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A recombinant *Lactobacillus plantarum* strain expressing the spike protein of SARS-CoV-2

Maopeng Wang [a,b,1], Tingting Fu [b,1], Jiayi Hao [b], Letian Li [b], Mingyao Tian [b], Ningyi Jin [b], Linzhu Ren [c,*], Chang Li [b,*]

[a] Institute of Virology, Wenzhou University, Chashan University Town, Wenzhou, 325035, China
[b] Research Unit of Key Technologies for Prevention and Control of Virus Zoonoses, Chinese Academy of Medical Sciences, Military Veterinary Institute, Academy of Military Medical Sciences, Changchun 130122, China
[c] Key Lab for Zoonoses Research, Ministry of Education, Jilin Provincial Key Laboratory of Animal Embryo Engineering, College of Animal Sciences, Jilin University, Changchun 130062, China

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ABSTRACT

Coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a global pandemic in the past four months and causes respiratory disease in humans of almost all ages. Although several drugs have been announced to be partially effective treatments for this disease, no approved vaccine is available. Here, we described the construction of a recombinant *Lactobacillus plantarum* strain expressing the SARS-CoV-2 spike protein. The results showed that the spike gene with optimized codons could be efficiently expressed on the surface of recombinant L. *plantarum* and exhibited high antigenicity. The highest protein yield was obtained under the following conditions: cells were induced with 50 ng/mL SppIP at 37 °C for 6–10 h. The recombinant spike (S) protein was stable under normal conditions and at 50 °C, pH = 1.5, or a high salt concentration. Recombinant L. *plantarum* may provide a promising food-grade oral vaccine candidate against SARS-CoV-2 infection.

1. Introduction

Coronavirus disease 2019 (COVID-19) has become a pandemic over the past five months, with 4,962,707 confirmed cases and 326,459 deaths reported in >215 countries, areas, or territories by 22 May 2020.

The pathogen of this disease is a newly identified virus, designated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which has high identity with bat SARS-CoV [1,2]. The virus belongs to the β-coronavirus genus and contains a positive-sense single-stranded RNA genome, which encodes at least 4 structural proteins and 7 non-structural proteins [1,2]. Among the viral proteins, the spike (S) protein is located on the viral surface and can bind to the cell-surface receptor angiotensin-converting enzyme 2 (ACE2) for entry and interact with the cellular serine protease TMPRSS2 for S protein priming [2,3]. Moreover, other groups have reported that the S protein can also recognize cellular CD147 (also known as Basigin or EMMPRIN) [4] and AGTR2 (angiotensin II receptor type 2) [5] for entry into human cells. These results indicate that the S protein serves as the main target of antibodies.

Lactic acid bacteria have become a potential oral bacterial vector to deliver DNA and proteins, as they are normally present in the intestine of humans and most animals and play beneficial roles in various gastrointestinal and inflammatory disorders [6–11]. Lactic acid bacteria are usually safe and stable in human gastrointestinal conditions, with relatively strong tolerance of gastric acid and bile salt, and can adhere well to colonic epithelial cells [12–14]. These bacteria also inhibit the adherence and growth of pathogens and induce cytokines [12–14]. Among lactic acid bacteria, *Lactobacillus plantarum* is widely recognized as a probiotic that can be used in food fermentation, vaccines and medicine [12–18]. Our previous studies also showed that L. *plantarum* CGMCC 1.557 (named Lp18 by our laboratory) is a promising probiotic strain due to its high adhesion to intestinal cells, strong anti-inflammatory and immunoregulatory functions [13,14,17,18]. Here, we report the construction and optimization of L. *plantarum* expression system for the SARS-CoV-2 S protein.

2. Materials and methods

2.1. Bacterial strain and growth media

The *Lactococcus lactis* strain NZ3900 was previously purchased from MoBiTec GmbH (Goettingen, Germany) and grown in M17 medium

* Corresponding authors.
E-mail addresses: renlz@jlu.edu.cn (L. Ren), lichang78@163.com (C. Li).
[1]These authors contributed equally to this word.

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(Difco, USA) supplemented with 0.5% glucose (GM17 medium) at 30 °C [11].

