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Genetically engineering of *Saccharomyces cerevisiae* for enhanced oral delivery vaccine vehicle

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ABSTRACT

As a series of our previous studies reported, recombinant yeast can be the oral vaccines to deliver designed protein and DNA, as well as functional shRNA, into dendritic cells (DCs) in mice for specific immune regulation. Here, we report the further optimization of oral yeast-based vaccine from two aspects (yeast characteristics and recombinant DNA constitution) to improve the effect of immune regulation. After screening four genes in negative regulation of glucan synthesis in yeast (*MNN9*, *GUP1*, *PBS2* and *EXG1*), this research combined HDR-based genome editing technology with Cre-loxP technology to acquire 15 gene-knockout strains without drug resistance-gene to exclude biosafety risks; afterward, oral feeding experiments were performed on the mice using 15 oral recombinant yeast-based vaccines constructed by the gene-knockout strains harboring pCMV-MSTN plasmid to screen the target strain with more effective inducing mstn-specific antibody which in turn increasing weight gain effect. And subsequently based on the selected gene-knockout strain, the recombinant DNA in the oral recombinant yeast-based vaccine is optimized via a combination of protein fusion expression (OVA-MSTN) and interfering RNA technology (shRNA-IL21), comparison in terms of both weight gain effect and antibody titer revealed that the selected gene-knockout strain (*GUP1*Δ*EXG1*Δ) combined with specific recombinant DNA (pCMV-OVA-MSTN-shIL2) had a better effect of the vaccine. This study provides a useful reference to the subsequent construction of a more efficient oral recombinant yeast-based vaccine in the food and pharmaceutical industry.

1. Introduction

Saccharomyces cerevisiae (*S. cerevisiae*) is an internationally recognized non-toxic and harmless edible microorganism widely used in the pharmaceutical, livestock and food industries [1–3]. And the glucan of *S. cerevisiae* plays an important role in the recognition and interacting between dendritic cells and yeast [4–6]. Yeast has been demonstrated to elicit immunologic responses in mammals and is readily engulfed by dendritic cells (DCs) and macrophages [7,8]. The phagocytosis of yeast by DCs is stimulated by the immunogenicity of yeast cell-wall components, such as β-1,3-D-glucan and mannan [9]. These components can transmit "danger signals", typically associated with microbial infection [10]. With the deepening of research on *S. cerevisiae*, the oral vaccine delivery system based on *S. cerevisiae* has been paid more and more attention by researchers because of its advantages over other oral vaccine delivery systems. Currently, yeast-mediated oral vaccine delivery systems, represented mainly by *S. cerevisiae* [11] and *Pichia pastoris* yeast [12]. Previous reports identified four genes, *GUP1* [13], *PBS2* [14], *EXG1* and *MNN9* [15], as negative regulators of yeast cell wall glucan production or glucan exposure [16], i.e., the glucan content or structure of yeast cell walls was significantly increased when the gene was in the deletion (null) state. Considering the key role of glucan in yeast-mediated oral vaccine delivery systems, knockout of the above four genes may increase the yeast glucan content and thus improve the effectiveness of yeast-mediated oral DNA vaccines (Fig. 1A).

With the rapid development of DNA vaccines, yeast-mediated oral DNA vaccines are of increasing interest to the community. Compared with traditional DNA vaccines, the use of recombinant yeast as an oral

