Heliyon 9 (2023) e22941

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Both recombinant *Bacillus subtilis* Expressing PCV2d Cap protein and PCV2d-VLPs can stimulate strong protective immune responses in mice

Yuxuan Zhang [a,b,1], Yao Wu [a,b,1], Chong Peng [a,b], Zixuan Li [a,b], Gang Wang [b], Hui Wang [b,c], Lanping Yu [b,c,**], Fangkun Wang [a,b,*]

[a] *Department of Veterinary Public Health, College of Veterinary Medicine, Shandong Agricultural University, Taian City, Shandong Province, China*
[b] *Shandong Provincial Key Laboratory of Animal Biotechnology and Disease Control and Prevention, Shandong Agricultural University, Taian City, Shandong Province, China*
[c] *Aquaculture Research Lab, College of Veterinary Medicine, Shandong Agricultural University, Taian City, Shandong Province, China*

ARTICLE INFO

Keywords:
PCV2d
Cap
VLPs
Bacillus subtilis
Vaccine

ABSTRACT

Porcine circovirus type 2 (PCV2) is one of the most serious pathogens in pig herds worldwide. The Capsid protein (Cap), a structural protein of PCV2, is involved in the host's immune response; it induces neutralizing-antibody production and has good immunogenicity. The main PCV2 subtype currently prevalent in the Chinese pig herd is PCV2d. In this study, We constructed a recombinant *Bacillus subtilis* (*B. subtilis*) capable of secreting Cap protein, named pHT43-Cap/*B. subtilis*; we concentrated the supernatant of the recombinant bacteria and observed virus-like particles (VLPs) of PCV2d formed by Cap protein under transmission electron microscopy, named PCV2d-VLPs. The immunocompetence of the pHT43-Cap/*B. subtilis* and PCV2d-VLPs were then assessed by oral administration and by intramuscular injection into mice, respectively. The results showed that the levels of PCV2d-Cap protein-specific IgG in the serum and of PCV2d-Cap protein-specific sIgA in the small intestinal fluid of pHT43-Cap/*B. subtilis* immunized mice were elevated compared to the control group, both of them highly significant ($p < 0.01$), and the corresponding serum-specific IgG antibodies were effective in neutralizing PCV2d virulence. The virus load in the liver of the immunized mice was significantly lower than that in the control group ($p < 0.01$), as was the virus load in the spleen and lungs of the immunized mice ($p < 0.05$). In addition, the serum levels of PCV2d-Cap-specific IgG in mice immunized with PCV2d-VLPs by intramuscular injection were significantly elevated compared to the control group ($p < 0.05$), and the viral load in all tissues was significantly lower in immunized mice ($p < 0.05$). In conclusion, the recombinant bacterium pHT43-Cap/*B. subtilis* can induce effective mucosal and humoral immunity in mice, PCV2d-VLPs can induce humoral immunity in mice, and both vaccines have good immunogenicity; these results provide a theoretical and material basis for the development of a new vaccine against PCV2d.

* Corresponding author. Department of Veterinary Public Health, College of Veterinary Medicine, Shandong Agricultural University, Taian City, Shandong Province, China.
** Corresponding author. Shandong Provincial Key Laboratory of Animal Biotechnology and Disease Control and Prevention, Shandong Agricultural University, Taian City, Shandong Province, China.
E-mail addresses: lanpingyu@sdau.edu.cn (L. Yu), wangfangkun@sdau.edu.cn (F. Wang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.heliyon.2023.e22941
Received 13 July 2023; Received in revised form 20 November 2023; Accepted 22 November 2023
Available online 28 November 2023

1. Introduction

Porcine circovirus (PCV) belongs to the genus *Circovirus* in the family Circoviridae and is the only mammalian circovirus [1]. Four serotypes—PCV1, PCV2, PCV3, and PCV4—have been identified in swine populations worldwide, although the most prevalent type is PCV2, which primarily targets lymphoid tissues and causes lymphocyte depletion and immunosuppression in pigs. Genome-wide phylogenetic analysis has shown that PCV2 genotypes can be divided into nine genotypes, PCV2a–PCV2i [2,3], and PCV2d is now the predominantly prevalent genotype in pig populations in China, the USA, Russia, Italy, and Korea [4–6]. PCV2d has greater virulence and can induce more severe clinical signs, viremia, and pathology in conventional manifestation in pigs [7,8].

The single-stranded cyclic PCV2 genome consists of 1766-8 nucleotides (nt), is approximately 1.7 kb in length, and contains eleven major viruses [9]. It contains eleven major open reading frames (ORFs), and the two most prominent functional ORFs are ORF1 and ORF2, which occur in opposite directions and account for approximately 93 % of the PCV2 genome [10]. ORF1 encodes two related proteins necessary for viral DNA replication: the Rep protein and the Rep' protein, which are essential to the proliferation of the PCV2 genome [11]. ORF2 encodes the Cap protein, the only structural protein that constitutes the Cap protein of the PCV2 icosahedral virus; the Cap protein is the major immunogenic protein containing a PCV2-specific neutralizing epitope that can participate in the host's immune response, which can affect the antigenicity, virulence, and pathogenicity of PCV2 [12]. Cap proteins expressed by recombinant systems are capable of self-assembling into virus-like particles (VLPs) [11], which are compatible with PCV2 natural viruses. Therefore, the Cap protein is a key protein in vaccine development.

In recent years, significant progress has been made in the field of research on PCV2 mucosal vaccines. Wang et al. constructed a recombinant *Lactococcus lactis* expressing the PCV2-Cap protein [13]; Li et al. constructed a recombinant *Lactobacillus casei* expressing the PCV2-Cap protein [14]; Wang Shubo et al. constructed recombinant *Lactobacillus royale* expressing PCV2-Cap protein [15]; and Silva et al. were able to express PCV2-Cap protein efficiently with the expression system of *Pichia pastoris* [16]. Related studies have also shown that mucosal vaccines using antigens alone, without the addition of adjuvants, often fail to induce a strong immune response or provide long-term protection, and adjuvants are therefore essential to enhancing the specific immune response to mucosal vaccines [17–19].

VLPs is a type of subunit vaccine. VLPs is highly structured protein complexes (about 20–100 nm in diameter) that self-assemble from one or more viral structural Cap proteins and resemble the appearance of natural viruses. Induces immune responses similar to those elicited by natural viruses [20,21]. At present, the research on PCV2-VLPs mainly uses *E. coli* expression system or insect baculovirus expression system to obtain a large amount of Cap protein, self-assembly into VLPs and purification to obtain high purity VLPs. In 2017, based on the *E. coli* expression system, Plaike Biological developed the first PCV2-VLPs vaccine, Yuanke Xin, which was successfully marketed in China; In 2021, PCV2-VLPs (recombinant baculovirus OKM strain) developed by Nanjing Agricultural University was granted a new veterinary drug registration certificate.

B. subtilis produces endospores in the absence of nutrients, and mature spores can survive for a long time in a dormant state [22]. *B. subtilis* can also survive in extreme temperatures and solvents while maintaining strong stability; these unique properties make *B. subtilis* easy to preserve and can deliver any foreign antigens or bioactive molecules to extreme environments and preserved excellent immunogenicity [23], such as intestinal mucosa [24]. Spores can germinate in the intestine of animals and promote the secretion of immunoglobulin A (sIgA) in the intestine, thereby inducing a strong mucosal immune response in the body [25–27]. *B. subtilis is* a non-pathogenic Gram-positive bacterium, and as a probiotic, it can improve the intestinal microenvironment, enhance growth performance, and strengthen the immunity of the body [28]. It has a good fermentation base and production technology, and it is easy to produce [29], compared with traditional systems such as *E. coli*, it does not require expensive purification processes to remove endotoxin, has low production costs, is easy to genetically manipulate [30], and is safe and non-toxic. It can secrete foreign proteins, survive in harsh environments that *E. coli* cannot adapt to, and can improve the intestinal health of animals. It has a well-defined genetic profile and is easy to produce, given its clearly-characterized genetics with no obvious codon bias. It is easy to administer, allowing immunization of animals via intranasal, sublingual, and oral routes [24]. The spores can play an adjuvant role to enhance the immune level of the organism [31]. Thus, *B. subtilis* has potential in the development of new and attractive vaccines in terms of efficacy, safety, needle-free injection, thermal stability, and cost.

