JAST (Journal of Animal Science and Technology) TITLE PAGE

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Yeast-based oral delivery of virus-like particles of the Foot-and-Mouth Disease Virus induces an immune response
Running Title (within 10 words)	Optimized production and immunogenicity of yeast-expressed FMDV VLPs
Author	Ngoc My Tieu Le[1#], Jeesun Chun[2#], Yo-Han Ko[2#], Geonmin Kim[2], Nguyen Ngoc Luong[3], and Dae-Hyuk Kim[2*] [#]These authors contributed equally to this work and share first authorship.
Affiliation	[1]Faculty of Biology-Agriculture and Environmental Science, University of Science and Education, The University of Danang, Danang, Vietnam. [2]Institute for Molecular Biology and Genetics, Department of Molecular Biology, Jeonbuk National University, Jeonju, Jeollabuk-do, Republic of Korea [3]Hue University, College of Sciences, Hue, Vietnam.
ORCID (for more information, please visit https://orcid.org)	Ngoc My Tieu Le (https://orcid.org/0009-0008-1858-1137) Jeesun Chun (https://orcid.org/0000-0003-0642-6769) Yo-Han Ko (https://orcid.org/0009-0006-7337-0768) Geonmin Kim (https://orcid.org/0009-0004-2726-1603) Nguyen Ngoc Luong (https://orcid.org/0000-0002-6123-7437) Dae-Hyuk Kim (https://orcid.org/0000-0002-9948-5313)
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources	This research was supported by Global - Learning & Academic research institution for Master's · PhD students, and Postdocs (LAMP) Program of the National Research Foundation of Korea (NRF) grant funded by the

ACCEPTED

State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	Ministry of Education (No. RS-2024-00443714) and the National Research Foundation of Korea (NRF) grant [NRF-2022R1A2C3005906].
Acknowledgements	We thank the Institute of Molecular Biology and Genetics at Jeonbuk National University for kindly providing the facilities for this research.
Availability of data and material	All data generated or analyzed during this study are included in this published article. Any additional datasets of this study can be available from the corresponding author upon reasonable request.
Authors' contributions Please specify the authors' role using this form.	D.H.K. wrote the manuscript; Y.H.K., J.C., G.K., and N.M.T.L performed the experiments; D.H.K., J.C., and N.N.L. contributed to experimental design and data analysis.
Ethics approval and consent to participate	The animal study was approved by Jeonbuk National University Institutional Animal Care and Use Committee (JBNU IRB NON2025-030-002). All experiments were performed in accordance with the Korea Experimental Animal Protection Act. The study was conducted in accordance with the local legislation and institutional requirements.
Declaration of generative AI	No AI tools were used in this article.

4

5 **CORRESPONDING AUTHOR CONTACT INFORMATION**

For the corresponding author (responsible for correspondence, proofreading, and reprints)	**Fill in information in each box below**
First name, middle initial, last name	Dae-Hyuk Kim
Email address – this is where your proofs will be sent	dhkim@jbnu.ac.kr
Secondary Email address	
Address	Jeonbuk National University, Jeonju, Jeollabuk-do, Republic of Korea

ACCEPTED

Cell phone number	+82)10-4274-3440
Office phone number	+82)63-270-3440
Fax number	

6

ACCEPTED

(Unstructured) Abstract (up to 350 words)

Virus-like particle (VLP)–based vaccines represent an innovative strategy that mimics the structural properties of native viruses while lacking infectious genetic material, thereby combining safety with potent immunogenicity. This platform is increasingly recognized as one of the most promising approaches to induce effective, long-lasting protective immunity. Foot-and-mouth disease virus (FMDV), a member of the *Picornaviridae* family, is the causative agent of a highly contagious vesicular disease affecting cloven-hoofed animals and poses significant economic threats worldwide. Previously, we demonstrated that FMDV VLPs were successfully expressed, processed, and assembled into empty virus particles in *Saccharomyces cerevisiae*, maintaining the stability and assembly efficiency after 3 days at 30 °C. The results indicate that the yeast-based platform provides a favorable environment for proper folding and self-assembly of capsid proteins into structurally intact VLPs. Furthermore, a two-step purification protocol comprising size-exclusion chromatography followed by sucrose density gradient ultracentrifugation effectively removed host cell proteins and other contaminants, yielding highly purified and structurally homogeneous VLP preparations. In the current study, we further enhanced the stability and assembly efficiency of FMDV VLPs by optimizing culture conditions in the yeast expression system. Moreover, initial oral administration in a mouse model induced robust systemic and mucosal immune responses, with generated antibodies cross-reacting with bacterially expressed VP1, confirming the preservation of key antigenic determinants. The immunogenicity of purified VLPs was further evaluated by parenteral administration, and the induced antibodies recognized bacterially expressed VP1, indicating that the purified VLPs preserved antigenic determinants. Therefore, we have demonstrated the potent immunogenicity of these optimized VLPs in mice following both oral and parenteral administration, thereby inducing systemic and mucosal immune responses. We establish a promising yeast-based VLP platform for scalable FMDV vaccine development.

ACCEPTED

Keywords (3 to 6): Foot-and-mouth disease virus; virus-like particle; *Saccharomyces cerevisiae*; oral immunization; immunogenicity; mucosal immunity;

Introduction

Several members of the *Picornaviridae* (https://picornaviridae.com) family of non-enveloped viruses are responsible for some important human and animal diseases [1]. Foot-and-mouth disease, caused by foot-and-mouth disease virus (FMDV), is endemic in certain regions, including extensive areas of Africa, Asia, and South America. It is among the most economically devastating diseases affecting cloven-hoofed livestock worldwide because it severely compromises animal health, reduces herd productivity, and culminates in strict trade restrictions across the livestock industry [2]. The FMDV virion is a spherical particle of approximately 25–30 nm in diameter. The particles are composed of a structural protein that encloses a positive-sense single-stranded RNA [(+)ssRNA] genome. The 8400-nucleotide-long RNA genome includes three parts: an open reading frame (ORF), an extended 5′ untranslated region (5′ UTR), and a 3′ untranslated region (3′ UTR), approximately 7000, 1300, and 90 nucleotides in length, respectively [3]. The ORF encodes four structural proteins (VP1, VP2, VP3, and VP4) and several nonstructural proteins involved in viral particle biogenesis and in modulation and evasion of the host immune response [4]. To form the mature virus, the capsid protein VP0 undergoes a second self-cleavage, yielding VP2 and VP4. VP1, VP2, and VP3 are exposed on the virus surface, whereas VP4 is located within the virus [5].

