Evaluation of the immunoprotection efficacy of *Riemerella anatipestifer* *fur*-deficient mutant as an attenuated vaccine

Mi Huang,[*,†,‡] Mafeng Liu,[*,†,‡] Jiajun Liu,[*,†,‡] Mingshu Wang,[*,†,‡] Renyong Jia,[*,†,‡] Dekang Zhu,[†,‡] Shun Chen,[*,†,‡] Xinxin Zhao,[*,†,‡] Qiao Yang,[*,†,‡] Ying Wu,[*,†,‡] Shaqiu Zhang,[*,†,‡] Qun Gao,[*,†,‡] Juan Huang,[*,†,‡] Xumin Ou,[*,†,‡] Sai Mao,[*,†,‡] Bin Tian,[*,†,‡] Di Sun,[*,†,‡] and Anchun Cheng[*,†,‡,1]

[*]*Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan 611130, PR China;* [†]*Research Centre of Avian Disease, College of Veterinary Medicine of Sichuan Agricultural University, Chengdu, Sichuan 611130, PR China; and* [‡]*Key Laboratory of Animal Disease and Human Health of Sichuan Province, Chengdu, Sichuan 611130, PR China*

ABSTRACT *Riemerella anatipestifer* (*R. anatipestifer*, **RA**) is an infectious pathogen that causes septicemia and polyserositis in ducks. Our previous studies showed that RA CH-1 Δ*fur* was significantly attenuated in ducklings, which highlights the potential of this strain as a live attenuated vaccine. In this study, it was shown that infection with 10^9 CFU of the *fur* mutant did not cause any clinical symptoms or significant histological lesions in 3-day-old ducklings and that the bacteria were readily cleared by the host within 3 d. Compared with the nonvaccinated group, the group inoculated with the mutant strain RA CH-1 Δ*fur* exhibited protection of ducklings against a high-dose (2.28×10^{10} CFU) challenge with the wild-type strain RA CH-1. Moreover, the average body weights and body weight gains of the Δ*fur*-inoculated group were not significantly affected by the challenge. Further analysis revealed that RA CH-1 Δ*fur* elicited higher IgY titers and that the serum antibody levels persisted for at least 49 d after immunization. Overall, our study showed that RA CH-1 Δ*fur* is a safe and effective vaccine candidate that is expected to play an important role in RA CH-1 infection prevention in the duck industry.

Key words: *Riemerella anatipestifer*, Fur, virulence, immunoprotection, attenuated vaccine

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INTRODUCTION

Riemerella anatipestifer (*R. anatipestifer*, **RA**), a gram-negative bacterium belonging to the family *Flavobacteriaceae*, mainly infects ducklings aged 2 to 8 wk and causes acute septicemia and infectious polyserositis (Sandu, 1991; Segers et al., 1993). Sick ducks will become defective or stiff and even die in severe cases, all of which cause serious economic losses in the duck industry (Ruiz, 2013). At present, more than 21 serotypes of RA have been reported, and new serotypes appear continuously; some of them are highly virulent, and there is no obvious cross-protection between each serotype (Pathanasophon et al., 1995, 2002). In addition, most of the RA isolates have been found to be resistant to a variety of antibiotics (Zhong et al., 2009; Huang et al., 2017; Zhang et al., 2017; Luo et al., 2018). Therefore, it is difficult to prevent and treat RA with existing vaccines and antibiotics, and there is an urgent need for new strategies to control the disease.

To reveal the pathogenic mechanisms and develop effective vaccines against RA infection, an increasing number of virulence factors, that is, OmpA, the TonB-dependent receptors TbdR1, B739_1343 and B739_1208, the ferric uptake regulator (**Fur**), and lipopolysaccharide biosynthesis proteins, have been identified in RA (Hu et al., 2011; Lu et al., 2013; Zou et al., 2015; Wang et al., 2017; Liu et al., 2018; Huang et al., 2021). OmpA of RA was identified as a predominant immunogenic outer membrane protein (Subramaniam et al., 2000). In RA serotype 2 strain Th4, the LD_{50} of a Δ*ompA* strain was attenuated more than 22 times in comparison with its parent strain (Hu et al., 2011). Iron acquisition within an iron-limited host is crucial to the virulence of RA. Since TonB systems are involved in iron acquisition and the virulence of *tonB1* or *tonB2* mutants is severely attenuated in ducks (Liao et al., 2015; Miao et al., 2015), TonB-dependent receptors are also reportedly involved in iron acquisition and host infection (Liao et al., 2015). Loss of the TonB-dependent receptor TbdR1 significantly attenuates the virulence of RA in vivo (Lu et al., 2013), and our previous

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[1]Corresponding author: chenganchun@vip.163.com

studies found that the LD_{50} of Δ*B739_1343* and Δ*B739_1208* strains decreased by 10^4-fold and 10-fold compared to that of the parent strain, respectively (Wang et al., 2017; Liu et al., 2018). However, most virulence factor-deficient strains have not been evaluated as attenuated vaccines.

In our previous study, we investigated the role of RA CH-1 Fur in maintaining iron homeostasis, oxidative stress resistance and pathogenesis (Huang et al., 2021). The absence of the *fur* gene is attenuated in RA as well as other bacteria, such as *Pasteurella multocida* (Liu et al., 2019b), *Staphylococcus aureus* (Horsburgh et al., 2001), *Listeria monocytogenes* (Rea et al., 2004), and *Vibrio cholerae* (Mey et al., 2005), which raised the possibility that the attenuated *fur* mutant could serve as a candidate attenuated vaccine against pathogenic bacterial infection. Indeed, a previous study revealed that a *Salmonella enterica* serovar Typhimurium TTSS-2-deficient *fur* mutant is safe for vaccination in immunocompromised mice and is immunogenic enough to confer protection against *Salmonella* infection (Vishwakarma et al., 2012). Regarding *Edwardsiella piscicida*, Swain et al. (2020) immunized zebrafish with an arabinose-regulated *fur* strain, and the results showed that systemic immunity genes were upregulated and that vaccination protected zebrafish from wild-type challenge, suggesting that this strain could be used as an effective immersion vaccine (Swain et al., 2020). In this study, we evaluated whether RA *fur* deletion strains could be used as an attenuated vaccine.