In this study, the codon preference of the Cap gene sequence was optimized, and the *B. subtilis* live vector vaccine expressing PCV2d Cap protein was successfully constructed, so that the Cap protein could be efficiently expressed. On this basis, PCV2d-VLPs was prepared by *B. subtilis* expression system for the first time. The recombinant bacterium pHT43-Cap/*B. subtilis* can induce effective mucosal and humoral immunity in mice and the PCV2d-VLPs can induce effective humoral immunity, our results provides a theoretical and material basis for the development of a new vaccine against PCV2d.

2. Materials and methods

2.1. Virus, bacterial strains, proteins, plasmids, antibodies, and animals

The PCV2d strain was generously donated by Professor Shang. The PCV2 Cap protein and polyclonal antibodies have been stored in our laboratory. *B. subtilis* WB800 N was purchased from Changsha U-Bio Biotechnology Co. The plasmid pUC57-Cap was synthesized by Beijing Liu he Hua da Gene Technology Co. Ltd. HRP-conjugated Affinipure Goat Anti-Mouse IgA was purchased from Wuhan Sanying Biotechnology Co., LTD. HRP-conjugated Affinipure Goat Anti-Mouse IgG was purchased from Beijing Kangwei Century Biotechnology Co., LTD.

The study shows that mice can be used as an animal model for studying porcine circovirus infection and evaluating the efficacy of PCV vaccines. (Cui et al., 2020; Deng et al., 2011; [13]. Therefore, mice were selected as the experimental model in this study. 70 4-week-old female BALB/c mice were purchased from Jinan Pengyue Experimental Animal Breeding Co.

2.2. Optimization of the PCV2d-Cap gene sequence

The sequence of the Cap gene was optimized for codon preference in reference to the 2020 GenBank public PCV2d (MH341497.1) isolate, to make it suitable for expression in the *B. subtilis* expression system; the protein encoded by the optimized gene demonstrated a 100 % match with the reference strain Cap protein coding sequence QCY50148.1. The optimized sequence was synthesized by Beijing Liu he Hua da Gene Technology Co., Ltd. and inserted into the pCU57 cloning vector, called pUC57-Cap.

2.3. Cloning and construction of pHT43-Cap/B. subtilis

The optimized plasmid vector pUC57-Cap and pHT43 were simultaneously double-cleaved with the restriction endonucleases *Bam*HI and *Sma*I; then, the target gene PCV2d-Cap and the vector plasmid pHT43 were ligated with T4 DNA Ligase; then, the recombinant plasmid was transformed into *E. coli* DH5α. The recombinant plasmid pHT43-Cap was transferred into *B. subtilis* WB800 using electrotransformation (2000 V, 5 ms, 200 Ω, 25 μF). The transformation was verified by the PCR method and shown to be successful.

2.4. Induced expression of pHT43-Cap/B. subtilis target proteins

Resuscitated pHT43-Cap*/B. subtilis* single colonies were inoculated in 3 mL of Cm^+ liquid LB under aerobic conditions overnight, and when the OD_{600} reading reached 0.8, the bacterial broth was divided equally into two groups: one was set as the induced group, with the addition of IPTG (50 mg/mL) to a final concentration of 1 mmol/L, and the other was set as the un-induced group and placed together in a shaker. The shakers were incubated at 37 °C and 220 rpm for 4 h. The target proteins were obtained using ultrasonic fragmentation and subsequently analyzed using Western blotting.

2.5. Western blotting

Protein samples were spiked onto 12 % SDS-PAGE for SDS-PAGE, and then the proteins were electroblotted on PVDF membranes pre-saturated with methanol (90A, 1 h). After they were transferred to the membrane, the membrane was washed three times with PBSD and then closed with 5 % BSA for 1 h. A PCV2-Cap polyclonal antibody was diluted with 2.5 % BSA at a ratio of 1:10,000 as primary antibody, then placed in overnight incubation at 4 °C. The secondary antibody, of murine origin, was diluted with 2.5 % BSA at a ratio of 1:20,000 and incubated with this secondary antibody for 1 h. The PVDF film was washed 5 times with PBST, removed and drained on absorbent paper, and placed in the developer; several drops of the developer solution were added evenly at the position of the target protein to start the exposure.

2.6. Preparation and identification of PCV2d-VLPs

After induction of 100 ml recombinant bacterial solution with IPTG (50 mg/mL) to a final concentration of 1 mmol/L for 4 h at 37 °C, the medium supernatant was removed by centrifugation at 4 °C. The medium supernatant was filtered through a 0.22 μm filter and concentrated in a (pretreated) ultrafiltration tube; before use, the ultrafiltration tubes were filled with ultrapure water, the water was passed over the membrane, and then the water was poured out in an ice bath for 5 min. The added protein solution was concentrated to 100 μL, and the protein concentration was determined using the BCA protein concentration assay kit; then, 10 μL of the concentrated PCV2d-Cap protein sample was aspirated onto the membrane surface of the copper mesh, stood for 1 min, and then dried on filter paper. Then, it was stained with 10 μL of the staining solution for 30–60 s and then dried on filter paper; the morphological structure of VLPs was directly observed under transmission electron microscopy.

2.7. Immunogenicity of oral pHT43-Cap/B. subtilis

Refer to Li's oral immunization program in Mice [32,33]. Seventy 4-week-old BALB/c mice were randomly divided into three groups of 23 mice each: the blank control group, the negative control group, and the test group. Immunization was given in triplicate, each for three consecutive days: oral immunization via gavage on days 1, 2, and 3; days 14, 15, and 16; and days 28, 29, and 30 respectively. The test group was immunized with the recombinant bacterium pHT43-Cap*/B. subtilis* 200 μL/time/animal (1×10^{10} CFU/mL) [34]; the blank control group was immunized with PBS 200 μL/time/animal; and the negative control group was immunized with the empty vector *B. subtilis* (pHT43*/B. subtilis*) (200 μL/time/animal).

The mice were fed the usual basal diet *ad libitum*, and 200 μL PCV2d wild virus ($10^{5.5}$ $TCID_{50}$/mL) was injected intraperitoneally into each mouse on day 14 after the third immunization round. Blood was collected from the eyes of 5 randomly selected mice on days 0, 14, 28, and 42, and serum was collected for specific antibody IgG levels, Western blotting, and virus neutralization. After blood collection, the mice were euthanized, whereupon 1–2 cm of small intestine was removed and the small intestine fluid was collected and stored at −20 °C for testing of the specific antibody sIgA level. On the 10th day after challenge, three mice each from the blank control

and the test groups were dissected, and the spleen, lungs, and liver of each mouse were collected. DNA was extracted from each tissue using a blood/cell/tissue genomic DNA extraction kit and was stored at −20 °C for determination of the viral load in each tissue sample.

2.8. Detection of antibodies by I-ELISA

Samples of mice serum and small intestine fluid were collected, and the specific IgG titer and the secretory immunoglobulin A (sIgA) antibody were determined by indirect ELISA. The purified *E. coli* prokaryotic expression PCV2-Cap protein was used as the antigen, which was diluted to a final concentration of 10 μg/mL with antigen coating solution; 100 μL was added to each well, incubated overnight at 4 °C, and washed 5 times with PBST after the antigen coating solution was discarded the next day. 200 μL of blocking solution was added to each well, which was closed for 1 h at 25 °C and washed 5 times. 100 μL of the diluted serum to be tested was added to each well (serum diluted 1:100), incubated for 1 h at 25 °C, and washed 5 times. The secondary antibody HRP-conjugated Affinipure Goat Anti-Mouse IgG or HRP-conjugated Affinipure Goat Anti-Mouse sIgA was added at 1:10,000 with an antibody diluent, diluted to 100 μL per well, and washed 5 times. 100 μL of TMB chromogenic solution per well was added; it was kept out of the light, covered with tin foil, and left for 15 min at 37 °C; then, 50 μL of termination solution per well were added and then read at $OD450_{nm}$ using an enzyme marker.