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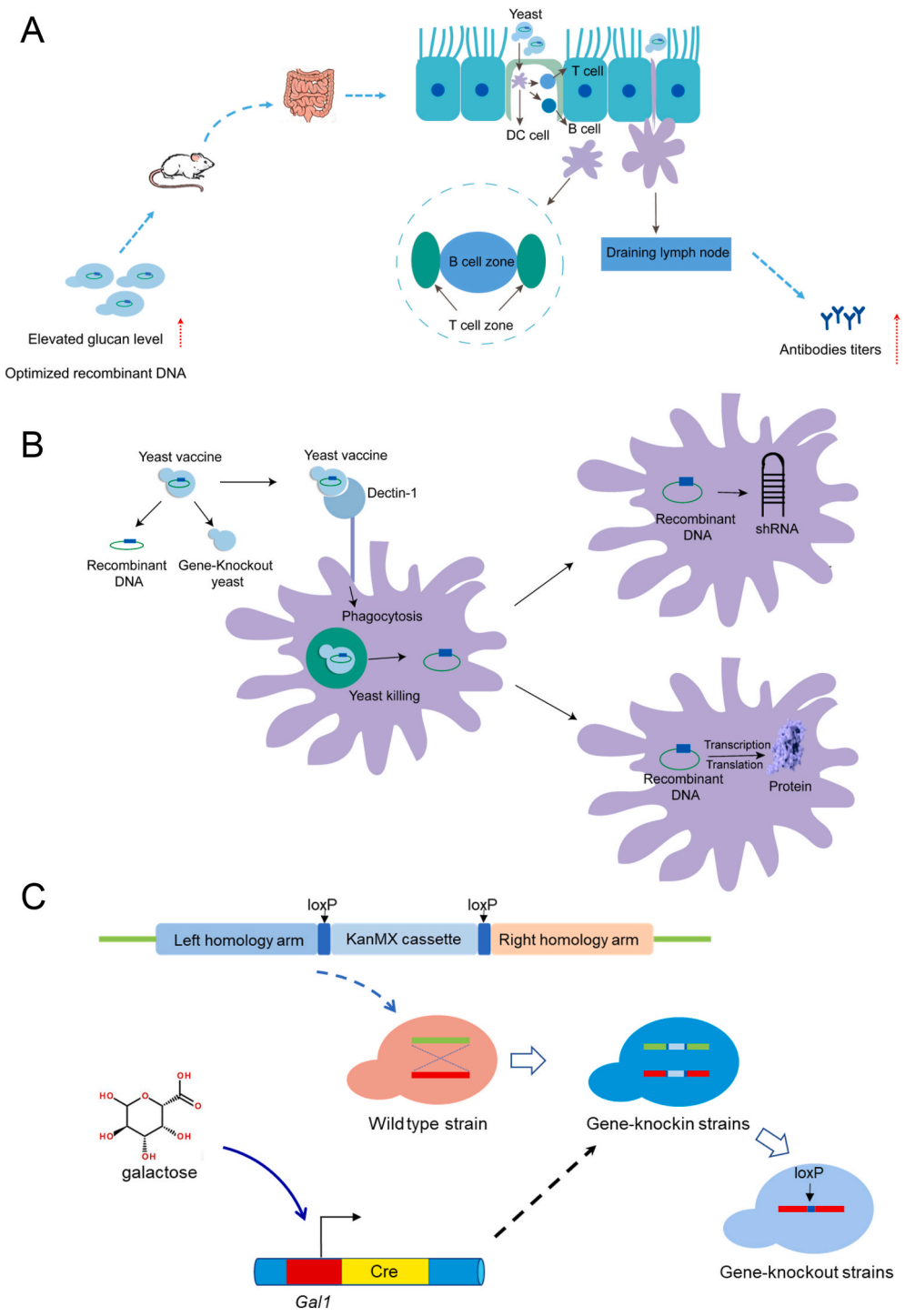

Fig. 1. The components of the oral yeast-based DNA vaccine and the schematic drawing for the function principle of the vaccine. A. The yeast carrying the eukaryotic expression vector of the target gene is orally fed into the small intestine portion of the mouse, and the antigen presenting cells (especially DC cells) located in the small intestine have receptors that can specifically recognize and bind to the yeast surface glucan. Yeast ruptures in DC cells, then releases the eukaryotic expression plasmid which can express the specific antigen. Subsequently, the antigen is presented to the body's immune system, thereby producing the specific antibodies. The figure illustrates the two optimization pathways used in this study: increasing glucan level and optimizing recombinant DNA, both of which improve subsequent antibody production; B. The composition of the oral yeast vaccine and the schematic diagram depicting its recognition mechanism by dendritic cells (DC) and the underlying principles of vaccine action; C. Schematic diagram of gene-knockout mediated by homologous arm donor DNA. After the linearized donor DNA being transformed into wild yeast, the original gene of the wild type strain is replaced by the *KanMX* gene expression cassette belonging to the donor DNA by homologous recombination mechanism, and then by using the characteristics of the Cre enzyme specifically recognizing the loxP site, the *KanMX* gene expression cassette was specifically excised to obtain a gene-knockout yeast strain without drug resistance-gene.

DNA vaccine does not require an additional endotoxin removal process, which greatly reduces the cost of vaccine production, and at the same time, the use of *S. cerevisiae* surface glucan and the surface of the DC cell Dectin-1 receptor can be specifically recognized [17] by the combination of the characteristics of the recombinant yeast-mediated DNA vaccine targeting intestinal immune cells. The effect of recombinant yeast-mediated DNA vaccines on intestinal immune cells is more precise, and the immune response triggered is within a controllable range. In addition, one study reported that oral yeast can be used as an adjuvant to enhance the humoral immune response triggered by DNA vaccines [18], while another study found that components such as β-glucan inherent in brewer's yeast can also play an immune adjuvant role during the action of DNA vaccines [19], which also makes the oral yeast DNA vaccine's ability to trigger specific immune responses is greatly enhanced. Yeast-derived β-glucan can also increase host immune defenses by activating the complement system and enhance macrophage and natural killer cell function [20], which also side-steps the ability of recombinant yeast DNA vaccines to enhance organismal immunity. Although recombinant yeast-mediated DNA vaccines have many advantages, they still face many obstacles now, which will require more efforts to optimise this oral vaccine system in the future.

