Evaluation of immunogenicity and protective efficacy of adjuvanted *Salmonella Typhimurium* ghost vaccine against salmonellosis in chickens

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**ABSTRACT**

**Background:** *Salmonella Typhimurium*, a non-host-adapted Gram-negative intracellular pathogen, is capable of infecting a variety of animal hosts and humans.

**Objective:** This study utilized the prime-booster immunization strategy using *Salmonella Typhimurium* – LTB (S. Typhimurium – LTB) ghost with the aim of inducing a robust immune response for the prevention of avian salmonellosis. In addition, the effect of Montanide™ ISA 70VG adjuvant on *S. Typhimurium* – LTB ghost vaccination was investigated.

**Animals and methods:** A total of 75 chickens were divided into three groups (n=25) for intramuscular immunization: group A (non-immunized control injected with sterile PBS), group B (immunized with *S. Typhimurium* – LTB ghost), and group C (immunized with *S. Typhimurium* – LTB ghost plus Montanide™ ISA70VG adjuvant).

**Results:** Compared with group A, the immunized chickens (groups B and C) exhibited increased titers of antigen specific plasma IgG and intestinal secretory IgA antibodies. In addition, group C showed enhanced induction of the humoral immune response compared to group B. The populations of splenic CD3+CD4+ and CD3+CD8+ T-cells increased significantly in both immunized groups. In addition, increased mRNA expression of the Th1 cytokines, IFN-γ and IL-2 were observed in *S. Typhimurium* antigen-stimulated peripheral blood mononuclear cells from groups B and C chickens. Chickens from both vaccinated groups showed significant protection against virulent *S. Typhimurium* oral challenge compared to non-vaccinated chickens and a lower challenge strain count was recovered from the internal organs of group C.

**Conclusions:** Injection of *S. Typhimurium-LTB* ghost with or without Montanide™ ISA70VG adjuvant is capable of inducing protective immunity against the virulent *S. Typhimurium* infection in chickens.

1. Introduction

*Salmonella enterica* serovar Typhimurium (S. Typhimurium), a Gram-negative intracellular pathogen, is a predominant *Salmonella* serovar associated with invasive non-typhoidal salmonellosis and usually causes foodborne diarrheal infections in humans (Gordon 2011). Infected chickens are the most likely zoonotic source of *S. Typhimurium* infection (Cheong et al. 2007). Various approaches have been used to control salmonellosis in poultry, including improved hygiene standards, improved animal husbandry, the use of prophylactic antibiotics, and vaccination. In addition, probiotics used in poultry feed are becoming accepted for control of specific enteric pathogens such as *Salmonella* (Tellez et al. 2012). Vaccination is one of the methods suggested to reduce the burden of *S. Typhimurium* in the poultry industry; it is expected to increase the resistance of chickens to infection and to prevent spread of bacterial micro-organisms to human society through the food production chain (Barrow 2007).

To vaccinate against non-typhoidal salmonellosis in chickens, the vaccine preparation should be safe and highly immunogenic (Desin et al. 2013). Bacterial ghosts (BGs) are non-living Gram-negative bacterial cell envelopes devoid of cytoplasmic contents while maintaining their cellular morphology and native surface antigenic structures including bio-adhesive properties (Jalava et al. 2002). BGs are produced by protein E-mediated lysis under the controlled expression of the PhiX174 lysis gene *E*, leading to the formation of a transmembrane tunnel in the cell envelope through which cytoplasmic contents are expelled (Witte et al. 1990). The coupling of immunomodulator molecules such as *Escherichia coli* heat-labile enterotoxin B subunit (LTB) in the architecture of the BG may enhance antigen presentation of the ghost antigens; this is because coupling LTB with the GM1 ganglioside receptor at the eukaryotic cell surface results in enhanced uptake of coupled antigens (Freytag & Clements 2005). In our previous study, we demonstrated the protective efficacy of a single
vaccination with a S. Typhimurium-LTB ghost vaccine to prevent S. Typhimurium infection (Jawale & Lee 2014a). However, a single dose of vaccine is usually associated with a shorter duration of immunity (Zepp 2010). More potent induction of immune responses may be important for long-term protection to be afforded by a vaccine and might be achieved by prime-booster vaccination strategy (Zepp 2010).