MATERIALS AND METHODS

Strains and Growth Conditions

RA strains RA CH-1 (serotype 1) and RA CH-2 (serotype 2) and clinically isolated RA strains RCAD-0421 (serotype 7), RCAD-0424 (serotype 10), and RCAD-0454 (serotype 11) used in this study were laboratory preserved. RA CH-1 Δ*fur* was constructed in a previous study according to the natural transformation-based knockout method in which *fur* was replaced by a spectinomycin resistance gene cassette (Huang et al., 2021). For each experiment, bacteria were grown routinely on LB agar supplemented with 5% sheep blood instead of standard rabbit blood for 1 d. Then, a monoclonal bacterial colony was selected and grown overnight in TSB at 37°C with shaking (180 rpm). Next, cultures (with starting OD_{600} adjusted to 0.1) were inoculated into fresh TSB at 37°C with shaking (180 rpm) until the exponential phase (OD_{600} approximately 1−1.5).

Bacterial Load in Blood and Different Tissues

To assess bacterial population changes in ducklings after inoculation with RA CH-1 Δ*fur*, RA CH-1 Δ*fur* was grown and cultured in 50 mL of TSB (with starting OD adjusted to 0.1) at 37°C with shaking for 6 h until the exponential phase, and then, the bacteria were collected by centrifugation at 9,485 × *g* for 10 min, resuspended in 10 mL of PBS and centrifuged again. This operation was repeated 3 times to wash the bacteria. The OD_{600} values of the bacterial suspensions were measured, and the suspensions were adjusted to 5×10^9 CFU/mL (1 $OD_{600} = 2 \times 10^9$ CFU) (Liao et al., 2015). Three-day-old ducklings ($n = 30$) were infected intramuscularly in a final volume of 200 μL containing 10^9 CFU of RA CH-1 Δ*fur*. At 1-, 2-, 3-, 4-, and 12-day postinfection, 6 surviving ducklings were randomly selected and euthanized by forced CO_2 inhalation. Heart-collected blood and liver, spleen, and brain tissue were collected in sterile Whirl-Pak bags (Nasco, B01245 WA, Madison, WI), weighed and added to PBS (0.1 g of sample added to 0.9 mL of PBS). All the samples were then transferred to sterile tubes, homogenized using MP FastPrep-24 (MP Biomedicals, Santa Ana, CA), serially diluted in PBS and plated on blood agar plates supplemented with 50 μg/mL kanamycin as described previously (Liu et al., 2019a) since *R. anatipestifer* is naturally resistant to kanamycin. The plates were incubated at 37°C overnight to determine the bacterial CFU.

To assess bacterial population changes of the wild-type strain RA CH-1 in immunized ducklings, RA CH-1 was administered to ducklings starting 12 d after preinjection with RA CH-1Δ*fur* or PBS. Briefly, 5×10^9 CFU/mL RA CH-1 Δ*fur* was prepared in the same manner and injected intramuscularly into 3-day-old ducklings ($n = 36$), with each duckling receiving 200 μL containing 10^9 CFU. Ducklings inoculated with PBS ($n = 36$) were used as a negative control. After 12 d, RA CH-1 was grown and cultured in 600 mL of TSB (with starting OD adjusted to 0.1) at 37°C with shaking for 4 h until the exponential phase, and then the bacteria were collected and washed in PBS using the method described above. The OD_{600} value of the bacterial suspensions was measured and adjusted to 1.14×10^{11} CFU/mL (57 OD units of cells, 1 $OD_{600} = 2 \times 10^9$ CFU). Each duckling was injected intramuscularly with 200 μL at a dose of 2.28×10^{10} CFU (100-fold LD_{50} per duckling; the LD_{50} values were calculated based on infection of 3-day-old ducklings with RA CH-1) (Liu et al., 2018). Six surviving ducklings were randomly selected at 1-, 2-, 3-, 4-, 5-, and 10-day postinfection. Extraction and treatment of all the tissues were performed in the same manner as described above. The data are expressed as CFU per gram of tissues.

Immunoprotection Assay

The strain used for immunization was RA CH-1 Δ*fur*, and the strains used for challenge were RA CH-1, RA CH-2, and clinically isolated RA strains RCAD-0421, RCAD-0424, and RCAD-0454, respectively. For immunization, 10^9 CFU/mL RA CH-1 Δ*fur* was grown and cultured using the method described above and was injected intramuscularly into 3-day-old ducklings

($n = 23$). Untreated ducklings ($n = 23$) served as a blank control, and ducklings inoculated with PBS ($n = 23$) were used as a negative control.

Twelve days after immunization, RA CH-1, RA CH-2, and clinically isolated RA strains RCAD-0421, RCAD-0424, and RCAD-0454 were prepared for challenge. Briefly, strains were grown and cultured in 600 mL of TSB (with starting OD adjusted to 0.1) at 37°C with shaking for 4 h until the exponential phase, and the bacteria were collected and washed in PBS using the method described above. The bacterial suspensions of all strains were adjusted to 100-fold LD_{50} per duckling, as RA CH-1 was adjusted to 2.28×10^{10} CFU/duckling (Liu et al., 2018), and RA CH-2, RCAD-0421, RCAD-0424, and RCAD-0454 were adjusted to 10^{10} CFU/duckling (data not shown). Forty-six ducklings (23 ducklings immunized with RA CH-1 Δ*fur*, 23 ducklings inoculated with PBS) were injected intramuscularly with the above dose of the challenge strains, and untreated ducklings ($n = 23$, without immunization and challenge) served as a blank control. Among them, 20 ducklings in each group were used for the immunoprotection assay. The moribund ducklings were euthanized and recorded as deceased on the day of euthanasia, and euthanasia and disease onset of animals were recorded daily for 10 d to evaluate the protection rate afforded by RA CH-1 Δ*fur*. The protection rate was calculated as described by Sandhu (Sandhu, 1979), with some modifications, as follows: [1 − (%morbidity in immunization group/%morbidity in PBS group)] × 100.

Clinical Observation and Histopathological Analysis

Clinical signs such as habits, appetite, mental status, and other clinical manifestations were observed once or more per day during the period of immunization and challenge. Meanwhile, histopathological changes were also observed in this period. Animals were euthanized via forced inhalation of CO_2 12 d after immunization and 10 d after challenge, and the hearts, livers, brains, and spleens were randomly collected from 3 ducklings in each group for the immunoprotection assay. Then, the specimens were fixed in 4% paraformaldehyde, embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin (**H&E**) using previously described procedures (Wen et al., 2019). The tissues from the nonvaccinated and unchallenged ducklings served as a blank control, and ducklings inoculated with PBS served as a negative control. The pathological changes were observed using a microscope (Olympus, Tokyo, Japan).

