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Immunoprotection against influenza H5N1 virus by oral administration of enteric-coated recombinant *Lactococcus lactis* mini-capsules

Han Lei [a,b,c], Yuhong Xu [b], Jian Chen [b], Xiaohui Wei [b], Dominic Man-Kit Lam [d,e,*]

[a] School of Life Science and Technology, Shanghai Jiao Tong University, Shanghai, 200240, P.R. China
[b] School of Pharmacy, Shanghai Jiao University, Shanghai, 20024, P.R. China
[c] School of Life Science and Food Engineering, Nanchang University, Nanchang, Jiangxi, 330047, P.R. China
[d] Torsten Wiesel International Research Institute, Sichuan University, Chengdu, P.R. China
[e] Institute of Creativity, Hong Kong Baptist University, Hong Kong

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ABSTRACT

Edible vaccines that can be made widely available and easily administered could bring great benefit to the worldwide battle against pandemic viral infections. They can be used not only for the vaccination of humans and domesticated animals, but also for wild herds and live stock which are otherwise difficult to vaccinate. In this study, we report the development of an edible mini-capsule form of live, non-persisting, recombinant *Lactococcus lactis* (*L. lactis*) vaccine against the highly virulent influenza H5N1 strain. Recombinant *L. lactis*-based H5N1 HA antigen expression constructs were made and shown to be able to induce higher levels of HA-specific serum IgG and fecal IgA antibody production after oral administration. The vectors were then formulated into a mini-capsule dosage form and fed to mouse. Four doses of oral administration rendered complete protection of the mouse against lethal challenges of H5N1 virus.

Introduction

The highly pathogenic avian influenza H5N1 virus is considered a great threat to worldwide human and animal health. This virus strain is highly susceptible to antigen drift and shift and has already caused several outbreaks in human subjects with very high mortality rate (Duan et al., 2008; Subbarao et al., 1998). Vaccination is considered the most desirable counteraction to prevent the spreading and rapid mutation of the virus. It is also highly preferable to develop vaccines for all the species affected to slow down the cross-species spreading. However, conventional influenza vaccines made of inactivated viruses could hardly be useful for the H5N1 strain because of difficulties in manufacturing and the general requirement of multiple injections to every subject (Bright et al., 2003; Johansson et al., 2006; Kilbourne et al., 2002). New vaccine preparations, including various subunit vaccines (Wei et al., 2008), DNA vaccines (Chen et al., 2008; Kong et al., 2006) and recombinant adenovirus vaccines (Gao et al., 2006; Hoelscher et al., 2006) are being examined, but they all require injection which would be impossible for wild birds and costly and troublesome for humans and farm animals. In this regard, safe and efficacious oral vaccines would be ideal since they can be added to the food or drinks of the subjects to be immunized.

There has been a long pursuit of edible vaccines that are safe, effective and convenient to use. We had previously proposed the use of edible plants such as tomato and potato to express antigens that could be taken orally for the vaccination of human and livestock animals (Lam and Shi, 1996; Lam et al., 1997; Mason et al., 1992). Recently, rice was used as the antigen expression and delivery vehicle and showed to convey immunity against cholera in mice (Nochi et al., 2007). The use of edible plants as expression and delivery vehicles for vaccines offer many advantages, for a variety infectious diseases, especially those whose pathogens are relatively conserved. This is because it normally takes over 1 year to produce stably expressed transgenic plants. In contrast, for diseases in which the infective viruses mutate or shift rapidly, such as the influenza viruses, expression vehicles that are both edible and can be transformed in a matter of weeks are much more desirable. It is mainly for this reason that we chose the lactic acid bacteria (LAB) which is generally regarded as safe (GRAS) and widely consumed or used in food products. This approach has been reported in studies using recombinant *Lactococcus lactis* encoding various antigens for oral administration (Cho et al., 2007; Gilbert et al., 2000; Robinson et al., 1997; Xin et al., 2003), resulting in a variety of immune responses thought to be related to the type of antigen, the amount of antigen expressed, and the duration of antigen expression in the gut (Gruzza et al., 1994; Klijn et al., 1995).

