Evaluation of Safety and Protective Efficacy of a \textit{waaJ} and \textit{spiC} Double Deletion Korean Epidemic Strain of \textit{Salmonella enterica} Serovar Gallinarum

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With an aim to develop a highly attenuated and strongly immunogenic distinguishable vaccine candidate, a \textit{waaJ} (a gene involved in the synthesis of lipopolysaccharide) and \textit{spiC} (a virulence gene) double deletion Korean epidemic strain of \textit{S. enterica} ser. Gallinarum (SG005) was constructed. Our results showed that the growth and biochemical characteristics were not altered by this double deletion. The double deletion strain contained dual markers. One was a bacteriological marker (rough phenotype) and the other was a serological marker helping distinguish infected chickens from vaccinated chickens. The double deletion strain showed good genetic stability and reduced resistance to environmental stresses \textit{in vitro}; furthermore, it was extremely safe and highly avirulent in broilers. Single intramuscular or oral immunization of 7-day-old broilers with the double deletion strain could stimulate the body to produce antibody levels similar to the conventional vaccine strain SG9R. In addition, against a lethal wild-type challenge, it conferred effective protection that was comparable to that seen in the group vaccinated with SG9R. In conclusion, this double deletion strain may be an effective vaccine candidate for controlling \textit{S. enterica} ser. Gallinarum infection in broilers.

Keywords: DIVA, high attenuation, \textit{waaJ}, \textit{S. enterica} ser. Gallinarum, double deletion, \textit{spiC}

INTRODUCTION

\textit{Salmonella enterica} serovar Gallinarum (\textit{S. enterica} ser. Gallinarum) causes fowl typhoid (FT), an acute septicemic disease that occurs in chickens of all ages. Chicken infections are characterized by severe hepatosplenomegaly accompanied with bronze liver, anemia, and septicemia. The mortality and morbidity of FT can be as high as 80\% (1). FT outbreaks have important economic significance in many areas, including Africa, Asia, the Middle East, and Central and South America (2). In Korea, \textit{S. enterica} ser. Gallinarum was first officially reported during an FT outbreak in 1992 and is now found nationwide. FT has become one of the most prevalent bacterial diseases.

Many strategies have been used to prevent \textit{S. enterica} ser. Gallinarum infection, including the establishment of strict biosecurity measures and the use of antibiotics and vaccines. The high costs of biosecurity measures limit their application in many developing countries, and long-term...
use of antibiotics can result in the emergence of multidrug-resistant strains. Vaccination seems to be the most effective strategy to control FT (1, 3). Until now, three major types of vaccines, namely inactivated vaccine, subunit vaccine, and live attenuated vaccine, have been developed to control Salmonella infections in the poultry industry. Inactivated and subunit vaccines can induce strong antibodies to eradicate extracellular bacteria; however, it is difficult for these antibodies to eliminate intracellular Salmonella; this goal can be achieved with the live attenuated strains of Salmonella (4). The live attenuated SG9R has been used as a commercial vaccine to control FT for nearly 60 years. However, some drawbacks, such as residual virulence in newly hatched chickens, insufficient protection, and vertical transmission, have been reported with its use (5, 6). Therefore, the development of a safe and effective S. enterica ser. Gallinarum vaccine remains a current research hotspot. Thus far, a number of deletion strains have been developed, and their protection efficiencies against FT have been reported (1, 7–14). However, none of the S. enterica ser. Gallinarum deletion strains have been found to have the feature to distinguish infected chickens from vaccinated chickens.

To construct a live attenuated S. enterica ser. Gallinarum vaccine with distinguishable capability, we firstly chose the waaj gene. This gene codes for the lipopolysaccharide (LPS) 1,2-glucosyltransferase, which is involved in the synthesis of LPS (15). Deletion of the waaj gene changes smooth LPS to rough LPS, which cannot react with antibodies against the O antigen. The spiC gene encodes an effector protein that is secreted by the Salmonella pathogenicity island-2 (SPI2) type III secretion system (T3SS), which plays important roles in Salmonella infections (16). Therefore, spiC was selected as the second target gene to be deleted.

In this study, we constructed a waaj and spiC double deletion Korean epidemic strain of S. enterica ser. Gallinarum and evaluated its safety and protection efficacy for use as a live attenuated distinguishable vaccine candidate for the prevention of FT.