Lactobacillus plantarum CGMCC 1.557 (Lp18) was purchased from the Institute of Microbiology, Chinese Academy of Sciences. Lp18 was grown in MRS medium (Merck, Darmstadt, Germany) at 37 °C [11].

2.2. Construction of expression plasmids

The codons of the spike gene from the SARS-CoV-2 isolate Wuhan-Hu-1 (GenBank: MN908947) were optimized according to the codon usage bias of Lp18. Then, the optimized *S* gene, named *tS*, was synthesized and linked with the sequence of the endogenous signal peptide 1320 (ALX04_001320) of L. *plantarum* at the 5′ terminus and the target peptide D (DCpep: FYPSYHSTPQRP) and HA genes at the 3′ terminus of the *tS* gene. Subsequently, the fragment, designated *1320-tSDH*, was amplified using the primers F01 and R01 and subcloned into pSIP411 (a gift from Qi) with *Nco* I and *Xba* I using the Gibson Assembly® Cloning Kit (NEB, USA) according to the manufacturer's instructions, producing the expression plasmid pLP-tS. Then, the plasmid was electrotransformed into competent L. *lactis* NZ3900 cells as described previously [11]. A positive colony was selected on a GM17 agar plate containing 10 μg/mL erythromycin (Sigma, USA) and verified by colony PCR using the primers F01 and R01, followed by sequencing analysis.

2.3. Transformation

Competent Lp18 cells were prepared according to a protocol described previously [11]. Briefly, precultured Lp18 cells were cultured in MRS medium containing 2% glucose at 37 °C. When the OD_{600} reached 0.3–0.5, the cells were centrifuged at 10,000 ×*g* for 10 min and washed with ddH_2O (distilled water) three times, followed by centrifugation at 10,000 ×*g* for 2 min. Then, the cells were resuspended in precooled electroporation buffer. Thereafter, 1 μg expression plasmid pLP-tS was incubated with 40 μL competent cells on ice for 20 min and electrotransformed into the competent cells in a 0.2-cm BTX cuvette by a single pulse with an apparatus (BTX) set at 1.75 kV and 5 ms. Then, the cells were plated on an MRS agar plate containing 10 μg/mL erythromycin (Sigma, USA) at 37 °C for 24–48 h. The positive colony, designated Lp18:S, was verified by colony PCR using the primers F01 and R01 and sequencing analysis.

2.4. Gene expression and protein purification

One milliliter of precultured Lp18:S cells was mixed with 100 mL MRS medium containing 10 μg/mL erythromycin, induced with SppIP (50 ng/mL, final concentration, Genscript, China) until the culture reached an OD_{600} of 0.3–0.5 and further cultured at 37 °C for 7–8 h. Then, the cells were centrifuged at 10,000 ×*g* for 2 min, washed with PBS twice, and lysed with 0.1 μm zirconia beads (1:3, Biospec, USA). The lysates were incubated with 5× loading buffer, boiled in a water bath for 5 min and evaluated by Western blot analysis.

2.5. Western blot (WB) analysis

Cell lysates obtained as described above were separated using 12% SDS-PAGE and transferred onto a PVDF membrane (Millipore, USA). Then, the membrane was incubated with PBST containing 5% skim milk for 2 h at room temperature, followed by incubation with a primary antibody (anti-HA tag rabbit polyclonal antibody, 1:2000, Proteintech, USA; anti-S1 rabbit monoclonal antibody, 1:500, Future Biotech, China; or anti-RBD mouse monoclonal antibody 13E10D5, 1:1000, Genscript, China) at 4 °C overnight and four washes with TBST. Thereafter, the membrane was reacted with an HRP-conjugated goat anti-rabbit IgG (H + L) (1:10,000, Zsbio, China) or HRP-conjugated goat anti-mouse IgG (H + L) secondary antibody (1:5000, Bioss, China) at room temperature for 1 h and washed with PBST four times. Subsequently, the bands were visualized with ECL reagent (Thermo Fisher Scientific, USA).