Vaccines remain the most effective tools for preventing and controlling viral outbreaks in livestock populations because they activate the immune system by prompting a specific immune response, thereby enhancing herd resistance to particular viral pathogens and safeguarding animal productivity. Various types of genetically engineered vaccines have been produced, including gene-deletion vaccines, peptide vaccines, and virus-like particles (VLPs). Among these, VLP-based vaccines have been shown to be among the safest and most effective [6]. VLPs have been developed as vaccine candidates from at least 30 different viruses that infect humans and animals [7], including porcine parvovirus (PPV), human papillomavirus type 16 (HPV16) L1, hepatitis C virus (HCV), norovirus, hepatitis B virus, and influenza virus [8 - 13]. Although VLPs lack a viral genome, they resemble natural viral particles in overall structure, size, and shape. These similarities enable VLPs to elicit protective immune responses. Additionally, the absence of a viral genome prevents the VLPs from replicating, thereby enhancing biosafety [14]. VLPs have been produced in several expression systems, including mammalian [15, 16], plant [17, 18], insect [19, 20], bacterial [21, 22], and yeast systems [23, 24]. VLP vaccines against HBV, HEV, and HPV have been used worldwide [25]. Since 1990, FMDV VLPs have been studied to assess the efficacy of vaccines that induce an immune response against FMDV in animals [25]. P12A and P12A3C have been the principal target sequences used to express FMDV VLPs in several expression systems, including baculovirus/Sf-9 insect cells, *Escherichia coli*, *Samia cynthia*

ACCEPTED

ricini, *Nicotiana benthamiana*, *Agrobacterium tumefaciens*, and *HEK-293A* and *BHK-21* cells [25]. Our previous work demonstrated that the viral structural polyprotein is processed by the viral 3C protease in *Saccharomyces cerevisiae* and that the resulting capsid subunits assemble into VLPs [26].

S. cerevisiae has several characteristics that make it an attractive platform for the cost-effective production and oral delivery of vaccine antigens for the livestock industry. As a well-established eukaryotic expression system, *S. cerevisiae* can efficiently express heterologous proteins and facilitates post-translational processing while offering a favorable safety profile for animal applications [27]. In particular, its generally recognized as safe (GRAS) status, capacity for high-level antigen expression, and intrinsic adjuvant properties [28] have supported its development as a functional feed additive for oral vaccine delivery [29]. These properties are particularly advantageous for mass vaccination through oral delivery in livestock farming, as oral administration via feed minimizes animal handling stress and injection-site lesions that can otherwise impair animal welfare and carcass quality. Furthermore, recombinant whole yeast can serve not only as an antigen-delivery vehicle but also as a nutritional supplement that supports gut health and animal production.

To advance a practical yeast-based VLP platform relevant to livestock disease management, we evaluated culture conditions, including temperature and incubation time for recombinant *S. cerevisiae*, to identify suitable conditions for VLP production. Moreover, as a proof of concept for oral livestock immunization, the immunogenic potential of the FMDV VLPs was evaluated following oral administration of VLP-expressing yeast cells to mice. Humoral and mucosal antibody responses were considerably greater than those in the negative control group, indicating enhanced systemic IgG and mucosal IgA production. Furthermore, parenteral immunization with purified VLPs elicited strong antigen-specific immune responses. Collectively, these findings support further investigation of FMDV VLP-expressing yeast cells as a dual-purpose functional feed additive for oral antigen delivery and the induction of immune responses against FMDV, offering a promising strategy to enhance livestock health, strengthen disease prevention, and sustain animal productivity.

Materials and Methods

Strains and culture media

Recombinant *Saccharomyces cerevisiae* engineered from the *S. cerevisiae* 2805 strain (*MATα pep4::HIS3 prb 1-δ Can1 GAL2 his3 ura3-52*) to express the P1, 2A, and 3C (P12A3C) of FMDV proteins (FMD serotype O virus (O1/Manisa/TUR/69, GenBank accession no. AY593823.1) was obtained from our previous study [26] and used for

ACCEPTED

94 further study. Culture media such as YEPD and uracil-deficient (ura$^-$) selective medium were prepared as described
95 previously [26].
96
97 **Optimized culture condition**
98 The selected recombinant clone was inoculated into 5 mL of uracil dropout liquid medium and cultured for 48 hours
99 at 30 °C, 200 rpm in a shaking incubator. Subsequently, 250 µL of this culture was transferred into 5 mL of YEPD
100 medium and cultured for an additional 12 hours under the same conditions. This culture was then inoculated into 40
101 mL of freshly prepared YEPD medium and grown at 30 °C, 25 °C, and 20 °C with shaking at 200 rpm. Cells were
102 harvested daily for 5 days and stored at -70 °C for further analysis.
103
104 **Purification of VLPs**
105 Bead-treated yeast cell lysates were centrifuged to remove cellular debris. Yeast cell lysates were precipitated with
106 PEG 8000 and NaCl at a final concentration of 7.5% and 0.5 M, respectively, and incubated overnight at 4 °C with
107 stirring. The overnight mix was centrifuged at 10,000 rpm for 0.5 hours at 4 °C. The precipitate was obtained by
108 centrifugation and resuspended in Tris–NaCl buffer (150 mM Tris-HCl, 150 mM NaCl, pH 7.8). The supernatant was
109 collected and filtered through a 0.2 µm filter before injection into the size exclusion chromatography (SEC) column.
110 The collected fractions were dialyzed in PBS and analyzed by SDS-PAGE and Western blot analysis. The supernatant
111 was subjected to ultracentrifugation at 119,000 ×*g* for 2 hours at 4 °C. The selected fractions from FPLC were loaded
112 onto a sucrose density step-gradient (10% to 25% sucrose, interval 5%) and subjected to further ultracentrifugation at
113 180,000 ×*g* for 4 hours at 4 °C. The collected fractions were dialyzed in PBS and analyzed by SDS-PAGE and Western
114 analysis. The structure of the virus particles was visualized on an H-7650 (Hitachi, Japan) transmission electron
115 microscope (TEM) using negative staining with 2% uranyl acetate solution.
116
117 **Western analysis**
118 A total of 50 µg of cell-free extract (CFE) protein from yeast transformation was loaded [26] into a discontinuous 12%
119 acrylamide/bis-acrylamide gel and separated at 60 mV for 15 min., followed by 80 mV until the front dye reached the
120 end of the separating gel. The gel was blotted onto a nitrocellulose filter membrane (Hybond™-C Extra, Amersham
121 Biosciences) and first incubated with FMDV antibodies or a Rabbit polyclonal antibody against FMDV VP1 (Biorbyt,
122 orb499995). After then, the target proteins (VP1, VP2, VP3) were detected using corresponding (anti-VP1, -VP2, -

ACCEPTED

VP3, and -3C) antibodies. Goat anti-mouse IgG conjugated to alkaline phosphatase was used as a secondary antibody (Sigma-Aldrich, USA). Color detection was performed by BCIP/NBT in TMN buffer (100 mM Tris-HCl, pH 9.5; 5 mM $MgCl_2$; 100 mM NaCl).