As a series of our previous studies reported, except for the traditional vaccine's usage for preventing disease [21], recombinant yeast can be the oral vaccines to deliver designed protein [22,23] and DNA [11,24], as well as functional shRNA (short hairpin RNA-*CD40* and *IL-21*) [25,26], into dendritic cells (DCs) in mice for specific immune regulation of the body's growth performance. As described previously, Myostatin (*MSTN*) plays a negative-regulating role on skeletal muscle differentiation [27]. Our previous studies found that *S. cerevisiae* expressing recombinant mstn protein can be orally fed to mice and rabbit for inducing the mstn-specific antibodies to achieve directed immune regulation of the animal production trait. In addition, based on our lab's previous research, the shIL21 can promote the effect of oral yeast-based DNA vaccine [28]. In addition, it was shown as early as 1997 that the chicken ovalbumin gene *OVA*, when expressed in fusion with the *IL-12* gene, induced a stronger immune effect than the sum of the individual proteins not fused [29] and based on our lab's previous research, recombinant yeast DNA vaccines carrying a fusion-expressed OVA gene have superior immunological efficacy [21,26,30]. Evidenced by above proofs, we assumed that oral yeast-based DNA vaccine could be enhanced by increasing the glucan level of yeast and optimizing recombinant DNA.

In this study, the original oral yeast-based vaccine was optimized using the combination of gene editing technology (the HDR repair and Cre-loxP technology combination) and DNA recombination platform of our group from different aspects, and the oral yeast-based vaccine with the better immune effect was obtained by comparative screening at the mammalian *in vivo* level. The successful optimization of the oral yeast-based vaccine in this study provides a platform for further developing oral DNA vaccines targeting for various disease of different animal species, and provide a useful reference for the study of the use of immunological methods to regulate the growth and developmental traits of different animals.

2. Materials and methods

2.1. The experimental design

According to the existing research, the schematic diagram of the oral yeast-based vaccine function was displayed in Fig. 1A and there are two important components of the oral yeast-based vaccine: the yeast and the recombinant DNA. In this study, we reformed or optimized these two components (Fig. 1A and B) according to different strategies: for the yeast itself, the elevated glucan level is the focus in the first part experiment; for the recombinant DNA, optimized recombinant DNA were constructed by fusion of the chicken *OVA* gene and co-expression of shRNA of mice *IL-21* gene.

2.2. Animals

In this study, we purchased a total of 96 female five-week Kunming mice from the Breeding and Research Centre of Xi'an Jiaotong University, China. All mice were divided into two batches. All mice weighed approximately 18 (±2). According to the approval by the Experimental Animal Manage Committee of Northwest A&F University (2011–31101684), mice-related animal experiments were performed in full compliance with regulations. Mice were housed in a specific pathogen-free (SPF) facility under normal 12-h light/12-h dark cycles. The temperature was maintained at 18–23 °C and humidity at 40–70%. Standard mouse food and water were used for all mice. Before the formal experiment, mice were given a week to adapt to the environment.

2.3. Genes screening and donor DNA preparation

In this part of the experiment, the four target genes (*MNN9*, *GUP1*, *PBS2* and *EXG1*) are mainly screened referring the Saccharomyces Genome Database (SGD) (https://www.yeastgenome.org/), which can inhibit glucan synthesis in yeast. Then, based on the selected genes, various combinations (Supplemental Table S1) are generated to provide theoretical support for the subsequent acquisition of different gene-knockout yeast strains.

Four donor plasmids containing homologous arms were constructed using the directional cloning. The left arms (~1 kb) and right arms (~1 kb) were cloned from the genome DNA of AH109 yeast with the primer pairs (LF/LR, RF/RR) separately. After gel extraction, the homologous arms and pBlue-HOL-KanMX vector (maintained in our lab) were digested with *Sal*I/*Kpn*I and *Sac*II/*Not*I separately at the meantime. After gel extraction, four donor plasmids were constructed by ligating these DNA products with T4 DNA ligase (NEB, America).

After that, refer to the published studies [31], we linearized the four donor plasmids to improve the efficiency of gene recombination. The specific steps of linearization treatment are as follows: four plasmids were digested with *Sac*II and *Kpn*I in a 37°C water bath for 4 h, then products were detected by agarose gel electrophoresis, and the target band was recovered by gel extraction. All primers were shown in Supplemental Table S2.

