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Salmonella Enteritidis ghost vaccine carrying the hemagglutinin globular head (HA1) domain from H1N1 virus protects against salmonellosis and influenza in chickens

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ABSTRACT

This study evaluated the attenuated *Salmonella* Enteritidis (SE) ghost strain JOL2114 (Δ*lon* Δ*cpxR* Δ*asd*), which displays on the bacterial surface the H1N1 hemagglutinin globular head portion (HA1; amino acid residues 63-286) on the bacterial surface for protective efficacy against *Salmonella* and H1N1 challenge in the chicken model, as the birds are the predominant reservoirs for both diseases. The ghost system enhanced the lysis process by converging two lysis processes found in bacteriophages: bacteriophage PhiX174 lysis gene *E* and holin-endolysin genes found in bacteriophage λ, complemented with accessory lysis-related proteins *Rz/Rz1*. The present lysis machinery resulted in complete lysis of host-attenuated SE strains in about 24 hrs of incubation under a non-permissible temperature of 42 °C in the absence of L-arabinose, an antisense inducer that blocks lysis gene expression during the growth phase. SE ghost JOL2114 surface display of HA1 was confirmed by Western blot analysis resulting in an immune-reactive band of 31 kDa in size. Chicken immunization via intramuscular and oral routes yielded both SE and HA1 antigen-specific immune responses. Protective humoral and cell-mediated immune responses were effectively elicited against both *Salmonella* and influenza challenge. This efficient strategy of ghost generation employs a dual system of phage lysis for biological generation of SE ghosts that preserves the surface antigenic architecture, offering a rapid and effective way to generate vaccines that could be deployed in urgent circumstances to protect against both *Salmonella* and influenza infection.

1. Introduction

Salmonella enterica subsp. *enterica* serovar Enteritidis (SE), a food-borne zoonotic pathogen, has been responsible for a substantial public health burden over the last two decades, mostly via egg consumption [1]. Although SE does not usually induce discernible systemic symptoms in chickens, the pathogen can easily enter the human food chain during the post-harvest processing. SE can invade beyond the gastrointestinal tract of chickens to colonize internal organs such as the liver, spleen, ovaries and oviducts, which are critical points of carcass contamination [2]. Further, the consumption of raw or undercooked shell eggs has contributed to the striking increase in SE food poisoning. The high prevalence of SE in commercial egg flocks has been associated with the incidence of human SE infection, since the strains isolated from the outbreaks are frequently related to poultry isolates [2]. Given that eggs can be contaminated via trans-ovarian (vertical) and trans-shell (horizontal) pathways [3], prevention strategies such as vaccination, rather than elimination strategies, are needed to directly reduce pathogen loads in laying hens.

Several attempts have been made to develop safe and effective vaccine candidates against SE infection [4–6]. Further, commercial vaccines are currently available based on live attenuated SE strains such as Layer AviPro® *Salmonella* VacE (Lohmann Animal Health, Cuxhaven, Germany) and Salmovac SE® (IDT Biologika, GmbH, Germany), which have been approved for poultry immunization. The vaccines have also been proved to be efficacious in compliance with the European Pharmacopoeia Monograph (EP) guidelines [7]. However, the live attenuated strain still has safety issues and poses risks from the vaccine strain circulating within the immunized flock for a longer period [8]. Thus, a novel vaccine platform providing both safety and immunogenicity is still required.

Bacterial ghosts (BGs, non-living bacteria) are novel, genetically inactivated vaccine platforms produced by expression of the lethal lysis gene *E* of PhiX 174 lytic bacteriophages [9,10]. The activation of lysis gene *E* mediates the formation of a transmembrane tunnel on the bacterial surface where all cytoplasmic contents are

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released due to osmotic pressure [11,12]. The empty bacterial envelopes possess immuno-stimulatory antigenic properties similar to their living counterparts that can elicit humoral and cell-mediated immunity. Thus, this novel BG vaccine system has been widely used and can be applied as a heterologous antigen delivery vector endowed with intrinsic adjuvant properties [13]. Furthermore, BGs can facilitate adaptive immune responses via pathways driving differentiation and maturation of dendritic cells (DCs), since BGs preserve immune-stimulatory surface components such as pathogen-associated molecular patterns (PAMPs), which are recognized by host pattern recognition receptors (PRRs) on DCs [14].