MATERIALS AND METHODS
Bacterial Strains, Plasmids, Media, and Growth Conditions
A wild-type Korean epidemic virulent strain A17-DW-005 (SG005) of S. enterica ser. Gallinarum with resistance toward ciprofloxacin (CIP), streptomycin (STR), gentamicin (GEN), nalidixic acid (NAL), sulfoxazole (FIS), and colistin (COL) was originally isolated from the liver of a 10-day-old broiler, which came from a broiler farm in Jeonbuk province in 2017. This strain was classified as type 1 by pulsed-field gel electrophoresis analysis (PFGE). Since type 1 has the highest proportion of all types, this strain was judged to be an epidemic strain. The suicide plasmid pRE112 and its host Escherichia coli (E. coli) χ7,213 were kept in our laboratory. pBluescript II SK (+) and pET-30 b (+) stored in our laboratory were used as intermediate vectors in this study. E. coli and S. enterica ser. Gallinarum were grown at 37°C in Luria–Bertani (LB) broth or on LB agar. When required, antibiotics were added to culture media at the following concentrations: ampicillin (Amp) at 100 µg/ml, kanamycin (Km) at 50 µg/ml, chloramphenicol at 25 µg/ml, and DL–α,e-diaminopimelic acid (DAP) at 50 µg/ml. LB agar containing 5% sucrose was used for sacB gene-based counterselection in allelic exchange experiments. A rough attenuated S. enterica ser. Gallinarum live vaccine SG9R (Intervet International, Boxmeer, The Netherlands) was used as control vaccine in this study.

Chickens
Ross broiler chickens were obtained from the Yangli Company (Cheonan, Choongnam, South Korea). Serum samples collected from all chickens in the present study were tested for Salmonella specific antibodies using a commercial ELISA kit (Biochek, cat# CK117, Crabethstraatt, Netherlands). Moreover, the antibodies level of Avian influenza and Newcastle disease were detected by hemagglutination-inhibition (HI) test virus. The test results were all negative. 10% of the chickens were randomly selected and euthanized. The chickens were dissected to check whether there were any lesions in the internal organs. Liver and fecal samples were aseptically collected for routine Salmonella and avian pathogenic Escherichia coli isolation and culture, and the follow-up Salmonella infection experiment was performed after the complete negative was confirmed. The chickens were used after adaptation for a week. To ensure the best environmental conditions, the conditions of the isolator (temperature, humidity, and ventilation) were continuously monitored. The chickens were taken care of and handled by a well-trained staff.

Ethics Statement
All experimental and animal management procedures were undertaken in accordance with the requirements of the Animal Care and Ethics Committee of Jeonbuk National University. The animal facility at Jeonbuk National University is fully accredited by the National Association of Laboratory Animal Care (approval number: JBNU 2020-0162).

Construction of Deletion Strains of S. enterica Ser. Gallinarum by Allelic Exchange
Construction of deletion strains was performed by the allelic exchange method using the suicide vector pRE112 as described previously (Supplementary Figure 1) (17). The 1,000-bp upstream fragment of the waaj gene was amplified from the genomic DNA of S. enterica ser. Gallinarum using a pair of primers (W1 and W2) by PCR (Supplementary Table 1). The PCR product was cloned into the XbaI and BamHI sites of the pBluescript II SK(+) vector, resulting in pSK-waaj-up. The 1,000-bp downstream fragment of the waaj gene was then PCR-amplified using a pair of primers (W3 and W4) and cloned into the BamHI and KpnI sites of pSK-waaj-up to obtain pSKΔwaaj. The 2,000-bp fragment, which included the upstream and downstream fragments of the waaj gene from the XbaI– and KpnI-digested plasmid pSKΔwaaj, was ligated into plasmid pRE112 to yield the suicide plasmid pREΔwaaj. The transfer of recombinant suicide plasmids into SG005 was accomplished...
by conjugation using *E. coli* χ7213 (pREΔwaaJ) as the plasmid donor. Strains containing single-crossover plasmid insertions (SG005waaJ::pREΔwaaJ) were isolated on plates containing chloramphenicol. The first homologous recombination was confirmed with the primers W5 and W6. A loss of the suicide vector after the second recombination between homologous regions (i.e., allelic exchange) was selected for by using the sacB-based sucrose sensitivity counterselection system. The second homologous recombination was confirmed with the primers W6 and W7. Primers W8 and W9 were used to confirm whether the full waaJ gene was removed from the genome. Primers W10 and W11 were used to confirm whether the suicide plasmid was removed from the genome (Supplementary Figure 2). This deletion strain was designated as SG005ΔwaaJ. Using the same method, deletion of *spiC* was introduced into SG005, and this deletion strain was designated as SG005ΔspiC. Notably, in the construction of the suicide plasmid pREΔspiC, pET-30 b (+) was used as an intermediate vector. Similarly, the deletion of *spiC* was introduced into SG005ΔwaaJ, and the double deletion strain was designated as SG005ΔwaaJΔspiC.

The complemented strains were constructed previously described with some modifications (18–20). The gene waaJ was PCR-amplified from the wild type strain SG005 using the primers pBR322-waaJ-F and pBR322-waaJ-R (Supplementary Table 1), which were designed based on the coding sequence of *waaJ* gene. After amplification, the DNA fragment was digested by BamHI and SalI and ligated to the plasmid pBR322. The resulting vector pBR322-waaJ was introduced into SG005ΔwaaJ by electroporation to produce the complemented strain SG005ΔwaaJ (pBR322-waaJ). Electroporation on the electroporator (Bio-Rad Laboratories, Hercules, CA, USA) was performed using optimized parameters (2.5 kV, 200 Ω, 25 µF, 5 ms). Similarly, the complemented strains SG005ΔspiC (pBR322-spiC) and SG005ΔwaaJΔspiC (pBR322-waaJ-spiC) were constructed. It should be pointed out that when constructing SG005ΔwaaJΔspiC (pBR322-waaJ-spiC), the waaJ gene and *spiC* gene were inserted into the plasmid pBR322 in tandem.