BGs maintain all surface proteins of the original bacterium in their original state and, therefore, possess all the structural, immunogenic, and bio-adhesive properties of the original bacterium (Eko et al. 1999; Jawale & Lee 2014b); however, as the genetically inactivated BG cannot replicate inside the immunized host, it might subject to rapid clearance by the host (Desin et al. 2013). Combination with a suitable adjuvant increases the persistence of the non-replicating BG antigens in order to induce long-lasting immune responses (Desin et al. 2013). Montanide™ ISA70 VG is water-in-oil (W/O) emulsion-based adjuvant comprising injectable mineral oil, refined emulsifier obtained from mannnitol, and purified oleic acid of vegetable origin (Dupuis et al. 2006).

With this research in mind, the present study attempted to optimize the protective efficacy of a S. Typhimurium-LTB ghost vaccine and evaluated the immune potential using prime-booster immunization strategy in chickens. In addition, we evaluated the effectiveness of Montanide™ ISA70 VG adjuvant in promoting protective immunity against S. Typhimurium infection. To assess the adjuvant effect, chickens were immunized with either S. Typhimurium-LTB ghosts combined with Montanide™ ISA70 VG adjuvant or S. Typhimurium-LTB ghosts alone. We then assessed the induction of immune responses and protective efficacies against virulent challenge.

2. Materials and method

2.1. Bacterial strains and oligonucleotides

The bacterial strains used are listed in Table 1. The Δods S. Typhimurium strain was grown at 37°C in Luria-Bertani (LB) broth containing 50 mg/mL of dianaminopimelic acid (DAP). All the bacterial strains were stored as frozen cultures in LB broth containing 20% glycerol at −80°C until use. The oligonucleotides used are listed in Table 2.

| Bacterial strains | Description | Reference |
|------------------|-------------|-----------|
| JOL1311          | S. Typhimurium, ods gene knockout strain | Lab stock |
| JOL401           | S. Typhimurium wild type | Lab stock |
| JOL1499          | JOL1311 containing pJHL187-LTB | Jawale and Lee (2014a) |

Table 2. Oligonucleotides used in this study.

| Oligonucleotide | Description | Reference |
|-----------------|-------------|-----------|
| TYPH-F          | 5’-TGTTACCTTTTTACCCCTGAA3’- | Jawale and Lee (2014a) |
| TYPH-R          | 5’-CCCTGAACCGCTTATATAT3’- | Jawale and Lee (2014a) |
| GAPDH-F         | 5’-GAACATCATCACCAGCCGCC3’- | Jawale and Lee (2014a) |
| GAPDH-R         | 5’-CGCAGGTTCGTCACA3’- | Jawale and Lee (2014a) |
| IFN-γ-F         | 5’-CAAGGCGCATACAAAA3’- | Jawale and Lee (2014a) |
| IFN-γ-R         | 5’-TTTCACCTCTCTCAGGCCAT3’- | Jawale and Lee (2014a) |
| IL2-F           | 5’-ATCTTGCTGTTGTTGTTG3’- | Jawale and Lee (2014a) |
| IL2-R           | 5’-TGGTCTAGTTGGTGTTG3’- | Jawale and Lee (2014a) |

2.2. Generation of a S. Typhimurium ghost carrying LTB and preparation of oil-adjuvanted ghost vaccine

We utilized our previously constructed plasmid vector pJHL187-LTB carrying the regulatory E-lysis system and the foreign protein delivery system carrying the eltB gene (Jawale & Lee 2014b). For generation of a S. Typhimurium ghost carrying LTB adjuvant protein, the JOL1311 strain was transformed with plasmid pJHL187-LTB and the resultant strain was named JOL1499. The ghost cells were generated from JOL1499 as described previously (Jawale & Lee 2014a). Final ghost cell pellets were resuspended in sterile 1X PBS (pH 7.4) and stored at −20°C until use. The oil-adjuvanted ghost vaccine was prepared by emulsifying the S. Typhimurium-LTB ghost suspension with Montanide™ ISA70 VG (Seppic, Puteaux, France) at a ratio of 30:70 (w/w). The final dose of the inactivated oil-emulsion S. Typhimurium-LTB ghost vaccine was 1 × 109 ghost cells per bird.