2.4. Culture and screen of gene-knockout yeast strains

The *S. cerevisiae* yeast strain AH109 (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4△, gal80△, lys2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2 URA3::MEL1UAS-MEL1TATA-LacZ MEL1*) (Clontech, America) was cultured in YEP medium or different defective YPD solid medium. In the first part of this study, according to the previous report [32], we introduced different DNA donors into yeast with LiAc method. Positive transforms were cultured on YEP media supplemented with G418 (Sigma, America). Then specific primers (Supplemental Table S2) were used to detect the positive yeast strains. After confirmation of the positive strains with drug resistance gene, we then used the inducible Cre-loxP technique to delete the drug resistance gene in the gene-knockout yeast strains (Fig. 1C). Finally, we constructed 15 modified oral yeast-based DNA vaccines using selected gene-knockout yeast strains and pCMV-MSTN plasmid.

In the second part of this study, three optimized recombinant DNA were generated referring to two different strategies (Fig. 2B). Afterward, based on the selected yeast strain (*GUP1*Δ*EXG1*Δ) screened in the first part of this study, we have prepared three optimized yeast-based vaccines: pCMV-OVA-MSTN; pCMV-MSTN-shRNA (IL-21); pCMV-OVA-MSTN-shRNA (IL-21). These three recombinant DNA plasmids were constructed referenced previous researches [21,26,30] and different detection results by different endonucleases (*Bam*HI、*Sal*I and *Not*I) were displayed in Supplemental Fig. 1C.

After 4–5 days of culture, the yeast count is performed using a hemocytometer plate, then the recombinant yeast is collected by

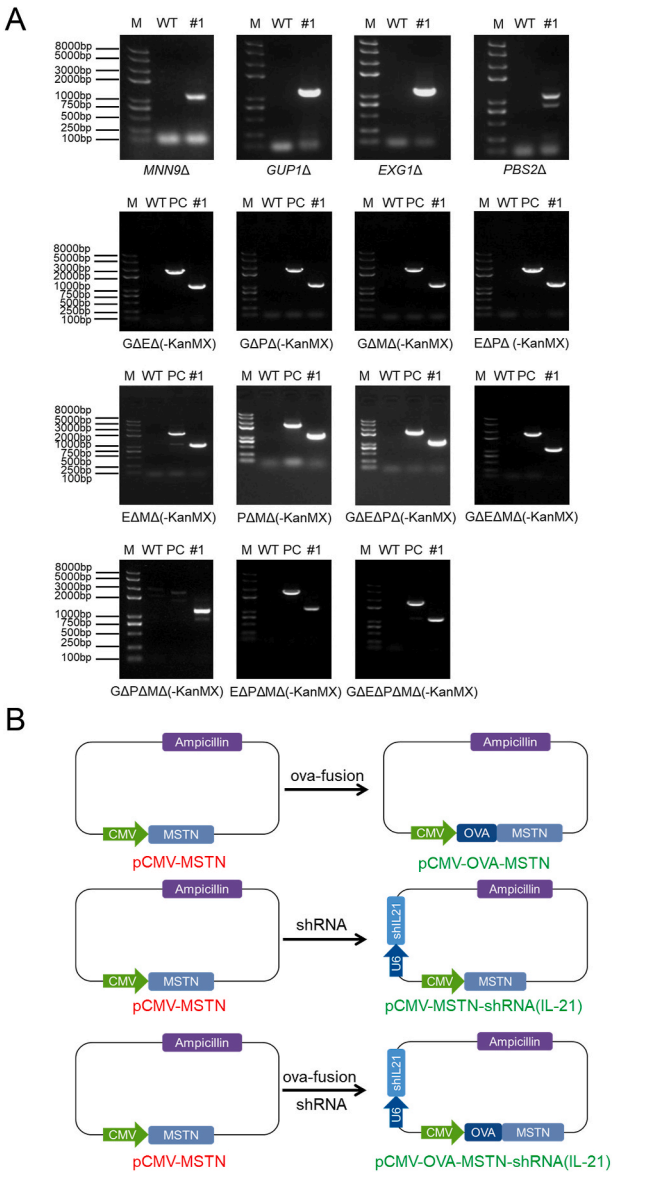

Fig. 2. The detection of gene-knockout strains without drug resistance-gene and three different strategies for optimizing recombinant DNA. A. The detection of different gene-knockout positive yeast strains without drug resistance-gene. The detection of positive clones was displayed in figure. M: Trans2k PlusII DNA Marker; WT: wild-type yeast strain group; PC: Positive single gene knockout yeast group; #1: gene-knockout group correspond to the labeled below the gel image; Δrepresents gene deletion; B. There were three optimized recombinant DNA: pCMV-OVA-MSTN, which was constructed by fusion of the chicken *OVA* gene and co-expressing shRNA of *IL-21* gene; pCMV-MSTN-shRNA (IL-21), which was constructed by co-expressing shRNA of *IL-21* gene; pCMV-OVA-MSTN-shRNA (IL-21), which was constructed by fusion of the chicken *OVA* gene and co-expression of shRNA of *IL-21* gene.

centrifugation at 5000 rpm, and diluted with a sterilized PBS buffer to a suitable concentration (5×10^9 cells/mL). After being dispensed into a 1.5 mL tube, it was placed in a 56 °C water bath for 1 h to kill the yeast, and finally stored at −80 °C until use [33].