2.6. Indirect immunofluorescence assay (IFA)

Lp18:S cells were cultured in MRS for 12 h, washed with PBS twice, and collected by centrifugation at 8000 ×*g* for 2 min. The pellet was mixed with a primary antibody (anti-HA tag rabbit polyclonal antibody, 1:100, Proteintech, USA) at 4 °C overnight, followed by three washed with PBS. Then, the bacteria were incubated with a FITC-conjugated secondary antibody (FITC-conjugated goat anti-rabbit IgG, 1:3000, Zsbio, China) at 37 °C for 30 min. After washing 4 times with PBS, 3 μL cells were fixed on a clean coverslip in the dark, followed by staining with 5 μL Antifade Polyvinylpyrrolidone Mounting Medium (Beyotime, Shanghai, China) on the coverslip. Thereafter, the coverslip was mounted on a clean slide, and the samples were examined by fluorescence microscopy.

2.7. Transmission electron microscopy

Lp18:S and Lp18 cells were induced with SppIP, collected, and washed with pure water twice by centrifuging for 10 min at 12,000 ×*g*. Then, the cells were negative stained with 2% phosphotungstic acid (PTA, Sigma, USA) for 2 min. Subsequently, the samples were examined using a Hitachi-7650 transmission electron microscope with 150-mesh Formvar grids at an accelerating voltage of 90 kV.

2.8. Flow cytometric analysis (FCM)

Lp18:S and Lp18 cells were induced with SppIP for 8 h, collected by centrifugation at 5000 ×*g* for 10 min and washed with PBS twice. The pellets were incubated with a primary antibody (anti-HA tag rabbit polyclonal antibody, 1:100, Proteintech, USA) at 4 °C overnight, followed by three washes with PBS. Then, the cells were incubated with a secondary antibody (FITC-conjugated goat anti-rabbit IgG, 1:3000, Zsbio, China) for 40 min at room temperature. After washing with PBS twice, the samples were examined using a flow cytometer.

2.9. Stability analysis

To evaluate the stability of the recombinant bacteria, Lp18:S cells were induced with SppIP for 8 h, followed by treatment at different temperatures (37 or 50 °C) for 20 min, different pH values (pH = 1.5 or 7) for 30 min, or different bile salt concentrations (0, 0.2 or 0.5%, Solarbio, China) for 2 h. Thereafter, the S protein expressed by the bacteria was evaluated by Western blot analysis.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad 6.0. Data are presented as the mean ± standard deviation (SD) of at least three replicates. $P < 0.05$ was considered to indicate a statistically significant difference between groups. Experiments were repeated at least three times.

3. Results

3.1. Construction of recombinant L. plantarum expressing the SARS-CoV-2 spike protein

Lactic acid bacteria are food-grade bacteria that are widely used for expressing target antigens in vaccine development [6–11]. However, the expression efficiency of a target protein may be affected by the codon usage bias of the bacteria chosen [11,19]. Therefore, we optimized the codons of the *S* gene of the SARS-CoV-2 isolate Wuhan-Hu-1 (GenBank: MN908947) according to the codon usage bias of L.

plantarum (Lp18) and synthesized the gene, followed by linking the sequence of the endogenous signal peptide 1320 (ALX04_001320) of *L. plantarum* to the 5′ terminus of the optimized *S* gene and the target peptide D (DCpep: FYPSYHSTPQRP) and HA genes to the 3′ terminus of the gene, resulting in a fragment named *1320-tSDH*. Then, the fragment was amplified using the primers F01 and R01 and subcloned into pSIP411 with *Nco* I and *Xba* I using the Gibson Assembly® Cloning Kit (NEB, USA), producing the expression plasmid pLP-tS. Thereafter, the expression plasmid was electrotransformed into competent Lp18 cells as described previously [11]. A positive colony, designated L. *plantarum* Lp18:S, was grown and verified by colony PCR using the primers F01 and R01 and sequencing analysis (Fig. 1A).

