Induction of immune responses after intragastric administration of mice with *Lactobacillus casei* producing porcine parvovirus protective antigen VP2

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Abstract *Lactobacillus casei* ATCC 393 was selected as an antigen delivery vehicle for mucosal immunization against porcine parvovirus (PPV) infection. A 64kDa fragment of PPV major protective antigen VP2 protein was used as the parvovirus antigen model. A recombinant lactobacilli expressing VP2 protein was constructed with plasmid pPG611.1, where expression and localization of the VP2 protein from the recombinant Lc393-rPPV-VP2 was detected via SDS-PAGE, Western blot and immunofluorescence. BALB/c mice oral immunized with the recombinant lactobacilli expressing VP2 protein were induced both local mucosal and systemic immune responses against PPV. The induced antibodies demonstrated neutralizing effects on PPV infection. These data indicated that the recombinant lactobacilli could be a valuable strategy for future vaccine development of PPV.

Keywords: porcine parvovirus; VP2 protein; *Lactobacillus casei*; oral vaccination

1. Introduction

Porcine parvovirus (PPV) characterized as a member of autonomous paroviruses, is a major cause of reproductive failure in swine, resulting in early embryonic death, fetal death, stillbirths, delayed return to estrus [1-4] and costs pig producers serious loss. The molecular features of PPV are similar to those of other autonomous paroviruses. PPV is a small, nonenveloped virus and the virion contains a 5kb linear, minus-polarity single-stranded DNA genome [5], which encapsidated within a simple icosahedron protein coat composed of three structural polypeptides VP1, VP2 and VP3 with masses of 84, 64, and 60 kDa, respectively [6]. The VP2 protein encompasses major antigenic domains of PPV and could induce PPV-neutralizing antibodies for neutralization of PPV infection [7-9]. Therefore, the VP2 protein plays a major role for PPV diagnosis and immune prophylaxis.
The mucosa tissues are particularly important for protection against diseases such as viral, bacterial and parasitic pathogens for which invade the mucosal system [10, 11]. Vaccines administered by parenteral routes generally fail to stimulate mucosal immune responses. The mucosal immunization has been proven to be effective approach against colonization of pathogens and further spread to the systemic circulation [12-14]. Therefore, it is necessary to develop efficient and safe antigen vectors that will be able to trigger mucosal and systemic immune responses. One promising approach relies on the use of live bacterial vehicles [15].

The potentiality of recombinant lactic acid bacteria (LAB) to deliver heterologous antigens to the mucosal immune system has been investigated during the last decade [16-22]. This approach offers a number of advantages over the traditional parenteral vaccination, such as non-invasiveness and the possibility to elicit both mucosal and systemic immune responses. In addition, lactobacilli have been used in a large variety of industrial food fermentation and preservation and are known for the beneficial effects on the health of humans and animals and considered “generally regarded as safe, GRAS” microorganisms. They represent an original alternative to the use of attenuated pathogenic bacterial carriers such as Salmonella, Vibrio and Mycobacterium[23-25]. Lactobacillus strains have been used as a host to express many bacterial and viral antigens and proved to elicit immune responses after inoculation by oral administration[17-20, 22], which make them be attractive candidates as antigens delivery carriers for the presentation to the mucosa with pharmaceutical interest, in particular vaccines. Furthermore, Lactobacilli could survive transit of the upper gastrointestinal tract and colonize the intestinal tracts [26, 27]. In addition, lactobacilli have been shown intrinsic adjuvant activity [28, 29].

In this study, the potentiality of using L. casei 393 to express heterologous parvovirus protein and it acts as an antigen delivery carrier for oral vaccination was analyzed. The 64kDa fragment of PPV capsid protein VP2 that encompasses the major antigenic domains critical for neutralization and a cell-surface expression system pPG611.1 was used in this study. The immunogenicity of the rLc393-rPPV-VP2 was analyzed post intragastric administration of live bacteria to the BALB/c mice. This is the first report on the cloning and expression of PPV antigen in Lactobacillus. Our data have indicated that intragastric inoculation of the rLc393-rPPV-VP2 could induce specific immune responses against PPV.
2. Material and methods

2.1 Bacterial strain and growth conditions

*Lactobacillus casei* ATCC 393 kindly gifted by Jos Seegers (NIZO, Netherlands), was anaerobically grown in MRS broth (Sigma) at 37°C without shaking. To analyze the protein expression, the rLc393-rPPV-VP2 was grown in basal MRS medium (10g peptone, 8g beef extract, 4g yeast extract, 2g potassium phosphate, 5g sodium acetate, 1ml Tween-80, 2g diammonium citrate, 0.2g magnesium sulfate, and 0.05g manganese sulfate, per liter) supplemented 2% xylose. Plating of bacteria used in this study was performed on MRS medium with 1.5% agar. Antibiotic concentration used for the selection of lactobacilli transformants was 10µg/ml of chloromycetin, Cm (Sigma).