2.5. Immunization

In the first part of this study, 72 mice were randomly divided into eighteen experimental groups (Supplemental Table S3). There are two repetitions for each experimental group and every repetition had two female mice. Experimental protocol was displayed as following: mice in different experimental groups were orally administrated with 5×10^8 recombinant gene-knockout yeast cells carrying pCMV-MSTN plasmid mixed with 100 μL of PBS for 8 weeks; mice in positive control group were orally administrated with 5×10^8 recombinant AH109 yeast cells carrying pCMV-MSTN plasmid mixed with 100 μL of PBS; mice in blank control group were orally administrated with 100 μL of PBS for 8 weeks; mice in negative control group were orally administrated with 5×10^8 recombinant yeast cells carrying naked plasmid (maintained in our lab) mixed with 100 μL of PBS for 8 weeks; all groups were orally administrated once every four days.

In the second part of this study, 24 mice were randomly divided into six experimental groups (Supplemental Table S4). There are two repetitions for each experimental group and every repetition had two female mice. Experimental protocol was displayed as following: mice in experimental groups were orally administrated with 5×10^8 recombinant yeast cells carrying different recombinant DNA mixed with 100 μL of PBS for 8 weeks; mice in negative control group were orally administrated with 100 μL of PBS for 8 weeks; mice in control group were orally administrated with 5×10^8 recombinant yeast cells carrying naked plasmid (maintained in our lab) mixed with 100 μL of PBS for 8 weeks; feeding interval is the same as above.

2.6. mstn expression in bacteria

In this part, the whole induction process was conducted according to previous reports [34]. The induced purified MSTN protein is used for coating the antigen in ELISA analysis experiments and western blotting. Firstly, the plasmid pET32-MSTN was transformed into *E. coli* strain Rosseta (DE3). Then recombinant bacteria were cultured with the LB medium until the OD600 of cells reached 0.6. The subsequent induction step was under the condition: 0.5 mM IPTG at 37 °C for 4 h in 2 mL medium. After that, induced bacteria were broken by sonication and made a centrifugation at 12,000 rpm for 3 min. Hereafter, 500 μL of 8 M urea was used to re-suspend the pellet. Afterward, re-suspended cells were broken again and made a centrifugation at 12,000 rpm for 10 min, and then the collected supernatant was purified with Ni-NTA Kit (Bai-Hui, China). The purified mstn protein was aliquoted and stored at −80 °C until use.

2.7. Weight measurement and statistical analysis

We measured the body weight of mice at two time points: 0-day and 56-day (from the beginning to the end), separately. Each mouse weight gain obtaining from their corresponding weight between these two time points was calculated statistically. Every group was consisted of four mice and we took the average of four samples for subsequent experimental analysis. The weight gain of mice was evaluated with ANOVA and expressed as means ± SE, and the unpaired, two-tailed independent Student's t-test were used to analyze the differences between control groups and immunized groups. A difference was significant if $p < 0.05$ was obtained.

2.8. ELISA assay

ELISA assay experiments were conducted using the mixed sera of every group as mentioned previously [32]. In the first part of this study, we first compared the weight gain and muscle growth conditions among all the experimental groups. After that, we further compared the difference between the screened three experimental groups. We set 7 dilutions (1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280) to conduct the ELISA assay. The previous purified mstn protein was used as the antigen protein in this experiment. Each antiserum dilution was assayed with three repeats and every repetition was also conducted for detection. The whole process was conducted as describe previously [35]. Briefly, after using the previous induced and purified mstn protein as the antigen for coating antigen, the sample was washed three times with PBS buffer.

Afterward, the sample were incubated with different mice serum being as the primary antibody at 4 °C overnights. Then PBS buffer was used to wash samples three times again and these samples were incubated with the HRP-conjugated goat anti-mouse antibody (1:2000, Beyotime, China) for 2 h. After four times washing with PBS buffer and adding the substrate solution, we read the absorbance (optical density) of each well with a plate reader.

In the second part of this study, to determine the better efficiency of recombinant DNA, we compared the antibodies titers between the four experimental groups. The experimental procedure refers to the above steps.

3. Results

3.1. Vectors construction and selection of positive gene-knockout strains

All resulted vectors were constructed and validated based on the experimental design (data not shown). With the PCR method as displayed in the Supplemental Fig. 1A, after deletion of drug resistance gene, we successfully screened 15 positive gene-knockout strains (Supplemental Table S1; Fig. 2A), which provides guarantee for follow-up experiments.

