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A novel subunit vaccine based on Fiber1/2 knob domain provides full protection against fowl adenovirus serotype 4 and induces stronger immune responses than a Fiber2 subunit vaccine

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ABSTRACT Outbreaks of hepatitis-hydropericardium syndrome (**HHS**) caused by fowl adenovirus serotype 4 (**FAdV-4**) have resulted in huge economic losses to the poultry industry in China since 2015. However, commercially available vaccines against the FAdV-4 infection remain scarce. In our study, subunit vaccine candidates derived from the bacterially expressed recombinant Fiber1 knob domain and Fiber2 knob domain fusion protein (termed as Fiber1/2 knob subunit vaccine) and Fiber2 protein (termed as Fiber2 subunit vaccine) of the FAdV-4 SDSX strain were developed. Immunogenicity evaluation showed that the Fiber1/2 knob subunit vaccine induced the production of antibodies at 7 d postvaccination (**dpv**), earlier than the Fiber2 subunit vaccine. Moreover, the neutralizing antibody level of the Fiber1/2 subunit vaccine group was higher than the Fiber2 subunit vaccine group, showing significant differences at 14, 21, and 28 dpv. Immune protection test results revealed that both Fiber1/2 knob subunit and Fiber2 subunit vaccines could protect chickens from death against FAdV-4 challenge, although the weight of chickens in the Fiber1/2 knob subunit vaccine group decreased less. Furthermore, analysis of plasma Glutamic oxaloacetic transaminase (**AST**) and blood glutamic pyruvic transaminase (**ALT**) levels suggested that the Fiber1/2 subunit vaccine can significantly inhibit liver damage caused by FAdV-4 infection and is more effective in blocking the pathogenicity of FAdV-4 in target organs. In addition, the Fiber1/2 knob subunit vaccine further reduced the viral load in different tissues and virus shedding in chickens than the Fiber2 subunit vaccine. Overall, the Fiber1/2 knob subunit vaccine was more effective than the Fiber2 subunit vaccine. These findings lay the foundation for the development of more effective FAdV-4 subunit vaccines.

Key words: FAdV-4, Fiber1 knob domain, Fiber2 knob domain, subunit vaccine, efficacy

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INTRODUCTION

Hepatitis hydropericardium syndrome (**HHS**), caused by fowl adenovirus 4 (**FAdV-4**), resulted in huge economic losses to the poultry industry worldwide (Li et al., 2017; Del Valle et al.,2020; Chitradevi et al., 2021; Mete et al.,2021; Lai et al.,2021). It was first reported in 1987 in the Ankara region near Karachi, Pakistan; therefore, it is also known as Ankara disease (Anjum et al., 1989). Later, the disease spread and epidemics of HHS appeared in many countries, including Russia, Japan, America, and South Korea (Schachner et al., 2018). FAdV-4 can be transmitted horizontally through fecal-oral route, and can also be transmitted vertically. FAdV-4 causes sudden onset of infection and death in a large number of chickens. The main pathological changes in infected chickens include pericardial effusion and hepatitis (Sun et al., 2019). In addition, the immune system and lymphoid tissues can be damaged, resulting in infection by various pathogens (Niu et al., 2019). In China, the FAdV-4 strain was first isolated from chickens with HHS in 2015 and since then has caused huge economic losses to the Chinese poultry industry (Zhao et al., 2015; Li et al., 2016; Ye et al., 2016).

Fowl adenoviruses (**FAdVs**) are nonenveloped viruses with a double-stranded DNA genome and a diameter of 70 to 90 nm, and belong to the genus *Aviadenovirus* and family *Adenoviridae* (De Luca et al., 2020). Based on restriction enzyme digestion patterns and serum cross-neutralization tests, FAdVs were divided into 5 species (FAdV-A to FAdV-E) and 12

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serotypes (FAdV-1 to 8a and 8b to 11). The capsid of the virus is mainly composed of 3 proteins: Hexon, Fiber, and Penton. All of FAdVs possess 2 Fibers pre Penton base, but the difference is that FAdV-1, FAdV-4 and FAdV-10 have 2 Fiber-encoding genes and only one Fiber-encoding gene in FAdV-B, FAdV-D and FAdV-E. (Hess, 2000; Hess, 2013). Fibers are thought to be a bridge between FAdV and host cells, which promote viral infection by attaching the viral capsid to the host cell surface and interacting with cellular receptors. For FAdV-4, the Fiber1 is closely related to viral replication and assembly, while Fiber2 interacts with the host nuclear protein α3 KPNA3/4 through its N terminal (Xie et al., 2021). Previous studies have revealed that both Fiber1 and Fiber2 of FAdV-4 are immunogenic and protect chickens against FAdV-4 infection. Fiber2 can provide better protection against FAdV-4 infection and induce rapid proliferation of CD4+ T cells (Chen et al., 2018). Fiber1 and Fiber2 of FAdV-4 are composed of a tail, stem, and knob from the N-terminus to the C-terminus. The knob domain is the functional region of the Fiber protein and is an epitope-enriched region that can induce a strong immune response in the body (Wang et al., 2020; Song et al., 2023).

Subunit vaccines have excellent immunogenicity and can avoid the risk of incomplete inactivation associated with whole-virus vaccines. It is a good choice for controlling HHS as numerous studies have demonstrated that the subunit vaccine provides protection against virulent FAdV-4 challenge (Ruan et al., 2018; Yin et al., 2021). However, current research on subunit vaccines mainly focuses on the Fiber2 protein of FAdV-4, and the protection of subunit vaccines based on the knob domain of Fiber1 and Fiber2 has not yet been studied.

In this study, the knob domains of Fiber1 and Fiber2 were fused using a flexible linker expressed in *E. coli* and prepared as a subunit vaccine (Fiber1/2 knob subunit vaccine). In the vaccination trial, the immune efficacy induced by the Fiber1/2 knob subunit vaccine was assessed and compared with a Fiber2 subunit vaccine by challenging with a virulent FAdV-4 strain in SPF chickens. Immune efficacies of both Fiber1/2 knob subunit and Fiber2 subunit vaccines were evaluated to determine whether the Fiber1/2 knob subunit vaccine provided superior protection against FAdV-4 infection. These findings would lay the foundation for the development of more effective vaccines against FAdV-4.