Oral administration of VLPs

Six-week-old BALB/c mice were divided into two immunization groups (n = 6 per group): P12A3C and negative control. The mice were fasted overnight (water was provided ad libitum) and individually fed P12A3C or a negative control by gastric gavage on days 0, 14, and 28. Each dose consisted of 1.6 g of yeast cells, corresponding to approximately 100 mL of culture. The cells had been cultured, frozen for storage, and thawed immediately before administration. The cells were resuspended to a final volume of 2 mL in PBS, split equally, and administered orally four times per dose. This dosage was selected based on our previous studies, in which the same amount of yeast was successfully used in mouse immunization experiments involving yeast-based subunit vaccine responses [26]. To investigate the memory response, each immunized mouse was injected intraperitoneally with 20 μg of purified VP1 expressed in *E. coli* 3 weeks after the final immunization. Blood and fecal pellets were collected from each mouse at 13, 26, 49, and 66 days after the first immunization. Serum and fecal levels of IgG and sIgA antibodies against VP1 were assessed by enzyme-linked immunosorbent assay (ELISA) as described previously [30].

Injection of purified VLPs

Each of the three mice received a subcutaneous injection of 0.2 mL of an emulsion containing 20 μg of purified VLP antigen per dose and complete Freund's adjuvant, administered with a 22-gauge needle. The emulsion was injected very slowly to avoid dislodging the needle from the syringe and to ensure the needle penetrated several millimeters into the subcutaneous tissue or peritoneal cavity, thereby minimizing leakage. After 2 weeks, immunize mice with 25 μg of antigen per animal emulsified in Incomplete Freund’s adjuvant. After 2 weeks, the mice were immunized a third time as described in step 2. After 2 weeks, the mice were bled, and Western blot analysis was performed to identify an antibody that recognized VP1.

Statistics analysis

Data are presented as the mean ± standard deviation (SD). Statistical differences in antibody responses between the VLP-expressing yeast and negative control groups at each sampling time point were analyzed using an independent-

ACCEPTED

samples *t*-test with SPSS software (IBM Corp., Armonk, NY). To control for multiple comparisons, the Bonferroni correction was applied. A Bonferroni-adjusted p-value < 0.01 was considered statistically significant.

Results

Optimized culture conditions.

VLP formation through capsid assembly is a highly coordinated process that can be sensitive to cellular metabolic activity, enzymatic efficiency, and culture conditions. Since our previous studies on intracellular protein expression suggested that lower temperatures during the culture period for protein production were beneficial for maintaining protein stability and enhancing the yield of difficult-to-express target proteins [26, 31], we measured the effect of temperature on VLP production. Densitometric analysis showed no significant differences in cross-reactive band intensities among cultures grown at 20, 25, and 30 °C (Figs. 1 and 2), suggesting that lower temperatures did not improve target protein expression under the conditions tested. We selected 30 °C as the temperature because cultures grown at this temperature produced significantly greater cell biomass than those grown at the lower temperatures tested (Fig. 2). This discrepancy may reflect distinct requirements for the correct folding of polyprotein and translational decoding by the 2A sequence, followed by proteolytic processing by the co-expressed 3C protease, and then multimeric capsid assembly of FMDV structural proteins. Our results showed no difference among temperatures, suggesting that the yeast optimal growth temperature, such as 30 °C, was sufficient to support protein expression, processing, and assembly.

Temporal expression of VLP was examined daily for 5 days. As shown in Fig. 3, VLP expression peaked 3 days after YEPD culture and remained stable until 5 days. Thus, the cultures were grown at 30 °C for 3 days to maximize antigen production prior to harvesting. Cells prepared under these optimized conditions, such as 30 °C for a 3-day culture, were subsequently used in the oral feeding experiments to evaluate immunogenicity. These optimizations ensured that downstream animal studies used yeast preparations with enhanced antigen content and integrity for immunoreactivity, thereby improving the reliability and consistency of the immunological assessments.

Purification of FMDV VLPs

Following initial purification of FMDV VLPs by size exclusion chromatography (SEC), the samples were further characterized by sucrose density gradient ultracentrifugation to assess particle integrity, homogeneity, and sedimentation behavior. This additional purification step enabled the separation of fully assembled VLPs from smaller

ACCEPTED

protein aggregates, partially assembled intermediates, and other host-derived contaminants, thereby improving the overall purity and structural consistency of the preparation. To directly evaluate particle morphology, the purified fractions were analyzed by SDS-PAGE, Western blot, and transmission electron microscopy (TEM).

In SDS–PAGE analysis (Fig. 4), all fractions that were not subjected to boiling prior to electrophoresis did not show clearly defined high molecular-weight bands corresponding to assembled capsid complexes. This observation is consistent with the fact that intact virus-like particles (VLPs) may not efficiently enter the resolving gel under non-denaturing or partially denaturing conditions or may remain as large multimeric assemblies that are poorly resolved [32]. However, when samples were heated to boiling in the presence of SDS-containing loading buffer, clear protein bands corresponding to capsid subunits became visible. Western blot analysis further confirmed these findings. In boiled samples, a prominent immunoreactive band corresponding to the VP3 capsid protein was consistently detected, with signal intensity markedly higher than in non-boiled samples. The stronger cross-reactive band observed on the membrane indicates that heat treatment effectively disrupted higher-order capsid assemblies, leading to dissociation of VLPs into their individual structural subunits. Once denatured and separated, these subunits were more readily resolved by electrophoresis and more accessible to antibody recognition during immunoblotting. Together, these results suggest that the recombinant FMDV VLPs maintain a multimeric capsid structure under non-boiled conditions but are susceptible to thermal disruption. Heating promotes degradation or dissociation of assembled particles into capsid subunits, particularly VP3, which becomes more detectable following denaturation. This behavior is consistent with the known structural lability of FMDV capsids [33] and further supports the conclusion that the purified material consisted of assembled VLPs that can be disassembled into constituent proteins under denaturing conditions.