Body Weight Measurement

The body weight changes were monitored in both experiments: i) ducklings ($n = 20$) inoculated with RA CH-1 Δ*fur*; ii) ducklings ($n = 20$) immunized with RA CH-1 Δ*fur* and then challenged with RA CH-1. In the first experiment, RA CH-1 Δ*fur* was grown and cultured in the same manner as described above. Then, a dose of 5×10^9 CFU/mL RA CH-1 Δ*fur* was prepared and inoculated into 3-day-old ducklings (10^9 CFU/duckling). Untreated ducklings ($n = 20$) and ducklings inoculated with PBS ($n = 20$) were both used as controls, and the average body weights of each group were recorded for 60 d. In the second experiment, immunization and challenge were performed in the same way as described above. The average body weights were recorded every 3 d for 12 d after immunization. After challenge with RA CH-1, the average body weights were recorded every 2 d for 10 d. Untreated ducklings ($n = 20$) and ducklings inoculated with PBS ($n = 20$) were both used as controls.

Determination of Serum Antibody Titers Via ELISA

An indirect ELISA was developed to measure the antibody titers according to a previously reported method (Liu et al., 2018). Blood samples were collected from group 1 (inoculated with RA CH-1 Δ*fur*) and group 2 (inoculated with PBS) at wk 1, wk 2, wk 3, wk 4, wk 5, wk 6, and wk 7, and the sera were prepared by centrifugation (10 min, 6,775 × *g*). For antigen preparation, RA strains RA CH-1 (serotype 1) and RA CH-2 (serotype 2) and clinically isolated RA strains RCAD-0421 (serotype 7), RCAD-0424 (serotype 10), and RCAD-0454 (serotype 11) were grown and cultured in 200 mL of TSB medium to the exponential phase, harvested via centrifugation (10 min, 9,485 × *g*) and washed twice with PBS. The bacterial pellets were resuspended in 25 mL of lysis buffer (20 mM Tris−HCl, pH 7.4, 10 mM EDTA, 1 mM TLCK) and lysed using a French press as described previously (Liu et al., 2018). Cellular debris was removed, and the protein concentration of the lysates was determined using a BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Then, 96-well ELISA plates were coated with the RA CH-1, RA CH-2, RCAD-0421, RCAD-0424, and RCAD-0454 lysate antigens in bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. The plates were then washed 3 times with PBS containing 0.1% Tween-20 (PBST) and blocked with 1% BSA in PBST at 37°C for 1 h. All antigens were prepared in duplicate. After blocking, serially diluted duck serum from group 1 (experimental group) and group 2 (control group) was added to the wells, followed by incubation at 37°C for 2 h. Thereafter, the plates were washed 3 times with PBST, and horseradish peroxidase (**HRP**)-conjugated goat antiduck IgY (ab112771, Abcam) was added. The plates were then incubated at 37°C for 1 h and washed 3 times with PBST, and TMB substrate solution (TIANGEN, Beijing, China) was added to each well. The reaction was stopped by adding H_2SO_4, and the plates were read at 450 nm using a microplate reader (Bio-Rad, Hercules, CA). The ratio of each test sample (experimental group OD_{450}: control group OD_{450}) was calculated. The highest dilutions of the sera with an OD_{450} value 2.1 times that of the control wells were used as the ELISA titers. The experiment

was performed using 3 independent experiments with 3 replicate samples for each experiment.

Ethical Approval

The animal study was reviewed and approved by the local animal welfare bodies and the Sichuan Agricultural University ethics committee. One-day-old ducklings were purchased from Grimaud farms in Chengdu (Sichuan, China) and housed at our animal facilities with free access to food and water. Animals were euthanized if a predetermined level of distress was reached before natural death, and pathological examination and tissue sampling were also performed after euthanasia.

Statistical Analysis

All experimental data are expressed as the mean ± standard deviation (**SD**). An independent Student *t* test was utilized to compare 2 groups, and one-way analysis of variance (**ANOVA**) was used to compare multiple groups. Comparisons were made between groups using the log-rank test with GraphPad Prism 8.00 (GraphPad Software, La Jolla, CA). A P value <0.05 was considered significant.

RESULTS

Propagation Dynamics of RA CH-1 Δfur in Ducklings

In our previous study, we demonstrated that the colonization ability of RA CH-1 Δ*fur* in ducklings was greatly diminished (Huang et al., 2021). To further investigate whether the attenuated strain RA CH-1 Δ*fur* could remain in ducklings, the bacterial loads in duckling organs (heart-collected blood, liver, spleen, and brain) were determined at 1 d, 2 d, 3 d, 4 d, and 12 d after injection with RA CH-1 Δ*fur* at a dose of 10^9 CFU per duckling. As shown in Figure 1, the number of recovered colonies ranged between 10^4 and 10^6 CFU per gram from the heart-collected blood, liver, brain, and spleen for RA CH-1 Δ*fur* at 1-day postinoculation. Then, at 2-day postinoculation, the amount of colonized RA CH-1 Δ*fur* in the liver, brain, and spleen decreased to 10^3 CFU/g and decreased to 10^2 CFU/g in heart-collected blood samples (Figure 1). At 3-day postinoculation, the number of detectable colonies in the heart-collected blood, liver, and spleen was significantly decreased, almost decreasing to 10 CFU/g, and no bacterial load was detected in brain tissues even in the undiluted tissue homogenates, which means it was below the limit of detection (Figure 1). After 4 d and 12 d, no viable bacteria were detected in the blood, liver, spleen, or brain under our assay conditions, and they were all below the assay limit of detection (Figure 1). These data suggested that the colonization ability of RA CH-1 Δ*fur* at a dose of 10^9 CFU in ducklings was significantly weakened, and the residual amount of RA CH-1 Δ*fur* in ducklings was very low during our assay.