2.2 Labeling of Lactobacilli with fluorescence dye cFDA-SE

*L. casei* 393 was labeled with 5’-(and 6’)-carboxyfluorescein diacetate succinimidyl ester, cFDA-SE (Molecular Probes, Sigma), a non-fluorescent membrane permeative ester which non-specific prokaryotic and eukaryotic intracellular esterase converts to a fluorescent derivative that in turn is then covalently linked to intracellular protein via the probe’s succinimidyl group[30]. In brief, a 100µM stock solution of cFDA-SE was prepared by being first dissolved in dimethyl sulfoxide (20µl) and then further diluted in ethanol (1ml: reagent grade). This solution was filter sterilized before being aliquoted and stored at -20°C. *L. casei* 393 was grown overnight at 37°C in MRS broth. The bacterial cultures were centrifuged at 4,000×g for 10min, and the pellets were washed twice in sterile PBS and adjusted to a concentration of 10¹⁰ CFU/ml before being labeled with cFDA-SE at 37°C for 25min. Fluorescent labelling was terminated by pelleting the bacteria. The labelled bacteria were washed twice in sterile PBS to remove excess cFDA-SE, resuspended in sterile PBS. The flow cytometry profile showed that about 99.5% of cells were labelled.

2.3 Adhesion study on animal

Seven-week-old BALB/c mice were obtained from the Laboratory Animal Center, Harbin Medical University of China. A group of 15 mice were dosed with approximately 10⁹ cFDA-SE-labeled lactobacilli by oral administration. Another group of 15 mice were orally fed with sterile PBS as control. Groups of three mice each were sacrificed on days 1, 2, 4, 6.
and 7, and then the duodenum, jejunum, ileum, and colon were extracted from each mouse. Individual section was cut longitudinally and any visible residual food particles or fecal material were removed from the intestine before being examined for the presence of adhering cFDA-SE-labeled Lactobacilli. This was performed by adding 150µl of PBS to every 1.0cm of tissue and dislodging microbes from the mucosal surface of the tissues. Cell extracts were fixed with formaldehyde (0.75%, v/v) prior to flow cytometry analysis [27].

2.4 Bile tolerance of Lactobacilli

1-ml cultures of L.casei 393 grown in MRS medium (OD$_{600}$ at 1.0) were pelleted and reinoculated in 10ml basal MRS medium supplemented with 0.05%, 0.1%, 0.2%, 0.3%, 0.4% of bile (w/v) respectively, grown at 37°C for 8h without shaking and enumerated the amount of survivable bacteria at 2-hour intervals via the plate method.

2.5 Construction of VP2 expression plasmid

All DNA manipulations were performed according to standard procedures [31]. The plasmid pPG611.1 used in this study is a kind of cell-surface expression vector containing SPUsp secretion signal peptide and anchor domain structure, which gifted by Jos Seegers (NIZO, Netherlands). A 1.75kb fragment of gene encoding PPV antigen VP2 was obtained by PCR from PPV genome. The oligonucleotides were: 5´-CGAGGATCC\[TA TGGTTCACTG-TTCGACGACCGCGAG-3´ (upper) containing a BamHI site and 5´-AGCTTCTCGAG\[CATGCTACCTGATTAACCGAGTAACTG-3´ (lower) containing an XhoI site. PCR amplification conditions were as follows: 95°C, 5min; 30 cycles of 94°C, 1min; 55°C, 1.2min; 72°C, 2min; 72°C, 10min for final extension. PCR product was digested by BamHI and XhoI, and inserted into the corresponding sites of pPG611.1 giving rise to pPG611.1-VP2 (Fig. 1).