Samples were negatively stained with 2% (w/v) uranyl acetate to enhance contrast and visualize capsid architecture. Electron micrographs of the partially purified viral preparation revealed uniform, empty, isometric particles with diameters of approximately 30 nm, consistent with the expected size of Foot-and-mouth disease virus (FMDV) capsids (Fig. 5). The observed particles exhibited well-defined boundaries and morphology characteristic of non-enveloped icosahedral virions, supporting the successful assembly of higher-order capsid structures. The absence of electron-dense cores within the particles further indicated that they were empty virus-like particles rather than genome-containing virions, as expected from a recombinant expression system lacking viral RNA. The overall structural appearance closely resembled that of native FMDV particles in terms of size and symmetry, suggesting that key aspects of capsid organization were faithfully reproduced in the heterologous host.

ACCEPTED

Collectively, these findings demonstrate that the P1 polyprotein precursor expressed in *Saccharomyces cerevisiae* was efficiently processed into the mature structural proteins (VP0, VP1, and VP3) by the co-expressed 3C protease at 30 °C. These processed capsid subunits (VP0, VP1, and VP3) were successfully assembled into VLPs at 30 °C. TEM data provide strong visual evidence that these self-assembling VLPs maintained morphological characteristics of FMDV VLPs. Therefore, the yeast expression system successfully recapitulated the essential steps of FMDV capsid maturation and assembly, yielding structurally authentic VLPs that promise further immunological and vaccine development studies.

Oral administration of VLPs

Because Foot-and-mouth disease virus (FMDV) primarily infects susceptible animals through mucosal surfaces, particularly the oral and respiratory epithelium, we further evaluated whether the recombinant VLPs could stimulate both mucosal and systemic immunity. To mimic the natural route of infection and to explore the feasibility of an oral vaccine strategy, mice were immunized by feeding yeast cells expressing FMDV VLPs. This approach also leverages the potential adjuvant and delivery properties of the yeast cell wall, which can protect antigens during gastrointestinal transit and promote uptake by mucosal immune tissues [34, 35].

Analysis of systemic humoral responses indicated that oral administration of 1.6 g of VLP-expressing yeast cells per dose, estimated to contain approximately 5 mg of VLPs based on a previous study [31], enhanced antigen-specific IgG production compared with that in the negative control group. Although no statistically significant difference was observed at 13 days after the primary feeding, a clear and significant increase in serum IgG titers was detected at 26 (after the second feeding) and 49 (after the final feeding) days. These findings suggest that repeated oral exposure to VLPs can induce a measurable and sustained systemic immune response, even if the initial response develops gradually (Fig. 6).

To further investigate the establishment of long-term immune memory, mice were subjected to an intraperitoneal antigenic challenge as a booster immunization (Fig. 6). Following this secondary exposure, animals that had originally received VLP-expressing yeast cells mounted a significantly stronger immune response compared with those receiving the mock transformant. This enhanced recall response supports the conclusion that primary oral immunization with FMDV VLPs was sufficient to prime immunological memory. Although mucosal sIgA titers declined over time in mice initially dosed with whole recombinant cells—and the differences were not statistically

ACCEPTED

significant at later time points—the robust systemic response following booster challenge suggests the successful induction of memory B-cell populations.

Taken together, these findings demonstrate that oral administration of yeast-delivered FMDV VLPs can stimulate both mucosal and systemic immune responses and can prime for long-term immunological memory. While further optimization may be required to improve the durability of mucosal sIgA responses, the results highlight the potential of this strategy as a needle-free vaccination approach targeting the primary route of FMDV infection.

Immunogenicity followed by the injection of the purified VLPs

In addition to oral administration, purified FMDV VLPs were injected subcutaneously into mice to assess their immunogenic potential. Following immunization, sera collected from the vaccinated animals were analyzed by Western blot analysis (Fig. 7). The immune sera consistently recognized a distinct band corresponding to the VP1 protein expressed in *Escherichia coli*, demonstrating that antibodies generated against the recombinant VLPs specifically bound to FMDV structural proteins. Notably, the reactivity pattern observed with these sera (Figs. 7B and 7C) was comparable to that obtained using a commercial anti-FMDV antibody preparation (Fig. 7A), suggesting that the immune response elicited by the recombinant VLPs targets relevant viral epitopes.

The detection of VP1 is particularly significant, as this capsid protein contains major immunodominant and neutralizing epitopes of FMDV and plays a central role in protective immunity. The ability of the induced antibodies to cross-react with bacterially expressed VP1 indicates that key antigenic determinants were preserved in the VLP formulation, despite the absence of viral genomic RNA. This supports the structural and antigenic authenticity of the recombinant particles. These results demonstrate that the engineered FMDV VLPs elicit a specific humoral immune response *in vivo*, with antigen recognition profiles similar to established anti-FMDV reagents.

Discussion (optional)

In our previous study, we demonstrated that *S. cerevisiae* supports high-level expression of FMDV structural proteins and efficient proteolytic processing of the P1 polyprotein precursor by the co-expressed viral 3C protease [26]. These findings confirmed that the yeast expression system could correctly generate the individual capsid proteins required for particle assembly. Building on this foundation, the present study focused on the stable expression of VLPs, purification of assembled VLPs, and their preliminary immunogenicity evaluation in a mouse model.

ACCEPTED

To obtain VLPs of sufficient purity and integrity for *in vivo* administration, we implemented a two-step purification strategy. First, size-exclusion chromatography was used to separate assembled particles from smaller protein fragments and host-derived contaminants. Subsequently, sucrose density gradient ultracentrifugation was performed to further enrich and concentrate fractions corresponding to correctly assembled VLPs. This sequential purification approach yielded preparations with adequate quality and quantity for immunization studies, as confirmed by biochemical and morphological analyses. In addition to optimizing purification procedures, we refined the culture conditions for VLP production. Yeast transformants expressing FMDV VLPs were cultivated under controlled conditions, and maximal antigen expression and stability were achieved at 30°C following a 3-day incubation. This optimized growth protocol improved VLP yield and reproducibility, ensuring sufficient material was available for subsequent animal experiments. Collectively, these improvements in both upstream production and downstream purification provided a robust platform for evaluating the immunogenic potential of recombinant FMDV VLPs *in vivo*.