The Inoculation of RA CH-1 Δfur Causes Neither Pathological Damage Nor Growth Defects in Ducklings

As an attenuated vaccine, RA CH-1 Δ*fur* should not affect the health of the host. First, the ducklings were observed daily for clinical signs after inoculation with 10^9 CFU of RA CH-1 Δ*fur*, and histological examination was also performed to validate the clinical data. Ducklings without any treatment and PBS-inoculated ducklings were used as the controls. As shown in Figure 2A, compared with the control ducklings, pathological results showed that there were no abnormal pathological

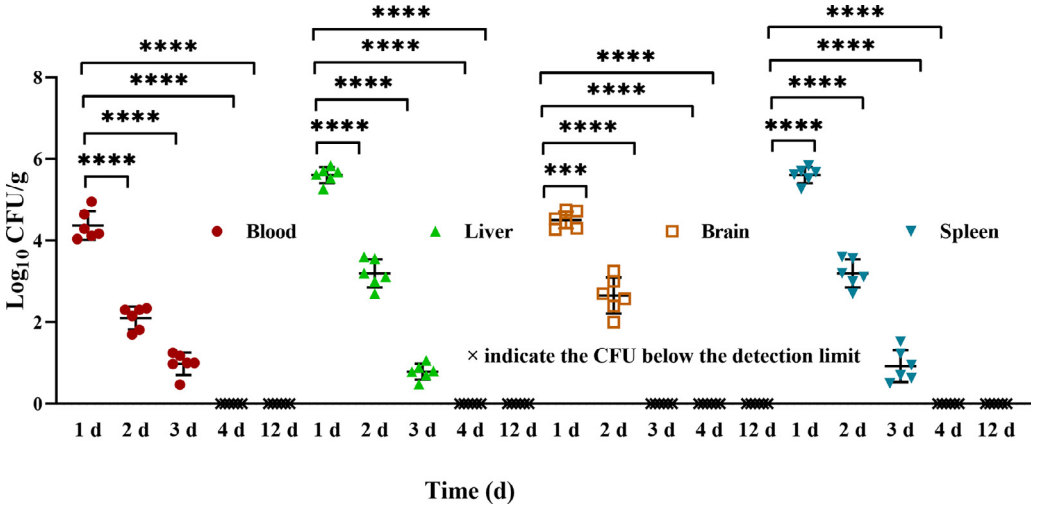

Figure 1. Colonization of RA CH-1 Δ*fur* in ducklings at 1-, 2-, 3-, 4-, and 12-day postinoculation. Doses of 10^9 CFU of RA CH-1 Δ*fur* were prepared and injected intramuscularly into 3-day-old ducklings. At 1-, 2-, 3-, 4-, and 12-day postinfection, the bacterial loads in the livers, spleens, brains, and blood from the hearts were recorded. Six ducklings at each time point were euthanized for these assays. The data points represent the CFU/g values for the indicated organs in individual ducklings. × indicates CFU below the detection limit. The error bars show the standard error for the average of the CFU/g values ($n = 6$) at each time point.

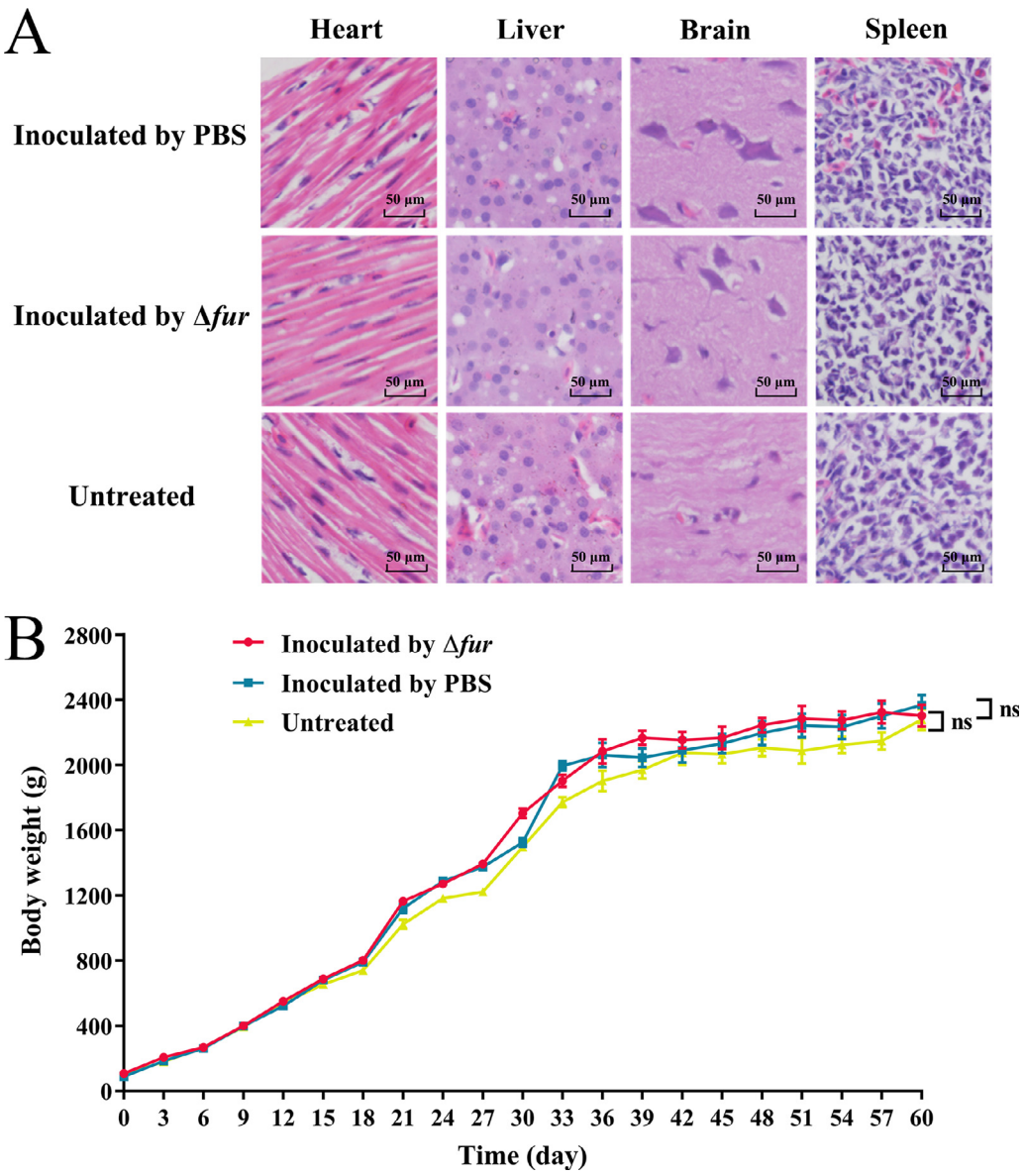

Figure 2. Pathological changes and average body weights in RA CH-1 Δ*fur*-inoculated ducklings before challenge. Three-day-old ducklings were inoculated with 10^9 CFU of RA CH-1 Δ*fur* or PBS, and untreated ducklings were used as blank controls. (A) The hearts, livers, brains, and spleens were randomly collected from 3 ducklings in each group on d 12, and H&E staining was performed on paraffin sections of these tissues. All images represent serial sections (scale bar, 50 μm). (B) From d 0 to d 60, the average body weights of ducklings in each group were recorded every 3 d. The error bars show the standard error for the average of the body weights ($n = 20$).

changes in heart, liver, spleen, or brain paraffin sections in RA CH-1 Δ*fur*-inoculated ducklings.