2.3. Immunization of chickens and virulent challenge

All experimental works involving animals were approved (CBU 2011-0017) by the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care. A total of 75 female Brown Nick layer Salmonella-free chickens were equally divided into groups A, B, and C (n = 25). Chickens were intramuscularly primed at five weeks of age and subsequently boosted at eight weeks of age. Group A chickens were used as a non-immunized control and were injected intramuscularly with sterile PBS (pH 7.4) at fifth and eighth weeks of age. Group B chickens were primed and boosted with 0.3 mL of ghost vaccine containing 1 × 109 ghost cells resuspended in PBS (pH 7.4). Group C chickens were primed and boosted with 0.3 mL of oil-adjuvanted vaccine containing 1 × 109 ghost cells. At 11 weeks of age, all chickens were orally challenged with 1 × 109 CFU of the S. Typhimurium virulent strain (JOL401).
2.4. Sample collection and antibody response of vaccinated chickens

For sample collection, five birds were randomly selected from each group. We collected blood samples from the wing vein of the birds with heparinized 1 mL syringes and centrifuged the blood to obtain plasma samples. The intestinal wash samples were collected by using the pilocarpine-based intestinal lavage method, as described previously (Matsuda et al. 2011). The samples were stored at −20 °C until use. The outer membrane protein (OMP) mixture isolated from JOL401 was used as a capture antigen. The OMP mixture was extracted from JOL401, as described previously (Kang et al. 2002). The indirect enzyme linked immunosorbent assay (ELISA) was carried out for detection of S. Typhimurium OMP-specific IgG and sIgA using a chicken IgG and IgA ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX, USA), as previously described (Nandre et al. 2011). The wells of a Microlon® ELISA plate were coated with 100 μL OMP at a concentration of 0.2 mg/mL. Wells were incubated with a 1:300 dilution of plasma for 1 h, followed by incubation with a 1:100,000 dilution of horseradish peroxidase (HRP) conjugated goat anti-chicken IgG (Bethyl Laboratories, Montgomery, TX, USA) for 1 h. The bounded HRP activity was measured using o-Phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA). The optical density at 492 nm was measured using an ELISA reader after the reaction was stopped with 50 μL 3.0 M sulfuric acid. The sIgA concentrations were quantified using procedures similar to those that were used to measure plasma IgG levels (except that the intestinal wash samples were diluted 1:5 and goat anti-chicken IgA HRP-conjugated antibody was used as secondary antibody).

2.5. Flow cytometric analysis of T-cell populations

The population of CD3+CD4+ and CD3+CD8+ T-cells in the spleen was measured one week post prime vaccination in five birds randomly selected from each group. The birds were euthanized, spleen tissues were collected, and white blood cells from spleen were isolated using the Histopaque-1077 protocol (Sigma-Aldrich, St. Louis, MO, USA). For staining of the isolated cells, 1 × 10^6 cells from each sample were resuspended in PBS supplemented with 0.5% bovine serum albumin (BSA) and 0.005% NaN3 (fluorescent activated cell sorting (FACS) buffer) incubated with an appropriate dilution of fluorescein isothiocyanate labeled anti-CD3, biotin-labeled anti-CD4, and phycoerythrin labeled anti-CD8 (Southern Biotech, Birmingham, AL) in the dark at 4 °C for 30 min following centrifugation at 1500 rpm for 5 min and washed with fluorescent activated cell sorting (FACS) buffer. The secondary staining of biotin-labeled anti-CD4 was accomplished by using allophycocyanin labeled streptavidin antibody. The stained cells were washed with FACS buffer and finally resuspended in 500 μL of PBS for FACS analysis. Data collection was performed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). A total of 10,000 events per sample were recorded and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