In the present study, we constructed a genetically inactivated SE ghost generated by concomitant expression of lysis gene *E* and an additional lysis gene cassette encoding holin and endolysin that can simultaneously express the globular head domain of the hemagglutinin (HA1) protein of A/Puerto Rico/8/1934(H1N1) (PR8) influenza virus as a candidate vaccine against both SE and H1N1 influenza virus infection. In this approach, the constructs employed a recently developed lysis vector, pJHL420, that harbors the lysis gene *E* and holin-endolysin system. Holin and endolysin, encoded by the *R* and *S* gene, respectively, are instrumental for programmed host bacterial cell lysis by double-stranded DNA λ phages. Recent research has revealed that incorporation of the *S* and *R* gene cassette into the gene *E* lysis system significantly increases the lytic capacity of *Salmonella*, along with immunogenicity induced by the resultant *Salmonella* ghost [13]. Furthermore, this strategy embodies the use of a novel SE ghost as a foreign antigen delivery vehicle, demonstrating its potential as a bivalent vaccine candidate. The HA1 gene was cloned into a foreign antigen delivery site of the lysis vector pJHL420 carrying the aspartate β-semialdehyde dehydrogenase (*asd*) gene [15]. The resultant plasmid pJHL420-HA1 was introduced into the Δ*lon*Δ*cpxR*Δ*asd* SE mutant, JOL1087. The *lon* and *cpxR* genes are associated with pathogenic trait of *Salmonella*. The *lon* gene is responsible for expression of invasion gene encoded on *Salmonella* pathogenicity island 1 (SPI-1), and the *cpxR* genes regulates type III secretion, motility and adherence [16]. Thus, the use of the attenuated mutant as a vaccine candidate can improve safety profile, and concurrently preserve its surface antigenic trait. The *asd* gene-based balanced lethal vector-host system was used to maintain the stability of the recombinant vector in the mutant strain. The co-expression of lysis gene cassettes was stringently regulated by a convergent promoter system composed of the arabinose-inducible araBAD promoter and the λpR promoter with a thermos-labile repressor, resulting in the programmed lysis of the SE mutant and expression of the target antigen. The SE ghost strain was simultaneously applied as a homologous vaccine candidate against *Salmonella* infection and a novel delivery platform for the heterologous target antigen. We further evaluated the immunogenicity and protective efficacy of the SE-BG vaccine candidate expressing the HA1 protein.

2. Material and methods

2.1. Bacterial strain, plasmids and growth media

Bacterial and viral strains and plasmids used in this study are shown in Table 1. Influenza A/Puerto Rico/8/34 (PR8) H1N1 virus [GenBank accession No. ADX99484.1] was used as a challenge virus, and passaged in embryonated chicken eggs. The 50% egg infective dose (EID50) of H1N1 was determined using the Reed and Muench method. The Δ*asd* Δ*lon* Δ*cpxR* SE mutant strains were cultured at 37 °C in either Luria-Bertani (LB) broth or LB agar supplemented with 50 μg/ml of diaminopimelic acid (DAP) (Sigma-Aldrich, St. Louis, MO).

Table 1
Bacterial strains and plasmids used for this study.

Strain/plasmids	Description	References
E. coli		
DH5α	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	Lab stock
BL21(DE3)	139(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^r)*end*A1 *nup*G F$^-$*ompT hsdSB* (rB$^-$mB$^-$)*dcm gal*λ(DE3) pLysS Cmr	Lab stock
χ 6212	F-λ-φ80 Δ(lacZYA-argF) endA1 recA1 hadR17 deoR thi-1 glnV44 gyrA96 relA1 ΔasdA4	Lab stock
Salmonella Enteritidis		
JOL1182	Wild type isolate from chicken, challenge strain	Lab stock
JOL1087	Δ*lon*Δ*cpxR*Δ*asd*, used as base vaccine strain	Lab stock
JOL2110	JOL1087 harboring pJHL420, a vector control	This study
JOL2114	JOL1087 harboring pJHL420-HA1	This study
Plasmids		
pJHL420	*asd*$^+$ vector, pBR ori plasmid harboring cI857/λP$_R$ promoter, araC P$_{araBAD}$, phiX174 lysis gene E, the R ghost cassette composed of S, R and R1/Rz genes,	[28]
pJHL420-HA1	pJHL420 harboring HA1 gene originated from A/Puerto Rico/8/1934(H1N1) (PR8) influenza virus	This study

2.2. Construction

An attenuated auxotrophic mutant strain of *S.* Enteritidis, JOL1087, was used as a delivery vehicle for the HA1 protein of the influenza A virus. JOL1087 was constructed by deleting the *lon*, *cpxR*, and *asd* genes from wild-type SE JOL394, as previously described. The *asd*$^+$ ghost plasmid pJHL420 carries a lysis gene cassette containing bacteriophage ϕX174 gene-*E* and holin-endolysin, a pBR origin, a multiple cloning site (MCS), and a C-terminal His-tag. The computationally codon optimized synthetic HA1 gene of the H1N1 subtype of influenza A virus (amino acid residues 63–286) was chemically synthesized (Bioneer). The gene fragment was cloned *in-frame* downstream of the transmembrane domains (TMD) of the outer membrane protein A (*ompA*) signal sequence (SS) of the lysis vector pJHL420. The resultant plasmid was subsequently introduced into JOL1087 by electroporation and designated JOL2114 for use as a vaccine candidate. The Δ*asd* Δ*lon* Δ*cpxR* SE strain carrying pJHL420, JOL2110 was used as a vector control. For the preparation of an inactivated ghost vaccine candidate, the JOL2114 strain was grown in LB broth supplemented with 0.2% L-arabinose at 28 °C until the culture reached the log phase. The culture was centrifuged and subsequently re-suspended in LB-broth, resulting in the removal of the remaining arabinose. To activate the lysis gene cassette in the ghost vector, the culture was incubated for ~48 h under agitation (200 rpm) at 42 °C. To assess the lysis efficiency of JOL2114 ghosts, 100 μl of the inoculations were collected at the end of the lysis process and spread onto LB plates. After 24 hr of incubation, cell viability was determined by measuring the number of colony-forming units (CFU).