2.9. Virus neutralization experiment

The PCV2d vaccine candidate serum was diluted in a DMEM medium at 10×, 20×, and 40× ratios, then filtered through a 0.22 μm filter to remove bacteria. The PCV2d virus was adjusted to 100 $TCID_{50}$/100 μL, and the virus and serum dilutions were mixed at 1:1 for 1 h at 37 °C and then inoculated in 12-well cell culture plates (using PK-15 cells) [35]: one well for normal cell control, one well for virus-positive control, and one well for each dilution of each of the remaining samples (washed with pre-warmed PBS twice before addition and once at 1 h after infection). These were incubated for 48 h in a CO_2 incubator at 37 °C by adding a cell maintenance solution; wall-cultured cells were digested with trypsin and processed into cell suspension, then the supernatant was poured out and cell genomic DNA was extracted using a kit.

2.10. Immunogenicity of intramuscular PCV2d-VLPs

Ten 5-week-old Kunming mice were randomly divided into immunized and blank control groups of 5 mice each, then immunized in two doses. The immunized group was immunized on day 1 with PCV2d-VLPs + Freund's complete adjuvant and on day 14 with PCV2d-VLPs + Freund's incomplete adjuvant at a dose of 100 μL (50 μg/each) + 100 μL [36]. On day 14, after the second immunization, blood was collected from the infraorbital vein of the mice, and serum levels of anti-PCV2d-Cap-specific antibody IgG were measured by indirect ELISA (same method described in section 2.2.7.8); on day 28 after the first immunization, each mouse was injected intraperitoneally with 200 μL of PCV2d virus ($10^{5.5}$ $TCID_{50}$/mL), and the spleen, lungs, and liver of each mouse were collected via dissection on day 10 after challenge.

2.11. Statistical analysis

All data are shown as mean ± S.D. Differences between the groups were analyzed using a one-way ANOVA test (IBM SPSS Statistics 26), with $p < 0.05$ considered significant and $p < 0.01$ considered highly significant. PCV2d Cap protein nucleic acid sequence Blast

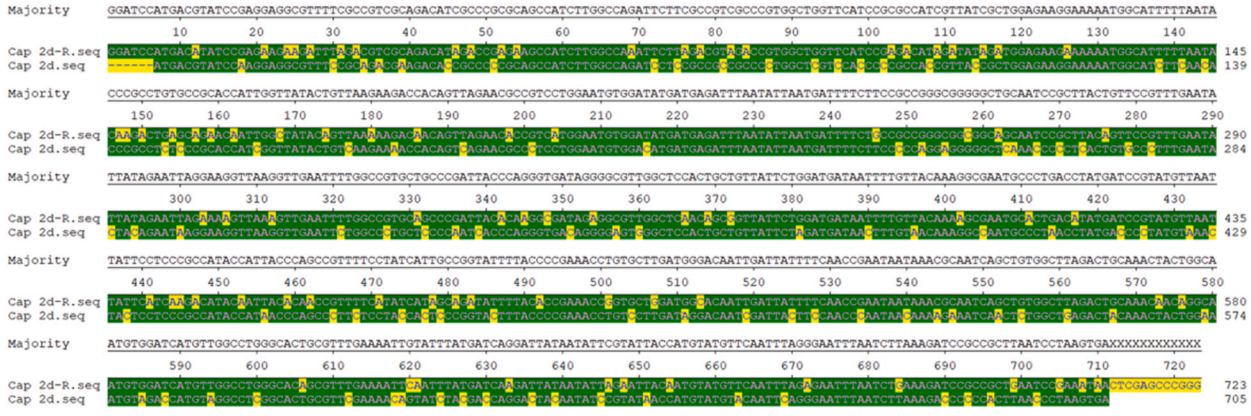

Fig. 1. Sequence optimization of PCV2d-Cap protein gene sequence The codon preference of Cap gene sequence was optimized according to the PCV2d (MH341497.1) isolate published in GenBank in 2020, to make it suitable for expression in a *B. subtilis* expression system. After optimization, 32.2 % of the bases were changed, encoding a total of 65 amino acids.

alignment and protein sequence antigenic determinant cluster analysis were conducted using the DNAstar software package. The SWISS-MODLE online software (https://swissmodle.expasy.org/) was used to predict and analyze the tertiary structure of the PCV2d Cap protein and the pHT43-Cap/*B. subtilis* expressing recombinant Cap protein (cCap).

3. Results

3.1. Optimization of PCV2d-Cap gene sequence

The codon preference of Cap gene sequence was optimized according to the PCV2d (MH341497.1) isolate published in GenBank in 2020, to make it suitable for expression in a *B. subtilis* expression system. After optimization, 32.2 % of the bases were changed, encoding a total of 65 amino acids (Fig. 1).

3.2. Construction and expression of recombinant B. subtilis

The plasmids pUC57-Cap and pHT43 linear vectors were double digested with *Bam*HI and *Sma*I after the Cap gene sequence was optimized. The pUC57-Cap plasmid was digested with two bands (2710 bp and 723 bp), with 723 bp being the target gene fragment (Fig. 2A), and the pHT43 vector had only one 8057 bp target band (Fig. 2A); the recombinant plasmid was named pHT43-Cap. Ten randomly-selected colonies were identified through PCR using the pHT43 sequencing primer. The plasmid was then subjected to double digestion with *Bam*HI and *Sma*I, and the results were as expected: a vector band of 8057 bp and a target fragment band of 723 bp (Fig. 2A).

The constructed recombinant plasmid pHT43-Cap was transformed into *B. subtilis* WB800 N using electroshock. The day after electrotransformation, six single colonies were randomly picked from the *Cm* + solid LB for shaking and expansion, and PCR identification was performed with pHT43 sequencing primers using the bacterial broth as a template; the PCR product of the successfully-identified pHT43-Cap recombinant plasmid was used as a positive control. The results are shown in Fig. 2A. The single colonies were all positive, proving that the recombinant bacterium was successfully constructed, and it was named pHT43-Cap/*B. subtilis*.

The recombinant pHT43-Cap/*B. subtilis* and the empty vector *B. subtilis* (pHT43/*B. subtilis*) control were inductively expressed; the