3.2. mstn expression in bacteria

According to previous reports [34,36,37], we adopted the established protocol to induce the mstn expression in bacteria. As shown in Supplemental Fig. 1B, the successful induction of mstn protein was obviously detected.

3.3. Effect of gene-knockout yeast-based vaccines on the weight gain and muscle growth

After lasting 56-day vaccination, we measured the body weight of mice at two time points: 0-day and 56-day (from the beginning to the end), separately. For the first part of this study, as shown in Fig. 3A, the weight gain (data was displayed in Supplemental Table S5) of several groups was slightly or significantly higher than positive control group when mice were at the same age with little weight difference at the start, indicating the strengthening role of different gene-knockout strains on inducing specific immune response. Moreover, the muscle growth condition was also compared between different groups. Herein, as displayed

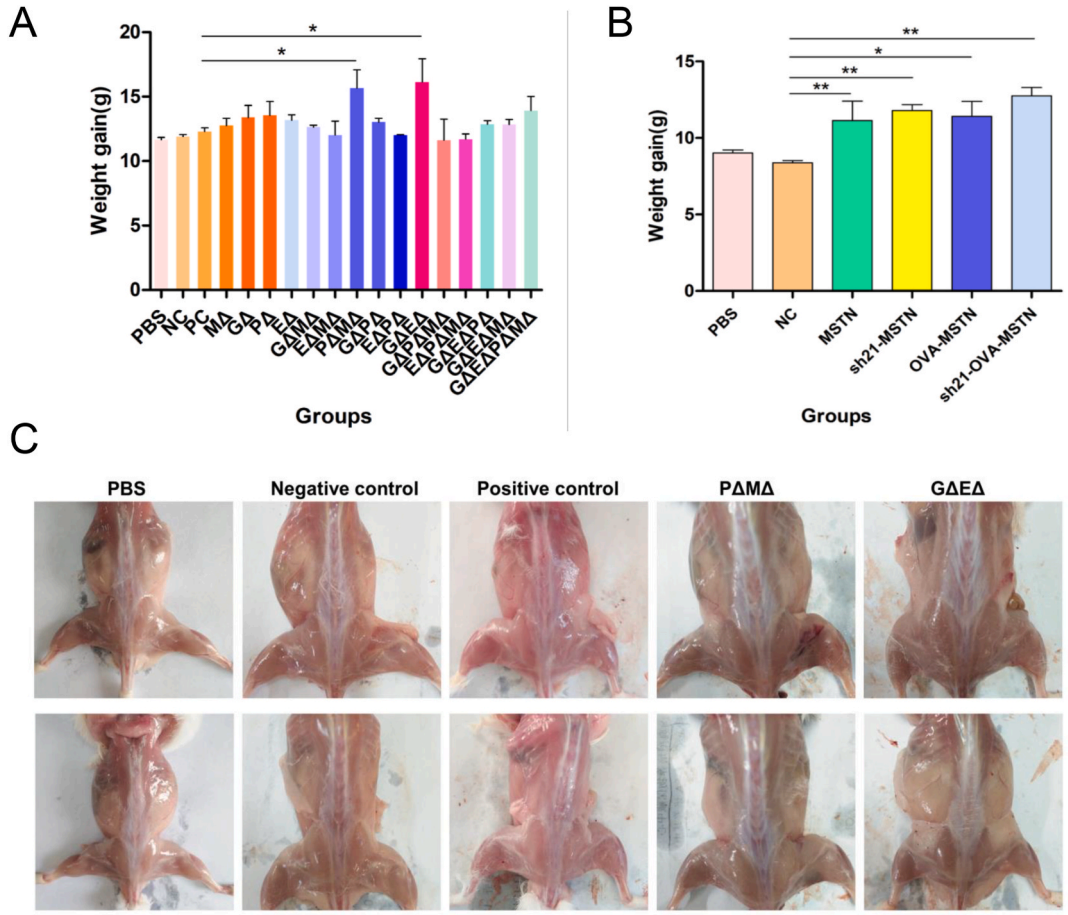

Fig. 3. Comparison of weight gain effects and mstn antibodies in serum of different experimental groups. A. After 56-day feeding, the weight gain of different groups was compared with the positive control. PBS: control group feeding orally with PBS buffer; NC: wild-type yeast group carrying naked plasmid; PC: wild-type yeast carrying *MSTN* gene eukaryotic expression plasmid. M, G, E and P stand for *MNN9*, *GUP1*, *EXG1* and *PBS2*, separately; Δ represents gene deletion. Asterisks "*" mark the significant differences between the experimental groups and control group; B. After 56-day feeding, the weight gain of different groups was compared with the negative control. PBS: control group feeding orally with PBS buffer; NC: *GUP1ΔEXG1Δ* yeast group carrying naked plasmid; MSTN: *GUP1ΔEXG1Δ* yeast group carrying pCMV-MSTN plasmid; sh21-MSTN: *GUP1ΔEXG1Δ* yeast group carrying pCMV-MSTN-shIL2 plasmid; OVA-MSTN: *GUP1ΔEXG1Δ* yeast group carrying the pCMV-OVA-MSTN plasmid; sh21-OVA-MSTN: *GUP1ΔEXG1Δ* yeast group carrying the pCMV-OVA-MSTN-shIL2 eukaryotic expression plasmid (Asterisks "*" mark the significant differences between the experimental groups and the control group; asterisks "**" mark the extremely significant difference between the experimental groups and the control group); C. Comparison of muscle morphology between different groups. PBS: control group feeding orally with PBS buffer; NC: wild-type yeast group carrying naked plasmid; PC: wild-type yeast carrying *MSTN* gene eukaryotic expression plasmid. M, G, E and P stand for *MNN9*, *GUP1*, *EXG1* and *PBS2*, separately; Δ represents gene deletion.

in Fig. 3C, it was found that the muscle growth condition of *MNN9ΔPBS2Δ* and *GUP1ΔEXG1Δ* groups were better than control groups, suggesting that these two gene-knockout yeast vaccines can effectively enhance the muscle growth of mice.

In the second part of this study, we also compared the weight gain difference (data was displayed in Supplemental Table S6) between different groups. It was found that the pCMV-OVA-MSTN-shRNA (IL-21) group has the highest weight gain than control group (yeast harboring the naked plasmid) when mice were at the same age with little weight difference at the start (Fig. 3B), indicating the better strengthening role of the optimized recombinant DNA on inducing specific immune response.