### Phenotype Identification

O9 antigen is one of the antigens produced on *S. enterica* ser. Gallinarum. *S. enterica* ser. Gallinarum with smooth phenotype have O9 antigen and therefore can be agglutinated by O9 antibody. Briefly, colonies from LB agar plates were mixed with 20 µl of phosphate-buffered saline (PBS) and 20 µl of the O9 factor rabbit antiserum (SSI® SALMONELLA ANTISERA, Denmark) on a glass slide. Mixtures were rotated for 1 min and observed for agglutination. The acriflavine agglutination test was performed to detect the rough phenotype characteristic of the deletion strains (21). Briefly, colonies of a 24-h culture prepared from the deletion strains on LB agar plates were mixed with 20 µl of 0.2% acriflavine on a glass slide. Mixtures were rotated for 1 min and observed for agglutination.

### Auto-Aggregation Assay

Auto-aggregation refers to the phenomenon that bacteria aggregate into clusters by themselves during the cultivation process. The stronger is the hydrophobicity of the cell surface, the higher is the auto-aggregation ability of the cell. The auto-aggregation assay was performed based on a previously described method with some modifications (22). Briefly, SG005, SG005ΔwaaJ, SG005ΔspiC, and SG005ΔwaaJΔspiC were statically grown in 5 ml of LB broth at 37°C for 24 h in 15-ml conical tubes. The upper 0.5 ml was carefully removed to measure its OD590 (recorded as OD590 prevortex). Then, the remaining culture in the tube was mixed by vortexing to resuspend the aggregated cells, and 0.5 ml of the suspension was removed, and its OD590 was measured (recorded as OD590 postvortex). The “percent aggregation” was calculated using the formula: 100% × (OD590 postvortex − OD590 prevortex)/OD590 prevortex.

### Stability Analysis

To determine the stability of the deletion strains, the liquid culture of each strain was continuously passaged 60 times every 12 h. Every 10 generations, DNA was extracted for PCR identification.

### Environmental Safety Evaluation

The sensitivity of SG005, SG005ΔwaaJ, SG005ΔspiC, and SG005ΔwaaJΔspiC to ultraviolet (UV) light and various disinfection agents was evaluated as described with some modifications (23, 24). Bacteria were grown to OD590 = 1.0 at 37°C with shaking at 200 rpm and were diluted in PBS to 10^9 colony forming units (CFU)/ml. One milliliter of suspension was then transferred to a sterile Petri dish and exposed to UV radiation (Sankyo denki UV G30TB: 30 W, distance: 45 cm) for 1 min. For testing oxidative stress and alkali and acid tolerance, each bacterial suspension and the stressor were mixed in equal volume and incubated at room temperature (RT, 25°C): 100-µM hydrogen peroxide (H2O2) for 5 min, 12.5-mM sodium hydroxide (NaOH) for 5 min, and 50-mM citric acid for 20 min. Finally, the number of viable bacteria was determined by plating serial dilutions onto LB agar plates. The experiment was performed three times.
To determine the 50% lethal dose (LD$_{50}$), SG005, SG005ΔwaaJ, SG005ΔspiC, and SG005ΔwaaJΔspiC were grown for 12 h at 37°C in LB broth. One milliliter of broth from each grown bacterium was inoculated into 100 ml of LB broth. After the inoculated strains were grown to OD$_{590}$ = 1.0, the bacterial cells were recovered by centrifugation for 10 min at 2,400 g at RT. The harvested cells were suspended in PBS to a concentration of 10$^{11}$–10$^{12}$ CFU/ml. The suspended strains were serially diluted by 10-fold. The 7-day-old chickens were intramuscularly (IM) injected or orally inoculated with each dose of these strains (10$^8$–10$^{11}$ CFU). Six chickens were used for each dose. Feed and water were not provided to the tested chickens for 4 h before and until 1 h after the treatment. The treated chickens were observed twice a day for 2 weeks after administration. Clinical scores were determined and recorded using a system as we have previously described (25). Briefly, disease severity was scored as follows: 0, normal; 1, depression and ruffled feathers; 2, depression, ruffled feathers, and respiratory distress; 3, abovementioned clinical signs plus anorexia, emaciation, and green-yellowish diarrhea; and 4, death. When the birds showed a clinical score of 3 (humane endpoint), they were euthanized immediately by cervical dislocation performed by trained veterinarians. LD$_{50}$ was calculated by the method of Reed and Muench (26).