The synthetic HA1 gene fragment was also inserted into the protein expression vector pET28a (+) plasmid, and subsequently transformed into BL21 cells. The recombinant, N-terminally 6 × His-tagged HA1 protein, purified as previously described, was validated by SDS-PAGE analysis and quantified using the Bradford assay. *In vitro* expression of HA1 in the ghost strains was confirmed by Western immunoblotting following the previously published protocol. Further, the morphological changes induced

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by lysis gene expression were visualized via scanning electron microscopy (SEM) after 24 h activation of the lysis genes following the previously published protocol.

2.3. Animal experiments

All animal experimentation was approved by the Chonbuk National University Animal Ethics Committee (CBNU2015-00085) and was carried out according to guidelines in the Korean Council on Animal Care and Korean Animal Protection Law, 2007; Article 13 (Experiments with Animals). Female, *Salmonella*-free 1-day-old layer chickens (Brown Nick) (n = 40) provided antibiotic-free food and water *ad libitum* were randomly divided into four groups. The chickens in Group vac_IM, and group vac_PO were immunized with JOL2114 ghost via an intramuscular (IM) and an oral route, respectively. The vaccination with a vector control (i.e. JOL2110 ghost cells) was also carried out in the chickens of a group VC via an IM route. The chickens in group NC received only 100 μl of sterile PBS and served as a negative control. The chickens were primed at 1 week of age and then boosted at 3 weeks of age. Serum and intestinal lavage samples were collected at a two-week interval as described elsewhere to examine the titers of antibodies specific to the target protein.

2.4. Indirect enzyme linked immunosorbent assay (ELISA)

Titers of total immunoglobulin (Ig) G and secretory IgA were assessed in the immunized birds and controls using indirect enzyme-linked immunosorbent assays (ELISA) according to the previously described method. Either purified HA1 antigen (0.5 μg/ml) or SE outer-membrane protein (5 μg/ml) were used to coat the ELISA plates to determine the generation of the antigen-specific antibody. The outer-membrane protein (OMP) was extracted from wild-type SE JOL1182, according to the previously reported method [17]. The end-point titers of serum IgG and secretory IgA antibodies were calculated by the optical density (OD) values at 470 nm.

2.5. T-cell responses elicited by the Salmonella ghost harboring the new ghost plasmid.

The proliferative capability of peripheral blood mononuclear cells (PBMCs) following *in vitro* vaccine stimulation was assessed by performing 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assays. Chicken PBMCs were isolated from 2 mL of blood samples each from five chickens in groups vac_IM and NC at week 2 post-immunization (PI) by density gradient centrifugation using Histopaque-1077® solution (Sigma, St. Louis, MO) following the manufacturer's instructions. The isolated PBMCs were incubated overnight in RPMI containing 10% FBS, 5% chicken serum at 37°C, 5% CO_2. Suspensions of 1×10^6 single cells were cultured in triplicate with JOL2114 ghost cells at a multiplicity of infection (MOI) of 10 at 37 °C in a 5% CO_2 incubator for 48 hr. Following *in vitro* stimulation, the MTT lymphocyte proliferative assay (LPA) and Real time PCR assay (qRT-PCR) for immunomodulatory cytokine gene expression was performed as previously published [18]. Changes in T-cell subpopulations were assessed in the primed PBMCs isolated from the birds in groups vac_IM, vac_PO and NC at week 2 PI using fluorescence-activated cell sorting (FACS) using a previously reported protocol. Briefly, the cells were stained with anti-CD3-PE, anti-CD4-FITC and anti-CD8-PerCP-Vio700 antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany), and the proportion of each T-cell subpopulation was determined using a MACSQuant® analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.6. Dendritic cell analysis

Chicken bone marrow-derived dendritic cells (chBMDCs) were obtained from the femurs and tibias of 6-week-old layer chickens (Brown Nick) as previously described [19]. Briefly, the aseptically sampled bone marrow was suspended in PBS and layered on Histopaque-111 (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation at 400*g* for 30 min, the total bone marrow-derived mononuclear cells isolated from the interface were incubated in RPMI supplemented with 50 ng/mL recombinant chicken granulocyte–macrophage–colony-stimulating factor (GM-CSF; Kingfisher Biotech Inc, Saint Paul, MN, USA), 25 ng/mL recombinant chicken IL-4 (Kingfisher Biotech), 10% chicken serum (Sigma-Aldrich), penicillin (10 units/ml) and streptomycin (10 μg/ml) at 41 °C with 5% CO_2. On day 7 of culture, the differentiated chBMDCs loosely adhered to the plate were collected, resuspended in RPMI, and then seeded into six-well cell culture plates. Subsequently, for infection with the vaccine candidate strains, the cells were treated with either JOL2114 ghost cells at 10 MOI for 24hr. Cells treated with lipopolysaccharides (LPS, 500 ng/ml, serotype O127:B8, Sigma-Aldrich, St. Louis, MO) and PBS were used as a positive and negative control, respectively. Expression of TLRs and immunomodulatory cytokines was evaluated in the pulsed chBMDCs using qRT-PCR with primer pairs obtained from a previous study [20,21].