3.4. Immune response of mice inducing from yeast-based DNA vaccines

The presence of mstn-specific antibodies was used for determining the induction of the specific immune response in mouse serum. In this experiment, equivalent purified mstn protein was used to conduct the ELISA assay. ELISA experiments were conducted as described previously [38]. According to the results shown in Fig. 4A, the antibody titers of *MNN9ΔPBS2Δ* and *GUP1ΔEXG1Δ* groups were significantly higher than positive control group, which indicated that these two strains can induce better immune response. In the second part of this study, we also found that the pCMV-OVA-MSTN-shRNA (IL-21) group has the highest antibody titer among the other groups, which was in consistent with above results (Fig. 4B).

4. Discussion

S. cerevisiae has been widely used in human life, particularly in the food industry. It is also commonly considered a food-grade, non-harmful microorganism. With the deepening of scientific research, scientists have found that recombinant *S. cerevisiae* can present a variety of foreign proteins or nucleic acids to the body through a variety of vaccination methods (injection or oral), thereby inducing the immune response [39–41]. This delivering system is also known as food-grade recombinant yeast vaccine [42]. In addition, the researchers also found that the yeast vaccine can be used to achieve regulation of animal body production traits by oral administration, including regulation of animal muscle growth through neutralization of mstn protein [43]. Based on above research reports, this study has optimized the existing oral yeast vaccine from two perspectives, and hopes to achieve more efficient immune regulation through optimized vaccines.

Long-term production practices have found that the glucan component on the surface of *S. cerevisiae* plays an important role in promoting the growth and development of the body and improving the body's immunity [6,44]. The subsequent research reports also found that the glucan component on the surface of *S. cerevisiae* mediates the interaction between antigen presenting cells (mainly including dendritic cells and macrophage) and yeast via their mutual recognition [45,46], and the recognition between antigen presenting cells and yeast is the key core of yeast vaccine. In this study, we used genome editing to directionally knock-out genes that inhibit glucan synthesis in yeast, thereby increasing the content of glucan on the surface of yeast, thereby enhancing the recognition interaction between antigen presenting cells and yeast, and finally promoting the effectiveness of yeast vaccines. The results of this experiment indicate that the two knockout yeast strain have better immunomodulatory effects. This also laid the foundation for the optimization and application of subsequent oral yeast-based vaccines. However, due to the lack of existing quantitative methods for glucan extraction, this study failed to quantify the glucan of the experimental gene knock-out yeast, which is yet to be further determined in the future.