Organ Colonization and Persistence

Bacterial persistence and clearance were analyzed by counting the number of the bacteria per gram of liver or spleen. Seven-day-old chickens were randomly divided into four groups (n = 12 for each group): SG005ΔwaaJ group, SG005ΔspiC group, SG005ΔwaaJΔspiC group, and PBS control group. The first three groups were injected intramuscularly with 1 × 10$^8$ CFU of SG005ΔwaaJ, SG005ΔspiC, and SG005ΔwaaJΔspiC, respectively. The last group was inoculated with an equal volume of PBS in the same way. Three chickens from each group were euthanized at 2, 6, 10, and 14 days post-inoculation (dpi), and liver and spleen samples were aseptically removed. The samples were weighed and suspended in 1 ml of PBS and then individually homogenized. The homogenates were diluted serially and subsequently plated on MacConkey agar (Difco Laboratories, Detroit, MI, USA) at 37°C. After overnight cultivation, the bacterial number was counted.

Vaccination Experiment

Sixty 7-day-old chickens were divided into five groups (n = 12 for each group) to evaluate the protective efficacy of the deletion strains. The group 1 (PBS negative control) and group 2 (PBS positive control) were intramuscularly inoculated with 500 μl of PBS (pH 7.2). The group 3 and group 4 were intramuscularly inoculated with 10$^8$ CFU of SG9R and SG005ΔwaaJΔspiC, respectively. The group 5 was orally inoculated with 10$^8$ CFU of SG005ΔwaaJΔspiC. After 4 weeks, except for the group 1, chickens in other groups were challenged intramuscularly with 10$^8$ CFU of virulent SG005. Deaths and clinical signs were recorded daily for 2 weeks. At the end of this period, all surviving chickens were euthanized. Clinical scores were determined and recorded following the method previously described (25).

Antibody Production Assay

A laboratory-made indirect enzyme-linked immunosorbent assay (ELISA) using heat-killed whole S. enterica ser. Gallinarum bacteria as the coating antigen was applied to quantify IgG in the serum. Briefly, bacterial antigen (10$^6$ CFU/well) was coated in a 96-well plate and incubated overnight at 4°C. After blocking, the sera samples collected on days 14, 21, and 28 were diluted (1:400) with 2% bovine serum albumin (BSA) and added to the wells. The wells were incubated with a 1:8,000 dilution of affinity-purified, peroxidase-labeled goat anti-chicken IgG (H+L) (KPL Inc., MD, USA) for 1 h. OD$_{450}$ was measured with a microplate reader VICTORX4 (PerkinElmer Inc., Waltham, MA, USA) after the reaction was stopped with 4.5 N H$_2$SO$_4$. The OD$_{450}$ average value of negative serum samples plus the standard deviation (SD) of three times was used as the cut-off value (27).

Capability of DIVA (Differentiation of Infected and Vaccinated Animals) Based Serodiagnosis

The serum plate agglutination (SPA) test was used to assess the DIVA capability of SG005ΔwaaJ, SG005ΔspiC, and SG005ΔwaaJΔspiC. Seven-day-old chickens were intramuscularly inoculated with each deletion strain. The sera were prepared on 14, 21, and 28 dpi by collecting blood from wing veins. The blood was allowed to coagulate for 1 h at RT, centrifuged for 10 min at 600 g, and then, the clean phase of serum was collected. Serum samples (30 μl) were mixed with an equal volume of S. enterica ser. Pullorum/Gallinarum standard antigen (Green Cross Veterinary Products, Yongin, South Korea), and the agglutination reaction was observed.

Statistical Analysis

Statistical analysis was performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). The data were analyzed by one-way analysis of variance (ANOVA). Differences were considered statistically significant at: *P < 0.05, **P < 0.01, ***P < 0.001.

RESULTS

Constructions of S. enterica Ser. Gallinarum Deletion Strains

The deletion of the waaJ gene in SG005 was confirmed by PCR using the primers W6 and W7 with the expected amplicon sizes of 2,490 and 1,476 bp for the wild type and deletion type of the waaJ gene, respectively. The deletion of the spiC gene in SG005 was confirmed by PCR using the primers S6 and S7 with the expected amplicon sizes of 2,003 and 1,619 bp for the wild type and deletion type of the spiC gene, respectively (Supplementary Figure 3). In the three deletion strains, the corresponding target gene and suicide plasmid could not be detected by PCR (Supplementary Figure 4). The sequencing results also showed that the deletion strains SG005ΔwaaJ, SG005ΔspiC, and SG005ΔwaaJΔspiC were constructed successfully (Supplementary Text 1).
Growth and Biochemical Characteristics

Growth curve analysis revealed no significant differences between the wild type and each deletion strain when cultured in LB broth at 37°C (Figure 1A). Results of biochemical tests including 2-nitrophenyl-β-D-galactopyranoside, L-arginine, L-lysine, L-ornithine, trisodium citrate, sodium thiosulfate, urea, L-tryptophane, L-tryptophane, sodium pyruvate, gelatin, D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, amygdalin, and L-arabinose were the same between wild type and each deletion strain, suggesting that deletions in these two genes did not alter the biochemical characteristics of SG005 (Supplementary Table 2).

Phenotype Identification

SG005 and SG005ΔspiC were agglutinated with O9 factor rabbit antiserum but not with acriflavine, indicating that they had a smooth phenotype. However, SG005ΔwaaJ and SG005ΔwaaJΔspiC were not agglutinated with O9 factor rabbit antiserum.

