Protects Mice against Lethal Challenge. *Bacterial Ghosts as an Oral Vaccine: a Single Dose of Escherichia coli O157: H7 Bacterial Ghosts Protects Mice agai. Infection and Immunity*, 73(8), 4810–4817. https://doi.org/10.1128/IAI.73.8.4810-4817.2005

16. Mayr, U. B., Haller, C., Haidinger, W., Atrasheuskaya, A., Bukin, E., Lubitz, W., & Ignatyev, G. (2005b). Bacterial Ghosts as an Oral Vaccine: a Single Dose of *Escherichia coli* O157:H7 Bacterial Ghosts Protects Mice against Lethal Challenge. *INFECTION AND IMMUNITY*, 73(8), 4810–4817. https://doi.org/10.1128/IAI.73.8.4810-4817.2005

17. MM, M., A, H., Ghazy A, A. E. K., S, S., & Fattah Amara, A. A. A. (2017). Klebsiella pneumoniae Ghosts as Vaccine Using Sponge Like Reduced Protocol. *Cellular & Molecular Medicine: Open Access*, 03(02), 1–8. https://doi.org/10.21767/2573-5365.100034

18. Park, H. J., Oh, S., Vinod, N., Ji, S., Noh, H. B., Koo, J. M., … Choi, C. W. (2016). Characterization of chemically-induced bacterial ghosts (BGs) using sodium hydroxide-induced *Vibrio parahaemolyticus* ghosts (VPGs). *International Journal of Molecular Sciences*, 17(11). https://doi.org/10.3390/ijms1711904

19. Rabea, S., Salem-Bekhit, M. M., Alanazi, F. K., Yassin, A. S., Moneib, N. A., & Hashem, A. E. M. (2018). A novel protocol for bacterial ghosts’ preparation using tween 80. *Saudi Pharmaceutical Journal*, 26(2), 232–237. https://doi.org/10.1016/j.jspj.2017.12.006

20. Sheweita, S. A. (2014). Evaluation the surface antigen of the *Salmonella typhimurium* ATCC 14028 ghosts prepared by “SLRP” *TheScientificWorldJournal*, 2014, 840863. https://doi.org/10.1155/2014/840863

21. Stokes, W. S. (2002). Humane Endpoints for Laboratory Animals Used in Regulatory Testing Moribund Condition as a Current Humane Endpoints for Safety Testing. *ILAR Journal*, 43, S31–S38.

22. Talebkhan, Y., Bababeik, M., Esmaeili, M., Oghalaei, A., Saberi, S., Karimi, Z., … Mohammadi, M. (2010). Helicobacter pylori bacterial ghost containing recombinant Omp18 as a putative vaccine. *Journal of Microbiological Methods*, 82(3), 334–337. https://doi.org/10.1016/j.mimet.2010.07.001

23. Vinod, N., Oh, S., Kim, S., Choi, C. W., Kim, S. C., & Jung, C. H. (2014). Chemically induced *Salmonella enteritidis* ghosts as a novel vaccine candidate against virulent challenge in a rat model. *Vaccine*, 32(26), 3249–3255. https://doi.org/10.1016/j.vaccine.2014.03.090

24. Vinod, N., Oh, S., Park, H. J., Koo, J. M., Choi, C. W., & Kim, S. C. (2015). Generation of a novel *staphylococcus aureus* ghost vaccine and examination of its immunogenicity against virulent challenge in rats. *Infection and Immunity*, 83(7), 2957–2965. https://doi.org/10.1128/IAI.00009-15

25. Wu, X., Ju, X., Du, L., Yuan, J., Wang, L., He, R., & Chen, Z. (2017). Production of Bacterial Ghosts from Gram-Positive Pathogen *Listeria monocytogenes*. *Foodborne Pathogens and Disease*, 14(1), 1–7. https://doi.org/10.1089/fpd.2016.2184
26. Yang, Y., Wan, C., Xu, H., Aguilar, Z. P., Tan, Q., Xu, F., ... Wei, H. (2013). Identification of an outer membrane protein of Salmonella enterica serovar Typhimurium as a potential vaccine candidate for Salmonellosis in mice. *Microbes and Infection, 15*(5), 388–398. https://doi.org/10.1016/j.micinf.2013.02.005

27. Yardeni, T., Eckhaus, M., & Morris, H. D. (2011). Retro-orbital_injections_in_mice lab Animal 2011.pdf. *Lab Anim (NY), 40*(5), 155–160. https://doi.org/10.1038/lab0511-155.Retro-orbital

### Tables

**Table 1:** The viable count of Salmonella in rats’ liver homogonate among different vaccinated groups.

| Vaccine type | Viable count CFU/gm |
|--------------|---------------------|
| PBS          | uncountable         |
| BG Oral      | $1.3 \times 10^5$   |
| BG+Alum Oral | $3 \times 10^4$     |
| BG SC        | 7000                |
| Alum         | uncountable         |
| BG+Alum SC   | 0                   |

$P$-value 0.0001 (One-way ANOVA).

### Figures
Figure 1

Scanning electron micrograph showing the perforating effect of tween 80 (7% w/v) on the cellular membrane of Salmonella's cells. The arrows are directed to some surface pores that resulted from incubation of tween 80 with Salmonella culture.
Figure 2

The OD of IgG titers produced versus time (days) among different vaccination groups: PBS: buffer solvent control group; Alum: alum control group; BG-oral: BG oral vaccinated group; BG + Alum-oral: orally vaccinated group by BG + alum; BG-sc: subcutaneously vaccinated group by BG; BG + Alum- sc: subcutaneously vaccinated group by BG + alum. P value $< 0.0001$ (One-way ANOVA).
The viable count of Salmonella in rats’ serum of different vaccinated groups, P value <0.0001 (one-way ANOVA).
The percentage of granulocytes among different vaccinated groups, P value <0.0001 (one-way ANOVA).

Virulence Challenge in Faeces

P value <0.0001
**Figure 5**

The viable count of Salmonella in rats’ faeces among different vaccinated groups, P value <0.0001 (One-way ANOVA).

(A) Survival Percentage of control (Alum/PBS) and BG-sc groups

(B) Survival Percentage of control (Alum/PBS) and BG+ Alum-sc groups

(C) Survival Percentage of control (Alum/PBS) and BG+Alum-Oral groups

(D) Survival Percentage of control (Alum/PBS) and BG-Oral groups

**Figure 6**
The survival percentage among all vaccinated groups; (A): survival percentage of control (alum/PBS) and BG-oral; (B): control (alum/PBS) and BG-alum-sc groups; (C): control (alum/PBS) and BG+ alum-oral groups; (D): control (alum/PBS) and BG-sc groups.

**Image not available with this version**

**Figure 7**

The apoptosis and/or necrosis activities of (A) control group (5%DMEM), (B) free ghost group (1 μg/ml), (C) free DOX (1 μg/ml), and (D) ghost combined DOX (1 μg/ml) on the HepG2 cells.