MATERIALS AND METHODS

Animals and Ethical Statement

Specific pathogen-free (**SPF**) chickens were purchased from Ringpu Biopharmaceutical Co., Ltd. (Baoding, China). The experiments were approved by the Animal Welfare and Ethics Committee of the Laboratory Animal Center of Hebei Agriculture University.

Cells, Viruses, Plasmids, and Antibodies

Leghorn male hepatoma (**LMH**) cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (1:1) (Gibco, NY) containing 10% premium fetal bovine serum (Aoqing, Beijing, China) at 37°C with 5% CO_2. The FAdV-4 strain SDSX1 (GenBank accession no. KY636400) was isolated, stored in our laboratory, and propagated in LMH cells. The plasmid pCold I, maintained in our laboratory, and used as a vector to efficiently expressed the Fiber1/2 knob and Fiber2 protein. A polyclonal antibody against Fiber2 of FAdV-4 were stored in our laboratory.

Expression and Purification of Fiber1/2 Knob

The optimized coding sequence of GSS + Fiber1 knob and Fiber2 knob were connected by a flexible linker (4*GGS) in the middle, which was synthesized and cloned into pCold I vector (pCold-Fiber1/2-knob). The coding sequence of the full length Fiber2 was also cloned into pCold I (pCold-Fiber2). Subsequently, pCold-Fiber1/2-knob and pCold-Fiber2 were transferred into *Escherichia coli* BL21 separately for expression of 2 fusion recombinant proteins as previously described (He et al., 2018). The protein was purified using Ni-agarose resin (Kangwei, Beijing, China) as previously described (Zhao et al., 2023). The subunit vaccines were prepared by emulsifying recombinant proteins with Freund's complete adjuvant at a volume ratio 1:1 which the protein content was calculated as 100 μg/mL.

Western Blot

Each of the purified proteins were combined with 5 × sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**SDS-PAGE**) sample loading buffer (Beyotime), boiled for 10 min, and separated via 10% SDS-PAGE. Subsequently, the samples were transferred onto polyvinylidene difluoride (**PVDF**) membranes and then blocked with 5% skim milk for 2 h at room temperature. After blocking, the membranes were incubated with mouse anti-His mAb (Biodragon, Suzhou, China) for 2 h at room temperature, washed with PBST 3 times, and subsequently incubated with horseradish peroxidase (**HRP**)-conjugated goat anti-mouse IgG (Biodragon, Suzhou, China) for 1 h at room temperature. Protein bands were detected using an ECL chemiluminescence system.

Immunization and Challenge Studies

Sixty 7-d-old SPF chickens were randomly divided into 3 test groups (n = 15 each) and a control group (n = 15). The 4 groups were then separated into different isolators. Chickens in group 1 (Fiber1/2 knob subunit vaccine), group 2 (Fiber2 subunit vaccine), and group 3 (**DMEM**) were vaccinated intramuscularly on d 7 and 21 with 0.2 mL of the corresponding vaccine or

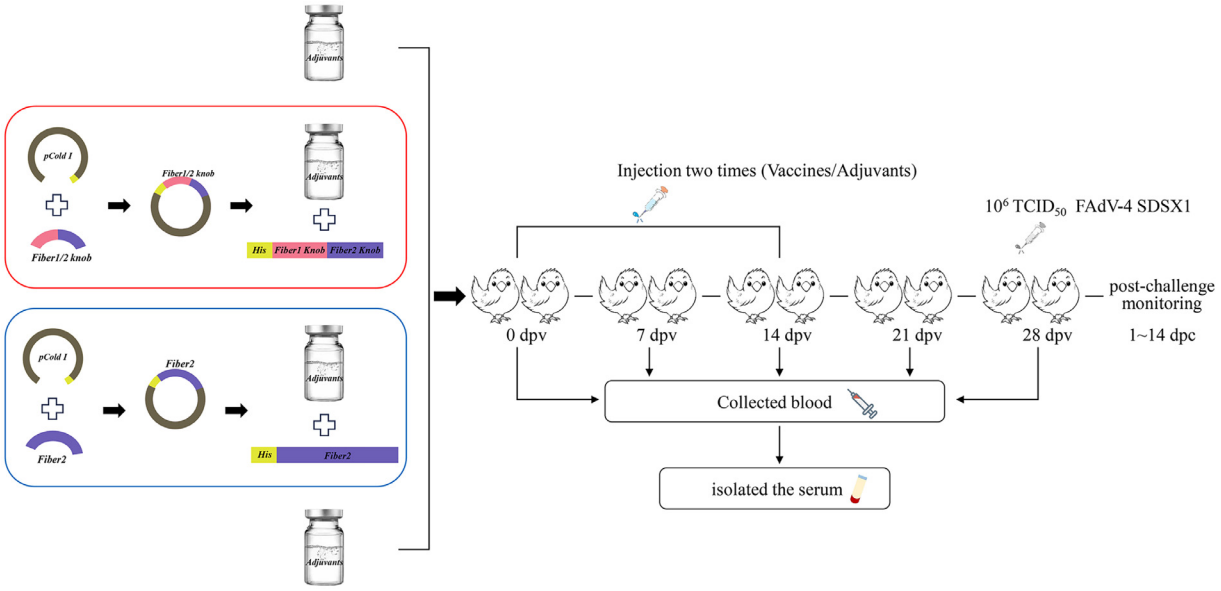

Figure 1. Experimental design of immunization and challenge studies. The purified protein was emulsified as shown on the left with adjuvant for the immunization test. Different procedures (vaccination, booster, and challenge) carried out on the chickens are indicated on the timelines (days postvaccination [**dpv**] and days post challenge [**dpc**]). Individual treatment schemes of each group are specified below. Arrows indicate blood sampling; syringes indicate vaccination and challenge.

DMEM. Serum samples from 15 chickens per group were collected at 7-, 14-, 21- and 28-d postvaccination (**dpv**) to detect specific neutralizing antibodies. At 28 dpv, chickens in test group 1 to 3 were challenged with 0.5 mL FAdV-4 SDSX strain (10^6 $TCID_{50}$). A flowchart of the immune protection experiment is shown in Figure 1.