The antibody titers elicited against conformational epitopes are comparable to those elicited by whole virions and naturally empty capsids [25]. Moreover, the oral and respiratory mucosae serve as natural routes of entry and primary sites of replication for FMDV [36]. Therefore, it is important to determine mucosal immune responses. We investigated immune responses in a mouse model after oral administration of yeast cells expressing FMDV VLPs. We compared responses to intact VLP-expressing yeast cells with responses to mock transformants containing an empty vector because intact FMDV particles (146S) are crucial for the induction of neutralizing antibodies [37]. The electron microscopy results (Fig. 5) suggested that the FMDV VLPs in yeast cells were intact and retained morphological characteristics of natural virus particles. The oral immunization elicited significant levels of humoral IgG and substantial mucosal sIgA antibodies. These results demonstrate humoral and mucosal antibody responses following oral administration of VLP-expressing yeast cells and support further investigation of this approach for the development of an edible vaccine against FMDV. *S. cerevisiae*, a GRAS organism with strong intrinsic adjuvant activity, is currently used as a feed additive. Virus neutralizing test (VNT) and experiments in livestock species susceptible to FMDV under tightly controlled conditions are required to evaluate the protective potential of this approach. Our studies demonstrated serotype-specific reactivity of the VLPs with antibodies in a commercially available detection kit (Fig. 1) [26]. These findings support the antigenicity of the VLPs but do not yet establish the neutralizing activity or protection efficacy of antibodies elicited *in vivo*. Future studies will include VNTs and experiments in livestock species susceptible to FMDV under tightly controlled conditions. However, the reason for the decrease in sIgA levels over time, especially after the third feeding, remains unclear. Although sIgA is the most

ACCEPTED

acid-resistant immunoglobulin isoform and is highly stable in mucosal secretions [38], FMDV VLPs are inherently acid labile and readily disassemble under acidic conditions. We hypothesize that repeated exposure to acid-sensitive VLP antigens may alter the efficiency of antigen presentation or induce local immune tolerance over successive feedings. Factors other than low pH, such as protease activity, may also contribute. Additionally, oral administration of whole *S. cerevisiae* cells may protect the antigen from gastric degradation and provide intrinsic adjuvant activity. However, limits on the amount of yeast cells that can be administered orally may restrict the antigen dose available for uptake in Peyer's patches [30]. We acknowledge that the sample size was small (n = 6 per group), although similar or smaller group sizes have been successfully employed in comparable studies [39, 40]. We have also conducted statistical analyses applying the Bonferroni correction for multiple comparisons ($p < 0.01$) and clarified the rationale for the sample size. Between-groups differences in IgG remained statistically significant at three scheduled sampling time points. For sIgA, while differences remained significant at one time point rather than two, the overall kinetic trend of elevated antibody production in the treated group was fully preserved. Given the large observed effect sizes (Cohen's d = 1.17-3.17), a post-hoc power calculation suggested that n = 6 provides sufficient statistical power to detect these substantial differences. The humoral immune response plays an important role in overcoming FMDV infection, with systemic neutralizing antibodies serving as key mediators for clearing the virus [41]. The rapid activation of B cells leads to an early antibody response, as evidenced by detectable serum IgM within 3–4 days after infection in cattle, followed by peaks of IgA and then IgG within the following 1–2 weeks [42-44]. Therefore, further studies are required to optimize the stability of FMDV VLPs in yeast cells and their oral administration by adjusting the VLP concentration per dose, increasing the number of doses, and adjusting the dose interval. Furthermore, we will focus on purifying the VLPs to produce large amounts of genome-free FMDV antigens.

Fully assembled virus-like particles represent the most structurally and antigenically complete form of the virus, preserving the conformational epitopes required to elicit a strong and protective immune response. In comparison, isolated viral proteins, partially assembled intermediates such as pentamers, or degradation products lack the same structural integrity and therefore induce significantly weaker immunogenicity [45]. Complete virus particles—including empty capsids—are consequently far more effective at eliciting robust immune responses than individual components or disassembled forms [46].

Unlike most other picornaviruses, Foot-and-mouth disease (FMD) virus particles exhibit marked structural instability [33]. Intact virions (146S), which represent the fully assembled and immunogenic form of the virus, readily dissociate into smaller pentameric subunits (12S) under relatively mild environmental stresses, including changes in

ACCEPTED

temperature, pH, or ionic conditions [47, 48]. This intrinsic lability distinguishes FMD virus from many related viruses whose capsids remain comparatively stable once assembled [33]. The dissociation of 146S particles into 12S pentamers has important immunological consequences. While the overall protein composition remains unchanged, the capsid's conformational integrity is disrupted, leading to the loss or alteration of key conformational epitopes required for strong neutralizing antibody responses. As a result, the immunogenicity of the dissociated 12S subunits is dramatically reduced compared with that of intact 146S virions. Although this reduction in immune potency is well documented, the precise mechanisms underlying the diminished immunogenicity—whether due to altered epitope presentation, reduced stability *in vivo*, impaired uptake by antigen-presenting cells, or other factors—remain incompletely understood [49]. For this reason, vaccine production processes must be optimized to ensure a high proportion of intact particles in the final formulation. Equally important is maintaining appropriate storage and handling conditions to preserve particle integrity, particularly by strictly adhering to the cold chain during distribution and vaccination campaigns. To support quality control, analytical systems have been developed to detect and quantify intact particles of specific serotypes, enabling reliable assessment of vaccine potency and stability [50, 51].

Our TEM analysis confirmed the presence of intact VLPs during our culture conditions and purification processes. In addition, our oral vaccine strategy is an excellent alternative to overcome the need to purify intact VLPs from cells. Given the known safety advantages of VLP-based platforms, such as the absence of infectious material and improved biosafety during production, these findings further support the feasibility of developing recombinant VLPs as a next-generation oral and injection-based vaccine candidate against FMD [6, 52]. Continued studies assessing neutralizing antibody titers, protective efficacy, and cross-serotype reactivity will be essential to fully establish their potential as an effective and safe alternative to conventional inactivated FMD vaccines.

ACCEPTED

Acknowledgments

We thank the Institute of Molecular Biology and Genetics at Jeonbuk National University for kindly providing the facilities for this research.