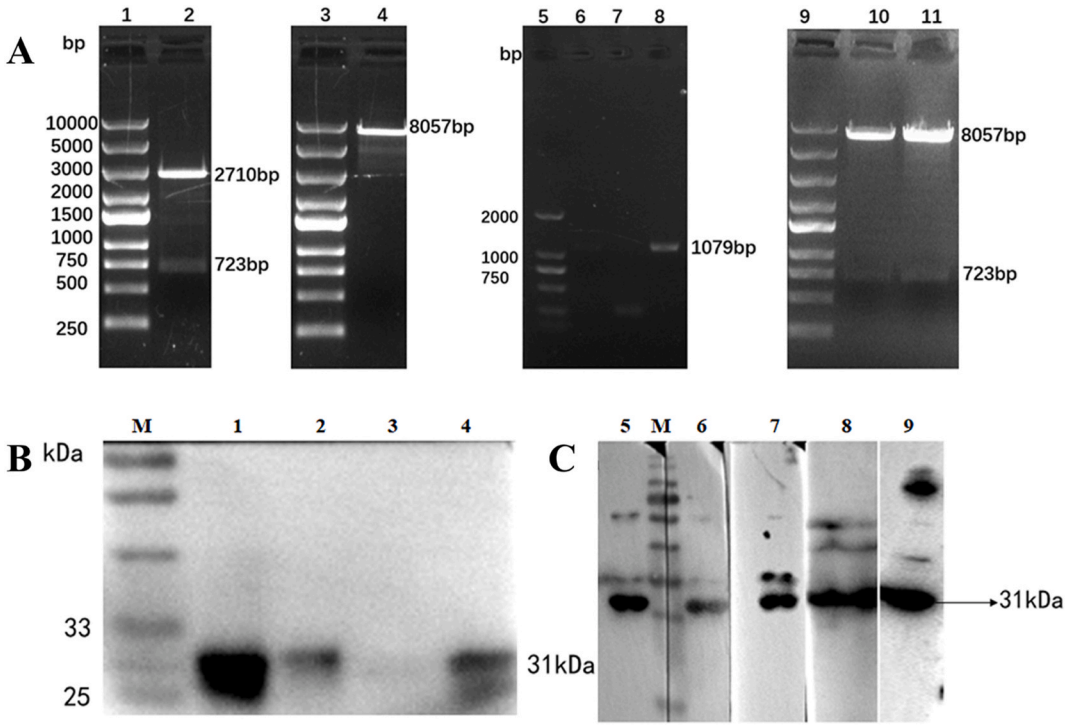

Fig. 2. Identification of recombinant *B. subtilis* expression. 2A. pHT43-Cap recombinant plasmid construction and identification. 1, 3, 9: DL2503 Marker; 2: pUC57-Cap plasmid; 4: pHT43 vector; 5: DL2501 Marker; 6: negative control; 8: pHT43 -Cap PCR product; 10, 11: pHT43-Cap recombinant plasmid. 2B. Determine the expression of recombinant protein by Western blot. 1: the sonicated bacterium (pHT43-Cap/*B. subtilis*); 2: the supernatant of the bacterium (pHT43-Cap/*B. subtilis*); 3: induced empty vector *B. subtilis* (pHT43/*B. subtilis*); 4: the concentrated supernatant of the recombinant bacterium after induction (pHT43-Cap/*B. subtilis*). 2C. The results showed that there was a specific band at 31 kDa. Determine the immune effect of mice by Western blot. M: protein molecular weight marker; 5: positive control; 6–9: triimmunized serum samples from 4 immunized mice.

concentrated supernatant of the recombinant, the sonicated bacteriophage, the bacteriophage supernatant, and the sonicated bacteriophage of pHT43/*B. subtilis* were identified by Western blotting. In comparison with pHT43/*B. subtilis,* the concentrated supernatant of the recombinant bacterium after induction, the sonicated bacterium, and the supernatant of the bacterium all showed protein bands of an expected size at 31 KDa (Fig. 2B), indicating that the recombinant cCap was successfully expressed and could be secreted in the culture supernatant. 100 mL of culture supernatant was concentrated by ultrafiltration to 100 μL, then the protein concentration was measured using the BCA kit. The whole protein secreted by pHT43-Cap/*B. subtilis* was measured at 30 mg/mL after ultrafiltration.

3.3. Identification of PCV2d-VLPs

The DNAStar protein structure analysis software Protean was used to analyze the antigenic determinants of the cCap protein. The cCap protein contained all the antigenic determinants of the PCV2d Cap protein (Fig. 3A). The coding sequences of the PCV2d Cap and cCap proteins were used to predict the protein structure using the SWISS-MODLE online software, and the results showed that the surface and tube structures were very similar (Fig. 3B). The results also showed that a large number of PCV2 VLPs with diameters of approximately 17 nm could be observed (Fig. 3C), indicating that the self-constituted VLPs of the cCap secreted by the recombinant bacterium were similar to the PCV2d Cap structure.

3.4. The immune effect of oral recombinant bacteria verified by western blotting

A purified *E. coli* prokaryotic expression of PCV2-Cap protein was used as loading protein sample, and PHT43-Cap/*B. Subtilis* candidate vaccine triple-immune serum was used as the primary antibody. Western blotting was used to verify the success of the oral immunization against recombinant bacteria in mice (Fig. 2C). The results showed that the target protein band at 31 KDa was consistent with the expected size, which proved that the mice were successfully immunized and could produce specific antibodies against PCV2d-Cap.

3.5. Oral vaccination induce a humoral immune response in mice

To assess the levels of antigen-specific antibodies in orally immunized mices, sera were collected from mice at different time points (days 0, 14, 28, and 42). The levels of specific IgG in the sera of immunized mice were measured by indirect ELISA, using PCV2-Cap protein as the antigen. The results, shown in Fig. 4A, indicate that as the number of immunizations increased, the IgG level of the orally immunized recombinant bacterium pHT43-Cap/*B. subtilis* gradually increased in the test group, with a fourfold increase in $OD450_{nm}$ at day 42; in a comparison with the PBS and the pHT43-Cap/*B. subtilis* groups, this difference was highly significant ($p < 0.01$). The difference between the control groups was not significant, demonstrating that oral administration of the recombinant bacteria induced a humoral immune response in mice.

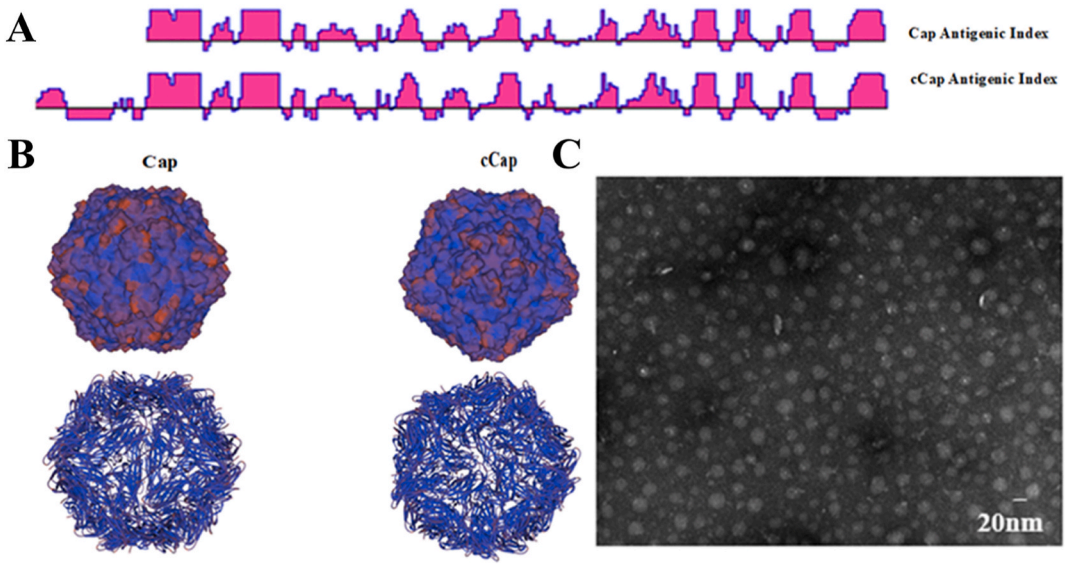

Fig. 3. Analysis of antigenic determinants of cCap protein. 3A. cCap protein structural analysis and identification of VLPs. Cap and cCap antigen index profiles. 3B. Stereo surface pattern and tubular pattern of Cap and cCap proteins. 3C. Transmission electron microscopy of cCap forming VLPs.

3.6. Oral vaccination induced sIgA

The intestinal fluid of mice was collected at different time points (days 0, 14, 28, and 42), and the level of specific sIgA in immunized mice was detected using indirect ELISA, with PCV2-Cap protein as the primary antibody. The results showed that the level of sIgA in the oral immunized recombinant pHT43-Cap/*B. subtilis* sIgA gradually increased in the test group (Fig. 4B); in a comparison with the PBS and pHT43-Cap/B. *subtilis* groups, this difference was highly significant ($p < 0.01$), as no significant change in antibody levels was observed between the control groups.

3.7. Virus neutralization experiment

The serum to be examined was mixed with the viral solution at a 1:1 ratio after multiplicative dilution, reacted at 37 °C for 1 h, connected to a 12-well cell culture plate, and incubated in a CO_2 incubator at 37 °C for 48 h. The cells were collected, genomic DNA was extracted, and the viral load was analyzed using fluorescence quantitative PCR (Fig. 4C). The IgG produced in the serum of mice after oral administration of the recombinant bacteria had a strong capability of neutralizing the virus.