Clinical Observation and Evaluation of Protective Efficacy

After the challenge, the clinical symptoms, mortality, and weight changes of all chickens in the different groups were monitored. If any chicken died during the experiment, they were immediately dissected. Blood samples were collected at 3-, 5-, 7-, and 10-d postchallenge (**dpc**) which were used for liver function tests. Cloacal swabs collected at 1, 3, 5, 7, 10, 14, and 21 dpc were used for the subsequent detection of shedding by qPCR according to a previous study (Wang et al., 2017). In addition, 3 chickens from each group were randomly selected for euthanasia and autopsy at 3, 5, 7, and 10 dpc. The hearts, livers, spleens, lungs, and kidneys were collected and weighed. Organ weight/body weight ratios were calculated. For each organ, one part was fixed in 4% paraformaldehyde for pathological analysis, and the other part was used for nucleic acid extraction to detect the tissue viral load by qPCR.

Serum Biochemical Parameter Analysis

The serum samples collected at each corresponding time after centrifugation (15 min, 3,000 rpm) were stored at -20°C, which were used to detect and record the changes in biochemical parameters. Biochemistry parameters (aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**)) were measured by an automatic serum chemistry analyzer (Chemray 420; Rayto, Shenzhen, China) according to the manufacturer's protocols. The damage caused by FAdV-4 could lead to changes in biochemical parameters.

DNA Extraction and Quantitative Real-Time PCR

Viral DNA was extracted from the tissues and cloacal swabs using a DNA extraction kit according to the manufacturer's instructions (TransGen Biotech, Beijing, China). The viral copy numbers in different tissues and anal swabs were assessed using quantitative real-time PCR, as previously described (Zhang et al., 2018).

Serum Neutralization Test

Sera of vaccinated chickens (Groups 1-3) collected at 7, 14, 21, and 28 dpv were tested for neutralization of the antibody against the FAdV-4 SDSX strain. The collected sera were inactivated at 56°C for 30 min. LMH cells were plated in 96-well plates which were used for neutralization. The assay was performed according to a constant virus-diluted serum method using 100 $TCID_{50}$/100 μL of FAdV-4 SDSX strain. The plates were cultured at 37°C in 5% CO_2 and investigated for CPE after 96 h as previously described (Xie et al., 2022).

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). Statistical significance was assessed using the Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Fusion Strategy and Expression and Purification of Fiber1/2 Knob and Fiber2

To develop a fusion protein of the Fiber1 knob domain and Fiber2 knob domain that enhances vaccine immunogenicity, we first constructed a 3-dimensional reconstruction of the Fiber1 knob domain (PDB ID: 7 × 5T) and Fiber2 knob domain (PDB ID: 7W83) using the SWISS-MODEL online server (https://swissmodel.expas.org/). A flexible linker was inserted between the Fiber1 and Fiber2 knob domains. The tandem fusion expression strategy of the Fiber1 knob domain and Fiber2 knob domain is shown in Figures 2A and 2B. The codon-optimized sequence of the tandem fusion of the Fiber1 knob domain and Fiber2 knob domain is shown in the supplementary material. The codon-optimized sequences of the Fiber1 and Fiber2 knob domains with linkers were inserted into the pCold-His expression vector and the recombinant plasmid was transformed into BL21 cells. These results suggest that Fiber1/2 knob was expressed in the form of inclusion bodies and that Fiber1/2 knob was purified using Ni-agarose resin. SDS-PAGE and western blotting results showed that only one band was detected in the purified Fiber1/2 knob, and the concentration of the purified Fiber1/2 knob reached 1 mg/mL (Figure 2C). The Fiber2 was also expressed and purified (Figures 2C and 2D). The clear band corresponding to Fiber1/2 knob and Fiber2 protein were detected by anti-His-monoclonal antibody in the Western blotting analysis (Figure 2D). Consequently, both Fiber1/2 knob and Fiber2 proteins were expressed and purified with high purity, and were used to develop corresponding subunit vaccines.

Antibody Response: IFA and SNT

To evaluate the immunogenicity and regular pattern of antibody production of the Fiber1/2 knob and Fiber2 subunit vaccines, the antibody levels in the serum collected at different time points were determined using IFA with LMH cells infected with FAdV-4. The results showed that the antibodies could be detected as early as 7 dpv in the Fiber1/2 knob subunit vaccine group, but not in the Fiber2 subunit vaccine group. In addition, specific green fluorescence was detected in