2.6 Generation of Lc393 transformants expressing PPV VP2 protein

Electroporation was carried out according to the method described by Josson et al [32] and made some modifications. In brief, 10µl recombinant plasmid DNA was added to 150µl Lcasei393, gently mixed and placed at 4°C for 5min. The mixer was subjected to a single electric pulse (25µF of 2500V/cm) and resuspended in MRS medium without antibiotic, incubated at 37°C anaerobically for 2h. Recombinant strains were selected on MRS-agar medium containing 10µg/ml Cm. The presence and integrity of the constructions carried by the transformants were checked by extraction of recombinant plasmid DNA following by
restriction analysis and sequencing.

2.7 Segregational and structural stability of pPG611.1-VP2

To assess the segregational stability of pPG611.1-VP2 in *L. casei* 393, the recombinant strain was grown in MRS medium at 37°C without Cm. Serial subcultures were performed by diluting (1:100) the culture in fresh broth without antibiotics and growing until mid-exponential phase (OD<sub>600</sub> at 1.0). After 70 generations, the percentage of Cm-resistant colonies was determined by plating 100μl culture dilutions onto MRS plates without Cm and onto MRS plates supplemented with 10μg/ml Cm. To determine the structural stability of recombinant plasmid, after 70 generations, several colonies were picked up from the MRS plate at random and analyzed by colony PCR using forward and reverse primers to detect the integrity of the VP2 gene in the recombinant plasmid.

2.8 Protein expression and Western blot, immunofluorescence analysis

To analyze the expression and localization of the VP2 fusion protein, overnight cultures of rLe393-rPPV-VP2 grown in basal MRS medium supplemented with xylose were collected by centrifugation at 12,000×g for 10min. The bacterial pellets were washed twice with sterile 50mM Tris-Cl, pH 8.0 and lysed in a Bead-Beater (Biospec, Bartlesville, USA) by vigorous shaking. The lysates were centrifuged at 15,000×g for 10min and the supernate were maintained at -20°C for further analysis. In a parallel experiment, the cells were treated with 10mg/ml of protease K (Sigma) at 37°C for 1h, washed thrice to remove the protease and then treated with lysozyme. The bacterial protein extracts and supernate were examined using 10% SDS-PAGE. Western blot analysis: Proteins were electrotransferred onto a nitrocellulose membrane and the immunoblots were developed using mouse anti-VP2 serum (the antiserum obtained from BALB/c mice immunized with Virus-Like Particles of PPV VP2 protein). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Sigma) diluted at 1:2000 was used and visualization of immunolabeled bands were then carried out using the Chemiluminescent Substrate reagent (Pierce) according to the manufacturer’s instruction.

Immunofluorescence was also used to analyze cell-surface display of VP2 protein: 1-ml overnight cultures of rLe393-rPPV-VP2 were pelleted, washed twice with sterile PBS, the pellets were resuspended in PBS containing mouse anti-VP2 serum and incubated at 37°C for 2h. The cells were centrifuged and washed thrice with sterile PBS to remove non-combination
antibodies. And then the pellets were incubated with FITC conjugated goat anti-mouse IgG (Sigma) containing 1% Evans blue (Pierce) at 37°C for 2h. Washed thrice with sterile PBS, the pellets were resuspended in sterile PBS and laid on a glass slide. The confocal microscopy was used to visualize fluorescence reaction on the cell surface of rLc393-rPPV-VP2. The wide-type *L. casei*393 was used as control.

2.9 Immunization

RLc393-rPPV-VP2 was cultured and centrifuged. Cell pellets were washed once with sterile PBS and resuspended in PBS to a concentration of \( \approx 1 \times 10^{10} \text{CFU/ml} \). The vaccine group of mice received dose of \( 1 \times 10^9 \) cells of rLc393-rPPV-VP2. The control mice were immunized with equivalent dose of Lc393-pPG611.1. The sham control group received 0.1ml doses of PBS. All the mice were immunized via oral administration. The immune protocol was administered on three consecutive days at days 0, 1 and 2. A booster immunization was given at days 14, 15 and 16 and a second booster was given at days 28, 29 and 30 [33].

2.10 ELISA analysis of serum and intestine mucus

Sera were prepared and stored at -20°C until required. The intestinal mucus of mice was extracted as described previously [34, 35]. Polystyrene microtitre plates were coated overnight at 4°C with the PPV propagated on swine testicular (ST) cells and the cultures of ST cell used as negative control antigen. The ELISA plates were washed thrice in PBS containing 1% Tween-20, and then saturated with PBS containing 5% skimmed milk at 37°C for 2h. Serum or intestinal lavage was diluted ten-fold in PBS containing 1%BSA using as primary antibodies. After being incubated at 37°C for 1h, the plated were washed thrice with PBS containing 1% Tween-20. Bound antibodies were detected using HRP conjugated goat anti-mouse IgA or IgG (Sigma), followed by colour development using o-phenylene diamine dihydrochloride (Sigma) as substrate. The absorbance was measured at 490nm.