References (Vancouver or NLM style)

1. Bolaños-Martínez OC, Rosales-Mendoza S. The potential of plant-made vaccines to fight picornavirus. Expert Rev Vaccines 2020;19:599–610. https://doi.org/10.1080/14760584.2020.1791090.
2. Dee SA, Bauermann FV, Niederwerder MC, Singrey A, Clement T, de Lima M, et al. Survival of viral pathogens in animal feed ingredients under transboundary shipping models. PLoS One 2018;13:e0194509. https://doi.org/10.1371/journal.pone.0194509.
3. Rahman MA, Zereen F, Rana ML, Hossain MG, Shimada M, Saha S. Foot-and-mouth disease in Asia. Virus Res 2025;351:199514. https://doi.org/10.1016/j.virusres.2024.199514.
4. Mason PW, Grubman MJ, Baxt B. Molecular basis of pathogenesis of FMDV. Virus Res 2003;91:9–32. https://doi.org/10.1016/s0168-1702(02)00257-5.
5. Xiao Y, Zhang S, Yan H, Geng X, Wang Y, Xu X, et al. The high immunity induced by the virus-like particles of foot-and-mouth disease virus serotype O. Front Vet Sci 2021;8:633706. https://doi.org/10.3389/fvets.2021.633706.
6. Kushnir N, Streatfield SJ, Yusibov V. Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development. Vaccine 2012;31:58–83. https://doi.org/10.1016/j.vaccine.2012.10.083.
7. Crisci E, Bárcena J, Montoya M. Virus-like particles: the new frontier of vaccines for animal viral infections. Vet Immunol Immunopathol 2012;148:211–25. https://doi.org/10.1016/j.vetimm.2012.04.026.
8. Antonis AF, Bruschke CJ, Rueda P, Maranga L, Casal JI, Vela C, et al. A novel recombinant virus-like particle vaccine for prevention of porcine parvovirus-induced reproductive failure. Vaccine 2006;24:5481–90. https://doi.org/10.1016/j.vaccine.2006.03.089.
9. Harro CD, Pang YY, Roden RB, Hildesheim A, Wang Z, Reynolds MJ, et al. Safety and immunogenicity trial in adult volunteers of a human papillomavirus 16 L1 virus-like particle vaccine. J Natl Cancer Inst 2001;93:284–92. https://doi.org/10.1097/00128360-200107000-00014.
10. Jeong SH, Qiao M, Nascimbeni M, Hu Z, Rehermann B, Murthy K, et al. Immunization with hepatitis C virus-like particles induces humoral and cellular immune responses in nonhuman primates. J Virol 2004;78:6995–7003. https://doi.org/10.1128/jvi.78.13.6995-7003.2004.
11. LoBue AD, Lindesmith L, Yount B, Harrington PR, Thompson JM, Johnston RE, et al. Multivalent norovirus vaccines induce strong mucosal and systemic blocking antibodies against multiple strains. Vaccine 2006;24:5220–34. https://doi.org/10.1016/j.vaccine.2006.03.080.

ACCEPTED

12. Joe CCD, Chatterjee S, Lovrecz G, Adams TE, Thaysen-Andersen M, Walsh R, et al. Glycoengineered hepatitis B virus-like particles with enhanced immunogenicity. Vaccine 2020;38:3892–901. https://doi.org/10.1016/j.vaccine.2020.03.007.
13. Quan FS, Huang C, Compans RW, Kang SM. Virus-like particle vaccine induces protective immunity against homologous and heterologous strains of influenza virus. J Virol 2007;81:3514–24. https://doi.org/10.1128/jvi.02052-06.
14. Balke I, Zeltins A. Use of plant viruses and virus-like particles for the creation of novel vaccines. Adv Drug Deliv Rev 2019;145:119–29. https://doi.org/10.1016/j.addr.2018.08.007.
15. Elfayres G, Paswan RR, Sika L, Girard MP, Khalfi S, Letanneur C, et al. Mammalian cells-based platforms for the generation of SARS-CoV-2 virus-like particles. J Virol Methods 2023;322:114835. https://doi.org/10.1016/j.jviromet.2023.114835.
16. Tan S, Dong H, Sun S, Guo H. Construction of foot-and-mouth disease virus like particles-induced expression vectors and screening of BHK-21 cell pools. Chin J Biotechnol 2023;39:4849–60. https://doi.org/10.13345/j.cjb.230114.
17. Marsian J, Fox H, Bahar MW, Kotecha A, Fry EE, Stuart DI, et al. Plant-made polio type 3 stabilized VLPs-a candidate synthetic polio vaccine. Nat Commun 2017;8:245. https://doi.org/10.1038/s41467-017-00090-w.
18. Jung JW, Zahmanova G, Minkov I, Lomonossoff GP. Plant-based expression and characterization of SARS-CoV-2 virus-like particles presenting a native spike protein. Plant Biotechnol J 2022;20:1363–72. https://doi.org/10.1111/pbi.13813.
19. Wang A, Gu L, Wu S, Zhu S. Duck hepatitis A virus structural proteins expressed in insect cells self-assemble into virus-like particles with strong immunogenicity in ducklings. Vet Microbiol 2018;215:23–28. https://doi.org/10.1016/j.vetmic.2017.12.020.
20. Mi Y, Xie T, Zhu B, Tan J, Li X, Luo Y, et al. Production of SARS-CoV-2 virus-like particles in insect cells. Vaccines (Basel) 2021;9:554. https://doi.org/10.3390/vaccines9060554.
21. Lee CD, Yan YP, Liang SM, Wang TF. Production of FMDV virus like particles by a SUMO fusion protein approach in *Escherichia coli*. J Biomed Sci 2009;16:69. https://doi.org/10.1186/1423-0127-16-69.

ACCEPTED

22. Mu S, Sun S, Dong H, Bai M, Zhang Y, Teng Z, et al. Potent protective immune responses to *Senecavirus* induced by virus-like particle vaccine in pigs. Vaccines (Basel) 2020;8:532. https://doi.org/10.3390/vaccines8030532.

23. Li HY, Han JF, Qin CF, Chen R. Virus-like particles for enterovirus 71 produced from *Saccharomyces cerevisiae* potently elicits protective immune responses in mice. Vaccine 2013;31:3281–87. https://doi.org/10.1016/j.vaccine.2013.05.019.

24. Sherry L, Swanson JJ, Grehan K, Xu H, Uchida M, Jones IM, et al. Protease-independent production of poliovirus virus-like particles in *Pichia pastoris*: Implications for efficient vaccine development and insights into capsid assembly. Microbiol Spectr 2023;11:e0430022. https://doi.org/10.1128/spectrum.04300-22.