2.6. Quantitative analysis of cytokine mRNA by real-time PCR

Three weeks after the booster vaccination, blood samples were collected from five birds in each group and suspensions of peripheral blood mononuclear cells (PBMCs) were prepared using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s protocol. Briefly, total 2 mL of the diluted blood sample was carefully loaded on the 2 mL of Histopaque-1077 and centrifuged at 400g for 30 min. The cell layer at the interface was collected and washed twice and resuspended in RPMI-1640 medium (HyClone Laboratories, South Logan, UT, USA). Sonicated bacterial cell protein lysate was prepared from JOL401. Briefly, the bacterial cell suspension was sonicated for 10 min and centrifuged at 5000g for 60 min at 4 °C. Supernatant containing the sonicated bacterial cell protein suspension was used as the antigen for stimulation of PBMC. The 1 × 10^6 viable PBMCs from each bird were added in the separate individual wells of 24-well tissue culture plates. The PBMCs in each well were stimulated with 4 μg/mL of sonicated bacterial cell protein lysate. The plates were incubated at 40 °C in a humidified 5% CO2 atmosphere for 72 h. Total RNA was extracted from the cultured cells by using Hybrid-R RNA extraction kit (GeneAll, South Korea) and treated with RQ1 RNase-Free DNase (Promega, WI, USA) for removal of genomic DNA contamination in the RNA preparation. The DNase-treated RNA was converted into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). The comparative Ct method was used for relative quantification of the IFN-γ and IL2 genes (Jawale & Lee 2014c). The housekeeping gene GAPDH was used as an endogenous control to normalize the expression of target cytokines.

2.7. Bacteriology for protection assay

We determined organ colonization of the wild type S. Typhimurium challenge strain (JOL401) 7 and 14 days post challenge; 10 chickens were euthanized at both time points from all three groups. The challenge strain load in internal organs was determined as described previously (Jawale & Lee 2014a). Aseptically collected liver, spleen, and caecal contents were weighed and homogenized in buffered peptone water (BPW, Becton, Dickinson and Company, MD, USA). The serial dilutions of the homogenized tissue suspensions were plated on brilliant green agar (BGA; Becton, Dickinson and
Company, MD, USA) and the plates were incubated at 37 °C for enumeration of bacterial count on direct culture. Overnight, incubated homogenates in BPW were inoculated in Rappaport–Vassiliadis R10 broth (Becton, Dickinson and Company, MD, USA) at 42 °C for 48 h for selective enrichment of JOL401. A loop of enrichment culture was streaked on BGA and incubated overnight at 37 °C. Samples positive on direct and enrichment cultures were confirmed by polymerase chain reaction (PCR) using S. Typhimurium-specific primers. The number of direct culture bacterial colonies was expressed as mean ± standard deviation of the log10 CFU/gm. Samples that were positive only after enrichment were counted as 1 CFU/gm.

2.8. Statistical analysis

All data are expressed as mean ± standard deviation. Analyses were performed with SPSS version 16.0 software (SPSS, Chicago, IL, USA). The Mann–Whitney U-test was used to analyze statistical differences in the immune responses and organ bacterial recovery between the immunized groups and the unimmunized control group. Differences were significant when P-values were ≤0.05.

3. Results

3.1. Development of antibody response after vaccination

The vaccination-induced humoral immune response was evaluated with the S. Typhimurium antigen-specific plasma IgG and intestinal sIgA. During the weeks after vaccination, the S. Typhimurium antigen-specific plasma IgG titers of chickens from both vaccinated groups were significantly elevated (Figure 1). The plasma IgG titers in both immunized groups were dramatically increased after booster vaccination. Group C chickens showed significantly higher antibody titers than group B chickens (P ≤ 0.05) (Figure 1). After prime and booster vaccinations, the intestinal sIgA titers of group B and C chickens were significantly elevated (Figure 1). Particularly after the booster vaccination, group C chickens had considerably higher intestinal sIgA titers than group B chickens.

3.2. Analysis of T-cell population after vaccination

For assessment of the cell-mediated immune response after vaccination, we quantified the T-cell subpopulation profiles in the spleen from both immunized and control groups by flow cytometry. CD3 is a marker of all T-cells, whereas CD4 and CD8 are markers for helper T-cells and cytotoxic T-cells, respectively. At one week post prime vaccination, the overall population of CD3+CD4+ and CD3+CD8+ T-cells in the spleen was evaluated with the flow cytometry. CD3 is a marker of

3.3. Cytokine gene expression after in vitro antigenic stimulation

For evaluation of the Th1 cytokine (IL-2 and IFN-γ) response, PBMCs prepared from vaccinated and non-vaccinated chickens were stimulated with the sonicated bacterial cell protein from JOL401. The
antigen-stimulated cultured cells from groups B and C showed significantly higher gene expression levels of IL-2 and IFN-γ cytokines than those from the non-vaccinated control chickens (P ≤ 0.05) (Figure 3).