In this study, we constructed two influenza H5N1 hemagglutinin (HA) antigen expression vectors based on a well-engineered nisinA-induced *L. lactis* expression strain. They either expressed the antigen in the cytoplasm (L2), or secreted the antigens (L3). These vectors were formulated with mucoadhesive polymers and packaged in

* Corresponding author. 1209 Shui On Center, 6 Harbour Road, Wanchai, Hong Kong, P.R. China. Fax: +852 2523 4817.
E-mail address: dlam.ibi@gmail.com (D.M.-K. Lam).

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enteric-coated mini-capsules (mm size). After oral administration of the enteric-coated antigen-secreted expression vector, the resulted immune responses were greatly improved, resulting in complete protection of the immunized mice from a lethal dose of viral challenge.

Results

Influenza H5N1 HA antigen gene expression by recombinant L. lactis

Three different *L. lactis* vectors were constructed and named L1 (empty vector), L2 (non-secretion) and L3 (secretion), respectively. Western blot analysis showed the L1 as a control vector which does not express any HA (Fig. 1C), and L2 expressed the HA protein (64 kDa) which was found in the cell lysate and no detection in supernatant (Fig. 1C). L3 had an usp45 signal sequence cloned before the HA gene, so expressed HAs were found in the cell lysate and the culture supernatant (Fig. 1C). These results supported that antigen proteins were correctly expressed and located.

Enteric capsule preparation and L. lactis release in simulated gastrointestinal (GI) environment

About 10^9 CFU of recombinant *L. lactis* vectors (10^9 CFU) were packaged in enteric capsules (mini-capsule), each measuring about 4×1 mm in size. The samples were named capsule-L1, capsule-L2 and capsule-L3, respectively. The capsule samples as well as solution samples were treated with simulated gastric fluid at pH 1.0 for 2 hours and then released in simulated intestinal buffer at pH 6.8 for 45 min. The resulted live *L. lactis* cell counts (CFU) were listed in Table 1. The capsule groups showed strong acid resistance and survived at pH 1.0 with a small loss of viable cell numbers, while the solution groups had drastically reduced live vector counts (>20000-fold) after the treatments. These results indicated enteric capsules are helpful and necessary for delivery of the recombinant *L. lactis* to the intestine escaping the degradation of stomach acid.

Table 1
The relative live *Lactococcus lactis* count after simulated gastric fluid treatment.

L. lactis vector	Live bacteria number (CFU)
L1	3.5×10^4
L2	3.6×10^4
L3	3.8×10^4
Capsule-L1	10^9
Capsule-L2	10^9
Capsule-L3	10^9

HA-specific IgG and IgA responses after oral administration

The mice were immunized four times at weeks 0, 2, 4 and 6 by oral administration of the recombinant *L. lactis* solutions or enteric capsules. Immunization dose was 10^9 CFU of the recombinant *L. lactis* in solutions or capsule forms containing the same amount of the