2.11 Neutralization ability of the induced antibodies

Intestinal fluids and serum samples obtained from the mice immunized with Lc393-rPPV-VP2 were evaluated using plaque reduction assay to determine the neutralization ability. Lavages and sera obtained from the mice fed with non-expressor strain or PBS were used as negative control. 50µl of samples in two-fold serial dilutions (from 1:2 to 512) were prepared in ninety-six cell plate. PPV adjusted to 200 TCID_{50} in 50µl of virus diluent (10%
concentrated Hank's balanced salt solution, 0.1% bovine serum albumin, pH 7.4) was added to the cell plate containing serially diluted serum or intestinal lavage. The antibody and virus mixture was mixed, incubated at 37°C for 1h. And then 100µl of swine testicular (ST) cell (used for virus infection) was added to the antibody-virus mixture, incubated in a 5% CO₂ incubator at 37°C for 5 days. The overlay medium was then discarded, after which the wells were washed thrice with sterile PBS, pH 7.4 and stained with 1% crystal violet solution. Differences in the number of plaques formed between treatments were examined for the level of significance by Student's t-test after analysis of variance.

3. Result

3.1 Colonization ability of L. casei 393

The colonization ability of L. casei 393 in the intestinal tracts was determined via oral administration of cFDA-SE-labeled Lactobacilli to mice and isolating the different regions of intestine from such mice post orogastric intubation. Flow cytometric analysis of cell extracts (Table 1) indicated that the lactobacilli were able to survive and adhere to different regions of intestinal tract. Adhesion was most prominent in ileum with 8.97±0.76×10⁶ cells being detected on day 1. By the seventh day, the amount of L. casei 393 that remained adherence to the intestinal mucosal were 39.38, 71.30, 76.59 and 11.79% of that on the first day in duodenum, jejunum, ileum, and colon, respectively (data not shown).

3.2 Bile tolerance of L. casei 393

Bile tolerance is a selection criterion for probiotics [36]. In normal intestine, the bile concentration ranges from 0.03% to 0.3% [37, 38]. The result of bile tolerance of L. casei 393 indicated that the Lactobacilli has a good property of tolerance to 0.05%~0.2% of bile. In 0.3% and o.4% of bile, the Lactobacilli could survive for 8h and 4h, respectively (Fig. 2).

3.3 Cell-surface expression of PPV antigen VP2

The gene encoding VP2 protein was amplified and cloned as a BamHI-XhoI fragment into the corresponding sites of expression plasmid pPG611.1, generating pPG611.1-VP2. The recombinant plasmid pPG611.1-VP2 was electroporated into L. casei 393. The segregational and structural stability of pPG611.1-VP2 in L. casei 393 was analyzed after 70 generations
subcultures in MRS broth without Cm' selection. The results indicated that the recombinant plasmid had good stability properties that no loss of the Cm-resistance phenotype and any structural rearrangement (data not shown).

The rLc393-rPPV-VP2 was grown overnight in basal MRS medium supplemented with either xylose or glucose. The lysates of the cells were analyzed by SDS-PAGE and Western blot using mouse anti-VP2 serum. Coomassie blue gel staining showed a 74kDa fusion protein was expressed in lysates of rLc393-rPPV-VP2 induced by xylose (Fig. 3. A: lanes 2, 3), but not when the same cells were grown in glucose (Fig. 3. A: lane 4) or wild-type *L. casei* 393 was grown in xylose (Fig. 3.A: lane 5). Similarly, an immunoreactive band was detected (Fig. 3.B: lane 1) via Western blot in a similar position as observed in the SDS-PAGE shown in the panel A and its expression was repressed when the rLc393-rPPV-VP2 were grown in MRS medium containing glucose (Fig. 3.B: lane 2). As the negative control, wild-type *L. casei* 393 did not display the corresponding immunoreactive band (data not shown). These results show that the xylose promoter from *L. casei* could efficiently induce the expression of PPV heterologous protein. However, the expression of VP2 protein was repressed by glucose.