### 3.4. Vaccination with S. Typhimurium ghost vaccines reduces colonization of wild-type S. Typhimurium in internal organs

The protective efficacy of the vaccination scheme was evaluated by bacterial counting of the challenge strain in the liver, spleen, and cecal contents. The chickens from both immunized and control groups were euthanized at 7 or 14 days after challenge. Groups B and C had significantly less colonization in the liver than group A and the differences were statistically significant at day 7 (Table 3). In comparison with group A, rapid clearance of the challenge strain was observed in groups B and C. The challenge strain load was significantly lower in the spleen and cecal contents at days 7 and 14 post-challenge (P ≤ 0.05). Group C had a significantly lower challenge strain load in the spleen and caecal content than group B at day 7 post-challenge (P ≤ 0.05) (Table 3).

### 4. Discussion

In the present study, we found that immunization with S. Typhimurium-LTB ghost plus Montanide™ ISA70 VG adjuvant increased the titers of antigen-specific plasma IgG and intestinal sIgA antibodies when compared to vaccination with S. Typhimurium-LTB ghost alone. However, both vaccinated groups showed a significant increase in antibody titers compared to non-vaccinated chickens. We also found a significantly increased population of CD4+ and CD8+ T-cells in spleens of the chickens from both the vaccinated groups compared to non-vaccinated chickens. In addition, we observed a significant increase in the gene expression of Th1 cytokines (IL-2 and IFN-γ) in the in vitro S. Typhimurium antigen-stimulated PBMCs of vaccinated groups compared to those from non-vaccinated group. Finally, both vaccinated groups showed significant protection against virulent S. Typhimurium infection compared to the non-vaccinated group. We recovered a considerably lower challenge strain count from the internal organs of chickens vaccinated with the S. Typhimurium-LTB ghost plus Montanide™ ISA70 VG adjuvant compared with chickens vaccinated with the S. Typhimurium-LTB ghost alone.

For production of the inactivated vaccines, we used controlled expression of the PhiX174 1ysis gene E because it enables the generation of the empty bacterial envelopes possessing functional and antigenic determinants in the natural conformation of their living homologues (Jalava et al. 2002). We incorporated LTB in the outer membrane to boost the immune potential of the S. Typhimurium ghost (Ekong et al. 2009; Jawale & Lee 2014b). The LTB binds to ganglioside receptors (GM1) on the surface of dividing eukaryotic cells, resulting in increased uptake of the coupled antigen by antigen-presenting cells (Lim et al. 2009).

The BG is capable of inducing humoral and cell-mediated immune responses (Jawale & Lee 2014c); however, its non-replicating nature may limit the persistence of the antigens in the vaccinated host and it may, therefore, be eliminated quickly (Desin et al. 2013). To overcome this issue, we combined BGS with the Montanide™ ISA70 VG adjuvant to enhance their vaccine potential. The Montanide™ ISA series of adjuvants are known to improve the vaccine efficacy via the induction of strong and long-lasting immune responses and are recommended for combination with bacterial, mycoplasma, viral, or parasite antigens (Aucouturier et al. 2006). Montanide™ ISA series adjuvants form a depot at the injection site, where the adjuvant protects the antigen from dilution, rapid

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**Figure 3.** Cytokine profile of in vitro stimulated PBMC cultures. Group A, non-immunized control; Group B, immunized with S. Typhimurium-LTB ghost; Group C, immunized with S. Typhimurium-LTB ghost plus Montanide™ ISA70VG adjuvant. PBMCs were collected from five randomly selected birds. The relative expression was determined by real-time PCR using the comparative Ct method. The values represent the relative expression of the cytokine normalized to GAPDH. The significant differences in cytokine expression between the control and immunized groups are indicated by asterisks (** indicates P ≤ 0.01, *** indicates P ≤ 0.001).