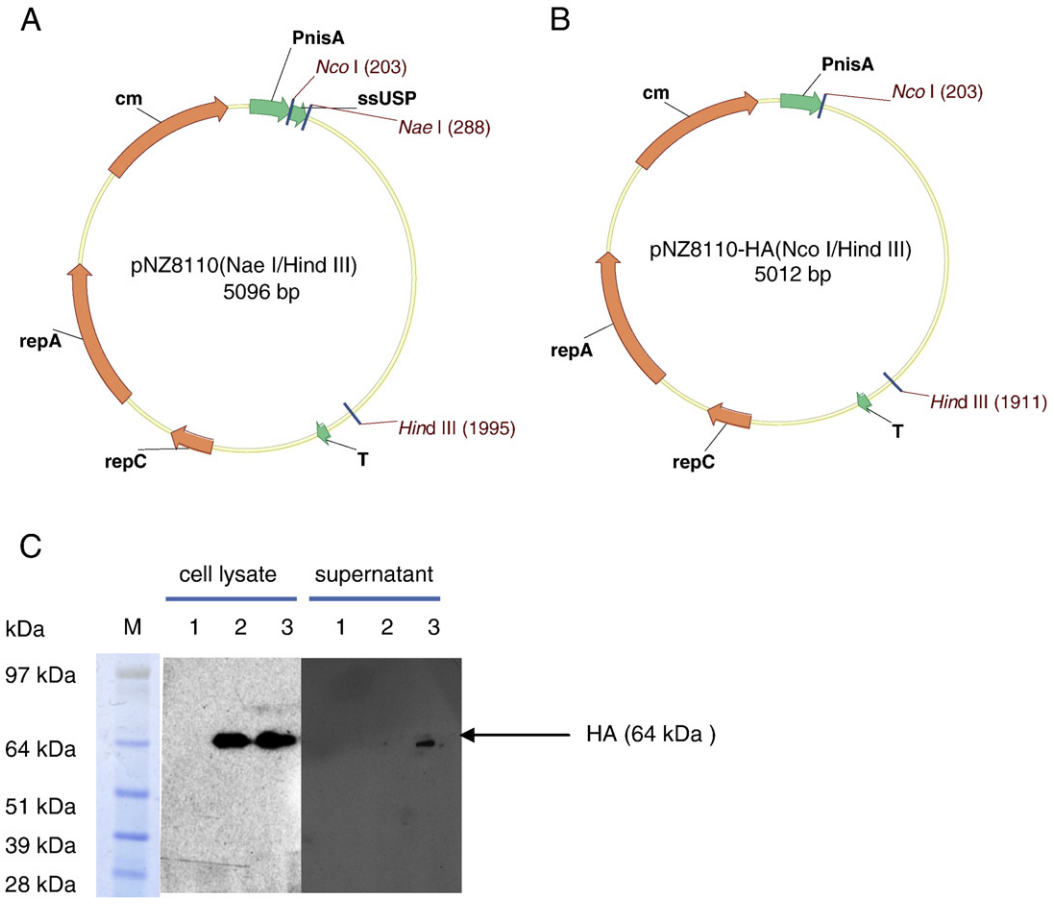

Fig. 1. Secretory and non-secretory vectors construct and expression of HA antigen gene on *Lactococcus lactis*. (A) A 1704-bp HA gene fragment was fused with the secretory expression plasmid pNZ8110 that contained secretion signal sequence ssUSP, to produce pNZ8110 (*Nae*I/*Hin*dIII). (B) After ssUSP was deleted by *Nco*I/*Hin*dIII digestion, non-secretory expression plasmid pNZ8110 (*Nco*I/*Hin*dIII). (C) Secretion and non-secretion expression on *L. lactis* were confirmed by western blot analysis in the cell lysate and supernatant, respectively. M: pre-staining marker, Lane 1: L1 (control), Lane 2: L2(non-secretion), Lane 3: L3(secretion).