The VP2 protein could not be detected in the supernate of overnight cultures of rLc393-rPPV-VP2 induced by xylose, even after 50-fold concentration in a Millipore Ultrafree-15 column (data not shown). The same cultures were incubated with lysozyme, which could release cell-anchored proteins, and the supernate were analyzed by western blot. As can be observed in Fig. 4, the induced rLc393-rPPV-VP2 was treated with lysozyme and an about 74kDa protein was released to the supernate (Fig. 4. lane 2), which could not be detected in supernate of the cells only treated with protease (Fig. 4. lane 4). Treatment with protease previous to lysozyme gives a single band about 50kDa (Fig. 4. lane 3). None of bands was observed in supernate from untreated cells (data not shown).

To confirm the cell-surface display of the VP2 fusion protein, immunofluorescence was used to analyze. The overnight cultures of rLc393-rPPV-VP2 expressing VP2 were grown in basal MRS medium supplemented with either xylose or glucose. The immunofluorescence was developed with the mouse anti-VP2 serum and FITC fluorescence conjugated goat anti-mouse IgG. The results indicated that there were green-yellow fluorescence on the cell surface of rLc393-rPPV-VP2 grown in xylose (Fig. 5B), but not in glucose, the bacteria were
red dyed by Evans blue (Fig. 5A). The wild-type *L. casei* 393 also did not display immunofluorescence reaction, the bacteria were red dyed by Evans blue (Fig. 5C).

The results of localization analysis of VP2 protein expressed from the rLc393-rPPV-VP2 by Western blot and immunofluorescence suggested that VP2 protein could be exposed outer side of the cell wall and part appeared to be embedded in the cell wall.

Furthermore, whether the rLc393-rPPV-VP2 subcultured 70 generations without Cm selection could still express the VP2 antigen was identified. The result shows that the recombinant lactobacilli could still express the interest protein (Fig. 6).

3.5 Immune responses of recombinant Lactobacilli induced by intragastric immunization

The mucosal immune response was studied by measuring the anti-PPV-VP2 IgA response in intestinal lavages post intragastric immunization. The concentration of mucosal IgA antibody against PPV-VP2 was determined via ELISA using the PPV cultures as coating antigen and the culture of ST cell used as negative control antigen. As the results showing, there was no substantial difference in mucosal IgA level between experimental group and control group prior to intragastric immunization, while oral immunization of recombinant Lc393-rPPV-VP2 elicited an antigen specific mucosal IgA response. After the second booster, high level of anti-PPV-VP2 IgA were obtained in intestinal lavages (Fig. 7) of mice immunized with rLc393-rPPV-VP2, whereas no anti-VP2 antibody was observed in the control groups of mice that received Lc393-pPG611.1 or PBS and used the ST cell culture as negative control antigen (data not shown).

Likewise, the concentration of anti-PPV-VP2 specific IgG from immunized mice was also determined. After the first booster, the mice that were orally administrated with rLc393-rPPV-VP2 were elicited a prompter and stronger level of anti-PPV-VP2 specific serum IgG on comparison to the mucosal IgA level induced. No significant elicitation of anti-PPV-VP2 antibodies was observed in the control groups that received Lc393-pPG611.1 or PBS (Fig. 8) and used the ST cell culture as negative control antigen (data not shown).

Taken together these results, the rLc393-rPPV-VP2 generated in this study was able to elicit both PPV specific systemic and mucosal antibody responses via oral administration.

3.6 Plaque reduction assay

Plaque reduction assays were performed to further detect whether the antibody responses
were specific against PPV VP2 protein. Results demonstrated that the presence of anti-PPV-VP2 IgA or IgG in the culture medium conferred statistically significant neutralizing effects \( (p<0.05) \) on PPV infection (Fig. 9). A near 77.2± 1.27% reduction in the number of plaques was consistently observed when plaque reduction assays were carried out using two to eight-folds diluted sera and 30.6±1.11% reduction of plaques using two to eight-folds diluted intestinal lavages, which obtained from the mice immunized with rLe393-rPPV-VP2. The inhibitory effect decreased gradually on further dilutions and reached a level similar to that of the buffer control or the control non-expressor strain at dilutions 1: 128 and 1:256 of intestinal lavage and sera, respectively.