3.8. Changes of viral load in mice tissues after oral vaccination

On the 10th day after challenge, the liver, spleen, and lungs of each mouse in the blank control and test groups were collected. The genomic DNA of each tissue was extracted, the concentration of the extracted genomic DNA was uniformly diluted to 5 ng/μL, and the viral load of each tissue was detected using fluorescence quantitative PCR. The viral loads in the spleen and lungs of the test group were significantly lower than those in the PBS blank control group (Fig. 5A and B), with approximately threefold and fourfold reductions, respectively ($p < 0.05$); the viral loads in the liver samples of the test group were lower by approximately 4.5-fold (Fig. 5C), a highly significant difference ($p < 0.01$).

3.9. Intramuscular injection induce a humoral immune response

In order to assess the level of antigen-specific antibodies, sera were collected from mice in the immunized and blank control groups on day 14 after the second immunization, and indirect ELISA was used to detect specific IgG in immune serum, with PCV2-Cap protein being used as the antigen (Fig. 4D). The $OD450_{nm}$ of the PCV2D-VLPs group was more than 2 times greater, which was significantly

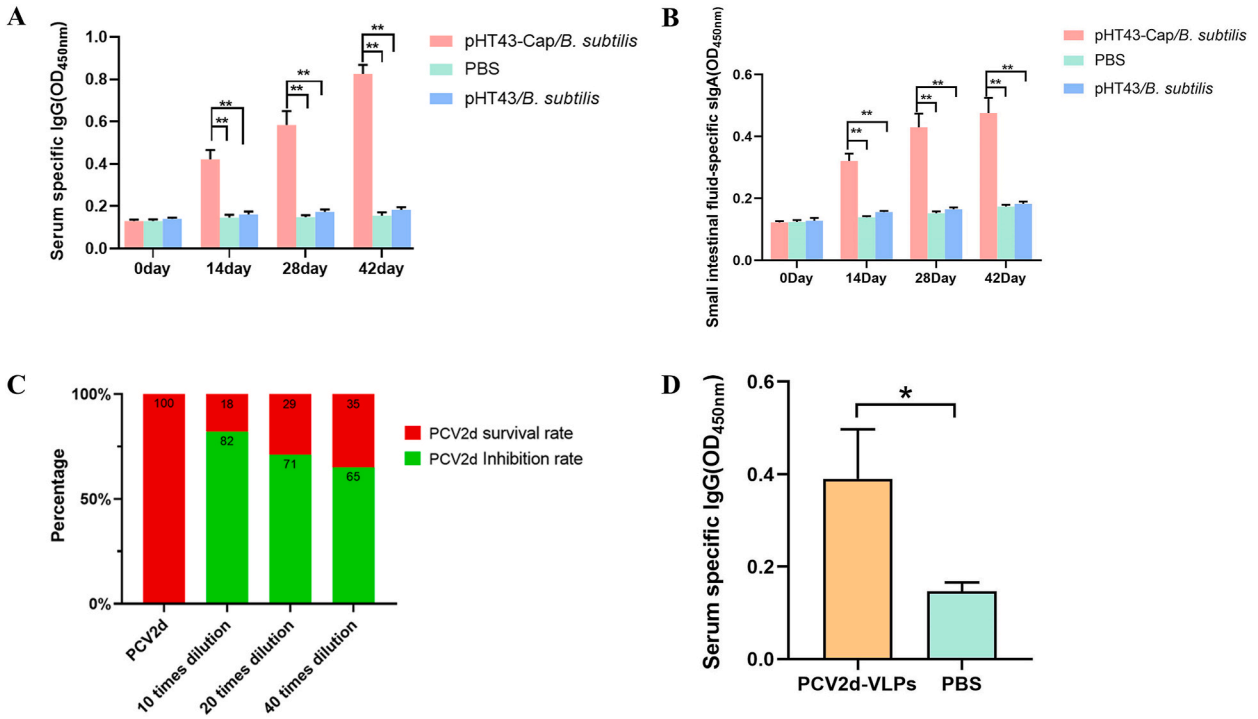

Fig. 4. Specific antibody levels in serum of immunized mice. 4A. Anti-Cap-specific IgG levels in the serum of mice immunized with recombinant bacteria. 4B. Anti-Cap-specific sIgA levels in the intestinal fluid of mice immunized with recombinant bacteria. 4C. Serum neutralizing antibody levels at different dilutions after immunizing mice. 4D. Anti-Cap-specific IgG levels in the serum of mice injected with PCV2d-VLPs. ** : $P < 0.01$, * : $P < 0.05$.

different from that of the blank control group ($p < 0.05$).

3.10. Changes of viral load in tissues of mice after intramuscular vaccination

On day 10 after the challenge, samples were collected from the liver, spleen, and lungs of each mouse, and the genomic DNA was extracted from the tissues. The extracted DNA was diluted at a concentration of 5 ng/μl, and the viral load of each tissue was detected using fluorescence quantitative PCR (Fig. 5D, E, and 5F). The results showed that the viral load in the tissues of the mice injected with PCV2d-VLPs was significantly lower than that in the tissues of the PBS blank control group—specifically, 2–3 times lower ($p < 0.05$).

4. Discussion

PCV-2 is the main pathogen of PCV-AD, a common, serious disease in swine herds, which can cause immunosuppression in the entire herd and is more likely to be complicated by or secondary to other infectious diseases, making epidemic prevention and control difficult. Huang et al. conducted an epidemiological survey and analysis of clinical specimens collected from multiple provinces in China in 2018–2020 and found that PCV2 positivity was as high as 53 % (3619/6872) [37], indicating that PCV2 is widespread in China and seriously endangering the swine industry. The Cap protein encoded by ORF2, the only structural protein of PCV2, contains PCV2-specific neutralizing epitopes and is an important target antigen in the development of vaccines against PCV-AD [13]. In 2006, the first commercial PCV2a vaccine was introduced in the USA, and PCV2 infection rates in swine herds were successfully reduced [38]. However, due to mutations in the amino acid sequence of the coat protein, the PCV2 genotype has evolved, and PCV2d genotype has become the main epidemic genotype in China and even in the world [39–41]. Compared to the classic PCV2a and 2 b, PCV2d can induce more severe clinical signs, viremia, and pathological manifestations in conventional pigs [7,8].

PCV primarily infects animals through the mucosal tissues of the respiratory and intestinal tracts [42–44], and protection against the disease is largely dependent on secretory sIgA antibodies [45]. However, the vast majority of current commercial vaccines are injectable vaccines that primarily induce neutralizing IgG antibodies in the serum; therefore, they may not play a substantial role in preventing PCV2 infection. Consequently, mucosal immunization is of great importance in the prevention and control of PCV2 [46]. Studies have shown that an effective mucosal immune response consists of mucosal sIgA and serum IgG [47]. sIgA is the basis of the mucosal immune system; it maintains mucosal homeostasis by regulating the composition of the intestinal flora and by limiting acute local inflammation caused by pathogen invasion and colonization [48,49]. IgG is the most abundant immunoglobulin in the blood and is essential to systemic immunity [50]. In addition, injectable vaccines are also subject to potential biosafety risks during use, including unstable potency, toxic residues, and impure toxoid cultures [51]. Therefore, a safe and effective mucosal vaccine is needed in order to control PCV2 infection.