2.7. Challenge determination of protective efficacy

Avian influenza A/Puerto Rico/8/34 (PR8) H1N1 virus was applied as a challenge virus. Five birds in each group were intranasally challenged with a sub-lethal dose of H1N1 virus (1×10^3 50% egg infective dose, EID_{50}) at week 6 PI. The chickens immunized with SE vector control (group_VC) were included in this challenge study. Cloacal swabs were taken in the infected birds on days 1, 3, 5, 7, 9 and 11 post-challenge (DPC). The shedding of challenge virus was determined by the virus copy number at the RNA level using qRT-PCR analysis for the viral HA gene as previously described [22]. Simultaneously, the remaining birds (n = 5) in each group were orally challenged with 1×10^9 of wild type SE strain JOL1182 at week 5 PI. The birds were euthanized 7 days post-challenge. To determine bacterial loads from feces, the samples were weighed and homogenized in 2 mL buffered peptone water. Each homogenate sample was plated on BGA and incubated at 37°C overnight. The number of bacterial colonies was expressed as the mean $\log_{10}$ CFU/g of samples.

2.8. Statistical analysis

Data are presented as mean ± standard deviation (s.d.). A paired *t*-test was used to evaluate significant differences in immune responses between the immunized and non-immunized groups. Statistical differences were considered significant when P values were <0.05.

3. Results

3.1. Construction of the SE strain inactivated by dual-lysis genes expressing HA1

The ghost plasmid pJHL420::*HA1* used in the present study was comprised of the bacteriophage PhiX174 lysis gene *E* and the *R* lysis gene cassette encoding lambda phage-derived holin-endolysin proteins. The *R* lysis gene cassette was located between the 5′ upstream anti-sense P_{araBAD} promoter and downstream to the 3′ *E* gene and λpR-CI857 promoter in the pJHL420 plasmid construct. Expression of the *R* gene occurred simultaneously with the *E*

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gene by the thermal induction mechanism in the absence of arabinose. The heterologous antigen HA1 of H1N1 was cloned into the plasmid infusion with an *Escherichia coli* OmpA secretion signal for efficient surface display of the antigen. The present ghost strain JOL2114 was grown to log phase at a permissible temperature of 28 °C in the presence of 0.2% L-arabinose to prevent the leaky expression of gene *E* and the holin-endolysin gene cassette. To generate gene *E* and holin-endolysin-induced *Salmonella* ghosts, cells were collected and washed with medium to remove residual L-arabinose. The cells were then re-grown at 42 °C for 48 hr to allow expression of lysis genes by eliminating cI857 repression of the genes. The efficacy of ghost generation was assessed by evaluating live cell numbers at the end of the induction period of 48 hrs, resulting in >99% reduction in viable cells (data not shown). To further confirm the completeness of lysis, SEM was conducted (Fig. 1I). The images demonstrated complete elimination of the bacterial cytoplasm with the cell envelope intact. Heterologous HA1 antigen expression was also regulated through a thermo-inducible λ_{PR} promoter; hence, co-expression of HA1 antigen occurred along with the lysis gene. Expression of the HA1 surface-displayed antigen was confirmed by Western blot analysis that resulted in a ~31-kDa immune-reactive band (Fig. 1II). The *asd*-complemented pJHL420 plasmid was stable in *Salmonella* due to a balanced lethal host-vector system mediated by the *asd* gene.

3.2. Production of antibodies specific to SE OMP or HA1 in immunized chickens

Immunization of chickens with JOL2114 bacterial ghosts was expected to generate SE- and HA1-specific antibodies in the immunized hosts. Antibody responses were measured in blood sera collected from each experimental group. To assess the SE-specific antibody response, chicken IgG concentrations were quantified and compared between the group vac_IM and NC. Levels of SE-specific antibody in group vac_IM chickens were significantly higher than in the negative control, with a peak response at the fourth week post-primary immunization (Fig. 2I). The HA1-specific antibody response was compared among the group vac_IM, group vac_PO, and the group NC. HA1-specific IgG antibody development was only observed in chickens immunized with JOL2114 via either an IM or oral route. Immune responses were higher with the IM route compared to the oral route, demonstrating an advantage of using the IM route for ghost delivery. Here too, the peak response was observed in the fourth week post-immunization (Fig. 2II).

3.3. T-cell-associated immune responses induced by immunization

To assess cell-mediated immune responses, lymphocyte proliferation was evaluated in PBMCs collected 2 weeks post-immunization from immunized (group vac_IM) and non-immunized chickens (group NC) (Fig. 3). When the cells were re-exposed to purified HA1 antigen, significantly higher cell proliferation was observed by MTT assay in the immunized chickens (Fig. 3). This finding demonstrates that the SE surface-displayed HA1 antigen effectively elicited CMI responses in immunized chickens. To further assess T-cell changes, $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cell populations were compared between the non-immunized group (group NC), IM immunized group (group vac_IM) and oral immunized group (group vac_PO). Cells were re-stimulated with