**Table 3.** Recovery of challenge strain from internal organs of chickens (n = 10 per group).

| Day | Group | Liver  | Spleen | Caecal contents |
|-----|-------|--------|--------|-----------------|
| 7   | A     | 3.02 ± 1.17 | 5.24 ± 0.89 | 6.79 ± 0.42 |
|     | B     | 1.47 ± 0.95*** | 2.86 ± 1.19*** | 5.72 ± 0.72*** |
|     | C     | 1.78 ± 1.03*  | 1.91 ± 0.71*** | 4.67 ± 1.45*** |
| 14  | A     | 1.47 ± 1.70 | 4.48 ± 0.96 | 6.45 ± 0.91 |
|     | B     | 0.6 ± 0.51  | 2.20 ± 1.63**  | 3.82 ± 2.00**  |
|     | C     | 0.7 ± 0.48  | 1.69 ± 1.11*** | 2.50 ± 1.98*** |

Note: The challenge strain isolation from the internal organs at 7th and 14th day post-challenge. Asterisks indicate the statistically significant difference between control and immunized groups (** P ≤ 0.05, *** P ≤ 0.01, **** P ≤ 0.001).

*Day post-challenge.

**Groups:** A: Non-immunized control; B: Immunized with 10⁶ S. Typhimurium LTB-ghost cells; C: Immunized with 10⁹ S. Typhimurium LTB-ghost combined with Montanide adjuvant.

**Challenge strain count:** log₁₀ CFU per gram of tissue.

**Statistically significant difference among the immunized groups B and C.**
degradation, and elimination by the host. We, therefore, included the Montanide™ ISA 70 VG adjuvant in the S. Typhimurium-LTB ghost vaccine preparation to enhance the immunogenicity of the ghost antigens for effective protection against the virulent S. Typhimurium challenge infection.

The sequential monitoring of plasma and intestinal antibodies revealed that the prime-boost immunizations with S. Typhimurium-LTB ghost induced a significant rise in plasma IgG and intestinal sIgA concentrations. The combination of ghost cells with Montanide™ ISA 70 VG adjuvant further enhanced the induction of antigen-specific antibody titers. Systemic antibodies are essential for targeting Salmonella residing in the extracellular space and are involved in the opsonization of bacteria, thereby, enhancing receptor-mediated uptake by macrophages (Dougan et al. 2011). As S. Typhimurium primarily targets the intestinal tract, mucosal immunity with an IgA-dominated antibody profile is more likely to be involved in defense against micro-organisms (Chappell et al. 2009). The anti-Salmonella antibodies in the intestinal mucus may restrict mucosal colonization of the virulent challenge strain and subsequent invasion of the bacteria to some extent (Pasetti et al. 2011). Group C chickens showed higher induction of antibody response, possibly due to the immunity-enhancing effect of Montanide™ ISA 70VG adjuvant.

As S. Typhimurium is an intracellular pathogen, the magnitude of vaccine antigen-induced cellular immune response is associated with clearance of the virulent organism (Withanage et al. 2005; Chappell et al. 2009). Measurement of T-cell subsets in the spleen was undertaken because of the reported association of CD4+ helper T-cells and CD8+ cytotoxic T-cells with the induction of acquired resistance to S. Typhimurium (Mittrucker & Kaufmann 2000; Withanage et al. 2005). We observed a significant increase in CD4+ and CD8+ T-cell populations one week after prime vaccination in groups B and C, which may correspond with increased activation of the cell-mediated immune system in response to vaccination with S. Typhimurium-LTB ghost antigen. CD4+ T-cells play a key role in resolution of S. Typhimurium infections by producing or inducing macrophage-activating cytokines such as IFN-γ and TNF-α (McSorley et al. 2000). CD4+ T-cells play a vital role in stimulating the B-cells for the production of pathogen-specific antibody via clonal propagation (Mittrucker & Kaufmann 2000). Cytotoxic CD8+ T-cells are involved in lysis of the Salmonella infected cells, thereby, making bacteria available for phagocytosis (Stenger et al. 1998). The Th1-type cytokines, IFN-γ and IL-2, are crucial for host resistance to Salmonella infection (Karem et al. 1996). In the present study, IFN-γ and IL-2 exhibited a clear response to in vitro antigenic stimulation of PBMCs in vaccinated chickens. The induction of high mRNA levels of IFN-γ and IL-2 in the in vitro S. Typhimurium antigen-stimulated PBMCs cultures from vaccinated chickens suggests that immunization with the S. Typhimurium-LTB ghost alone or in combination with Montanide ISA 70 VG adjuvant induced a Th1-type immune response in the immunized host. IFN-γ-mediated T-cell responses play an important role in protection against Salmonella infection in avian hosts, particularly in the clearance of the systemic stages of infection (Karem et al. 1996). IFN-γ likely functions in host defense against Salmonella in the activation of macrophages by inducing NO production (Eckmann et al. 1996) and major histocompatibility complex (MHC) class II expression (Kaspers et al. 1994).