In this study, *B. subtilis* was chosen to be a mucosal vaccine delivery vector, due to its ability to efficiently secrete and express

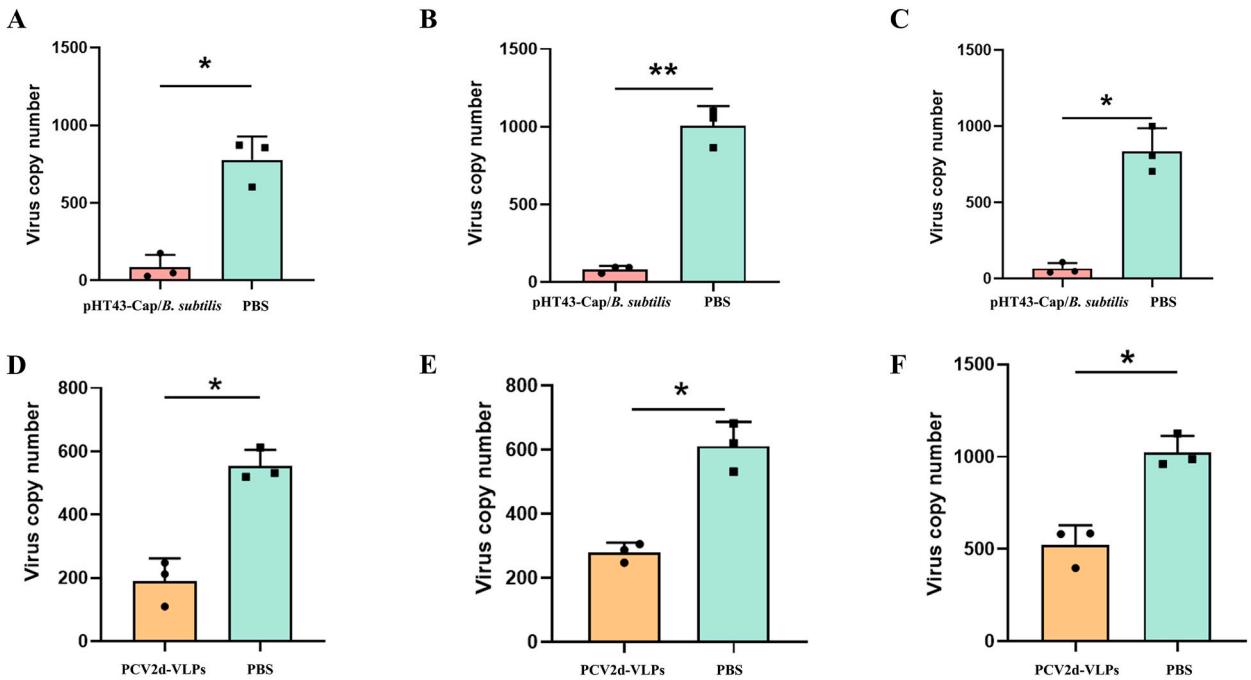

Fig. 5. Viral loads in organ of immunized mice after challenge. Differences in viral loads in 5A. spleen, 5B. lung and 5C. liver after challenge in mice immunized with recombinant bacteria. Differences of viral loads in 5D. spleen, 5E. lung and 5F. liver after c hallenge in mice immunized with intramuscular injection of PCV2d-VLPs. ** : $P < 0.01$, * : $P < 0.05$.

heterologous proteins and maintain their biological activity [52–54]. *B. subtilis* can form spores under harsh conditions to stimulate the proliferation of immune cells in intestinal lymphoid tissues and produce a strong immune response [31]. Therefore, we sought to use the *B. subtilis* expression system to express the PCV2d-Cap protein and thus develop a candidate for an oral vaccine candidate against PCV2d.

Mouse models are effective tools for the in vivo study of PCV viral replication and pathogenesis [55,56]. In this study, we successfully constructed recombinant pHT43-Cap/*B. subtilis* and confirmed its efficient expression of the PCV2d-Cap protein using immunoblotting, with clear and specific reactive bands of the secreted protein concentrated in the bacterium and the medium. The experiments showed that pHT43-Cap/B. *subtilis* caused a sustained increase in PCV2d-specific sIgA antibodies in the small intestinal fluid and of PCV2d-specific IgG antibodies in the serum, with highly significant differences compared to the control group in mouse model. This is very consistent with Li's datas [33]. It is also consistent with the pig in vivo test results that the levels of IgA in the piglets inoculated with pHT43-Cap/B. *subtilis* remained elevated throughout the experiment [50]. After the challenge, viral loads in the liver, spleen, and lungs were significantly lower than those in the control group, indicating that oral immunization with this recombinant bacterium was effective in inducing mucosal and systemic immune responses and in producing antibodies to neutralize the PCV2d virus. The recombinant *B. subtilis* expressing PCV2d-Cap constructed in this study is expected to become a candidate for a new probiotic vector vaccine, given its advantages of non-toxicity, immune specificity, and regulation of protein expression.

VLPs is morphologically and immunologically similar to their natural counterparts; their formation without the involvement of viral genetic material avoids the risk of viral replication or proliferation and they are effective in stimulating humoral and cellular immunity. Therefore, they are widely used in novel vaccine design, such as SARS-CoV-2 VLPs vaccines and foot-and-mouth disease virus (FMDV) VLPs vaccines [57–60]. Current studies on PCV2-VLPs have worked on obtaining large amounts of Cap proteins through *E. coli* expression systems or insect baculovirus expression systems, self-assembling them into VLPs, and then purifying them to obtain higher-purity VLPs. It has also been shown that prepared PCV2d-VLPs can induce broadly cross-neutralizing antibodies against multiple genotypes (2a, 2 b, 2d) and can provide protection in dual infection with PCV2d and PRRSV [61].

In this study, the supernatant of the recombinant medium was heavily concentrated, and the VLPs of PCV2d formed by Cap protein Cap proteinwas obtained (Fig. 2C). Mice were intramuscularly immunized with adjuvant, and the results showed that the level of specific IgG was significantly improvement in the serum of PCV2d-VLPs immunized mice on day 14 after the second immunization, indicated that the humoral immunity is stimulated. And the PCV2d-VLPs significantly reduced the viral load in the tissues of the mice, this indicated its good potential to be a vaccine. We can optimize the process, select a suitable immune adjuvant, and obtain another candidate VLPs protein vaccine.

In summary, a recombinant *B. subtilis* expressing PCV2d-Cap and PCV2d-VLPs was successfully constructed in this study. Mice orally immunized with pHT43-Cap/*B. subtilis* and mice intramuscularly immunized with PCV2d-VLPs showed systemic and mucosal immune responses: cCap effectively produced neutralizing antibodies, and after attacking the virus, it significantly reduced the viral load of each organ. However, the effect of the immune response in mice may be different from that of direct immunization in pigs, so the practical application of this vaccine needs to be validated in pigs.

Funding

This work was supported by the Shandong Province Key Research and Development Program-Major scientific and technological innovation (2022CXGC020711) and the Modern agricultural shrimp and crab industry technology system in Shandong Province (SDAIT-13-02).

Ethics statement

The study protocol and the mice studies were approved by the Animal Care and Use Committee of Shandong Agricultural University, Tai'an, China. Sample collection was carried out in accordance with the approved guidelines of the Ethics Committee of Shandong Agricultural University.

Data availability statement

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

CRediT authorship contribution statement

Yuxuan Zhang: Writing - original draft, Methodology, Data curation. **Yao Wu:** Writing - original draft, Software, Methodology, Data curation. **Chong Peng:** Methodology, Formal analysis. **Zixuan Li:** Software, Data curation. **Gang Wang:** Resources, Conceptualization. **Hui Wang:** Supervision, Project administration. **Lanping Yu:** Writing - review & editing, Investigation, Funding acquisition, Conceptualization. **Fangkun Wang:** Writing - review & editing, Supervision, Resources, Funding acquisition, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22941.