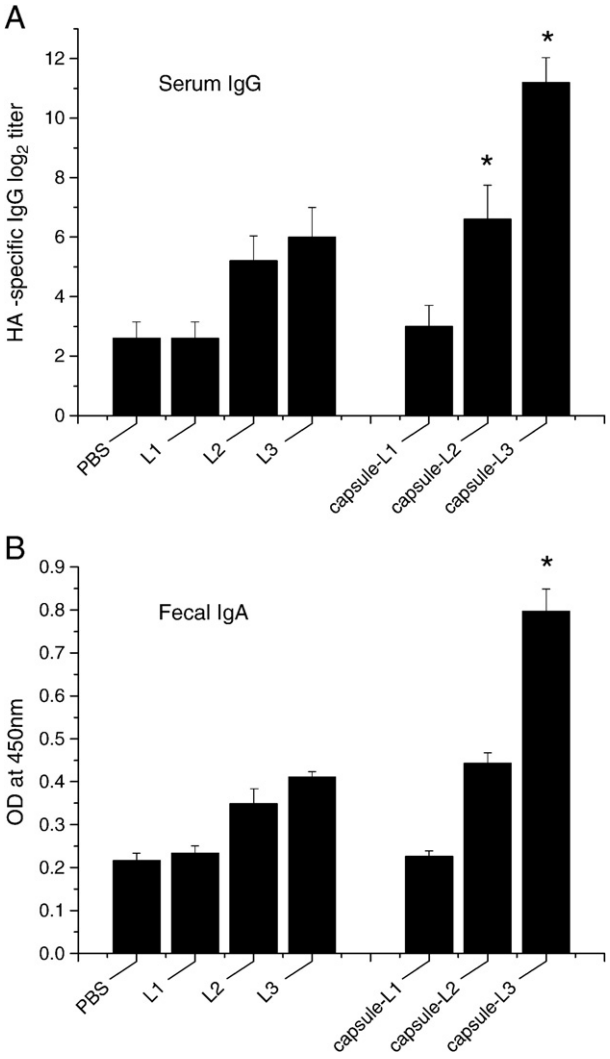

Fig. 2. HA-specific antibody titers detected by ELISA. Mice were orally immunized with enteric capsules and non-enteric live bacteria. (A) HA-specific serum IgG was determined by ABS-ELISA using recombinant HA protein as a coating antigen. (B) HA-specific mucosal IgA was determined from the fecal pellets. *Statistically significant differences relative to the PBS, L1 and capsule-L1 controls (*$p<0.05$). Data are given as mean ± SD of duplicate experiments. $n=5$ mice per group.

recombinant *L. lactis* solutions (10^9 CFU). HA-specific serum IgG and fecal IgA levels were measured 10 days after the last immunization dosing.

The mean $\log_2$ titers of serum IgG of all the tested groups were shown in Fig. 2A. All the HA expressing vectors (solution and capsules) resulted in significant production of HA-specific serum IgG, while PBS and L1 samples did not. In general, encapsulated groups (capsule-L2 and capsule-L3) had higher titers than the corresponding solution groups. The group dosed with capsule-L3 reached the highest antibody titer, 11.2 ± 0.837, and significantly higher than capsule-L2, 6.6 ± 1.14.

To examine the HA-specific mucosal immune response, the production of mucosal IgA antibody was examined using fecal pellets (Fig. 2B). Again, the enteric capsule groups all developed significant IgA antibody responses. The capsule-L3 gave the best results.

Neutralizing antibody titers in mice

The neutralizing antibody titers in each treatment groups were measured using the microneutralization (MN) method. Neutralization titers values of the enteric capsule groups (capsule-L2, and capsule-L3) were all higher than 80, suggesting good neutralization activities of the antibodies generated (Table 2).

Table 2
Neutralization assay of H5N1 virus with immune serum ($n=5$ mice per group).

Mice orally immunized with	Neutralization titer of serum (IC_{50})
PBS	<10
L1	<10
L2	47
L3	68
Capsule-L1	<10
Capsule-L2	80
Capsule-L3	148

Antigen specific T cell responses

We also used the IFN-γ ELISpot assay to examine the HA-specific T cell response resulted from recombinant *L. lactis* after oral administration. Splenocytes (10^6 cells) from each treatment group were collected and stimulated with 10 μg/ml of HA epitope peptide (ISVGTSTLNQRLVP). The resulted IFN-γ expressing T cell numbers were counted and plotted in Fig. 3. Again, there were HA-specific T cell responses generated by oral administration of enteric-coated recombinant *L. lactis*. The encapsulated groups (capsule-L2 and capsule-L3) were much more effective than the respective solution groups. These results revealed capsule-L2 and capsule-L3 not only induced humoral and mucosal immune, but also produced cellular immune.

H5N1 virus challenge experiment

Two weeks after the final immunization, the mice were intranasally challenged with lethal doses of highly pathogenic H5N1 viruses and closely monitored for 14 days for weight loss and mortality. After viral challenge, all mice experienced certain levels of body weight loss (Fig. 4A), but mice immunized with capsule-L3 gradually recovered after 8 days and 100% survival. In contrast, the naïve mice (PBS treated) and mice immunized with the empty plasmid vector (L1 and capsule-L1), all died within 10 days of challenge (Fig. 4B).