To determine whether inoculation with RA CH-1 Δ*fur* has an effect on the growth of ducklings, we tested the body weight changes of the ducklings within 60 d after inoculation with 10^9 CFU of RA CH-1 Δ*fur*. Before starting the experiment, the initial average body weight of each group was calculated, and ducklings were weighed every 3 d during the experiment. Uninoculated ducklings were used as a blank control, and ducklings inoculated with PBS were used as a negative control. As shown in the curves for average body weight in Figure 2B, the body weights did not differ among groups prior to the initiation of the experiment, and there was no significant difference in body weights between the ducklings in each group during the whole experimental stage, demonstrating that inoculation with 10^9 CFU of Δ*fur* had no effect on body weight growth in ducklings.

Immunization With RA CH-1 Δfur *Provides Protection Against Challenge With the Parent Strain*

Next, we further evaluated whether inoculation with RA CH-1 Δ*fur* was able to protect against the highly virulent parent strain RA CH-1. First, the clinical symptoms and pathological changes of ducklings were observed after challenge. Compared to unchallenged ducklings, ducklings preinoculated with RA CH-1 Δ*fur* did not show any symptoms postchallenge. However, challenged ducklings preinoculated with PBS exhibited

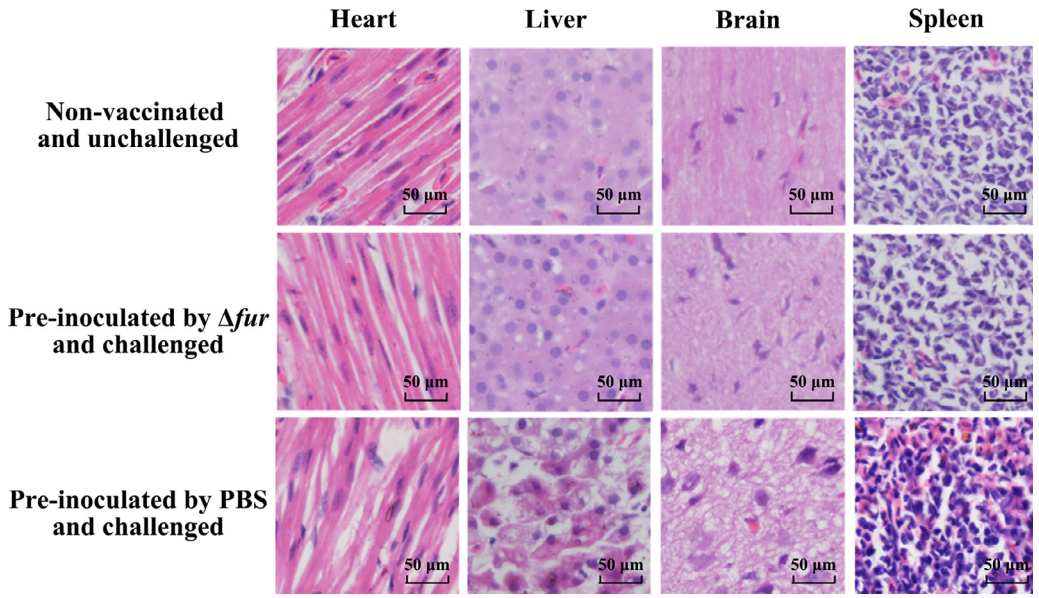

Figure 3. Pathological changes in ducklings after challenge with RA CH-1. Three-day-old ducklings were inoculated with 10^9 CFU of RA CH-1 Δ*fur* or PBS and challenged with RA CH-1 after 12 d. Nonvaccinated and unchallenged ducklings were used as controls. Ten days after the challenge, 3 ducklings from each group were euthanized, and the hearts, livers, brains, and spleens were randomly collected. H&E staining was performed on the paraffin sections of these tissues, and all images represent serial sections (scale bar, 50 μm).

a series of symptoms, such as tiredness/low energy, poor appetite, opisthotonos, and even death. After dissecting PBS-inoculated ducklings, we revealed that serosal surfaces harbored fibrinous pericarditis and exudate, the brain showed congestion and dilation of blood vessels, and the liver and spleen were moderately enlarged and appeared congested, with hemorrhagic foci (Supplementary Figure S1). Simultaneously, pathological section results in these tissues showed that myocardial fibers were broken, liver and spleen pathological changes were mainly revealed as cellular swelling/necrosis and inflammatory cell infiltration, and numerous vacuolated spaces and macrophages appeared in brain tissues (Figure 3). It is worth mentioning that neither gross lesions nor pathological lesions were observed on almost all collected organs in RA CH-1 Δ*fur*-inoculated ducklings after challenge, and only minor lesions were observed in brain tissue sections (Figure 3).

Furthermore, the average body weights of challenged ducklings preinoculated with RA CH-1 Δ*fur* were not significantly different from those of unchallenged ducklings (Figure 4), and due to severe disease of the ducklings inoculated with PBS and challenged with RA CH-1, we were unable to obtain valid body weight measurements in this group (data not shown). Finally, the surviving lesion-negative ducklings were used for the calculation of the protection rate. For ducklings preinoculated with PBS, the mortality and morbidity were 85 and 90%, respectively, after challenge (Table 1), and these ducklings almost all died within 1 to 4 d. Conversely, the mortality of ducklings preinoculated with RA CH-1 Δ*fur* was 0%, and the morbidity was 10%

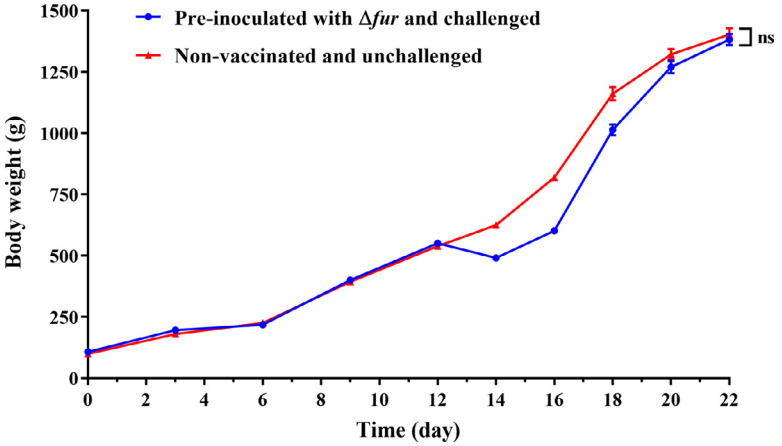

Figure 4. The average body weights of challenged ducklings. Three-day-old ducklings were inoculated with RA CH-1 Δ*fur*, and the average body weights of the ducklings were recorded every 3 d for 12 d. On d 12, vaccinated ducklings were challenged with RA CH-1, and the average body weights were recorded every 2 d for 10 d. Nonvaccinated and unchallenged ducklings were used as controls. The error bars show the standard error for the average of the body weights ($n = 20$) on each day.