BG-based vaccines efficiently confer protection against Gram-negative bacterial infections (Chaudhari et al. 2012). In this study, we assessed the ability of an intramuscular administration of S. Typhimurium-LTB ghost with Montanide™ ISA 70 VG adjuvant or ghost cells alone to induce immune protection in chickens against a virulent S. Typhimurium infection. The degree of protection afforded by vaccine candidates against a virulent challenge was judged by the pathogen colonization load in the liver, spleen, and caecal contents of the infected birds. Unvaccinated chickens showed a high degree of S. Typhimurium colonization throughout the challenge study, while chickens vaccinated either with the ghost plus Montanide™ ISA 70 VG adjuvant or the ghost alone showed a statistically significant reduction in virulent S. Typhimurium count, although different degrees between groups were noted. A statistically lower challenge strain count was recovered from the spleen and caecal contents of chickens vaccinated with the ghost plus Montanide ISA™ 70 VG adjuvant compared to those from chickens vaccinated with ghost alone. This might be because the degree of protection appears to be associated with high levels of antigen-specific antibodies and cellular responses (Withanage et al. 2005). These findings suggest that S. Typhimurium-LTB ghost plus Montanide™ ISA 70 VG adjuvant intramuscularly administered to chickens is able to increase the resistance of birds, significantly protecting them from the virulent S. Typhimurium infection.

5. Conclusions
Prime-booster immunization regimen tested in this study is capable of inducing humoral and cell-mediated immune responses and can afford protection against S. Typhimurium challenge by enabling a remarkable reduction of bacterial colonization in the systemic tissues. The results from this study suggest that vaccination of chickens with the S. Typhimurium-LTB ghost vaccine with or without combination with Montanide™ ISA 70VG adjuvant may induce protective immunity against avian salmonellosis.
Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Aucouturier J, Ascarateil S, Dupuis L. 2006. The use of oil adjuvants in therapeutic vaccines. Vaccine. 245:S2/44—S2/45.

Barrow PA. 2007. Salmonella infections: immune and non-immune protection with vaccines. Avian Pathol. 36:1—13.

Chappell L, Kaiser P, Barrow P, Jones MA, Johnston C, Wigley P. 2009. The immunobiology of avian systemic salmonellosis. Vet Immunol Immunopathol. 128:53—59.

Chaudhari AA, Jawale CV, Kim SW, Lee JH. 2012. Construction of a Salmonella Gallinarum ghost as a novel inactivated vaccine candidate and its protective efficacy against fowl typhoid in chickens. Vet Res. 43:44.

Cheong HJ, Lee YJ, Hwang IS, Kee SY, Cheong HW, Song JY, Kim JM, Park YH, Jung JH, Kim WJ. 2007. Characteristics of non-typhoidal Salmonella isolates from human and broiler-chickens in southwestern Seoul, Korea. J Korean Med Sci. 22:773—778.

Desin TS, Koster W, Potter AA. 2013. Salmonella vaccines in poultry: past, present and future. Expert Rev Vaccines. 12:87—96.

Dougan G, John V, Palmer S, Mastroeni P. 2011. Immunity to salmonellosis. Immunol Rev. 240:196—210.

Dupuis L, Ascarateil S, Aucouturier J, Ganne V. 2006. SEPPIC adjuvants. J Control Release. 85:171—181.

Eckmann L, Frier J, Kagnoff MF. 1996. Genetically resistant (lty) and susceptible (ltyb) congenic mouse strains show similar cytokine responses following infection with Salmonella Dublin. J Immunol. 157:2894—2900.

Eko FO, Witte A, Huter V, Kuen B, Fischel-Ladani S, Haslberger A, Katinger A, Hensel A, Szostak MP, Resch S, et al. 1999. New strategies for combination vaccines based on the extended recombinant bacterial ghost system. Vaccine. 17:1643—1649.

Ekong EE, Okenu DN, Mania-Pramanik J, He Q, Ijetsejume JU, Ananaba GA, Lyn D, Black C, Eko FO. 2009. A Vibrio cholerae ghost based subunit vaccine induces cross-protective chlamydial immunity that is enhanced by CTA2B, the non-toxic derivative of cholera toxin. FEMS Immunol Med Microbiol. 55:280—291.