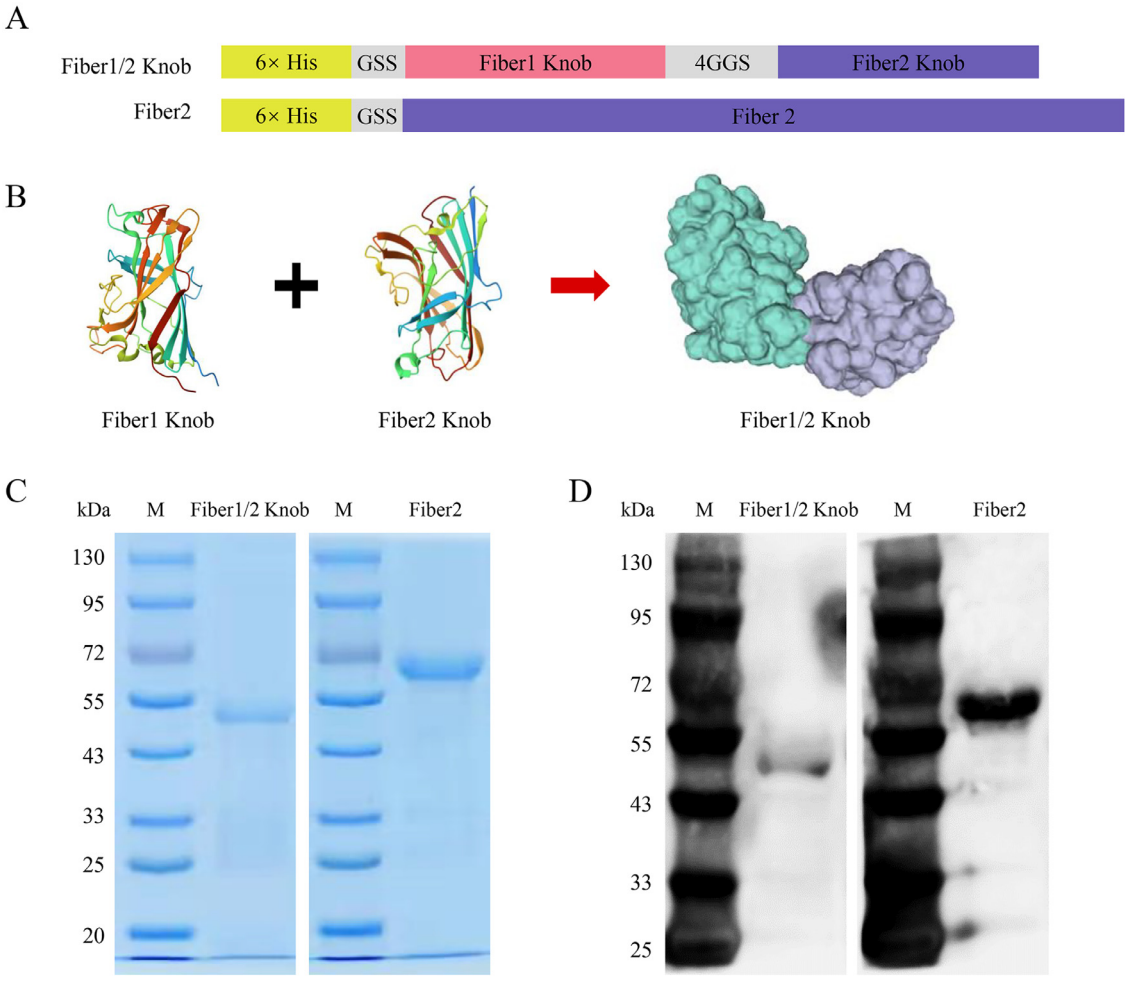

Figure 2. Expression and purification of recombinant proteins. (A) Construction of target protein expression plasmids for *E. coli* expression systems. (B) Ribbon representation of a monomer of the FAdV-4 Fiber1 and Fiber2 knob and the cartoon image of Fiber1 knob's C-terminal domain connected to Fiber2 knob's N-terminal domain by a flexible linker. (C) SDS-PAGE analysis of the purified recombinant proteins. (D) Verification of recombinant protein expression by Western blot analysis using anti-His-monoclonal antibody.

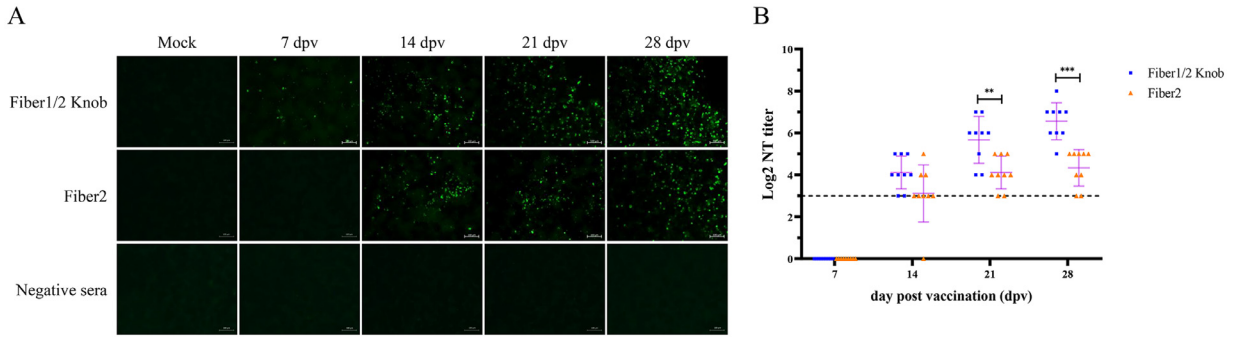

Figure 3. Regular pattern of antibody production after inoculation with Fiber1/2 knob and Fiber2 subunit vaccines. (A) Production of antibodies were detected in chickens inoculated with the 2 vaccines by IFA at 7, 14, 21, and 28 dpv. (B) Detection of neutralizing antibody levels in chickens after vaccination. Asterisks indicate statistical significance of the differences between the Fiber1/2 knob group and the Fiber2 *(**$P < 0.01$, ***$P < 0.001$)*.

FAdV-4-infected LMH cells using chicken serum vaccinated with the Fiber1/2 knob vaccine and Fiber2 vaccine at 14, 21, and 28 dpv by IFA (Figure 3A). These results suggest that the Fiber1/2 knob subunit vaccine can induce antibodies earlier than the Fiber2 subunit vaccine. In addition, the neutralizing activity (NT) of sera from inoculated chickens was tested. The results revealed that specific neutralizing antibodies in the vaccine group were induced at 14 dpv, whereas NT was not detected in the negative control chickens. However, the mean neutralizing antibody levels in the chickens in each group showed a time-dependent increase. The average NT titers for sera at 14, 21, and 28 dpv were 4.1, 5.6, and 6.5 in chickens immunized with the Fiber1/2 knob subunit vaccine, and 3.3, 4.1, and 4.3, respectively, in chickens immunized with the Fiber2 subunit vaccine (Figure 3B). The mean neutralizing antibody levels were higher in group Fiber1/2 knob subunit vaccine group than in group Fiber2 subunit vaccine group at all time points, and were significantly higher at 21 and 28 dpi (Figure 3B, $P < 0.01$; $P < 0.001$). These results suggest that the Fiber1/2 knob subunit vaccine can induce higher levels of neutralizing antibodies than the Fiber2 subunit vaccine.

Clinical Protection

A challenge protection test was performed to evaluate the protective efficacy of the experimental subunit vaccines. Chickens in groups 1 to 3 (vaccinated and unvaccinated) were challenged with a lethal dose of FAdV-4 at 28 dpi. The chickens in the vaccinated group 1 (Fiber1/2 knob subunit vaccine), group 2 (Fiber2 subunit vaccine), and control group 4 (unvaccinated and unchallenged) were alert and active throughout the experimental period. Chickens in group 3 (unvaccinated and challenged) showed signs of ruffled feathers, crouching, huddling together, and almost complete loss of appetite at 2 dpc, and started dying at 3 dpc. All chickens in group 3 died at 7 dpc (Figure 4A). Chickens in the control and vaccinated groups survived and appeared to be in a good mental state, exhibiting active behavior and a normal appetite. Although the body weight changes of the Fiber1/2 knob subunit vaccine group and the Fiber2 subunit vaccine group showed a similar trend, the weight of the chickens in the Fiber1/2 knob subunit vaccine group decreased to a lesser extent (Figure 4B). These results demonstrated that the Fiber1/2 knob subunit vaccine possessed better protective efficacy.