Table 1. Animal challenge experiment with RA CH-1.

Group	Immunization	Challenge strain[1]	No. of deaths (total)	No. showing morbidity (total)	Mortality (%)	Morbidity (%)	Protection[2] (%)
1	PBS	RA CH-1	17 (20)	18 (20)	85%	90%	-
2	RA CH-1 Δ*fur*	RA CH-1	0 (20)	2 (20)	0%	10%	89%
3	-	-	0 (20)	0 (20)	0	0	-

[1]The challenge strain dose was 2.28×10^{10} CFU.
[2]The protection rate was calculated as [1 − (% morbidity in group 2/% morbidity in group 1)] × 100.

(Table 1). The calculation of the morbidity of ducklings preinoculated with PBS and RA CH-1 Δ*fur* showed that vaccinated ducklings were 89% protected from the challenge. These results indicated that the attenuated RA CH-1 Δ*fur* elicited excellent protection against RA CH-1 in the host, and neither pathological damage nor weight loss was observed at the same time.

The Colonization Ability of RA CH-1 Was Decreased in Vaccinated Ducklings

Since vaccinated ducklings were protected against challenge with the parent strain, it was hypothesized that the colonization ability of RA CH-1 was decreased in vaccinated ducklings. To test this hypothesis, 6 ducklings that were preinoculated with RA CH-1 Δ*fur* or PBS were randomly chosen for challenge. Then, the ducklings were euthanized, and the tissues were collected at different stages for bacterial colonization analysis. After challenge, compared with that in PBS-inoculated ducklings, the number of colonies of RA CH-1 in RA CH-1 Δ*fur*-inoculated ducklings were significantly decreased (Figure 5). The bacterial loads of RA CH-1 in the heart-collected blood, liver, spleen and brain of vaccinated ducklings were almost eliminated at 10 d (Supplementary Figure S2). These results revealed that ducklings preinoculated with RA CH-1 Δ*fur* had a

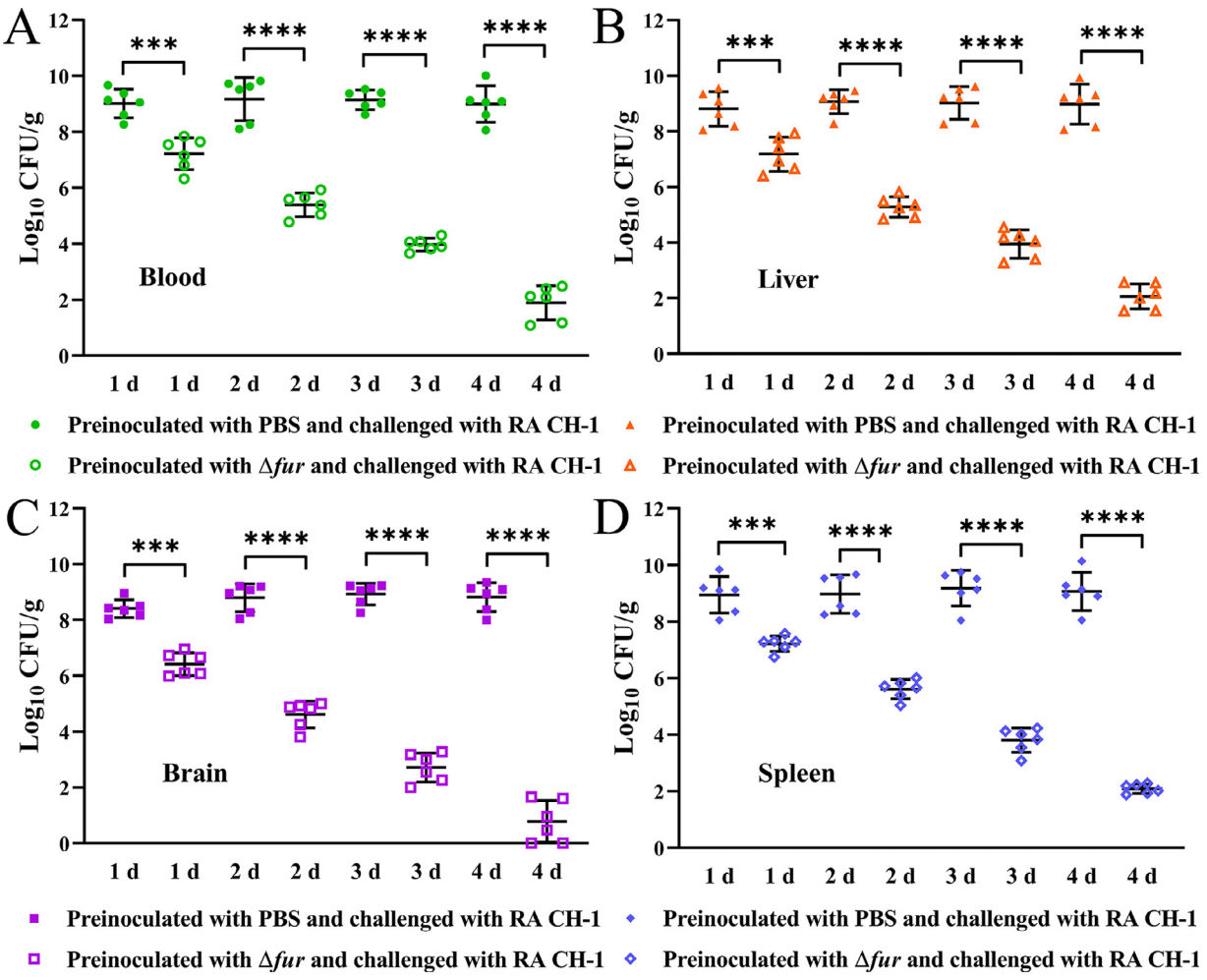

Figure 5. Colonization of RA CH-1 in ducklings preinoculated with RA CH-1 Δ*fur* or PBS. Three-day-old ducklings were inoculated with 10^9 CFU of RA CH-1 Δ*fur* or PBS and challenged with 2.28×10^{10} CFU of RA CH-1 after 12 d. Bacteria were isolated from the livers, brains, spleens, and heart-collected blood of ducklings that were preinoculated with PBS (solid symbols) and RA CH-1 Δ*fur* (hollow symbols) at various time points after challenge. The data points represent the CFU/g values for the indicated organs in individual animals, and the error bars show the standard error for the average of the CFU/g values ($n = 6$) at each time point. Differences were assessed for statistical significance with one-way analysis of variance (ANOVA).

stronger scavenging ability for RA CH-1 and that vaccinated ducklings essentially eliminated RA CH-1 infection, which resulted in an increase in the survival rate when challenged with RA CH-1.