4. Discussion

For many pathogens, the initial infection mainly occurs at the mucosa tissues of intestines. Therefore, it is important to develop vaccines that elicit protective immune responses to prevent the infection and replication of the pathogens at the mucosa via mucosal immunization. Furthermore, the vaccines that could also induced specific immunity in the systemic lymphoid tissues are highly desirable, for most viral infections gained entrance through the mucosal surfaces will become systemic. A great deal of research is currently being explored for oral application focused on the development of adequate mucosal vaccines with various vaccine delivery systems. In contrast, among the available approaches to stimulate efficient mucosal responses, using lactobacilli carriers to delivery vaccine antigens, probably, constitutes one of the most successful strategies [15].

The use of lactic acid bacteria as live vehicle for the delivery of antigens for mucosal immunization or other therapeutic molecules has been proposed. However, only a few of systems have been described using *Lactobacillus casei* strains as a carrier for expressing heterologous viral antigens in a form that can be presented to and processed by the immune system of the mammalian host [16, 18-20]. In this study, we engineered for the first time, using *L. casei* 393 to express the porcine parvovirus VP2 protein, which without being compromised its antigenic properties and could be recognized by specific antiserum. Furthermore, the recombinant plasmid pPG611.1-VP2 in *L. casei* 393 has a good segregational
and structural stability without showing any structural rearrangement.

IgA is the predominant antibody at the mucosal surface as it is locally produced at a level that exceeds that of all of other immunoglobulins [39, 40]. Therefore, an efficient PPV oral vaccine will have to induce a specific mucosal IgA response. We evaluated the immunogenicity of rLc393-rPPV-VP2 used BALB/c mice as animal model. The rLc393-rPPV-VP2 was shown to be able to elicit both mucosal and systemic immune responses after intragastric administration. The oral administration regime was used in this study, consisted of three sets of three successive daily doses of the rLc393-rPPV-VP2 used as the experimental vaccine. This protocol was adapted from the procedure of Challacombe [33], who found that this pattern of immunization was consistently effective when particulate oral vaccines were used to immunize mice. Three successive daily doses of recombinant bacteria were required in order to ensure that systemic antibody response to PPV could be elicited in all mice received recombinant strain intragastrically.

In order to confirm the efficacy of the induced antibodies in inhibiting the virus, we tested whether intestinal lavages and sera could inhibit the infection of ST cells in plaque reduction neutralization assay. Serum and intestinal samples collected from mice immunized with rLc393-rPPV-VP2 demonstrated statistically significant inhibition. As a delivery system, being able to adhere to and colonize the intestinal tract is important and desirable. In this study, we investigated the colonization potentiality of *L. casei* 393 in mouse intestine by using cFDA-SE. It was shown that 7.44×10^6 to 8.97×10^6 of the orally fed *L. casei*393 was able to adhere to the intestinal tract of mice, where the percentage of *L. casei*393 that remained in the intestinal tract after the initial attachment as of day 1 varies from 11.79% to 76.59%. In particular, the amounts of *L. casei*393 that remained adherence were maintained at high percentage of 71.3-87.49% and 76.59-87.74% in the jejunum and ileum, respectively. The recombinant strain generated in this study also demonstrated similar adherence and colonization ability as its native counterpart (data not shown). Bile tolerance is a selection criterion for probiotics [36]. The lactobacilli remained visible for 8 h after being incubated in MRS medium containing bile concentration of 0.3% (w/v). The delivery system of *L. casei* 393 used in this study was able to adhere to and colonize the intestinal tracts and tolerate to bile, which make it be attractive candidate as antigens delivery carries for the presentation to
the mucosa with pharmaceutical interest, in particular vaccines.

In the present study, *L. casei* 393 has been demonstrated to be able to survive the transit of the upper gastrointestinal tract and was able to express and secrete heterologous parvovirus protein that induced specific immune responses against the antigen within the murine intestine milieu when orally administrated. Currently, our research is under way to confirm the immune protection of the recombinant lactobacillus expressing VP2 protein for pigs and the mechanism of how the antigen was able to transude into the intravascular compartment to induce systemic immune responses will also be further investigated. Nevertheless, with the probiotic effects and the harmless nature of *Lactobacillus casei* 393 would make it more appropriate an oral vaccine carrier to delivery heterologous antigens for oral vaccine.

Acknowledgements

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Fig. 1. Map of pPG611.1-VP2. Cm resistance determinant; repA and repC replication elements; SP, signal peptide obtained from the lactococcal Usp45 protein containing xylose operon promoter; VP2, VP2 gene obtained from PPV genome DNA by PCR; Anchor, the cell wall anchor motif obtained from Streptococcus pyogenes M6 protein.