Plasma AST and ALT

AST and ALT were the 2 main marker proteins of liver injury. Our results showed that AST and ALT levels in the unvaccinated and challenged group were significantly higher than those in the negative control and vaccinated groups at 3, 5, and 7 dpc (Figures 5A and 5B, $P < 0.05$).

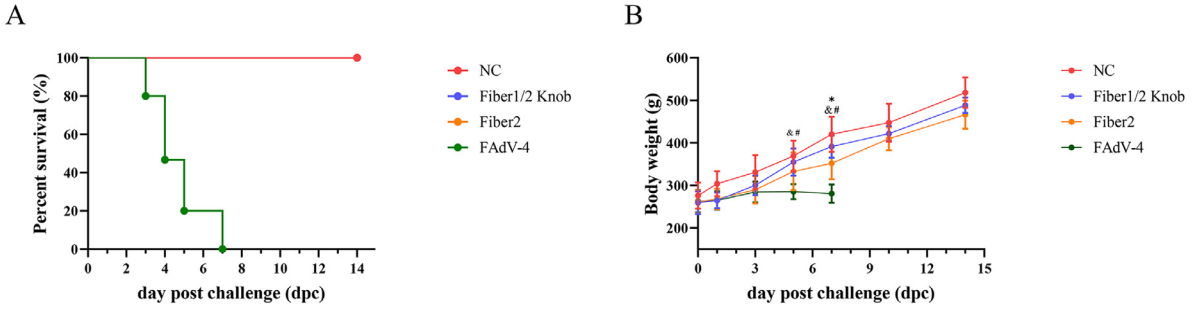

Figure 4. The survival curve and body weight of chickens in each group after the challenge. (A) Survival curves of SPF chickens following the challenge with virulent FAdV-4 strain SDSX1; mortality of chickens was recorded for 14 d. (B) Body weight was measured at different times during the challenge. Symbol above bars indicate significant differences (*, Fiber2 vs Fiber1/2 knob; &, Fiber1/2 knob versus challenge control; #, Fiber2 group versus challenge control). The data are presented as the mean ± SD. Student's *t*-test was used to determine the statistical significance *($P < 0.05$)* of differences.

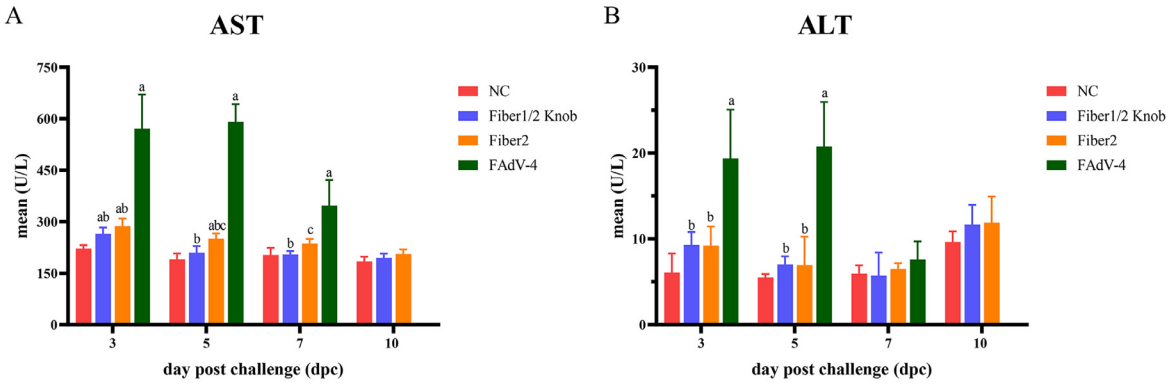

Figure 5. Changes in serum biochemical markers in chickens infected with FAdV-4. Mean and standard deviation of plasma AST (A) and plasma ALT (B) for each experimental group during the time period after challenge. Lowercase letters above bars indicate significant differences (a, versus negative control; b, versus challenge control; c, versus Fiber1/2 knob group). The data are presented as the mean ± SD. The statistical significance of differences was determined using Student's *t*-test and significance was assessed at $P < 0.05$.

Gross Lesions and Histopathologic Analysis

To further assess the protective efficacy of the subunit vaccines, the chickens were subjected to necropsy. Macroscopic lesions of different organs in the unvaccinated and challenged group displayed typical signs of HHS, including flaccid and spotty bleeding on the heart, focal necrosis of the liver, swelling congested on the spleen, bloated bleeding in the lungs, and congested kidneys, whereas the vaccinated chickens displayed normal healthy organs that were not distinguishable from the negative control group (Figure 6). However, the wet organ-to-body weight ratios of the liver, spleen, and kidney of chickens in the Fiber2 vaccine group were significantly higher than those in the Fiber1/2 knob vaccine group, particularly at 5 and 7 dpc (Figure 7, $P < 0.05$). Histopathological analysis revealed that chickens from the unvaccinated and challenged group presented extensive and severe microscopic lesions in the liver, such as lymphocytic infiltration, necrosis, and degeneration of hepatocytes with vacuolization. Furthermore, swollen cells, loose cytoplasm, and inflammatory cell infiltration were observed in the spleens, lungs, and kidneys of the chickens in the unvaccinated and challenged group. This was accompanied by hydropic degeneration of myocardial Fibers in the heart (Figure 8).