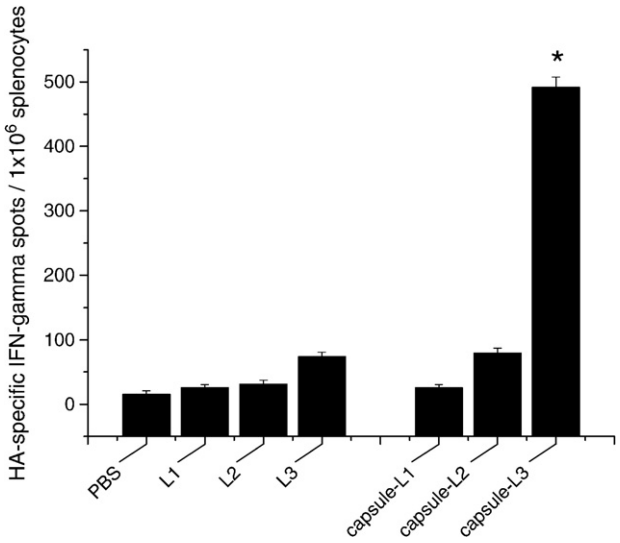

Fig. 3. Cell-mediated immune responses induced by enteric-coated recombinant *Lactococcus lactis*. *Statistically significant differences relative to the PBS, L1 and capsule-L1 controls. Data are represented as mean ± SD of triplicate experiments. $n=5$ mice per group.

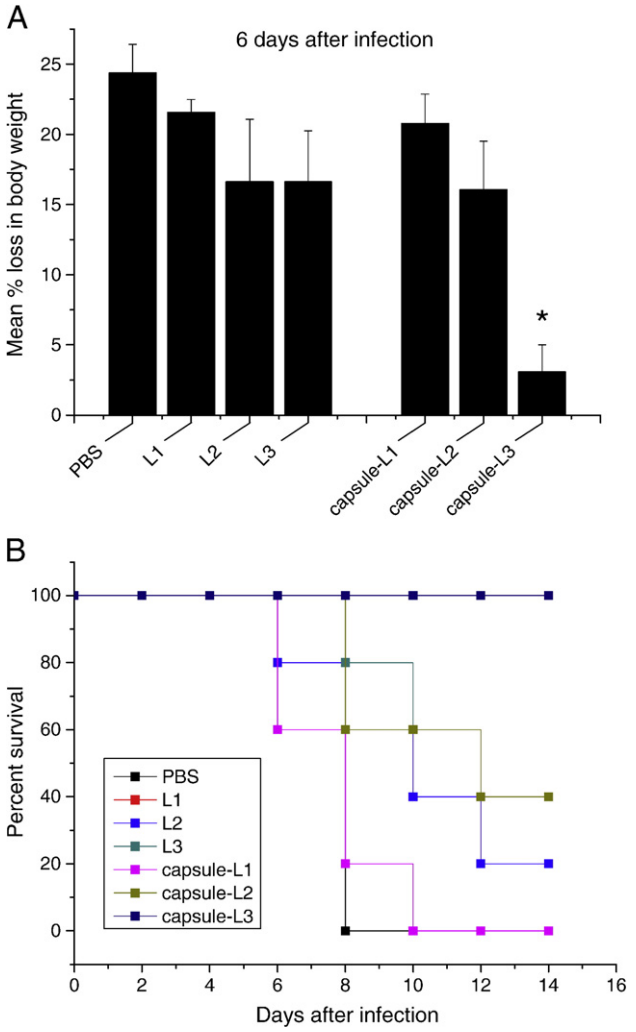

Fig. 4. Immune protection against H5N1 virus lethal challenges after oral deliveries of different vaccine preparations. Mice were infected intranasally with H5N1 virus 2 weeks after the last immunization. (A) Mean weight loss (%) of mice 6 days after infection. (B) Percent survival of mice 0–14 days after infection. *Statistically significant differences relative to the PBS, L1 and capsule-L1 controls. $n = 5$ mice per group.