Determination of Serum Antibody Levels of RA CH-1 Δfur

To examine whether the protection observed in RA CH-1 Δ*fur*-vaccinated ducklings was due to an antibody response, serum IgY antibody titers against RA CH-1 antigen were determined by indirect ELISA as described previously (Liu et al., 2018). To assess the durability of the responses, antibody titers were assessed at wk 1, 2, 3, 4, 5, 6, and 7 following vaccination. As shown in Figure 6, antibody titers were detected at wk 1 postvaccination, the levels increased throughout the whole vaccination period, and the high titers of antibodies were maintained for at least 7 wk. These results indicated that vaccinated ducklings developed specific humoral immunity, which was detectable for at least 7 wk after vaccination.

Immunization With RA CH-1 Δfur *Was not Effective Against Other Serotypes of RA*

We next determined whether vaccination with RA CH-1 Δ*fur* would provide cross-protection against other serotypes of RA. Ducklings were preinoculated with 10^9 CFU of RA CH-1 Δ*fur* or PBS and challenged with 10^{10} CFU of RA CH-2 (serotype 2), RCAD-0421 (serotype 7), RCAD-0424 (serotype 10), or RCAD-0454 (serotype 11) after 12 d. Ten days after challenge, the morbidity and mortality of the vaccinated ducklings and PBS-inoculated ducklings were recorded. As shown in Table 2, after challenge with RA CH-2 and RCAD-0454, the mortality and morbidity of vaccinated ducklings and PBS-inoculated ducklings were both 100%, suggesting that vaccination with RA CH-1 Δ*fur* does not provide protection against RA CH-2 and RCAD-0454. Furthermore, 10.53 and 11.11% protection were observed in vaccinated ducklings challenged with RCAD-0421 and RCAD-0424, respectively. Meanwhile, the lysates of RA CH-2, RCAD-0421, RCAD-0424, and RCAD-0454 were used as the coating antigen for detecting the IgY antibody titers in the serum of vaccinated ducklings and the control ducklings inoculated with PBS. The results showed that the antibody titers against RA CH-2, RCAD-0421, RCAD-0424, and RCAD-0454 were lower than those against RA CH-1 (data not shown). These findings suggest that vaccination with RA CH-1 Δ*fur* did not provide sufficient protection in response to other serotypes of RA, which was potentially related to the weak antibody responses elicited by these antigens.

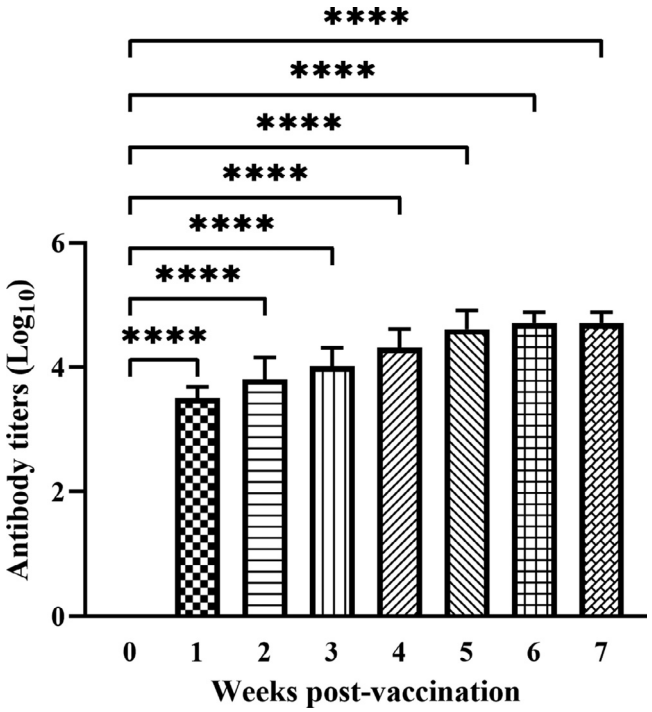

Figure 6. Serum antibody titers of ducklings prevaccinated with RA CH-1 Δ*fur* against RA CH-1. Serum was collected from the vaccinated ducklings at 1-wk intervals from wk 1 to wk 7 postvaccination, and serum antibodies against RA CH-1 were quantified using an indirect ELISA. The ratio of each test sample (experimental group OD_{450}: control group OD_{450}) was calculated. The highest dilutions of the sera with an OD_{450} value 2.1 times that of control wells were recorded and graphed. The error bars show the standard error for the average of the antibody titers at each time point.

DISCUSSION

Once bacterial diseases emerge, the application of a large quantity of antibiotics is the main method to decrease massive economic losses, but their massive use also causes the development of antibiotic-resistant bacteria, which is one of the most critical treatments for public health. Currently, vaccination is considered the most ideal method of controlling bacterial disease. Specifically, live bacterial vaccines may be an eligible substitution for antibiotics due to their advantages of inducing longer and more intense immune responses and being stable and affordable (Frey, 2007). RA, a pathogen that has become a threat to the duck industry of China, is known to be resistant to diverse antibiotics and without cross-protection between serotypes (Pathanasophon et al., 1995, 2002). Precise prevention and control of RA may be achieved through the development of live bacterial vaccines. In recent years, a large number of virulence factors of RA have been confirmed (Chang et al., 1998; Crasta et al., 2002; Zou et al., 2015; Zhao et al., 2016; Wang et al., 2017; Hu et al., 2011), and as such, an attenuated strain has the potential to be a vaccine. Furthermore, the mutation of global regulators may be preferable over single virulence factors for targeting live attenuated vaccines because global regulators control a cascade of genes that are related to bacterial virulence, which may improve safety and immunogenicity (Miller et al., 1993; Asensio et al., 2008; Zhao et al., 2016).