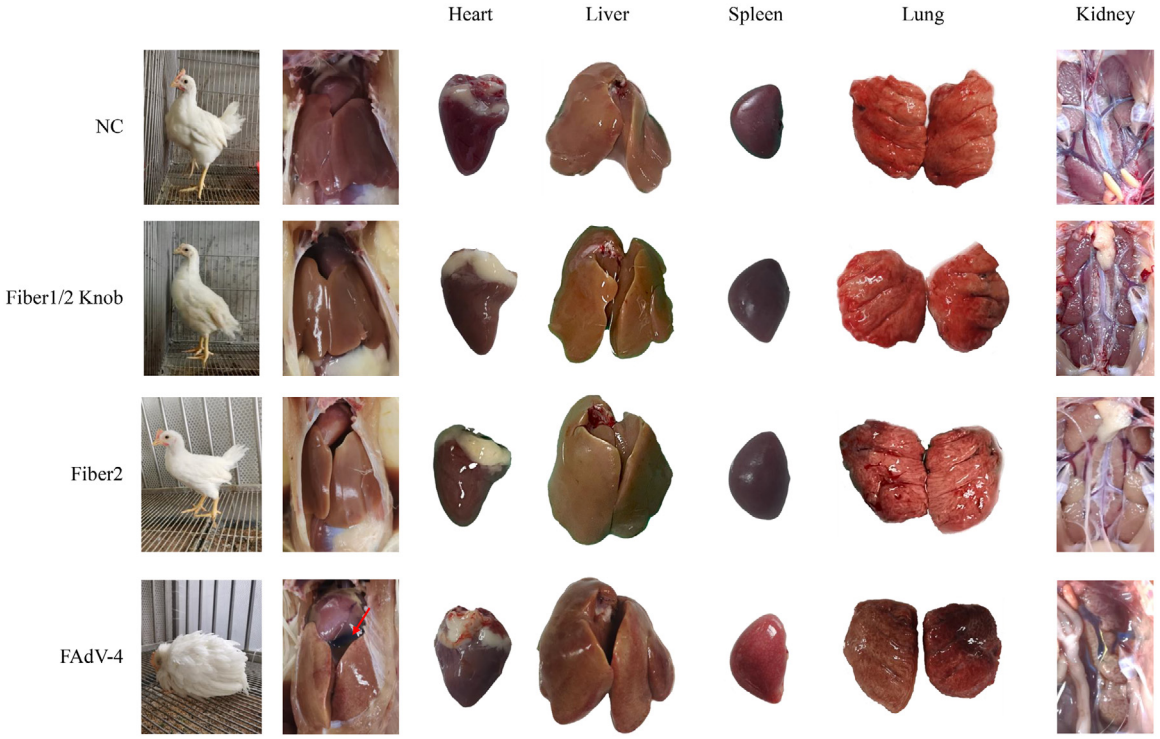

Figure 6. Clinical symptoms and pathological changes of SPF chickens in different groups after the challenge. Clear pericardial effusion, swollen livers with blood spots, enlarged spleens with congestion, dark -colored lungs with plaque-like necrotic focus, and kidneys exhibiting edemas with bleeding were observed in the challenge control group, while no clinical symptoms and pathological changes were observed in the surviving chickens in the negative control and vaccinated/challenged groups.

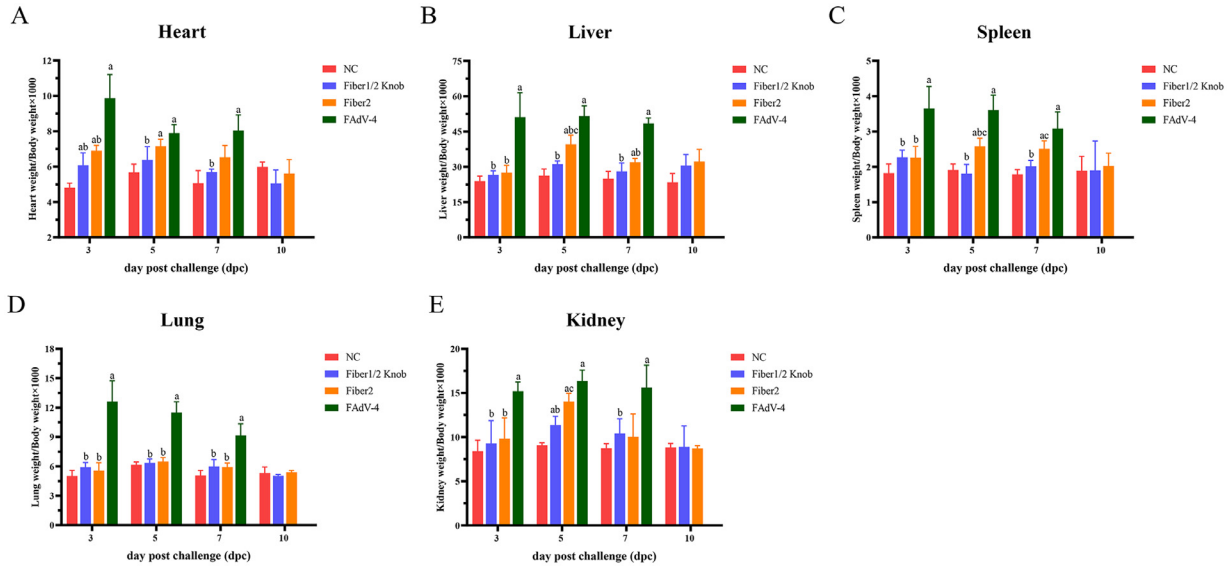

Figure 7. Organ weight / body weight ratios at specified times post-challenge. All SPF chicken body weights and organ weights were recorded for each experimental group during the time period after the challenge. (A) Heart weight (HW) / body weight (BW) × 1,000. (B) Liver weight (LW)/body weight (BW) × 1000. (C) Spleen weight (SW)/body weight (BW) × 1,000. (D) Lung weight (SW)/body weight (BW) × 1000. (E) Kidney weight (KW)/body weight (BW) × 1,000. Lowercase letters above bars indicate significant differences (a, versus negative control; b, versus challenge control; c, versus Fiber1/2 knob group). The data are presented as the mean ± SD. The statistical significance of differences was determined using Student's *t*-test and significance was assessed at $P < 0.05$.

Comparatively, no lesions were observed in the negative control group and the Fiber1/2 knob vaccine group. However, minor lesions were observed in the Fiber2 vaccine group (Figure 8). These results reveal that both Fiber2 subunit and Fiber1/2 knob subunit vaccines could protect chickens against FAdV-4, but the Fiber1/2 knob subunit vaccine was more effective in blocking the pathogenicity of FAdV-4 in target organs, thereby providing effective protection against FAdV-4.