To evaluate the expression of the S protein in the recombinant strain, Lp18:S cells were induced with SppIP (50 ng/mL, final concentration), and the levels of the S protein were analyzed by Western blot analysis. As shown in Fig. 1B, a 150-kD band could be detected using an anti-HA tag rabbit polyclonal antibody, an anti-S1 rabbit monoclonal antibody or anti-RBD mouse monoclonal antibody 13E10D5 as the primary antibody, indicating that the codon-optimized S protein was successfully expressed in the L. *plantarum* Lp18:S strain. Furthermore, both Lp18:S cells and Lp18 cells were examined using a transmission electron microscope (TEM) and an indirect immunofluorescence assay (IFA). The results showed that the morphology of the bacteria was not affected by the viral S protein, while numerous filamentous proteins were present on the surface of the recombinant bacteria Lp18:S (Fig. 1C) and exhibited high-efficiency reactivity (Fig. 1D). In addition, the positive rates of the bacteria expressing the S protein induced by SppIP were analyzed by flow cytometry. The results showed that the positive rate of S protein expression in recombinant Lp18 was approximately 37.5%, while it was 2.5% in the parental strain (Fig. 1D). These results suggest that the viral S protein can be efficiently displayed on the surface of the recombinant L. *plantarum* Lp18:S strain, which can be used for further research.

3.2. Construction of recombinant L. plantarum expressing the SARS-CoV-2 spike protein

Previously, we found that relatively high expression yields for proteins can be achieved by optimizing the parameters of the expression system [11]. Therefore, recombinant *L. plantarum* Lp18:S was optimized for induction time (2 to 22 h), inducer concentration (10, 50, 100, and 150 ng/mL) and bacterial passage (passage 1 to 10). As shown in Fig. 2A, the levels of the S protein were significantly enhanced in the SppIP-induced group compared with the other groups. The highest levels of the S protein were obtained by induction with up to 50 ng/mL SppIP for 8 h at 30 °C (Fig. 2B-C). In addition, the passage of bacteria, at least in the first 5 passages, had no significant effect on protein expression. As the passage number increased, it was difficult to maintain a consistent inoculation amount. Due to different induction states, some changes occurred. However, there were still sufficient amounts of protein specifically detected by Western blot analysis (Fig. 2D).

To evaluate the stability of the recombinant protein, Lp18:S cells were induced with SppIP for 8 h, followed by treatment at different temperatures (37 or 50 °C) for 20 min, different pH values (pH = 1.5 or 7) for 30 min, or different bile salt concentrations (0, 0.2 or 0.5%)