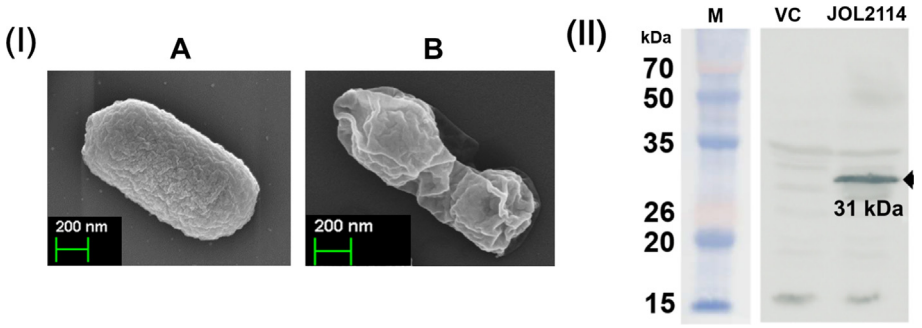

Fig. 1. Characterization of JOL2114, *S.* Enteritidis ghost cells expressing HA1 gene. **(I)** Electron microscopy observation on morphology of the ghost cells following the activation of the lysis gene cassette. Arrowheads indicate the transmembrane lysis tunnels. A, Intact *S.* Enteritidis before lysis; B, JOL2114 ghost cells after 24 hr of lysis. **(II)** HA1 protein expressed in JOL2114 validated by western blot analysis. The arrow shows the band at ~ 31 kDa, the expected size of the target protein fused with OmpA. Lane M, size marker; lane VC, a pellet of JOL2110 as a vector control; lane JOL2114, a pellet of JOL2114.

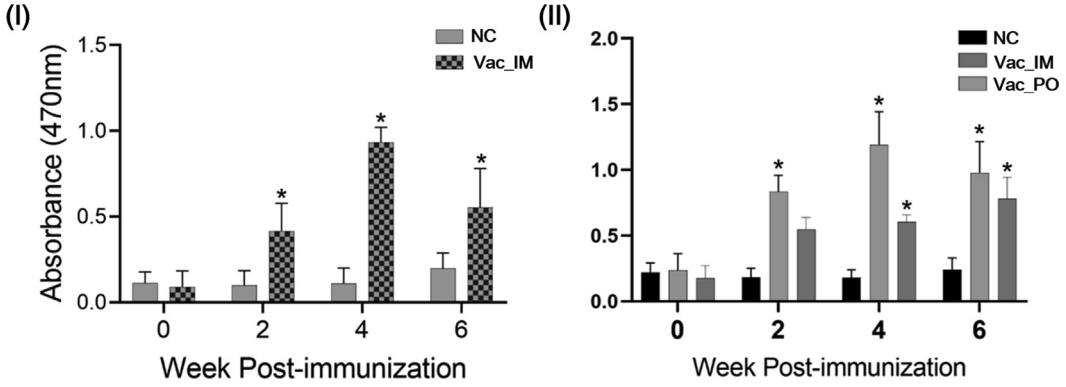

Fig. 2. Humoral immune responses specific to either HA1 or *S.* Enteritidis (SE) elicited by immunization with JOL2114 in chickens. Titers of serum IgG against **(I)** SE OMP or **(II)** HA1-antigen by ELISA in immunized chickens. Group NC, negative controls; group Vac_IM, chickens immunized with JOL2114 ghost cells via an intramuscular route; group Vac_PO, chickens immunized with JOL2114 ghost cells via an oral route. Bars indicate the mean of all chickens (n = 10) in each group and vertical lines show the standard deviation (s.d.). PI: post-immunization, $^*P < 0.05$, (vs. control group NC).

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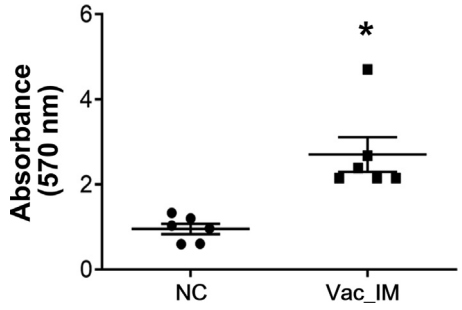

Fig. 3. Lymphocyte proliferative responses against JOL2114 ghost cells. An MTT assay was performed in PBMCs isolated from non-immunized (group NC, n = 5) and immunized (group vac_IM, n = 5) chickens at week 2 PI. The bars indicated the mean absorbance values in each group. Error bars show s.d. *$P < 0.05$ (vs. control group NC).

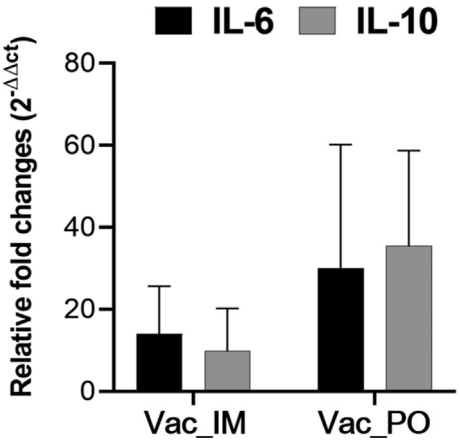

Fig. 5. The cytokine mRNA transcript level in *in vitro* stimulated PBMCs isolated from chickens in the negative control group (NC) and immunized groups via an IM route (vac_IM) or oral route (vac_PO) as measured by qRT-PCR. The mRNA transcript level of cytokines was assessed with gene specific primers. Results are expressed as relative fold change ($2^{-\ \Delta\Delta Ct}$) in mRNA transcription levels of stimulated PBMC from immunized group compared to the negative control group. Gene expressions were normalized to GAPDH. Each fold change value represents the mean ± s.d. of five individual values.

purified HA1 antigen and surface-expressed $CD4^+$ and $CD8^+$ markers were assessed by FACS analysis. Immunization of chickens with JOL2114 ghost significantly induced larger $CD4^+$ and $CD8^+$ T-cell populations compared to the non-immunized control group. However, no significant difference between the IM and oral route of immunization was observed (Fig. 4I and II).