Viral Loads in Target Tissues and Cloacal Swabs

The heart, liver, spleen, lungs, and kidneys were collected to determine the viral DNA copy number by qPCR. The results showed that the viral DNA copy numbers in the heart, liver, spleen, lung, and kidney were significantly lower in the vaccinated and challenged groups than that in the unvaccinated and challenged

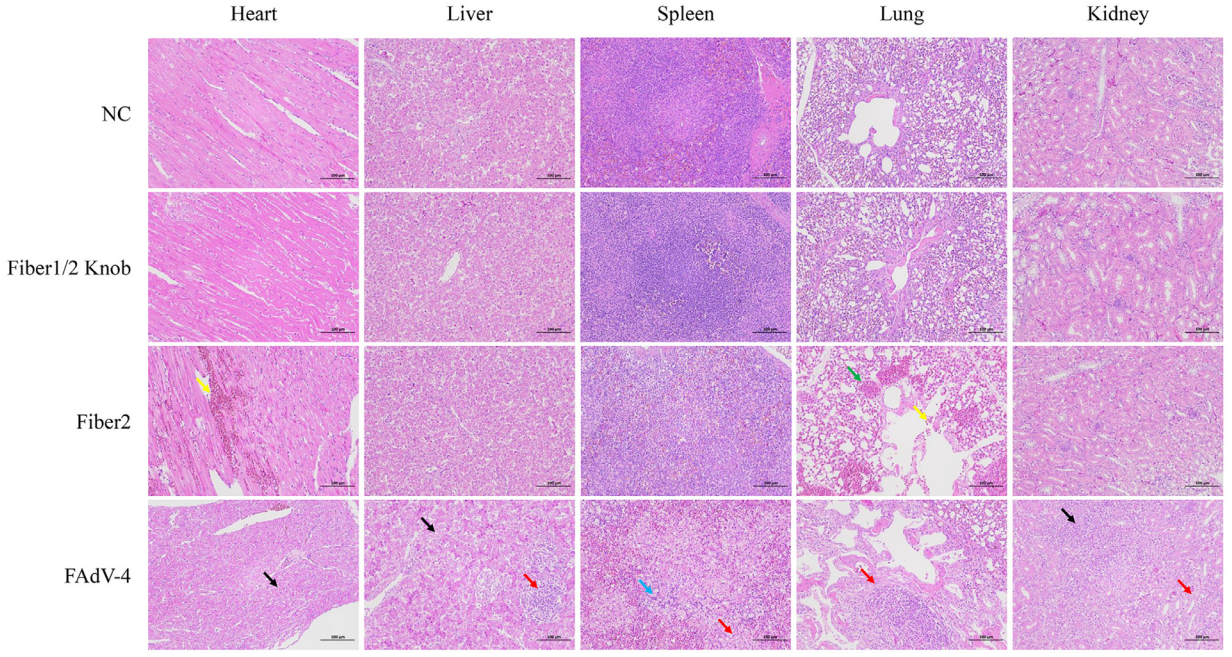

Figure 8. Representative histological changes in the analyzed organs of chickens from negative control, vaccinated/challenged, and challenge control groups (H&E stain, magnification × 200, scale bar =100 μm). The organs from the challenge control group present massive inflammatory cell infiltration (marked by red arrow heads), large areas of degeneration (black arrows) and nuclear swelling (blue arrow), while mild bruising was found in the hearts and lungs from the Fiber2 subunit vaccine group (yellow arrows). No obvious pathological damage was found in the negative control and Fiber1/2 knob subunit vaccine groups.

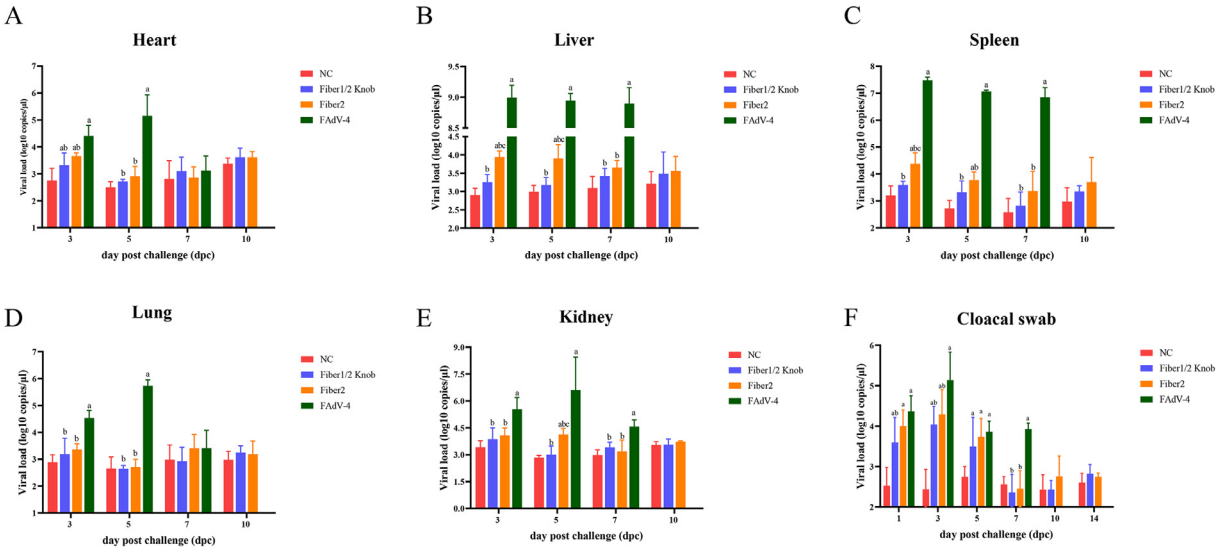

Figure 9. Viral loads and viral shedding were determined by quantitative real-time PCR (**qPCR**). Viral loads in the heart (A), liver (B), spleen (C), lung (D) and kidney (E) tissues from the challenged chickens. Viral shedding in cloacal swabs (F) from the challenged chickens. Lowercase letters above bars indicate significant differences (a, versus negative control; b, versus challenge control; c, versus Fiber1/2 knob group). The data are presented as the mean ± SD. The statistical significance of differences was determined using Student's *t*-test and significance was assessed at $P < 0.05$.

group (Figures 9A−9E, $P < 0.05$). In addition, the overall viral load in different tissues of chickens immunized with the Fiber1/2 knob subunit vaccine was lower than that of chickens immunized with the Fiber2 subunit vaccine, especially the viral loads in the liver, spleen, and kidney at 3 and 5 dpc, which were significantly lower in the Fiber1/2 knob vaccine group (Figure 9B, 9C, and 9E, $p < 0.05$). The viral loads in the cloacal swabs collected on different days were lower in the Fiber1/2 knob subunit vaccine group than Fiber2 subunit vaccine and unvaccinated groups (Figure 9F). These results revealed that the Fiber1/2 knob subunit vaccine could reduce the viral load in different tissues and virus shedding in chickens more efficiently than the Fiber2 subunit vaccine.