25. Ren M, Abdullah SW, Pei C, Guo H, Sun S. Use of virus-like particles and nanoparticle-based vaccines for combating picornavirus infections. Vet Res 2024;55:128. https://doi.org/10.1186/s13567-024-01383-x.

26. Le NMT, So KK, Chun J, Kim DH. Expression of virus-like particles (VLPs) of foot-and-mouth disease virus (FMDV) using *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 2024;108:81. https://doi.org/10.1007/s00253-023-12902-9.

27. Zhao M, Ma J, Zhang L, Qi H. Engineering strategies for enhanced heterologous protein production by *Saccharomyces cerevisiae*. Microb Cell Fact 2024;23:32. https://doi.org/10.1186/s12934-024-02299-z.

28. Elghandour MMY, Tan ZL, Abu Hafsa SH, Adegbeye MJ, Greiner R, Ugbogu EA, et al. *Saccharomyces cerevisiae* as a probiotic feed additive to non and pseudo-ruminant feeding: a review. J Appl Microbiol 2020;128:658–674. https://doi.org/10.1111/jam.14416.

29. Shin MK, Yoo HS. Animal vaccines based on orally presented yeast recombinants. Vaccine 2013;31:4287–4292. https://doi.org/10.1016/j.vaccine.2013.07.029.

30. Bal J, Jung HY, Nguyen NL, Park J, Jang YS, Kim DH. Evaluation of cell-surface displayed synthetic consensus dengue EDIII cells as a potent oral vaccine candidate. Microb Cell Fact 2018;17:146. https://doi.org/10.1186/s12934-018-0994-8.

31. So KK, Le NMT, Nguyen NL, Kim DH. Improving expression and assembly of difficult-to-express heterologous proteins in *Saccharomyces cerevisiae* by culturing at a sub-physiological temperature. Microb Cell Fact 2023;22:55. https://doi.org/10.1186/s12934-023-02065-7.

ACCEPTED

32. Leung RLC, Robinson MDM, Ajabali AAA, Karunanithy G, Lyons B, Raj R, et al. Monitoring the Disassembly of Virus-like Particles by ^{19}F-NMR. J Am Chem Soc 2017;139:5277–80. https://doi.org/10.1021/jacs.6b11040.s001.

33. Li H, Liu P, Dong H, Dekker A, Harmsen MM, Guo H, et al. Foot-and-mouth disease virus antigenic landscape and reduced immunogenicity elucidated in atomic detail. Nat Commun 2024;15:8774. https://doi.org/10.1038/s41467-024-53027-5.

34. Baldassano S, Accardi G, Vasto S. Beta-glucans and cancer: the influence of inflammation and gut peptide. Eur J Med Chem 2017;142:486–92. https://doi.org/10.1016/j.ejmech.2017.09.013.

35. Zhang L, Zhang T, Wang L, Shao S, Chen Z, Zhang Z. In vivo targeted delivery of CD40 shRNA to mouse intestinal dendritic cells by oral administration of recombinant *Saccharomyces cerevisiae*. Gene Ther 2014;21:709–14. https://doi.org/10.1038/gt.2014.50.

36. Alejo DM, Moraes MP, Liao X, Dias CC, Tulman ER, Diaz-San Segundo F, et al. An adenovirus vectored mucosal adjuvant augments protection of mice immunized intranasally with an adenovirus-vectored foot-and-mouth disease virus subunit vaccine. Vaccine 2013;31:2302–9. https://doi.org/10.1016/j.vaccine.2013.02.060.

37. Pay TW, Hingle PJ. A potency test method for foot and mouth disease vaccine based on the serum neutralizing antibody response produced in cattle. Vaccine 1992;10:707–13. https://doi.org/10.1016/0264-410x(92)90093-y.

38. Brandtzaeg P. Secretory IgA: Designed for Anti-Microbial Defense. *Front Immunol*. 2013;4:222. https://doi.org/10.3389/fimmu.2013.00222.

39. Kou Y, Zhang S, Chen J, Shen Y, Zhang Z, Huang H, et al. A mouse protozoan boosts antigen-specific mucosal IgA responses in a specific lipid metabolism- and signaling-dependent manner. Nat Commun 2024;15:7914. https://doi.org/10.1038/s41467-024-52336-z.

40. Howe SE, Konjufca VH. Per-oral immunization with antigen-conjugated nanoparticles followed by sub-cutaneous boosting immunization induces long-lasting mucosal and systemic antibody responses in mice. PLoS One 2015;10:e0118067. https://doi.org/10.1371/journal.pone.0118067.

41. Robinson L, Knight-Jones TJ, Charleston B, Rodriguez LL, Gay CG, Sumption KJ, et al. Global Foot-and-Mouth Disease Research Update and Gap Analysis: 6-Immunology. Transbound Emerg Dis 2016;63 Suppl 1:56–62. https://doi.org/10.1111/tbed.12534.

ACCEPTED

42. Collen T, Pullen L, Doel TR. T cell-dependent induction of antibody against foot-and-mouth disease virus in a mouse model. J Gen Virol 1989;70(Pt 2):395–403. https://doi.org/10.1099/0022-1317-70-2-395.

43. Juleff N, Windsor M, Lefevre EA, Gubbins S, Hamblin P, Reid E, et al. Foot-and-mouth disease virus can induce a specific and rapid $CD4^+$ T-cell-independent neutralizing and isotype class-switched antibody response in naive cattle. J Virol 2009;83:3626–36. https://doi.org/10.1128/jvi.02613-08.

44. Pacheco JM, Arzt J, Rodriguez LL. Early events in the pathogenesis of foot-and-mouth disease in cattle after controlled aerosol exposure. Vet J 2010;183:46–53. https://doi.org/10.1016/j.tvjl.2008.08.023.

45. Doel TR, Chong WK. Comparative immunogenicity of 146S, 75S and 12S particles of foot-and-mouth disease virus. Arch Virol 1982;73:185–91. https://doi.org/10.1007/bf01314726.

46. Belsham GJ. Towards improvements in foot-and-mouth disease vaccine performance. Acta Vet Scand 2020;62:20. https://doi.org/10.1186/s13028-020-00519-1.

47. Harmsen MM, Fijten HP, Westra DF, Coco-Martin JM. Effect of thiomersal on dissociation of intact (146S) foot-and-mouth disease virions into 12S particles as assessed by novel ELISAs specific for either 146S or 12S particles. Vaccine 2011;29:2682–90. https://doi.org/10.1016/j.vaccine.2011.01.069.