3.4. TLRs and immunomodulatory cytokine expression in the primed ch-PBMCs

The expression of immunomodulatory cytokine responses was evaluated between the IM and oral route of immunized chicken groups. Th-2 type cytokines IL-6 and IL-10 were evaluated in PBMCs (Fig. 5). In addition, chicken dendritic cells were stimulated and tested for TLR-4 and TLR-5 responses in bone marrow-derived dendritic cells collected from the birds immunized with bacterial ghosts. Results revealed the engagement of immunomodulatory cytokine responses in PBMCs and particular engagement of TLR-4 and TLR-5 activation in dendritic cells (Fig. 6).

3.5. Challenge against SE wild type

To investigate the protective efficacy of ghost vaccination against wild type SE challenge, chicken groups immunized either via IM or oral route were challenged at 6 weeks post-immunization with the wild type SE strain. The efficacy of protection was assessed by quantifying the fecal shedding of wild type SE in on days 1, 5 and 11 post-challenge. A significant reduction in fecal bacteria shedding was observed in both IM and oral JOL2114-immunized chickens compared to the non-immunized group (Fig. 7).

3.6. Challenge against H1N1 strain

Whether JOL2114 immunization led to protection against H1N1 virus injection was investigated by a challenge study with primed chickens 6 weeks after primary immunization. Viral shedding in feces was evaluated by determining the viral copy number using an RT-PCR based method. Immunization of chickens with JOL2114 bacterial ghosts via either an IM or oral route resulted

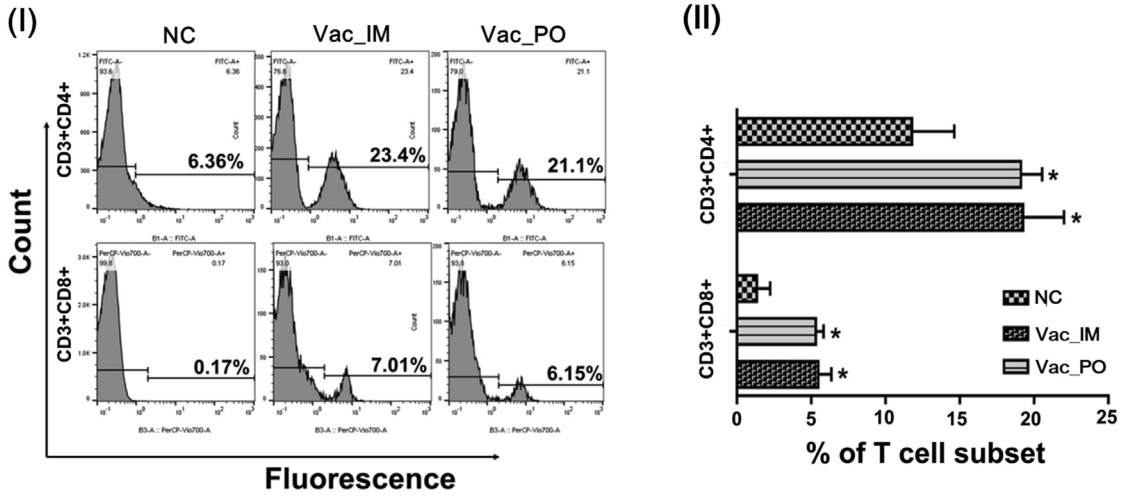

Fig. 4. Flow cytometric analysis of T lymphocyte populations. **(a)** Representative flow cytometry scatter dot plots for $CD3^+CD4^+$, $CD3^+CD8^+$ T cell populations isolated from chPBMC of non-immunized and the immunized birds. The subpopulations are expressed as a percentage of the gated cells. **(b)** $CD3^+CD4^+$, $CD3^+CD8^+$ T cell subsets in non-immunized and immunized birds. Group NC, negative controls (n = 5); group vac_IM, chickens immunized with JOL2114 ghost cells via an intramuscular route (n = 5); group vac_PO, chickens immunized with JOL2114 ghost cells via an oral route (n = 5). The data are presented as the mean ± s.d. *$P < 0.05$ when the values were compared with the negative control group.

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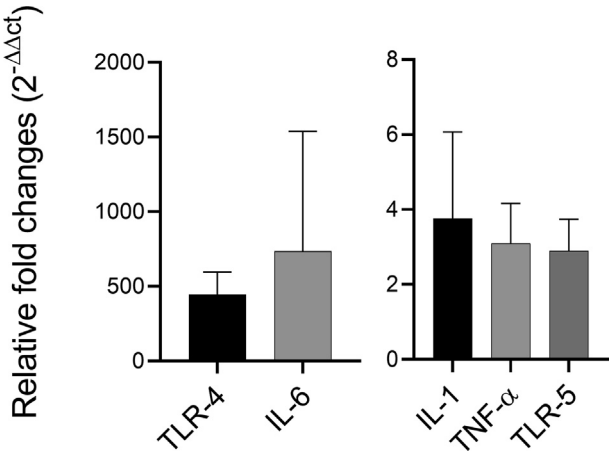

Fig. 6. Cytokine mRNA upregulated in DCs co-cultured with JOL2114 ghost cells. Relative fold changes were calculated based on the $2^{-\Delta\Delta CT}$ method. *$P < 0.05$.