**Figure 1**

Growth curves, phenotypic characterization, and auto-aggregation assay. (A) Growth curves of wild-type SG005 and the deletion strains. SG005, SG005ΔwaaJ, SG005ΔspiC, and SG005ΔwaaJΔspiC showed a similar growth curve in LB broth. (B) Phenotype identification. Agglutination was examined with O9 factor rabbit antiserum and acriflavine. Pictures were taken within 3 min. (C) Visual auto-aggregation of SG005 (1), SG005ΔwaaJ (2), SG005ΔspiC (3), and SG005ΔwaaJΔspiC (4). (D) Auto-aggregation (%). Bacterial cultures were statically grown in LB broth for 24 h at 37°C. Statistical analysis was done using one-way ANOVA. ***P < 0.001. Each experiment was repeated three times.
antiserum but were agglutinated with acriflavine, indicating that they had a rough phenotype (Figure 1B). The complemented strains SG005ΔwaaJ (pBR322-waaJ), SG005ΔspiC (pBR322-spiC) and SG005ΔwaaJΔspiC (pBR322-waaJ-spiC) were all agglutinated with O9 factor rabbit antiserum but not with acriflavine. Therefore, complementary analysis proved that the deletion of the waaJ gene was the direct cause of the bacteria changing from smooth to rough.

Auto-Aggregation Assay
Auto-aggregation was tested in LB broth (Figure 1C), and the aggregation percent was calculated using OD_{590} measurements from these cultures. Both SG005 and SG005ΔspiC showed ~5% aggregation, whereas SG005ΔwaaJ and SG005ΔwaaJΔspiC demonstrated 84 and 79% aggregation, respectively (Figure 1D).

Stability
The deletion Strains were serially passaged 60 times in LB medium, and the absence of waaJ and spiC genes was confirmed by PCR, indicating that each deletion strain had good genetic stability (Figure 2).

Bacterial Resistance to Environmental Stress
After UV irradiation, the survival rates for SG005, SG005ΔwaaJ, SG005ΔspiC, and SG005ΔwaaJΔspiC were 48, 37, 45, and 37%, respectively (Figure 3A). A significant difference (P < 0.001) was seen between ΔwaaJ strains (SG005ΔwaaJ and SG005ΔwaaJΔspiC) and non-ΔwaaJ strains (SG005 and SG005ΔspiC), indicating that the deletion of the waaJ gene increased the sensitivity of the strain to UV. In terms of oxidative stress, SG005ΔwaaJ and SG005ΔwaaJΔspiC with survival rates of 52 and 50% showed reduced resistance compared to SG005 and SG005ΔspiC with survival rates of 59 and 60% (P < 0.001) in H2O2 (Figure 3B). For alkali endurance, the survival rates for SG005, SG005ΔwaaJ, SG005ΔspiC, and SG005ΔwaaJΔspiC were 68, 51, 67, and 49%, respectively. A significant difference was noted between ΔwaaJ strains and non-ΔwaaJ strains (P < 0.001; Figure 3C). For acid endurance, the survival rates for SG005, SG005ΔwaaJ, SG005ΔspiC, and SG005ΔwaaJΔspiC were 34, 31, 35, and 31%, respectively. Statistical analysis revealed a difference between ΔwaaJ strains and non-ΔwaaJ strains (P < 0.05; Figure 3D). However, deletion of spiC gene had no effect on bacterial resistance to these environmental stresses.

Determination of LD_{50}
To confirm the safety of the deletion strains, the virulence of wild and deletion strains was evaluated in 7-day-old chickens. The IM LD_{50} of SG005ΔwaaJ and SG005ΔspiC was about 10^{8}-fold higher than that of the wild strain. The IM LD_{50} of SG005ΔwaaJΔspiC was about 10^{12}-fold higher than that of the wild strain. The oral LD_{50} of the three deletion strains SG005ΔwaaJ, SG005ΔspiC, and SG005ΔwaaJΔspiC was about 10^{5}-fold higher than that of the wild strain SG005 (Table 1).
FIGURE 3 | Survival rates of SG005 and deletion strains in different environmental stresses. (A) UV irradiation for 1 min. (B) Hydrogen peroxide (100 µM for 5 min). (C) Sodium hydroxide (12.5 mM for 5 min). (D) Citric acid (50 mM for 20 min). Statistical analysis was done using one-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. Each experiment was repeated three times.

TABLE 1 | Chicken LD50 for the wild-type SG005 and deletion strains.

| Strain             | LD50a (CFU) | IMb | Oralc |
|--------------------|-------------|-----|-------|
| SG005              | <10^1       | 1.6 x 10^5 |       |
| SG005ΔwaaJ         | 1.1 x 10^9  | >10^10 |       |
| SG005ΔspiC         | 5.7 x 10^9  | >10^10 |       |
| SG005ΔwaaJ/ΔspiC   | >10^11      | >10^11 |       |

aLD50: 50% lethal dose.
bChickens were inoculated intramuscularly (IM) with SG005 and the deletion strains.
cChickens were inoculated orally with SG005 and the deletion strains.