Fur is a global regulatory protein that is responsible for a diversity of cellular functions. Fur controls the transcription of many genes involved in iron metabolism, oxidative stress resistance, metabolic processes and

Table 2. Animal challenge experiment with RA CH-2, RCAD-0421, RCAD-0424, and RCAD-0454.

Group	Immunization	Challenge strain[1]	No. of deaths (total)	No. showing morbidity (total)	Mortality (%)	Morbidity (%)	Protection[2] (%)
1	PBS	RA CH-2	20 (20)	20 (20)	100%	100%	-
2	RA CH-1 Δ*fur*	RA CH-2	20 (20)	20 (20)	100%	100%	0%
3	PBS	RCAD-0421	18 (20)	19 (20)	90%	95%	-
4	RA CH-1 Δ*fur*	RCAD-0421	15 (20)	17 (20)	75%	85%	10.53%
5	PBS	RCAD-0424	17 (20)	18 (20)	85%	90%	-
6	RA CH-1 Δ*fur*	RCAD-0424	14 (20)	16 (20)	70%	80%	11.11%
7	PBS	RCAD-0454	20 (20)	20 (20)	100%	100%	-
8	RA CH-1 Δ*fur*	RCAD-0454	20 (20)	20 (20)	100%	100%	0%
9	-	-	0 (20)	0 (20)	0	0	-

[1]The challenge strain dose was 1×10^{10} CFU.
[2]The protection rate was calculated as [1 − (% morbidity in vaccinated group/% morbidity in control group)] × 100.

virulence, suggesting a key role for this protein in pathogenic bacteria (Hall and Foster, 1996; Hassett et al., 1996; Oglesby et al., 2005; Troxell and Hassan, 2013; Fillat, 2014; Husain et al., 2014; Huang et al., 2021). Although the precise mechanism for the observed attenuation of *fur* mutants is not clear, evidence indicates that the loss of *fur* may result in a reduction in the activity of enzymes required for protection against ROS, which are products of the host, and Fur controls the expression of some virulence factors or controls the activity of enzymes related to bacterial catabolism, which may contribute to a decrease in virulence (Bury-Mone et al., 2004; Harrison et al., 2013; Husain et al., 2014; Huang et al., 2021). The above properties suitably explain why the *fur* mutant could be used as an attenuated vaccine. In *Pseudomonas fluorescens* strain TSS, the *fur* deletion strain was found to be used as a live vaccine to protect fish against infections by *P. fluorescens* and *Aeromonas hydrophila* (Wang et al., 2009). In *Salmonella enterica* serovar Typhimurium, an SPI-2-deficient *fur* mutant was proven to be safe for vaccination and to provide sufficient immunogenicity to confer protection against *Salmonella* infection (Vishwakarma et al., 2012). In addition, a *fur* mutant of *Edwardsiella ictaluri* was attenuated and could trigger systemic immunity in zebrafish (*Danio rerio*) and catfish (*Ictalurus punctatus*), conferring immune protection against *Edwardsiella ictaluri* infection (Santander et al., 2012).

In RA, Fur is involved in iron homeostasis and oxidative stress resistance, and mutation of the *fur* gene significantly decreases virulence (Guo et al., 2017; Huang et al., 2021). In this study, the safety and efficacy of RA CH-1 Δ*fur* as a live attenuated vaccine against RA infection were evaluated in ducklings. First, the colonization of RA CH-1 Δ*fur* in ducks was detected. As shown in Figure 1, within 4 d after infection, the number of bacterial clones detected in ducks decreased gradually every day, and no viable bacteria were detected after d 4 (Figure 1). Simultaneously, ducklings inoculated with RA CH-1 Δ*fur* did not show any clinical signs or significant lesions, and the growth of the ducklings was not affected before challenge. These results confirmed that RA CH-1 Δ*fur* is safe as an attenuated vaccine. Subsequently, we explored the immunoprotective efficacy of RA CH-1 Δ*fur* and found that inoculation with RA CH-1 Δ*fur* provided 89% protection against infection with the parent strain RA CH-1 (Table 1). Compared with ducklings preinoculated with PBS, the vaccinated ducklings did not show any clinical symptoms or severe pathological changes and grew well after challenge (Figures 3 and 4, Supplementary Figure S1). To explore the reason underlying this result, the colonization ability of RA CH-1 was examined in challenged ducklings that were preinoculated with RA CH-1 Δ*fur* or PBS. As Figure 5 shows the colonization ability of RA CH-1 indeed declined in vaccinated ducklings. Furthermore, inoculation with RA CH-1 Δ*fur* was found to produce a high level of IgY titers in the serum through 7 wk when RA CH-1 was used as an antigen (Figure 6), which means that short-term colonization of RA CH-1 Δ*fur* in ducklings induced a long-term immune response and that the immune memory elicited by RA CH-1 Δ*fur* persisted and provided long-lasting protection against challenge with the parent strain. However, RA CH-1 Δ*fur* was unable to provide cross-protection to other serotypes, such as RA CH-2, RCAD-0421, RCAD-0424, and RCAD-0454 (Table 2), and when they were used as the coating antigen, the IgY antibody titers in the serum of vaccinated ducklings were lower than those for RA CH-1 in the whole analysis (data not shown). These results revealed that RA CH-1 Δ*fur* could provide effective protection against RA CH-1 but could not protect against other serotypes of RA, such as RA CH-2, RCAD-0421, RCAD-0424, and RCAD-0454.

In summary, inoculation with RA CH-1 Δ*fur* is safe for ducklings and could elicit an intense immune response, which suggests that it could be used as a live attenuated vaccine for protecting ducklings against RA, especially RA CH-1. However, immunization with RA CH-1 Δ*fur* did not provide cross protection against challenge with other serotypes of RA. The main reason for this is that there is no cross-protection between *R. anatipestifer* serotypes (Sandhu and Leister, 1991; Loh et al., 1992; Pathanasophon et al., 1995). To provide effective protection against *R. anatipestifer*, existing inactivated vaccines that can achieve this effect are made by combining multiple inactivated serotype strains of RA (Liu et al., 2013). Therefore, further experiments are needed to assess whether the *fur*-deficient strains in other serotypes could be used as vaccines. In this way, multivalent vaccines will help to achieve better cross-protection. Moreover, the addition of adjuvants to vaccines may also improve protection. For all the above reasons, we believe that Δ*fur* strains can be used as a monovalent or

multivalent vaccine against various pathogens, at least against RA, which will be a focus of research on live attenuated vaccines in the future.

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DISCLOSURES

The authors declare that they have no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102450.

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