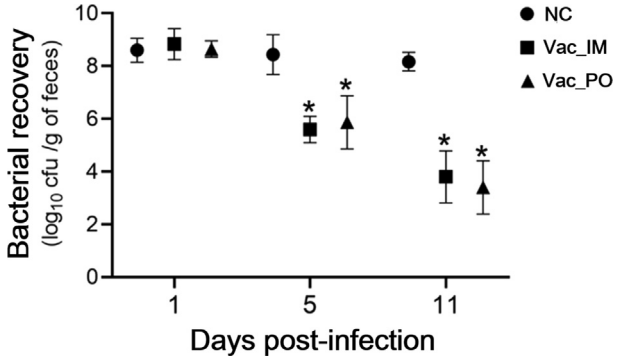

Fig. 7. Bacterial load in surviving chickens post-challenge. Chickens immunized with JOL2114 ghost cells or PBS were challenged at 6 weeks post-immunization with a virulent SE strain JOL1182, and bacterial recovery from feces was recorded. Group NC, negative controls; group vac_IM, chickens immunized with JOL2114 ghost cells via an IM route. Each data point represents mean ± s.d. of 5 chickens per group.

in a significant reduction in viral copy number compared to the non-immunized and vector control groups on days 9 and 11 post-infection (Fig. 8), indicating the potential protective efficacy of the vaccine constructs.

4. Discussion

The present study investigated the ability of a *Salmonella* Enteritidis-based influenza vaccine to elicit efficient antigen-specific immune responses and protection against a lethal challenge with H1N1 in the chicken model. One considerable obstacle in influenza vaccine development is the huge extent of genetic diversity among virus strains, leading to antigenic variations. Thus, the natural immunity developed against one vaccine strain can be obsolete against another strain, resulting in no protection against the heterologous strain [23]. Therefore, vaccine development is an annual process that requires huge investment and a long timeline for the generation of attenuated virus candidates and ultimately constructing a vaccine suitable against field strains [24]. As an alternative to conventional vaccine development programs, *Salmonella*-mediated live vaccines provide a highly innovative approach that can be rapidly deployed in a pandemic. This strategy utilizes *Salmonella*'s intrinsic ability to induce mucosal and cell-mediated immunity [25]. Further, bacterial ghosts offer a highly

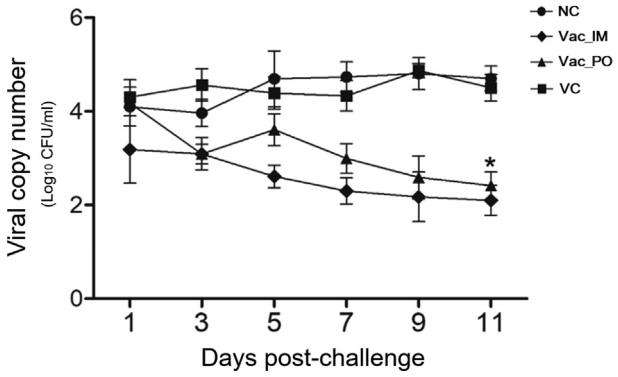

Fig. 8. Post sub-lethal challenge H1N1 virus copy number in feces of chickens. Group NC, negative controls; group vac_IM, chickens immunized with JOL2114 ghost cells via an IM route; group vac_PO, chickens immunized with JOL2114 ghost cells via an oral route, group VC, chickens immunized with vector control, JOL2110 ghost cells via an IM route; Feces were collected from each group (n = 5) at days 1, 3, 5, 7, 9 and 11 post H1N1 sub-lethal challenge, and viral titers were determined by the Reed and Muench method. The H1N1 viral copy number was also determined at the RNA level by qRT-PCR assay. The experiments were performed in duplicate and the results are present as mean ± s.d. *$P < 0.05$.

safe option due to their structure as non-viable cell envelopes that preserve mosaic antigenic display on the cell surface. To take advantage of these features, we sought to develop a novel *Salmonella* ghost vaccine displaying the globular head region of HA1 of the H1N1 influenza strain, which was prevalent during the 2009 outbreak [26]. Herein, we evaluated the potential of *Salmonella* Enteritidis ghosts expressing HA1 to elicit protective immunity against both salmonellosis and influenza challenge using chickens as the ideal model organism.

The protection provided by the globular head region of the influenza virus HA1 surface glycoprotein has been widely characterized in novel influenza vaccine platforms [27]. In the present study, the codon-optimized HA1 open reading frame (63-286 amino acid residues) was cloned into the ghost plasmid pJHL420 carrying holin-endolysin and phage PhiX174 lysis gene *E* for improved lysis efficacy [28–30]. Previous investigations have demonstrated incorporation of lethal genes relevant to bacteriophage lysis such the nuclease gene A encoded by *Staphylococcus* species [30]. In the present lysis mechanism, the holin-endolysin proteins were sequentially expressed along the lysis gene *E*. The holin and endolysin genes were in fusion with *Rz/Rz1* elements known to promote the degradation of the cell wall by making a physical connection between the inner and outer membranes of the cell envelope [31]. This efficient *Salmonella* lysis strategy promoted temperature-induced lysis of SE, resulting in a complete elimination of live bacteria in the growth medium in 24 hrs of growth at a non-permissible temperature (42 °C). The onset of lysis occurred as early as 6 hrs into temperature induction, as evidenced by OD_{600} reduction with time. More importantly, the generated bacterial ghosts were immunogenic in both oral and IM routes, delivering significant antigen-specific protective immune responses against both the SE and HA1 antigens. The present plasmid construct was transformed into the SE strain JOL1087, which was attenuated by deleting the two major virulence-related genes Δ*lon* Δ*cpxR* for a resultant SE strain highly defective in intracellular survival and prolonged existence in animal hosts.