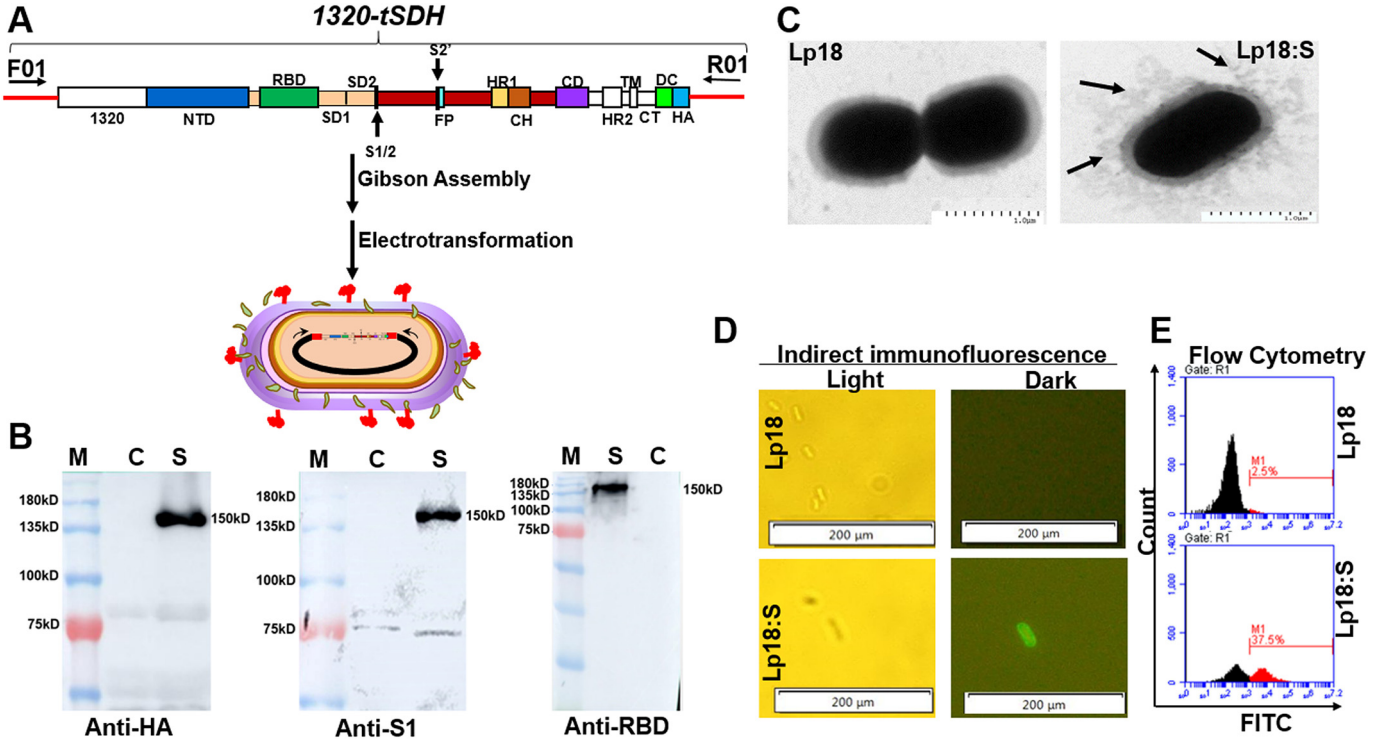

Fig. 1. Construction of a recombinant L. *plantarum* strain expressing the SARS-CoV-2 spike protein. (A) Schematic diagram of the construction of the recombinant L. *plantarum* strain expressing the SARS-CoV-2 spike protein. (B) Identification of the S protein. Western blotting was performed using an anti-HA tag rabbit polyclonal antibody (left panel, anti-HA, 1:2000), anti-S1 rabbit monoclonal antibody (middle panel, anti-S1, 1:500), or anti-RBD mouse monoclonal antibody 13E10D5 (right panel, 1:1000) as the primary antibody and an HRP-conjugated goat anti-rabbit IgG (H + L) (1:10000, Zsbio, China) or HRP-conjugated goat anti-mouse IgG (H + L) antibody (1:5000, Bioss, China) as the secondary antibody. M, protein marker; C, sample extracted from the un-induced bacteria was used as control; S, spike. RBD, receptor binding domain of S protein. (C) TEM. Both L. *plantarum Lp18* (Lp18) and recombinant L. *plantarum Lp18* (Lp18:S) were induced with SppIP and negatively stained with 2% PTA (Sigma, USA), followed by examination using a TEM (Hitachi-7650). (D) IFA. Bacteria (Lp18 and Lp18:S) were incubated successively with an anti-HA tag rabbit polyclonal antibody (1:500, Proteintech, USA) and a FITC-conjugated secondary antibody (1:3000, Zsbio, China). Then, the cells were fixed with 4% formaldehyde on a clean coverslip in the dark, followed by the addition of 5 μL Antifade Polyvinylpyrrolidone Mounting Medium (Beyotime, Shanghai, China) on the coverslip. Thereafter, the samples were examined by fluorescence microscopy. (E) FCM. Flow cytometry was performed using an anti-HA tag rabbit polyclonal antibody (1:2000, Proteintech, USA) as the primary antibody and a FITC-conjugated secondary antibody (1:200, Zsbio, China). Positive rates were calculated by subtracting control rates.