Organ Colonization and Persistence

The results of bacteria persistence and clearance in organs were shown in Figure 4A. SG005ΔwaaJΔspiC colonized and persisted in the liver and spleen for at least 10 days. Within these 10 days, the number of SG005ΔwaaJΔspiC gradually decreased. At 14 dpi, SG005ΔwaaJΔspiC could not be isolated from the liver and spleen. However, SG005ΔwaaJ and SG005ΔspiC could still be isolated, indicating that the double deletion strain could be cleared more quickly than the single deletion strains.

Protective Efficacy Against Virulent S. enterica Ser. Gallinarum Challenge

Chickens that were intramuscularly inoculated with 10^8 CFU of SG9R and SG005ΔwaaJΔspiC showed 83 and 92% protection against S. enterica ser. Gallinarum challenge, respectively. Chickens that were orally inoculated with 10^8 CFU of SG005ΔwaaJΔspiC showed 83% protection. All chickens died in the PBS-positive control group (Figure 4B). Depression, anorexia, and diarrhea were observed in the PBS-positive control group, whereas no clinical signs were observed in the SG005ΔwaaJΔspiC group.
Humoral Immune Response Generated by the Deletion Strains

Statistical analysis showed a significant difference in the humoral immune response in chickens immunized with the deletion strains compared with the PBS control group. Serum IgG levels at each post-immunization time point were significantly higher in the immunized groups than in the PBS control group. The IgG level of the SG005ΔwaaJΔspiC group immunized by intramuscular injection oral administration was not significantly different from that of the SG9R group at 3 and 4 weeks after inoculation ($P > 0.05$; Figure 4C).
**Differentiation of Serum Antibodies to S. enterica Ser. Gallinarum**

Antibodies to S. enterica ser. Gallinarum were detected by the SPA test using the SG005 antigen. Sera collected from SG005ΔwaaJ- or SG005ΔwaaJΔspiC-immunized chickens at different time intervals were identified as SPA-negative (Figure 4D). However, sera collected from SG005- or SG005ΔspiC-immunized chickens were identified as SPA-positive. Overall, SG005ΔwaaJ and SG005ΔwaaJΔspiC showed evident distinguishable capability.

**DISCUSSION**

Many shortcomings of SG9R vaccine limit its application. The residual virulence of SG9R caused liver and spleen lesions in day-old broilers and brown layers, and a single point mutation of the waaJ gene can make SG9R lose DIVA capability. Moreover, SG9R old broilers and brown layers, and a single point mutation of the residual virulence of SG9R caused liver and spleen lesions in day-old chickens. Consequently, the waaJ gene may reduce the virulence more significantly and the DIVA effect in waaJ deletion strain may be more obvious than that of waaL deletion strain. Secondly, through whole-genome sequencing, it was found that there was a point mutation in the waaJ gene of the commercial vaccine strain, which prevented the protein encoded by waaJ from being synthesized. LPS defect caused by a point mutation in the waaJ gene may be one of the major mechanisms of vaccine strain attenuation (28). The commercial vaccine has been used for decades, and its immunogenicity is widely recognized by the poultry industry. Therefore, this reminded us that deleting the waaJ gene may be a good choice when developing new vaccines.

The growth and biochemical properties were not altered by deletions of waaJ or spiC or both genes from S. enterica ser. Gallinarum, suggesting that both genes may not be related to the bacterial metabolic pathway (Figure 1A and Supplementary Table 2). S. enterica ser. Gallinarum with smooth phenotype has O9 antigen and can therefore be agglutinated by O9 antibody. Our result showed that SG005 and SG005ΔspiC were agglutinated with O9 factor rabbit antisera but SG005ΔwaaJ and SG005ΔwaaJΔspiC were not, indicating that the waaJ deletion strain did not contain O9 antigen. We performed the acriflavine agglutination test, which is used to detect the rough phenotype of bacteria (21). Our results were consistent with our expectations. SG005ΔwaaJ and SG005ΔwaaJΔspiC were agglutinated with acriflavine, whereas SG005 and SG005ΔspiC were not, indicating that deletion of the waaJ gene directly caused the LPS to change from smooth to rough (Figure 1B). In addition, both SG005ΔwaaJ and SG005ΔwaaJΔspiC showed evident auto-agglutination (Figures 1C,D). This is because the increased hydrophobicity of the rough Salmonella surface makes the cells aggregate and settle. In brief, the deletion of waaJ and spiC genes had no effect on the growth and biochemical characteristics of the bacteria, and the deletion of the waaJ gene not only changed the phenotype of the bacteria from a smooth type to a rough type but also made the bacteria exhibit obvious auto-aggregation.

The sensitivity of strains to different environmental stresses is an important indicator for testing the environmental safety of vaccines. As an important component of LPS, the O antigen structure may prevent antibacterial agents, such as disinfectants, from reaching the target sites in the cell wall to some extent, thereby improving its environmental resistance. The destruction of the LPS structure could reportedly reduce the resistance of bacteria to environmental stresses (24). In our study, the waaJ deletion strains (SG005ΔwaaJ and SG005ΔwaaJΔspiC) showed reduced resistance to UV, oxidative stress, and alkalins and acids, suggesting that the integrity of LPS is instrumental in the resistance of bacteria to environmental stresses (Figure 3). Similar results have been reported for S. enterica ser. Pullorum, S. enterica ser. Enteritidis, and E. coli (21, 24, 34). These results indicated that the deletion of the waaJ gene significantly increased the sensitivity of bacteria to environmental stress.