Table 1. The amount of cFDA-SE-labeled *L. casei* 393 isolated from different intestinal tracts

<table>
<thead>
<tr>
<th>Days</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Colon</th>
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<tr>
<td></td>
<td>Average cells on per intestinal tract</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>7.44±0.75 E+06</td>
<td>8.71±0.69 E+06</td>
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<tr>
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<td>6.21±0.57 E+06</td>
<td>6.87±0.59 E+06</td>
<td>9.96±1.02 E+05</td>
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Fig. 2. The bile tolerance of *L. casei* 393. Using the plate method to enumerate the amounts of Lactobacilli survived in different concentration of bile. Results were mean values of amount of bacteria ±S.E.M.

Fig. 3. Expression of VP2 protein in rLc393-rPPV-VP2. Total cell lysates were analyzed by SDS-PAGE and Western blot with specific antiserum. A: Coomassie blue gel staining shows the expression of a 74kDa fusion protein in lysates from rLc393-rPPV-VP2 induced by xylose (lanes 2, 3), but not in glucose (lane 4) or wild-type *L. casei* 393 was grown in xylose(lane 5); lane 1. MW, molecular mass marker. B: An immunoreactive band was detected (lane 1) in a similar position as observed in the SDS-PAGE shown in the panel A. No immunoreactive bands were observed in the cell lysates from recombinant cells induced by glucose (lane 2).
Fig. 4. Localization analysis of VP2 expression from the Lc393-rPPV-VP2 by Western blot. Lane 1, total lysates of xylose-inducible cells expressing VP2 fusion; lane 2, supernate of the same cells treated with lysozyme; lane 3, supernate of cells treated with protease followed by lysozyme; lane 4, supernate of cells only treated protease.

Fig. 5. The immunofluorescence reaction of VP2 protein on the cell surface of recombinant Lc393-rPPV-VP2. A: the rLc393-rPPV-VP2 was grown in MRS medium containing glucose, no fluorescence on cell surface and the bacteria were red dyed by Evans blue; B: The rLc393-rPPV-VP2 was induced by xylose, there were green-yellow fluorescence reaction on the surface of the bacteria; C: the wide-type L. casei 393 was induced by xylose, the result of immunofluorescence was negative and the bacteria were red dyed by Evans blue.
Fig. 6. Expression and identification of VP2 protein in rLc393-rPPV-VP2 subcultured 70 generations without Cm selection. lane1. MW, molecular mass marker; lane 3. Coomassie blue gel staining shows a 74kDa fusion protein was expressed from rLc393-rPPV-VP2 subcultured 70 generations without Cm selection induced by xylose in a similar position as observed in the SDS-PAGE shown in the Fig. 3 (panel A), but not in glucose (lane2); lane 4. An immunoreactive band of protein expressed by rLc393-rPPV-VP2 subcultured 70 generations was detected in a same position as in lane 3. No immunoreactive bands were observed in the cell lysates from the recombinant cells induced by glucose (lane 5).

Fig. 7. Anti-VP2 specific IgA level in intestinal lavage after intragastric immunization. The mice received three consecutive doses of $10^9$ Lc393-rPPV-VP2, three times at 2-week interval. Control mice received $10^9$ Lc393-pPG611.1, while negative group received PBS. Intestinal lavages that were collected on days 18, 32, 46 after the first immunization, were analyzed via ELISA using PPV as the coating antigen. Bars represent the IgA titer ±S.E.M in each group.
Fig. 8. Anti-PPV-VP2 serum IgG response induced after intragastric immunization with rLe393-rPPV-VP2. Sera from groups of mice immunized orally with $10^9$ rLe393-rPPV-VP2 and equivalent doses of Lc393-pPG611.1 and negative control sera from mice received with PBS were analyzed for the presence of anti-PPV-VP2 specific IgG by ELISA, using the PPV as the coating antigen. Bars represent the IgG titer ± S.E.M in each group.
Fig. 9. Inhibition of viral plaque formation by (A) intestinal lavages and (B) sera prepared from mice immunized with rLc393-rPPV-VP2. Maximum reduction in number of plaques, expressed as a percentage of plaques obtained for the negative control samples, using (A) intestinal lavages or (B) sera collected from mice fed with Lc393-rPPV-VP2 was 31.2±1.09% and 80.5±1.13%, respectively. Results are mean values and standard errors of triplicates.