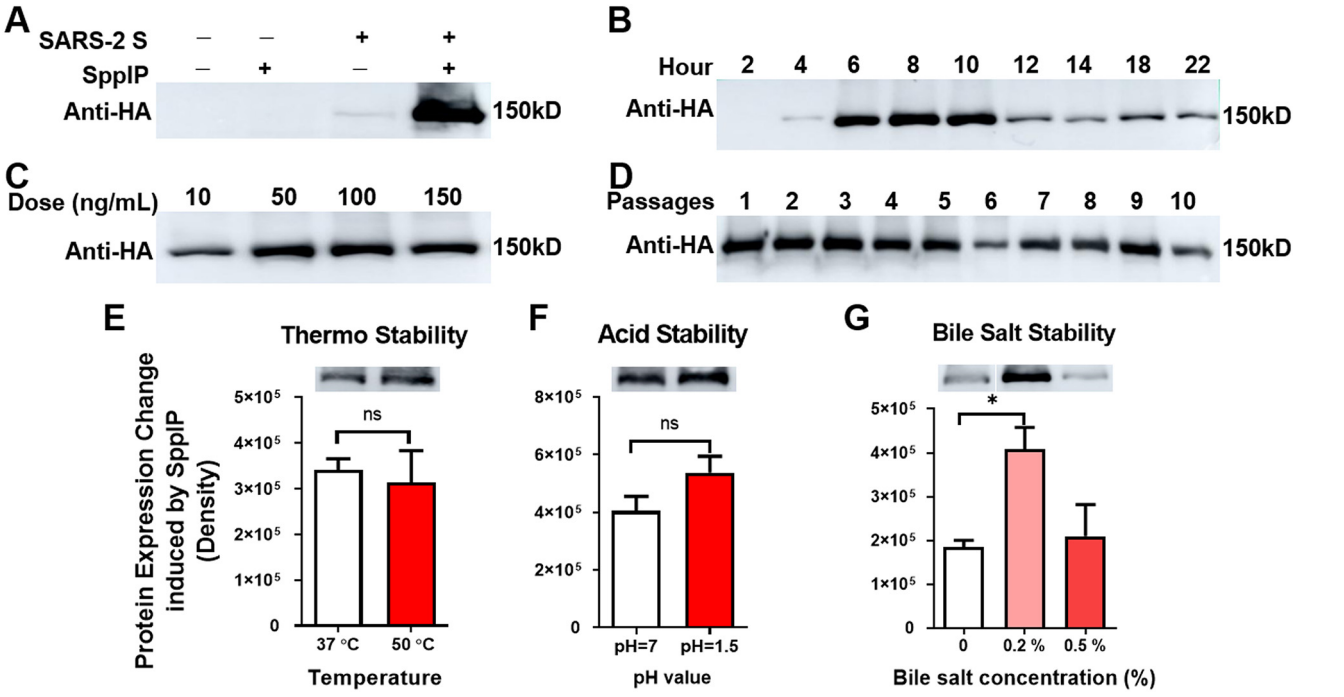

Fig. 2. Features of recombinant L. *plantarum*. The bacteria were cultured under the indicated conditions and evaluated by Western blot analysis using anti-HA tag rabbit polyclonal antibody (1:2000, Proteintech, USA) as the primary antibody and HRP-conjugated goat anti-rabbit IgG (H + L) antibody (1:10000, Zsbio, China) as the secondary antibody. (A) Significant induction of the SARS-CoV-2 S protein in recombinant Lp18:S. (B-D) Optimization of L. *plantarum* expression system by analysis of different induction times (B), SppIP concentrations (C), and bacterial passages (D). (E-G) Stability analysis of the S protein at the indicated temperature (E), pH (F), and bile salt concentration (G). Immunoblotting and grayscale analyses were performed to determine the levels of the S protein. *, $P < 0.05$. ns, not significant.

for 2 h. The results showed that the recombinant S protein was stable at 50 °C for 20 min or pH = 1.5 for 30 min, with no significant differences between the normal group and the treated group (Fig. 2E-F). Moreover, the level of the S protein was significantly increased in the bacteria treated with 0.2% bile salt compared with the bacteria treated with the other bile sale concentrations (Fig. 2G). These results suggest that the recombinant S protein expressed in the L. *plantarum* system is stable in an environment with a high temperature, acidity, and bile salt concentration. Therefore, *L. plantarum* expressing the recombinant S protein may be used orally as a food-grade vaccine.