The induction of bacterial lysis and expression of HA1 was a simultaneous process controlled by the temperature-sensitive λ_{PR} promoter. Expression of the HA1 antigen on the surface of bacterial ghosts was confirmed by Western blot analysis displaying an immune reactive band of ~31 kDa. To assess whether the SE ghost JOL2114 could generate protective immune responses against

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influenza challenge, we carried out an experiment using chickens as the model organism. This choice is particularly relevant, since these livestock birds are the dominant reservoirs for circulating influenza viruses within the human community [32]. Successful mitigation of circulating influenza in livestock birds may directly contribute to the reduced risk of human pandemic breakouts.

Salmonella Enteritidis bacterial ghost JOL2114-mediated antigen-specific humoral and cellular immune responses demonstrated a correlation with protective efficacy against the influenza challenge in a route-dependent manner. In the previous study [33], the ghost vaccine candidate elicited a sufficient humoral immune response containing high IgG titers when injected via an IM or SC route compared to those via an oral route. The IM route appears to be a better choice for ghost vaccination, probably due to enhanced antigen presentation compared to the oral route. The gastric environment encountered via the oral route is likely too harsh for inactive bacterial ghosts to survive [34]. Thus, we evaluated the vaccine efficacy against SE infection mainly in the chicken injected via an IM route. The efficacy against HA1, the heterologous antigen delivered by SE strain was assessed in chickens in both groups, vac_IM and vac_PO for the comparison study. Unlike other inactivated *Salmonella* vaccines, biologically prepared ghosts retain all relevant antigens found on live bacteria that can be recognized by antigen-presenting cells, enhancing both humoral and cellular immune responses [35]. The results of chicken immunization corroborated previous findings that ghost immunization via IM and oral routes elicits significant HA1 antigen-specific IgG responses compared to non-immunized controls. Furthermore, the antibody responses generated via the IM route were not only higher, but also appeared earlier. Such humoral immunity can be beneficial to eliminate invading pathogens such as *Salmonella* or influenza virus during the early phase of infection where complement killing plays a significant role.

Cell-mediated immune responses play a pivotal role in host protection against intracellular pathogens [36–38]. Thus, protection against the influenza virus essentially requires CMI responses to contain an ongoing infection effectively. Herein, we observed significantly higher HA1 antigen-specific cell proliferative responses in JOL2114 ghost-immunized chickens compared to controls. Interestingly, ghost immunization caused an increase in both CD4+ and CD8+ T-cell populations, indicating the engagement of both the Th1 and Th2 immune responses that play a major role in cell-mediated adaptive immunity. Digging deeper into the ghost-induced antigen-specific immune responses, we observed engagement of both the TLR-4 and TLR-5 pathways in modulating HA1-specific protective immune responses. Such induction may occur due to the mutual effects of *Salmonella*-encoded pathogen-associated molecules such as lipopolysaccharides and flagellin along with the surface-displayed HA1 antigen. In this regard, Th1 immune responses (marked by indicator cytokines TNF-α) play a significant role in the elimination of intracellular viral activity. Th2 type responses (marked by IL-6 and IL-10) may complement the antiviral defense by inducing antibody responses against the ongoing viral infection. The true protective efficacy of SE ghost immunization was seen in the challenge assay, which resulted in a significant reduction in fecal viral shedding in immunized chicken groups by day 11 post-infection.

One major advantage of utilizing *Salmonella* as a vaccine vector is its ability to induce strong anti-*Salmonella* immunity in addition to the intended immune responses delivered by the targeted antigen (HA1). Therefore, SE ghosts delivering HA1 antigen actually act as a bivalent vaccine that may protect immunized hosts against both salmonellosis and influenza infection. Usually, the vector derived anti-*Salmonella* immunity is a stronger response than that against the delivered heterologous antigen. In the present study, successful elicitation of anti-*Salmonella* immune responses was evidenced by significantly higher IgG responses in chicken serum against SE outer membrane proteins in SE immunized groups compared to the control groups. Here too, the IM route of immunization resulted in a superior immune response over the oral route, ultimately leading to significantly lower bacterial shedding in feces for IM immunized chickens.

In conclusion, the present study demonstrates that the immunization of chickens with *Salmonella* Enteritidis ghosts with surface-displayed HA1 antigen provides dual protection against salmonellosis and influenza infection by robust stimulation of both humoral and cellular immune responses. The lysis strategy incorporated in the ghost utilizing holin-endolysin and gene *E* mediated lysis promotes rapid generation of bacterial ghosts while preserving the antigenicity of the envelope. Further studies are needed to determine efficacy and broad cross protection against both diseases under field conditions.

5. Author's contributions

WG, conceived and designed the experiment. WG, carried out the experiments, WG and AS analysis and generation of figures. WG, AS and JHL manuscript preparation and revision of the final version. All authors have read and reviewed the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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