Discussion

For viral infections, such as the H5N1 influenza virus which affects humans as well as many animal species, it is highly desirable to develop safe and efficacious edible vaccine formulations that can be produced economically and applied conveniently. In addition, since influenza viruses naturally infect through the mucosa, it is also highly desirable to develop vaccines that can induce mucosal immune responses as well as systemic responses. Mucosal vaccine delivery by the oral route is considered the most convenient method, but most viruses and protein antigens are quickly degraded in the gastrointestinal system. They might also not be immunogenic enough to elicit significant or clinically important immune responses.

Different antigen carrier systems have been proposed, including recombinant plants, bacteria or virus-based vectors for the production and presentation of the antigens (Cho et al., 2007; Mason et al., 1992). In addition, various polymer and lipid microspheres have been used for the protection and controlled release of protein antigens in the gut. In this study, we have combined these two approaches to produce an oral vaccine that is effective against H5N1 infection in mice and probably in chicken as well. The recombinant *L. lactis* vectors were ideal to produce large quantities of antigens and deliver them orally to the gut. Considering that the gastric environment would still be somewhat hostile to *L. lactis* viability, we have developed an enteric-coated polymer capsule formulation of a small enough size (mm) to be ingested even by mice or chickens. Our results indicate that this formulation indeed produces a great improvement on the overall immunogenicities of the vaccines, resulting in complete protection and survival of mice injected with an otherwise lethal dosage of H5N1 virus.

For the production and delivery of antigens, genetically engineered live vector systems have many advantages in manufacturing and processing. About 20 years ago, Lam and his colleagues initiated the plant-based vaccine development and showed the feasibility of using plants to express HBV surface proteins and viral particles to be used as vaccines (Lam and Shi, 1996; Lam et al., 1997; Mason et al., 1992). More recently, there was a study using rice grain as the delivery vehicle. The protein antigens were shown to be well protected and maintained stable in rice without requirements for refrigeration (Nochi et al., 2007). Good stability of oral vaccines under ambient conditions is clearly important for the distribution of vaccines to remote areas of the world.

Alternatively, bacteria-based systems such as *Salmonella, Bortedella and Listeria* spp. have also been studied extensively as antigen expression and delivery carriers. Most of them were originally pathogenic strains so they may be more immunogenic or express stronger immune responses. In contrast, the lactic acid bacteria (LAB)-based vectors are considered safer, but may not be as immunogenic for the human immune system. Some studies have suggested the ability of certain LAB vectors to persist in the GI tract is critical for the effectiveness of vaccines. Grangette et al. (2002) conducted a direct comparison of *Lactococcus plantarum*, a persisting LAB, and *L. lactis*, a non-persisting LAB and found *L. plantarum* to be more effective at eliciting antigen-specific immunity. Many other studies employed *Lactococcus casei*-based vectors which could also persist in human GI microbiota. However, the use of persisting bacteria might not be desirable as vehicles for edible vaccines as special consideration would be needed for biocontainment purposes (Steidler et al., 2003).

The uniqueness of the present study is the successful creation of an edible vaccine against an influenza virus (H5N1) using a non-persisting *L. lactis* (Kimoto et al., 2003) as the carrier, loading them on mucoadhesive polymers and packaging them in enteric-coated mini-capsules. We showed that although the viability of *L. lactis* is rapidly diminished in the gastrointestinal tract, the antigens they carried and produced shortly after they were administered and protected temporarily by the encapsulation were sufficient to induce significant mucosal and systemic immune responses and allow all the mice to survive the lethal in mice and chickens, challenge of H5N1 injection.

It is possible that the vector systems themselves had specific immune stimulation effects. It has been shown that LAB can initiate inflammatory responses and activate monocytes and other antigen presenting cells (Mercenier et al., 2003). In our study, different immune responses resulted from similar vector systems which differed only in antigen expression designs were observed. The mini-capsule form of the HA antigen expression and secretion vector (capsule-L3) gave the best response in every aspect after. For avian influenza strains, the analysis of sera from mice supports the use of a neutralizing titer of ≥ 80 as an efficacy endpoint (Eichelberger et al., 2007). In this study, the neutralization titer of capsule-L2 was 80 and the survival rate was 40% after H5N1 infection. In contrast, neutralization titer of capsule-L3 was 148 and provided 100% protection against H5N1 virus challenge. Similar plans for H5N1 challenges in chickens are in progress pending regulatory approvals.