4. Discussion

To date, no effective medicines or approved vaccines are available for COVID-19. Although several groups have reported the development of inactivated vaccines, subunit vaccines or mRNA-based vaccines for SARS-CoV-2 virus infection [20–22], most of these vaccines are in clinical trials and are generally provided by injection. Notably, the use of these vaccines is expensive and complicated, requiring professional administration. However, oral vaccines are cost effective, easy to administer, easy to store, and widely accepted to be biofriendly [23–25]. Moreover, it has been reported that oral vaccines induce strong antigen-specific IgG responses, mucosal IgA responses, and Th1/Th17 responses [26–28], thus reducing or preventing the infection and replication of viruses in the respiratory tract and intestinal mucosa. Therefore, the development of an effective oral vaccine for COVID-19 is still urgently needed.

In recent years, lactic acid bacteria, as food-grade bacteria, have been widely used for expressing target antigens in oral vaccines [6–11,27,28]. Furthermore, antigens displayed on the surface of L. *plantarum* can induce a significant immune response and provide efficient protection against infection [27,28]. In this study, we constructed a recombinant L. *plantarum* Lp18:S strain expressing the S protein of SARS-CoV-2 *via* Gibson assembly and homologous recombination. We previously found that codon optimization could improve the expression level of a target protein and a target peptide could enhance the transport of the target protein to the cell surface [11]. Therefore, to enhance expression efficiency, codons of the S protein were optimized according to the codon usage bias of L. *plantarum*, and both a target peptide and an endogenous signal peptide were linked at the 3′ terminus and 5′ terminus of the S protein, respectively. The results demonstrated that the S protein could be efficiently displayed on the bacterial surface, forming a large number of irregularly folded peptide chains, and exhibit high reactivities with a rabbit polyclonal antibody (anti-HA tag) and SARS-CoV-2 spike-specific monoclonal antibodies (Fig. 1).

For oral vaccines, the stability of the antigen protein is important. Therefore, we analyzed the stability of the S protein expressed on the surface of the bacteria. The results showed that the antigenic protein had the characteristics of thermal stability and acid stability and that the expression level was obviously increased in the presence of 0.2% bile salt (Fig. 2), suggesting that recombinant L. *plantarum* Lp18:S can be used as an oral vaccine against SARS-CoV- 2 infection.

Moreover, several groups have reported that antibodies against SARS-CoV and Middle East respiratory syndrome (MERS) spike proteins may trigger antibody-dependent enhancement (ADE) but not alter the expression profile of proinflammatory genes in human macrophages [29–31]. However, Lou et al. found that low levels of antibodies induced by an inactivated SARS-CoV vaccine might not induce ADE in *rhesus macaques* [32]. Similarly, whether SARS-CoV-2-specific vaccines can induce ADE is also controversial [20,33–35]. Encouragingly, Qin and colleagues reported that a purified inactivated SARS-CoV-2 vaccine could induce SARS-CoV-2-specific neutralizing antibodies in mice, rats and nonhuman primates, providing effective protection against SARS-CoV-2 challenge in *macaques* without observable ADE effects [20]. Therefore, whether oral vaccines developed with the recombinant L. *plantarum* strain created in this study can induce ADE will be further evaluated in animal models and human clinical trials in subsequent studies.

Further studies are in progress to analyze the feasibility, safety, and efficacy of recombinant L. *plantarum* as a practical oral vaccine *in vivo*.

5. Conclusions

In conclusion, we constructed a recombinant L. *plantarum* strain expressing the S protein of SARS-CoV-2, which could be efficiently displayed on the bacterial surface with high stability at 50 °C for 20 min or pH = 1.5 for 30 min. The highest levels of the S protein were obtained by treatment with 50 ng/mL SppIP and 0.2% bile salt for 8 h at 30 °C. Recombinant L. *plantarum* Lp18:S is a potential mucosal vaccine candidate tolerant to gastrointestinal conditions.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author statement

Conceptualization: Ningyi Jin; Methodology: Maopeng Wang, Tingting Fu; Formal analysis: Jiayi Hao; Validation: Letian Li; Data Curation: Mingyao Tian and Chang Li; Writing - Original Draft: Linzhu Ren and Maopeng Wang; Writing - Review & Editing: Linzhu Ren and Chang Li; Funding acquisition: Ningyi Jin, Linzhu Ren and Chang Li; Supervision: Ningyi Jin and Chang Li.

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Declaration of competing interest

All authors have approved the final version of the manuscript. The authors declare no conflicts of interest.

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