The deletion of the waaJ gene was reported to not only confer the DIVA capability to the smooth type strains but also reduce the virulence. The deletion of the waaJ gene consequently obstructed the expression of a part of the outer core and the
O antigen of LPS. LPS is an important virulence factor, and partial deletion of LPS will directly reduce bacterial virulence. It has been reported that S. enterica ser. Typhimurium deletion strain 11310 (\textit{waaJ}+) was significantly attenuated (about 10^{5} -fold) in mice (29). S. enterica ser. Enteritidis deletion strain SEM1C\textit{waaJ} had a LD_{50} at least 100 times higher than that of the parental strain in chickens (35). The \textit{spiC} gene is closely related to the virulence of bacteria. It has been reported that the LD_{50} of S. enterica ser. Pullorum S06004\textit{spiC} was 200 times higher than that of the parent strain (23). Furthermore, the LD_{50} of S. enterica ser. Enteritidis C50041\textit{spiC} was reportedly 900 times higher than that of the wild-type strain (16). The LD_{50} of S. enterica ser. Typhimurium\textit{spiC} was 10^{5} times higher than that of the parent strain in mice (16). Our research results were similar to these research results. Our result showed that the oral LD_{50} of both SG005\textit{waaJ} and SG005\textit{spiC} was about 10^{5} -fold higher than that of the wild strain SG005 (Table 1). In addition, our results showed that when the 7-day-old broilers were administered intramuscularly, SG005\textit{waaJ}\textit{spiC} was the least virulent among the three deletion strains, followed by SG005\textit{spiC} and finally SG005\textit{waaJ} (Table 1). In addition, it should be noted that SG005\textit{waaJ}\textit{spiC} was highly avirulent (IM LD_{50} increased at least by 10^{10} -fold) because it did not cause mortality even in response to a high-dose challenge (10^{11} CFU). Furthermore, our results showed that the oral LD_{50} of SG005\textit{waaJ}\textit{spiC} (>10^{11} CFU) was higher than that of SG9R (5 × 10^{10} CFU) (36). Taken together, these results indicated that SG005\textit{waaJ}\textit{spiC} was extremely safe for broilers.

After intramuscular inoculation with SG005\textit{waaJ}\textit{spiC}, the chickens did not show any of the clinical signs, and the weight of the inoculated chickens did not differ from that of the negative control group. This suggested that SG005\textit{waaJ}\textit{spiC} did not cause any obvious damage to the chickens and was quite safe. In the colonization experiment, our results showed that SG005\textit{waaJ}\textit{spiC} could exist in the chicken for at least 10 days and could not be detected on 14 dpi (Figure 4A). Some research reports have pointed out that the temporary residence of bacteria in the internal organs of chickens was essential for inducing protective immunity (37). In addition, SG005\textit{waaJ}\textit{spiC} was cleared faster from the liver and spleen than the two single deletion strains SG005\textit{waaJ} and SG005\textit{spiC} (Figure 4A). In previous experiments using chicken embryos to evaluate the virulence of S. enterica ser. Gallinarum, we have reported that the higher was the virulence of the strain, the stronger was its invasiveness into the liver. In this colonization experiment, since SG005\textit{waaJ}\textit{spiC} had the lowest virulence had the weakest invasiveness to chickens, it was eliminated first. According to the above results, it can be interpreted that the inoculation of SG005\textit{waaJ}\textit{spiC} did not cause any clinical signs and had no effect on body weight and that it could colonize in the chicken for at least 10 days.

Generally, the stronger is the virulence of the bacteria, the higher is the immunogenicity. Therefore, the construction of live attenuated \textit{Salmonella} vaccine needs to balance attenuation and immunogenicity. According to the results of the LD_{50} assessment, the virulence reduction of SG005\textit{waaJ}\textit{spiC} was verified, and herein, the protective efficacy was also verified. SG005\textit{waaJ}\textit{spiC} provided protection similar to SG9R (92 vs. 83%, respectively) by intramuscular injection (Figure 4B). More importantly, through oral immunization, the protection of SG005\textit{waaJ}\textit{spiC} could reach 83%, indicating that this double strain can be developed as an oral vaccine. In addition, severe clinical signs of FT, such as ruffled feathers, anorexia, somnolence, and greenish diarrhea, were observed in the PBS group 2 (positive control), whereas no obvious clinical signs were observed in the SG005\textit{waaJ}\textit{spiC} group. For the vaccine strain SG9R, two times of immunization were required, and subcutaneous injection and intramuscular injection were the main ways of vaccination (6). However, for SG005\textit{waaJ}\textit{spiC}, only a single intramuscular injection or oral immunization can achieve an immune effect no less than that of the vaccine strain SG9R. Taken together, these results indicated that attenuated SG005\textit{waaJ}\textit{spiC} could confer effective protection against acute systemic FT infection.