References

[1] B.M. Meehan, F. McNeilly, D. Todd, S. Kennedy, V.A. Jewhurst, J.A. Ellis, L.E. Hassard, E.G. Clark, D.M. Haines, G.M. Allan, Characterization of novel circovirus DNAs associated with wasting syndromes in pigs, J. Gen. Virol. 79 (Pt 9) (1998) 2171–2179.
[2] G. Franzo, J. Segalés, Porcine circovirus 2 (PCV-2) genotype update and proposal of a new genotyping methodology, PLoS One 13 (2018), e0208585.
[3] G. Franzo, J. Segalés, Porcine circovirus 2 genotypes, immunity and vaccines: multiple genotypes but one single serotype, Pathogens 9 (2020).
[4] S. Dei Giudici, A. Lo Presti, P. Bonelli, P.P. Angioi, G. Sanna, S. Zinellu, F. Balzano, F. Salis, M. Ciccozzi, A. Oggiano, Phylogenetic analysis of porcine circovirus type 2 in Sardinia, Italy, shows genotype 2d circulation among domestic pigs and wild boars, Infect. Genet. Evol. 71 (2019) 189–196.
[5] Z. Hou, H. Wang, Y. Feng, M. Song, Q. Li, J. Li, Genetic variation and phylogenetic analysis of Porcine circovirus type 2 in China from 2016 to 2018, Acta Virol. 63 (2019) 459–468.
[6] Y. Wang, L. Noll, N. Lu, E. Porter, C. Stoy, W. Zheng, X. Liu, L. Peddireddi, M. Niederwerder, J. Bai, Genetic diversity and prevalence of porcine circovirus type 3 (PCV3) and type 2 (PCV2) in the Midwest of the USA during 2016-2018, Transbound Emerg Dis 67 (2020) 1284–1294.
[7] L. Guo, Y. Fu, Y. Wang, Y. Lu, Y. Wei, Q. Tang, P. Fan, J. Liu, L. Zhang, F. Zhang, L. Huang, D. Liu, S. Li, H. Wu, C. Liu, A porcine circovirus type 2 (PCV2) mutant with 234 amino acids in capsid protein showed more virulence in vivo, compared with classical PCV2a/b strain, PLoS One 7 (2012), e41463.
[8] T. Opriessnig, C.T. Xiao, P.F. Gerber, P.G. Halbur, S.R. Matzinger, X.J. Meng, Mutant USA strain of porcine circovirus type 2 (mPCV2) exhibits similar virulence to the classical PCV2a and PCV2b strains in caesarean-derived, colostrum-deprived pigs, J. Gen. Virol. 95 (2014) 2495–2503.
[9] R. Wei, J. Xie, S. Theuns, H.J. Nauwynck, Changes on the viral capsid surface during the evolution of porcine circovirus type 2 (PCV2) from 2009 till 2018 may lead to a better receptor binding, Virus Evol 5 (2019) vez026.
[10] S. Wang, C. Xin, X. Wu, J. Shi, Z. Peng, P. Sun, Y. Wang, S. Xu, Y. Yang, F. Zhang, J. Li, Genetic characterization of porcine circovirus type 2 from 2013 to 2018 in Shandong province, China, Vet. Med. Sci. 6 (2020) 76–81.
[11] P. Nawagitgul, I. Morozov, S.R. Bolin, P.A. Harms, S.D. Sorden, P.S. Paul, Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein, J. Gen. Virol. 81 (2000) 2281–2287.
[12] F. Correa-Fiz, G. Franzo, A. Llorens, E. Huerta, M. Sibila, T. Kekarainen, J. Segalés, Porcine circovirus 2 (PCV2) population study in experimentally infected pigs developing PCV2-systemic disease or a subclinical infection, Sci. Rep. 10 (2020), 17747.
[13] L. Wang, D. Zhao, B. Sun, M. Yu, Y. Wang, Y. Ru, Y. Jiang, X. Qiao, W. Cui, H. Zhou, Y. Li, Y. Xu, L. Tang, Oral vaccination with the porcine circovirus type 2 (PCV-2) capsid protein expressed by Lactococcus lactis induces a specific immune response against PCV-2 in mice, J. Appl. Microbiol. 128 (2020) 74–87.
[14] F. Li, X. Wang, R. Ma, W. Wu, F. Teng, X. Cheng, Y. Jiang, H. Zhou, L. Wang, L. Tang, X. Qiao, Y. Li, Oral Immunization with Lactobacillus Casei Expressing the Porcine Circovirus Type 2 Cap and LTB Induces Mucosal and Systemic Antibody Responses in Mice, 2021. Viruses 13.
[15] S. Wang, Y. Xu, Z. Chen, Z. Mei, W. Cui, Y. Jiang, H. Zhou, L. Wang, X. Qiao, F. Li, L. Tang, In mice, analysis of immune response induced by recombinant Lactobacillus royale expressing porcine circovirus type 2 cap protein, Journal of Animal Husbandry and Veterinary Medicine 51 (2020) 2238–2249.
[16] J.G. Silva, E.C. Coimbra, A.L. Jesus, F.C. Mariz, K.M. Silva, Z.I. Lobato, A.C. Campos, L.C. Coutinho, R.S. Castro, A.C. Freitas, Secretory expression of porcine circovirus type 2 capsid protein in Pichia pastoris, J Virol Methods 207 (2014) 226–231.
[17] S. Apostólico Jde, V.A. Lunardelli, F.C. Coirada, S.B. Boscardin, D.S. Rosa, Adjuvants: classification, modus operandi, and licensing, J Immunol Res 2016 (2016), 1459394.
[18] T. Azegami, Y. Yuki, R. Nakahashi, H. Itoh, H. Kiyono, Nanogel-based nasal vaccines for infectious and lifestyle-related diseases, Mol. Immunol. 98 (2018) 19–24.
[19] S.H. Kim, Y.S. Jang, The development of mucosal vaccines for both mucosal and systemic immune induction and the roles played by adjuvants, Clin Exp Vaccine Res 6 (2017) 15–21.
[20] D.T. Le, K.M. Müller, In Vitro Assembly of Virus-like Particles and Their Applications, vol. 11, Life, Basel, 2021.
[21] D. Yan, Y.Q. Wei, H.C. Guo, S.Q. Sun, The application of virus-like particles as vaccines and biological vehicles, Appl. Microbiol. Biotechnol. 99 (2015) 10415–10432.
[22] S. Lee, B.R. Belitsky, D.W. Brown, J.P. Brinker, K.O. Kerstein, J.E. Herrmann, G.T. Keusch, A.L. Sonenshein, S. Tzipori, Efficacy, heat stability and safety of intranasally administered Bacillus subtilis spore or vegetative cell vaccines expressing tetanus toxin fragment C, Vaccine 28 (2010) 6658–6665.
[23] H. Sun, Z. Lin, L. Zhao, T. Chen, M. Shang, H. Jiang, Z. Tang, X. Zhou, M. Shi, L. Zhou, P. Ren, H. Qu, J. Lin, X. Li, J. Xu, Y. Huang, X. Yu, Bacillus subtilis spore with surface display of paramyosin from Clonorchis sinensis potentializes a promising oral vaccine candidate, Parasit Vectors 11 (2018) 156.
[24] H. Amuguni, S. Tzipori, Bacillus subtilis: a temperature resistant and needle free delivery system of immunogens, Hum Vaccin Immunother 8 (2012) 979–986.
[25] G. Casula, S.M. Cutting, Bacillus probiotics: spore germination in the gastrointestinal tract, Appl. Environ. Microbiol. 68 (2002) 2344–2352.
[26] M. Ceragioli, G. Cangiano, S. Esin, E. Ghelardi, E. Ricca, S. Senesi, Phagocytosis, germination and killing of Bacillus subtilis spores presenting heterologous antigens in human macrophages, Microbiology (Read.) 155 (2009) 338–346.
[27] H. Duc le, H.A. Hong, N.Q. Uyen, S.M. Cutting, Intracellular fate and immunogenicity of B. subtilis spores, Vaccine 22 (2004) 1873–1885.
[28] V. Jacquier, A. Nelson, M. Jlali, L. Rhayat, K.S. Brinch, E. Devillard, Bacillus subtilis 29784 induces a shift in broiler gut microbiome toward butyrate-producing bacteria and improves intestinal histomorphology and animal performance, Poultry Sci. 98 (2019) 2548–2554.
[29] J. Jeżewska-Frąckowiak, K. Seroczyńska, J. Banaszczyk, G. Jedrzejczak, A. Żylicz-Stachula, P.M. Skowron, The promises and risks of probiotic Bacillus species, Acta Biochim. Pol. 65 (2018) 509–519.
[30] J.A. Lebron, J.J. Wolf, C.V. Kaplanski, B.J. Ledwith, Ensuring the quality, potency and safety of vaccines during preclinical development, Expert Rev. Vaccines 4 (2005) 855–866.
[31] F.W. van Ginkel, R.J. Jackson, Y. Yuki, J.R. McGhee, Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues, J. Immunol. 165 (2000) 4778–4782.
[32] Q.Y. Li, M.M. Xu, H. Dong, J.H. Zhao, J.H. Xing, G. Wang, J.Y. Yao, H.B. Huang, C.W. Shi, Y.L. Jiang, J.Z. Wang, Y.H. Kang, N. Ullah, W.T. Yang, G.L. Yang, C. F. Wang, Lactobacillus plantarum surface-displayed influenza antigens (NP-M2) with FliC flagellin stimulate generally protective immune responses against H9N2 influenza subtypes in chickens, Vet. Microbiol. 249 (2020), 108834.
[33] W. Li, J. Li, X. Dai, M. Liu, A. Khalique, Z. Wang, Y. Zeng, D. Zhang, X. Ni, D. Zeng, B. Jing, K. Pan, Surface Display of porcine circovirus type 2 antigen protein cap on the spores of bacillus subtilis 168: an effective mucosal vaccine candidate, Front. Immunol. 13 (2022), 1007202.
[34] B. Hu, Construction and Experimental Immunization of Asia 1 and O FMDV Composite Multi-Epitope Oral Vaccine, Jilin University, 2010.