The influenza HA antigen gene (AY950232 in GenBank) we cloned in our vectors is from A/chicken/Henan/12/2004(H5N1). The enteric-coated mini-capsule dosage forms could result in significant

humoral and systemic immune responses and provide 100% protection from H5N1 virus challenge. The nisinA-induced recombinant *L. lactis* antigen expression vectors we used are very flexible in design and can be optimized for antigen expression and presentation. It is also a well-engineered stable system for large scale production (Bahey-El-Din et al., 2008; De Ruyter et al., 1996). The polymer mini-capsule formulation we developed was also easy and inexpensive to manufacture. Although it is a simple design, the improvement in vaccine efficacy was highly significant.

Materials and methods

Construction of recombinant L. lactis expressing HA gene

The *L. lactis* expression plasmid pNZ8110 was purchased from NIZO, Netherlands. The HA gene fragment (1704 bp) was amplified from pGEM-HA (kindly supplied by Prof. Ze Chen, Wuhan, China) by PCR using the following primer pairs with *Nae*I or *Hin*dIII sites underlined (forward primer: 5'tctgccggcgagaaaatagtgcttctt3', reverse primer: 5'cccaagcttttaaatgcaaattctgcattgtaacg 3'). The resulting *Nae*I/*Hin*dIII fragment was cloned into pNZ8110 containing the secretion signal ssUSP (Fig. 1A). Another plasmid was constructed by replacing the forward primer with the following primer containing an *Nco*I site underlined (5'catgccatggagaaaatagtgcttctt3'). The resulting *Nco*I/*Hin*dIII fragment was sub-cloned into pNZ8110 so that the ssUSP was deleted (Fig. 1B).

These plasmids were then individually transformed into the *L. lactis* NZ9000 stains by electroporation. The most highly expressed clones were selected and banked. The *L. lactis* vector transfected with the empty plasmid pNZ8110 was designated as L1. The vector transfected with the HA expressing plasmid without the ssUSP sequence was designated as L2. The one transfected with the HA expressing plasmid containing the ssUSP sequence was designated as L3.

All the *L. lactis* stains were cultured at 30 °C in M17 medium supplemented with 0.5% (wt/vol) glucose. Chloramphenicol was used at a concentration of 10 μg/ml.

Western blot analysis

The HA antigen expression levels in the *L. lactis* cultures were analyzed by Western blot analysis. NisinA were added into the culture medium to the final concentration of 1 ng/ml to induce antigen expressions. The cultures were maintained for 3 more hours and the supernatants were harvested. The harvested *L. lactis* cells pellets were washed three times with 500 μl sterile phosphate-buffered saline (PBS), and resuspended. Aliquots of the samples (either cell suspensions or supernatants) were mixed with 6× loading buffer and boiled 10 min. Extracts were run on SDS–PAGE (10% acrylamide) and transferred to polyvinylidene difluoride membrane (PVDF, Millipore, USA). Protein was detected using polyclonal mouse anti-HA antibody followed by affinity-purified horseradish peroxidase (HRP)-conjugated anti-mouse IgG (R&D Systems). The membrane was radiographed on X-film using the ECL Western Blotting Detection System according to the manufacture's recommendations (Pierce, USA).

Preparation and analysis of the enteric-coated mini-capsule forms of L. lactis vectors

L1, L2 and L3 were *p*repared into solutions with the concentration of 10^{11} colony-formation units (CFU)/ml, respectively. Ten microliters of the recombinant *L. lactis* solution containing 1×10^{9} CFU was mixed with 0.5 mg of BSA and methyl cellulose (MC), air dried and packaged into enteric-coated capsules and named capsule-L1, capsule-L2, capsule-L3, respectively.