Live attenuated \textit{Salmonella} vaccine can stimulate the body to produce humoral immunity, which is essential for preventing \textit{Salmonella} infection. In this study, we used indirect ELISA to check the serum IgG antibody to assess the level of specific humoral immune response induced by different deletion strains. The results showed that the antibody level of the SG005\textit{waaJ}\textit{spiC} group immunized by intramuscular injection was not significantly different from that of the SG9R group (P > 0.05; Figure 4C). This indicated that SG005\textit{waaJ}\textit{spiC} could provide antibody levels comparable to SG9R. A previous similar study reported that S. enterica ser. Pullorum S06004\textit{spiC} \textit{waaL} (\textit{waaL}, also a gene involved in the synthesis of LPS) led to a significant antibody increase in broilers (21). In brief, SG005\textit{waaJ}\textit{spiC} induced a strong humoral immune response. The disadvantage of this study was that we did not evaluate the cellular immune responses induced by the deletion strains. The cellular immunity induced by attenuated \textit{Salmonella} is very important in the evaluation of vaccines, because \textit{Salmonella} is an intracellular pathogen. Unfortunately, we did not test this indicator. Since this indicator can only be tested in animal experiments, we plan to test the cellular immune response through cell proliferation experiments in the next animal experiment.

It has been described that some LPS deficient mutants (\textit{waaJ}, \textit{waaL}, \textit{rfaH}, \textit{rfbH}, \textit{rfgB}, \textit{et al.}) were used in \textit{Salmonella} DIVA vaccine research (22, 29, 30, 34, 38). It was reported that immunization of piglets with the \textit{waaJ} or \textit{waaL} mutants of S. enterica ser. Typhimurium resulted in the induction of a serological response lacking detectable antibodies against LPS, which allowed a clear differentiation between serums from pigs immunized with the \textit{waaJ} or \textit{waaL} strains and serums from pigs infected with the wild type strain (30). There was also a report showing that vaccination of swine with the \textit{rfaH} (a gene encoding the RfaH antiterminator that prevents premature termination of long mRNA transcripts) mutant conferred protection against challenge with virulent S. enterica ser. Typhimurium but did not interfere with herd level monitoring for \textit{Salmonella} spp (38). \textit{rfaH} and \textit{rfgB} (both genes are related to LPS biosynthesis) mutants of S. enterica ser. Enteritidis and S. enterica ser. Pullorum S06004\textit{spiC} \textit{waaL} were also reported.
to have DIVA features (22, 34). Our results showed that the antibodies produced by SG005ΔwaaJ and SG005ΔwaaJΔspiC did not react with S. enterica ser. Pullorum/Gallinarum standard antigen, indicating that these two deletion strains have good DIVA capability (Figure 4D). In addition, the limitations of the DIVA need to be pointed out in the present study. For example, when chickens are infected with a low level of wild strains in the field, the resulting serums may not agglutinate with the standard antigen, because the sensitivity of plate agglutination test is lower than that of ELISA (39). Immunization of animals with these deletion strains will not interfere with the Salmonella monitoring program. The vaccine with DIVA capability has the characteristic of distinguishing between infected and immunized animals, and its application to the prevention and control of livestock and poultry diseases is of great significance.

Additionally, although we have verified the deletion strains by PCR and sequencing, to perfect verification of our candidate, we will conduct of characteristics study using southern blot, whole-genome sequencing, immunological study, and field trials.

In this study, we lacked novelty in the selection of target genes, because waaJ deletion strains and spiC deletion strains have been reported on many other Salmonella species (16, 21, 23, 29, 30, 35). However, the waaJ and spiC double-gene deletion strain has not been reported in S. enterica ser. Gallinarum. Another important aspect was that we used a Korean epidemic strain, which was selected through PFGE analysis and antimicrobial sensitivity testing from many clinical isolates. Finally, we want to re-explain the purpose of our experiment. We hope to use the advantages of the commercial vaccine strain (removing the waaJ gene to produce DIVA effects and at the same time reduce virulence) and make up for the disadvantages (removing spiC to further eliminate residual virulence) to develop a new vaccine based on a Korean epidemic strain of S. enterica ser. Gallinarum.

CONCLUSION

In summary, we have demonstrated that the rough attenuated S. enterica ser. Gallinarum vaccine candidate SG005ΔwaaJΔspiC had attributes required in a potent vaccine, such as high attenuation, good immunogenicity, and distinguishable capability. Therefore, this double deletion strain—SG005ΔwaaJΔspiC—may be an effective vaccine for controlling S. enterica ser. Gallinarum infection in broilers.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Ethics Committee of Jeonbuk National University.

AUTHOR CONTRIBUTIONS

J-FZ, MK, and H-KJ contributed to conception and design of experiments. S-YC, KS, Y-JL, and J-YP contributed to acquisition, analysis, and interpretation of data. J-FZ, MK, S-YC, BW, and H-KJ drafted and/or revised the article. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.756123/full#supplementary-material

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