Freytag LC, Clements JD. 2005. Mucosal adjuvants. Vaccine. 23:1804—1813.

Gordon MA. 2011. Invasive nontyphoidal Salmonella disease: epidemiology, pathogenesis and diagnosis. Curr Opin Infect Dis. 24:484—489.

Jalava K, Hensel A, Szostak M, Resch S, Lubitz W. 2002. Bacterial ghosts as vaccine candidates for veterinary applications. J Control Release. 85:17—25.

Jawale CV, Lee JH. 2014a. Characterization of a Salmonella Typhimurium ghost carrying an adjuvant protein as a vaccine candidate for the protection of chickens against virulent challenge. Avian Pathol. 43:506—513.

Jawale CV, Lee JH. 2014b. Salmonella enterica serovar enteritidis ghosts carrying the Escherichia coli heat-labile Enterotoxin B subunit are capable of inducing enhanced protective immune responses. Clin Vaccine Immunol. 21:799—807.

Jawale CV, Lee JH. 2014c. Characterization of adaptive immune responses induced by a new genetically inactivated Salmonella Enteritidis vaccine. Comp Immunol Microbiol Infect Dis. 37:159—167.

Kang HY, Srinivasan J, Curtiss R III. 2002. Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated Salmonella enterica Serovar typhimurium vaccine. Infect Immun. 70:1739—1749.

Karem KL, Kanangat S, Rouse BT. 1996. Cytokine expression in the gut associated lymphoid tissue after oral administration of attenuated Salmonella vaccine strains. Vaccine. 14:1495—1502.

Kaspers B, Lillehoj HS, Jenkins MC, Pharr GT. 1994. Chicken interferon mediated induction of major histocompatibility complex class II antigens on peripheral blood monocytes. Vet Immunol Immunopathol. 44:71—84.

Kim JG, Kim DA, Chung HJ, Kim TG, Kim JM, Lee KR, Park SM, Yang MS, Kim DH. 2009. Expression of functional pentameric heat-labile enterotoxin B subunit of Escherichia coli in Saccharomyces cerevisiae. J Microbiol Biotechnol. 19:502—510.

Matsuda K, Chaudhari AA, Lee JH. 2011. Evaluation of safety and protection efficacy on cpxR and lon deleted mutant of Salmonella Gallinarum as a live vaccine candidate for fowl typhoid. Vaccine. 29:668—674.

McSorley SJ, Cookson BT, Jenkins MK. 2000. Characterization of CD4+ T cell responses during natural infection with Salmonella Typhimurium. J Immunol. 164:986—993.

Mittrucker HW, Kaufmann SHE. 2000. Immune response to infection with Salmonella Typhimurium in mice. J Leukoc Biol. 67:457—463.

Nandre RM, Chaudhari AA, Matsuda K, Lee JH. 2011. Immu nogenicity of a Salmonella Enteritidis mutant as vaccine candidate and its protective efficacy against Salmonellosis in chickens. Vet Immunol Immunopathol. 144:299—311.

Pasetti MF, Simon JK, Sztein MB, Levine MM. 2011. Immunology of gut mucosal vaccines. Immunol Rev. 239:125—148.

Stenger S, Hansson DA, Teitelbaum R, Dewan P, Niazi KR. 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. Science. 282:121—125.

Tellez G, Pixley C, Wolfenden RE, Layton SL, Hargis BM. 2012. Probiotics/direct fed microbials for Salmonella control in poultry. Food Res Int. 45:628—633.

Withanage GS, Wigley P, Kaiser P, Mastroeni P, Brooks H, Powers C, Beal R, Barrow P, Maskell D, McCon nell I. 2005. Cytokine and chemokine responses associated with clearance of a primary Salmonella enterica serovar Typhimurium infection in the chicken and in protective immunity to rechallenge. Infect Immun. 73:5173—5182.

Withi A, Wanner G, Blasi U, Halfmann G, Szostak M, Lubitz W. 1990. Endogenous transmembrane tunnel formation mediated by phiX174 lysis protein E. J Bacteriol. 172:4109—4114.

Zepp F. 2010. Principles of vaccine design—lessons from nature. Vaccine. 28:C14—C24.