[35] W. Liu, Study on the Immunogenicity of Recombinant PEDV-S1 Bacillus Subtilis, Heilongjiang Bayi Agricultural Reclamation University, 2017.
[36] Y. Li, Development of Porcine Circovirus Type 2 Virus-like Particles Vaccine and Evaluation of Immune Protection Efficacy, Yangzhou University, 2019.
[37] Y. Huang, X. Chen, Y. Long, L. Yang, W. Song, J. Liu, Q. Li, G. Liang, D. Yu, C. Huang, X. Tang, Epidemiological analysis from 2018 to 2020 in China and prevention strategy of porcine circovirus type 2, Front. Vet. Sci. 8 (2021), 753297.
[38] Z. Afghah, B. Webb, X.J. Meng, S. Ramamoorthy, Ten years of PCV2 vaccines and vaccination: is eradication a possibility? Vet. Microbiol. 206 (2017) 21–28.
[39] W. Gong, H. Du, T. Wang, H. Sun, P. Peng, S. Qin, H. Geng, Z. Zeng, W. Liang, H. Ling, C. Tu, Z. Tu, Epizootiological surveillance of porcine circoviruses in free-ranging wild boars in China, Virol. Sin. 38 (2023) 663–670.
[40] Z. Ma, M. Liu, Z. Liu, F. Meng, H. Wang, L. Cao, Y. Li, Q. Jiao, Z. Han, S. Liu, Epidemiological investigation of porcine circovirus type 2 and its coinfection rate in Shandong province in China from 2015 to 2018, BMC Vet. Res. 17 (2021) 17.
[41] H.K. Maity, K. Samanta, R. Deb, V.K. Gupta, Revisiting Porcine Circovirus Infection: Recent Insights and its Significance in the Piggery Sector, vol. 11, Vaccines, Basel, 2023.
[42] J. Segalés, Porcine circovirus type 2 (PCV2) infections: clinical signs, pathology and laboratory diagnosis, Virus Res. 164 (2012) 10–19.
[43] I. Shibata, Y. Okuda, S. Yazawa, M. Ono, T. Sasaki, M. Itagaki, N. Nakajima, Y. Okabe, I. Hidejima, PCR detection of Porcine circovirus type 2 DNA in whole blood, serum, oropharyngeal swab, nasal swab, and feces from experimentally infected pigs and field cases, J. Vet. Med. Sci. 65 (2003) 405–408.
[44] M. Yan, L. Zhu, Q. Yang, Infection of porcine circovirus 2 (PCV2) in intestinal porcine epithelial cell line (IPEC-J2) and interaction between PCV2 and IPEC-J2 microfilaments, Virol. J. 11 (2014) 193.
[45] J. Wang, L. Huang, C. Mou, E. Zhang, Y. Wang, Y. Cao, Q. Yang, Mucosal immune responses induced by oral administration recombinant Bacillus subtilis expressing the COE antigen of PEDV in newborn piglets, Biosci. Rep. 39 (2019).
[46] T. Azegami, Y. Yuki, H. Kiyono, Challenges in mucosal vaccines for the control of infectious diseases, Int. Immunol. 26 (2014) 517–528.
[47] X. Wang, L. Wang, X. Huang, S. Ma, M. Yu, W. Shi, X. Qiao, L. Tang, Y. Xu, Y. Li, Oral Delivery of Probiotics Expressing Dendritic Cell-Targeting Peptide Fused with Porcine Epidemic Diarrhea Virus COE Antigen: A Promising Vaccine Strategy against PEDV, 2017. Viruses 9.
[48] P. Brandtzaeg, Mucosal immunity: induction, dissemination, and effector functions, Scand. J. Immunol. 70 (2009) 505–515.
[49] M. Tsuji, K. Suzuki, K. Kinoshita, S. Fagarasan, Dynamic interactions between bacteria and immune cells leading to intestinal IgA synthesis, Semin. Immunol. 20 (2008) 59–66.
[50] S. Zhang, C. Mou, Y. Cao, E. Zhang, Q. Yang, Immune response in piglets orally immunized with recombinant Bacillus subtilis expressing the capsid protein of porcine circovirus type 2, Cell Commun. Signal. 18 (2020) 23.
[51] Y. Wang, Y. Miao, L.P. Hu, W. Kai, R. Zhu, Immunization of mice against alpha, beta, and epsilon toxins of Clostridium perfringens using recombinant rCpa-b-x expressed by Bacillus subtilis, Mol. Immunol. 123 (2020) 88–96.
[52] P. Lv, Y. Song, C. Liu, L. Yu, Y. Shang, H. Tang, S. Sun, F. Wang, Application of Bacillus subtilis as a live vaccine vector: A review, J. Vet. Med. Sci. 82 (2020) 1693–1699.
[53] P. Lv, X. Zhang, M. Song, G. Hao, F. Wang, S. Sun, Oral administration of recombinant Bacillus subtilis expressing a multi-epitope protein induces strong immune responses against Salmonella Enteritidis, Vet. Microbiol. 276 (2023), 109632.
[54] C. Peng, Y. Zhang, L. Chen, Z. Li, P. Lv, P. Wang, N. Li, F. Wang, Bacillus subtilis expressing duck Tembusu virus E protein induces immune protection in ducklings, Microb. Pathog 185 (2023), 106419.
[55] J. Li, X. Yuan, C. Zhang, L. Miao, J. Wu, J. Shi, S. Xu, S. Cui, J. Wang, H. Ai, A mouse model to study infection against porcine circovirus type 2: viral distribution and lesions in mouse, Virol. J. 7 (2010) 158.
[56] L. Ren, X. Chen, H. Ouyang, Interactions of porcine circovirus 2 with its hosts, Virus Gene. 52 (2016) 437–444.
[57] X. Gao, Y. Xia, X. Liu, Y. Xu, P. Lu, Z. Dong, J. Liu, G. Liang, A perspective on SARS-CoV-2 virus-like particles vaccines, Int. Immunopharm. 115 (2023), 109650.
[58] H. Liu, Z. Teng, H. Dong, J. Li, S. Waheed Abdullah, Y. Zhang, J. Wu, H. Guo, S. Sun, Poly(I:C) and CpG improve the assembly of foot-and-mouth disease virus-like particles and immune response in mice, Virology 579 (2022) 94–100.
[59] D. Wang, J. Mai, B. Lei, Y. Zhang, Y. Yang, N. Wang, Structure, Antigenic Properties, and Highly Efficient Assembly of PCV4 Capsid Protein. Front Vet Sci 8 (2021), 695466.
[60] P.C. Wu, W.L. Lin, C.M. Wu, J.N. Chi, M.S. Chien, C. Huang, Characterization of porcine circovirus type 2 (PCV2) capsid particle assembly and its application to virus-like particle vaccine development, Appl. Microbiol. Biotechnol. 95 (2012) 1501–1507.
[61] S.J. Kang, S.M. Bae, H.J. Lee, Y.J. Jeong, M.A. Lee, S.H. You, H.S. Lee, B.H. Hyun, N. Lee, S.H. Cha, Porcine Circovirus (PCV) Genotype 2d-Based Virus-like Particles (VLPs) Induced Broad Cross-Neutralizing Antibodies against Diverse Genotypes and provided Protection in Dual-Challenge Infection of a PCV2d Virus and a Type 1 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). Pathogens, vol. 10, 2021.