Studies have shown that fusion of antigen with chicken ovalbumin (*OVA*) gene can effectively improve the antigenicity of antigens [29,47]; In 2014, it was found that oral recombinant yeast can target intestinal

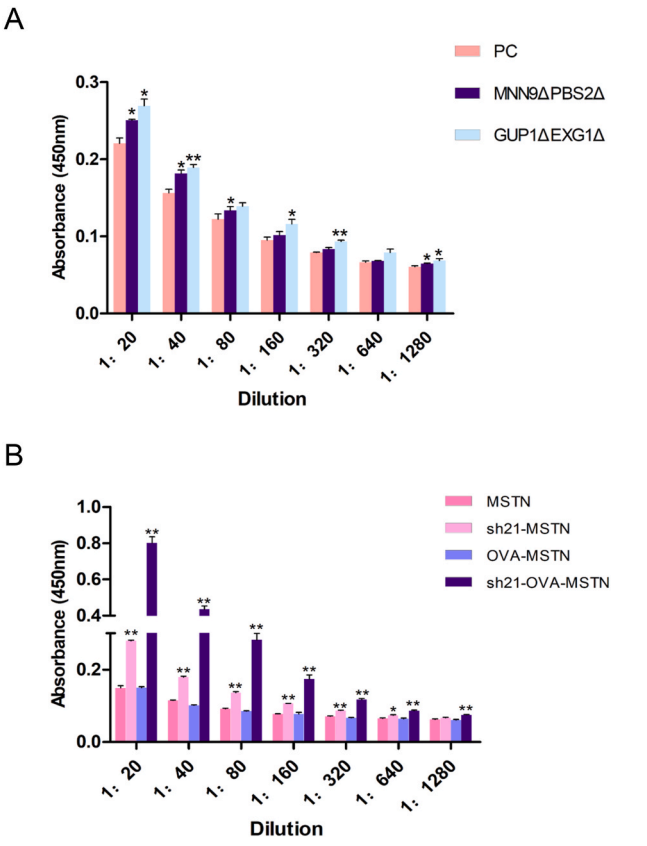

Fig. 4. Comparison of antibody titers between different experimental groups. A. mstn-specific antibody levels of mice vaccinated with different DNA vaccines were determined by ELISA. Data are means for three assays (three repeats from every group) and presented as the means ± SE. Asterisks "*" mark the significant differences between the experimental groups and the control group; ANOVA for comparisons of multiple treatment groups within individual experiments. Asterisks "**" mark the extremely significant difference between the experimental groups and the control group. PC: wild-type yeast carrying *MSTN* gene eukaryotic expression plasmid; *MNN9ΔPBS2Δ*: *MNN9ΔPBS2Δ* yeast group carrying pCMV-MSTN plasmid; *GUP1ΔEXG1Δ*: *GUP1ΔEXG1Δ* yeast group carrying pCMV-MSTN plasmid; B. mstn-specific antibody levels of mice vaccinated with different DNA vaccines were determined by ELISA. Data are means for three assays (three repeats from every group) and presented as the means ± SE, ANOVA for comparisons of multiple treatment groups within individual experiments. Asterisks "*" mark the significant differences between the experimental groups and the control group; Asterisks "**" mark the extremely significant difference between the experimental groups and the control group. MSTN: *GUP1ΔEXG1Δ* yeast group carrying pCMV-MSTN plasmid; sh21-MSTN: *GUP1ΔEXG1Δ* yeast group carrying pCMV-MSTN-shIL2 plasmid; OVA-MSTN: *GUP1ΔEXG1Δ* yeast group carrying the pCMV-OVA-MSTN plasmid; sh21-OVA-MSTN: *GUP1ΔEXG1Δ* yeast group carrying the pCMV-OVA-MSTN-shIL2 eukaryotic expression plasmid.

DC cells and successfully introduce shRNA into the cells, thereby interfering with the expression of *CD40* gene in DC cells [41]. Based on above researches, to improve the immunogenicity of the antigenic protein in the yeast vaccine, we made a fusion expression of the *OVA/MSTN* genes and co-expressed the shRNA expression cassette of the *IL21* gene with the *MSTN* gene expression cassette. The results show that combined usage of OVA-MSTN fusion expression and shRNA (IL-21) have the best immune effect compared with other optimized oral recombinant yeast vaccines, which is beneficial for further optimization and application of oral yeast vaccine.

In conclusion, this study optimized the existing oral yeast-based DNA vaccine from different angles, and screened two strains with stronger immunity. Based on the selected strains, the recombinant DNA was

further optimized. In consideration of the mammalian model used in the experiments, we hope that the optimized yeast-based vaccine can be applied to more animals or humans. This study will also provide a solid foundation and useful reference for the development and application of subsequent edible yeast vaccines.

Ethics statement

The animal study was reviewed and approved by Animal Ethics Committee of Northwest A&F University (2011–31101684).

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CRediT authorship contribution statement

Baoquan Han: and. **Feng Yue:** Data curation, Writing – original draft. **Xiaojun Zhang:** Visualization, Investigation. **Kun Xu:** Supervision. **Zhiying Zhang:** Conceptualization, Methodology, Software, Writing – review & editing. **Zhongyi Sun:** and, and. **Lu Mu:** and. **Xiaoyu Li:** Software, Validation.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2024.109425.

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