DISCUSSION

HHS caused by FAdV-4 is characterized by sudden onset, high morbidity, and high mortality, and is mainly observed in 3 to 6 week chickens (Liu et al., 2016). This has caused significant economic losses to the poultry industry worldwide. Since 2013, the number of HHS cases in chickens has been increasing in China, epidemics have occurred in the Liaoning, Hebei, Henan, and Shandong provinces especially since 2015, and have spread rapidly (Li et al., 2016). Therefore, there is an urgent need to develop safe and effective vaccines for controlling this disease.

Inactivated and live vaccines against FAdV-4 have been developed in many countries (Schonewille et al., 2010; Pan et al., 2017). However, inactivated and live vaccines face risks of incomplete inactivation and reversion to virulence. In addition, the production process is complicated, and the production costs are high. Embryo-adapted and cell culture-derived viruses were used to optimize the manufacturing technique of inactivated vaccines, and the results showed that both cell culture-derived and embryo-adapted-derived vaccines can protect chickens from FAdV-4 infection. However, cell-culture-derived vaccines induced higher and earlier humoral immune responses than embryo-adapted-derived vaccines, indicating that cell-culture-derived vaccines are better candidates for manufacturing inactivated vaccines (Du et al., 2017). However, it is well known that inactivated vaccines are derived from virulent strains and biosafety threats from their potential pathogenic components have been presented to the poultry industry. Live-attenuated vaccines also have safety issues. Although chickens immunized with live vaccines can induce high levels of neutralizing antibodies and be protected against FAdV-4 infection, live attenuated vaccines may reverse to virulence (Schonewille et al., 2010; Zhang et al., 2021a; Zhang et al., 2021b). Compared to inactivated and live vaccines, subunit vaccines are safer and more effective, and researchers have made notable attempts to develop subunit vaccines against FAdV-4 (Liu et al., 2022).

As Fiber2 is an important epitope structural protein of FAdV-4, it is a prime candidate for developing an effective subunit vaccine. Numerous studies focusing on Fiber2 have been reported. At present, the baculovirus and *E. coli* expression systems have been used to express Fiber2 and the immune effects have been evaluated through immunization and challenge studies in chickens. Fiber2 subunit vaccine can provide complete protection against FAdV-4 infection (Schachner et al., 2014; Ruan et al., 2018). For Fiber2 subunit vaccine, it induced a remarkable increase in the level of Th1 type cytokines (IL-2 and IFNγ) and Th2 type cytokines (IL-4 and IL-6). All of these revealed that Fiber2 subunit vaccine could induce the cellular immune responses and humoral

immune response (Yin et al., 2021). However, Fiber1 is also immunogenic and has a protective effect against FAdV-4, it can induce high level of neutralizing antibodies in immunized chickens (Wang et al., 2018; Watanabe et al., 2023). Both Fiber2 and Fiber1 knob domains are their main functional areas and are key domains for their immune effects (Song et al., 2023). Based on this evidence, a subunit vaccine of the Fiber1 and Fiber2 knob domain (Fiber1/2 knob vaccine) was developed in our study and we believe that the Fiber1/2 knob vaccine can induce better cellular immunity and humoral immunity response as it combined the major immune domains of Fiber1 and Fiber2.

Previous studies have shown that an H7N9 subunit vaccine designed using a combination of highly immunogenic epitopes and linkers could result in a strong immune response (Hasan et al., 2019). Meanwhile, our study results suggested that a recombinant porcine IFN-α and IL-2 fusion protein (**rPoIFNα+IL-2**) has both the antiviral effect of IFNα and the pro-proliferation effect of IL-2 (Zhao et al., 2023). These results suggest that multiple epitopes or structural domains expressed in tandem play coordinating roles. Therefore, in this study, the coding sequence of the knob domain of Fiber1 and Fiber2 was connected by a flexible linker and expressed in *E. coli* to develop a subunit vaccine (Figure 1A). As the Fiber2 subunit candidate vaccine provided complete protection against FAdV-4 challenge (Ruan et al.,2018), the immunogenic and protective effects of the Fiber1/2 knob vaccine were compared with the Fiber2 subunit vaccine. As expected, antibodies were detected as early as 7 dpv in the Fiber1/2 knob vaccine group, but not in the Fiber2 vaccine group. Therefore, the Fiber1/2 knob subunit vaccine induced antibodies earlier than the Fiber2 subunit vaccine (Figure 3A). In addition, chickens immunized with the Fiber1/2 knob vaccine induced higher levels of neutralizing antibodies compared to those with the Fiber2 vaccine, and there was a significant difference at 14 and 20 d after immunization (Figure 3B). Although both Fiber1/2 knob vaccine and Fiber2 vaccine can protect infected chickens from death, chickens vaccinated with Fiber1/2 knob vaccine lost less weight after being infected with FAdV-4 compared to those with Fiber2 vaccine (Figures 4A and 4B). In addition, chickens vaccinated with the Fiber1/2 knob vaccine showed reduced viral shedding and alleviated clinical symptoms and pathological damage after infection with FAdV-4 (Figures 5−9). Therefore, the knob domain of Fiber1 and Fiber2 of FAdV-4 may be a better choice for developing a subunit vaccine against FAdV-4.

In summary, a fused protein containing the knob domain of Fiber1 and Fiber2 of FAdV-4 was expressed and purified. The fused protein was developed as a subunit vaccine candidate that induced higher levels of neutralizing antibodies and provided better protection against FAdV-4 infection than the Fiber2 subunit vaccine. The present findings can help accelerate the development of safer and more effective subunit vaccines for HHS control in China.

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DISCLOSURES

The authors declare 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.2024.103888.

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