For the analysis of the capsule integrity, the capsules were immersed in simulated gastric fluid at pH 1.0 with low speed agitation for 2 hours and then dropped into phosphate buffer at pH 6.8 for 45 min to release the encapsulated contents. Viable cells were counted by gradient dilution methods.

Animals and animal immunizations

Six-week-old female BALB/c mice were purchased from China SLC, Shanghai, China. The mice were housed in the specific pathogen-free (SPF) Animal Center of Shanghai Jiao Tong University. They were divided into 5 mice per experimental group. Before each dose, they were fasted for 6 h and then administer 10 μl of the recombinant *L. lactis* solution or 1 capsule containing 10^{11} CFU of the respective recombinant *L. lactis* vectors using a 21-gauge feeding tube. Immunizations were repeated at 2, 4 and 6 weeks after the initial dosing.

ELISA assay

Sera were collected 10 days after the last dosing. HA-specific antibody responses were detected by avidin-biotin system (ABS)-enzyme-linked immunosorbent assay (ELISA) (Katz et al., 1997). The 10-μg/ml influenza A virus (A/chicken/Henan/12/2004(H5N1)) recombinant HA protein was used to coat 96-well microplates. At the same time, fecal pellets (50 mg) for each group of five mice were collected and suspended in 250 μl sterile PBS, centrifuged down at 15,000× rpm for 10 min, and the supernatants tested for IgA by indirect ELISA. The mean antibody titer was expressed as the highest dilution that yielded an optical density greater than twice the mean plus one standard deviation of that of similarly diluted negative control samples.

IFN-γ ELISpot assay

The IFN-γ ELISpot assay was performed one week after the final immunization using an ELISpot kit for mouse IFN-γ as recommended by the manufacturer (R&D Systems, USA). Briefly, mouse IFN-γ microplate was added 200 μl/well of sterile culture media and incubated for 20 min at room temperature. After aspirating the culture media from the wells, the plates were added 100 μl of 1×10^{6} splenocytes per well. The 10 μg/ml of HA-specific peptide (ISVGTSTLNQRLVP) was used as stimuli for 48 h in a humidified 37 °C CO_2 incubator. Control wells were not stimulated with HA-specific peptide. After incubation, each well was aspirated and washed, the plates were treated sequentially with biotinylated anti-mouse IFN-γ antibody, alkaline phosphatase conjugated streptavidin and the substrate solution to reveal the spots. The developed microplate could be analyzed by counting spots using a dissection microscope.

Neutralization assay

Determination of endpoint neutralizing antibody titers was performed by microneutralization assay, as previously described (Rowe et al., 1999). Briefly, serial 2-fold dilutions of sera treated with receptor-destroying enzyme (RDE) from *Vibro cholerae* were mixed and incubated with 35 μl 100 50% tissue culture infective doses ($TCID_{50}$) of H5N1 virus, then added to Madin–Darby canine kidney (MDCK) cells and incubated for 1 h. The H5N1 virus-infected MDCK cells were further cultured for 72 h at 37 °C in the presence of 5% CO2, and the neutralizing titer was determined by hemagglutination test. For the HA test, 50 μl of 0.5% cock red blood cells was added to 50 μl of cell culture supernatant and incubated at room temperature for 30 min. The $TCID_{50}$ was determined on the basis of the Reed–Muench method (Rohm et al., 1995). The neutralization titer (IC_{50}) was defined as the reciprocal of the antiserum dilution at which H5N1 virus entry was 50% inhibited.

H5N1 virus challenge

For the challenge experiment, mice were anesthetized and intranasally challenged with 20 μl 10 × 50% lethal dose (LD_{50}) H5N1 virus 2 weeks after the last immunization. After infection, the mice were weighed and monitored for signs of illness for 14 days. The challenge experiments were strictly performed under biosafety level-3-plus enhancement conditions.

Statistical analysis

Statistical analysis of the experimental and control data were performed by one-way factorial analysis of variance. *P*-values less than 0.05 were considered as a statistical significance.

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