48. Martin-Acebes MA, Rincon V, Armas-Portela R, Mateu MG, Sobrino F. A single amino acid substitution in the capsid of foot-and-mouth disease virus can increase acid lability and confer resistance to acid-dependent uncoating inhibition. J Virol 2010;84:2902–12. https://doi.org/10.1128/jvi.02311-09.

49. Malik N, Kotecha A, Gold S, Asfor M, Ren J, Fry EE, et al. Structures of foot and mouth disease virus pentamers: Insight into capsid dissociation and unexpected pentamer reassociation. PLoS Pathog 2017;13:e1006607. https://doi.org/10.1371/journal.ppat.1006607.

50. Yang M, Holland H, Clavijo A. Production of monoclonal antibodies against whole virus particles of foot-and-mouth disease virus serotype O and A and their potential use in quantification of intact virus for vaccine manufacture. Vaccine 2008;26:3377–82. https://doi.org/10.1016/j.vaccine.2008.04.062.

51. Harmsen MM, Seago J, Perez E, Charleston B, Eblé PL, Dekker A. Isolation of single-domain antibody fragments that preferentially detect intact (146S) particles of foot-and-mouth disease virus for use in vaccine quality control. Front Immunol 2017;8:960. https://doi.org/10.3389/fimmu.2017.00960.

52. Song H, Waheed Abdullah S, Yin S, Dong H, Zhang Y, Tan S, et al. Virus-like particle-based multipathogen vaccine of FMD and SVA elicits balanced and broad protective efficacy in mice and pigs. Vaccine 2024;42:3789–801. https://doi.org/10.1016/j.vaccine.2024.04.092.

ACCEPTED

Figures

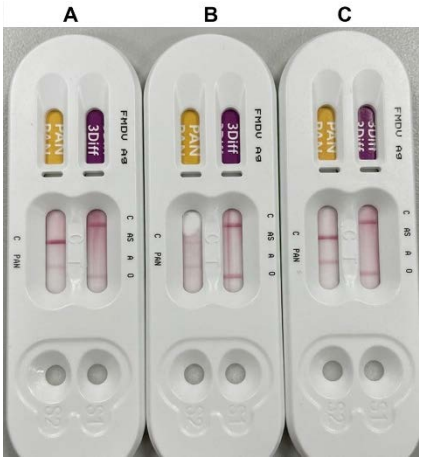


Fig. 1. FMDV detection using a commercially available FMDV diagnostic kit. Assess the thermostability of the yeast-produced VLPs; A-C**:** represent results of immune responses using cultures grown at 20 ˚C, 25 ˚C, and 30 ˚C, respectively**.** 50 µg of total soluble protein from transformant #1 was used for each reaction. The left and right response panels in the kit represent the infection and the serotype, respectively.

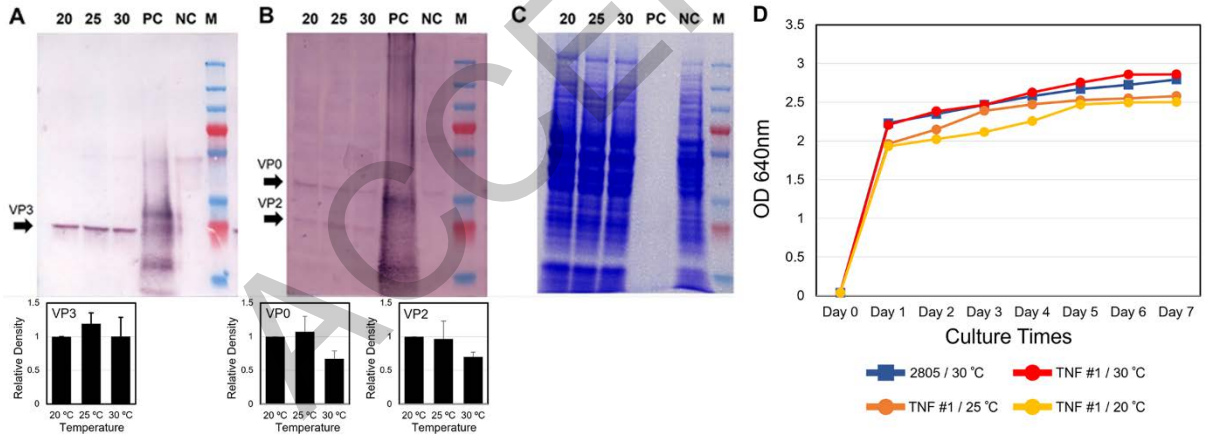


Fig. 2. Analysis of VLPs production from cultures at different temperatures. Protein preparations were obtained from cultures grown for 3 days at 20 ˚C, 25 ˚C, and 30 ˚C, and anti-VP2 and anti-VP3 antibodies were used with the protein samples, which were boiled prior to gel loading. 50 µg of total soluble protein from transformant #1 at 3 different temperatures was used for each lane. Western blot analysis using VP3 antibody (A) and VP2 antibody (B) was performed after boiling the protein samples prior to loading the gel. PC and NC contain protein preparations from *E. coli* M15 transformed with VP3 and a mock (vector only) transformant, respectively. Arrows indicate the

ACCEPTED

corresponding capsid VP3 (A), VP2, and VP0 (VP4+VP2) (B) proteins of VLPs of FMDV. (C) The Coomassie Brilliant Blue-stained twin gel was used as a control.

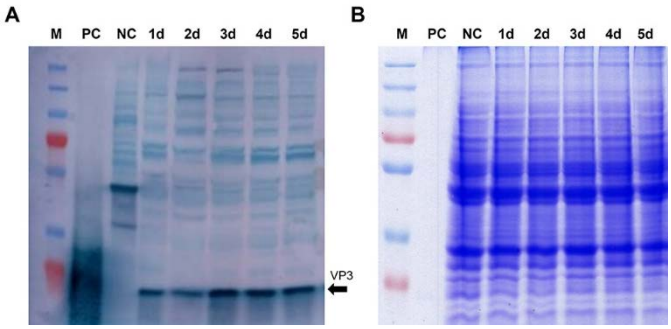


Fig. 3. Temporal expression of VLPs. Proteins were prepared from cultures after 1, 2, 3, 4, and 5 days at 30 ℃. (A) Western blot analysis was conducted using an anti-VP3 antibody, with the protein samples being boiled prior to loading the gel. 50 μg of total soluble protein from transformant #1 was used for each lane. PC and NC contain protein preparations from *E. coli* M15 transformed with VP3 and a mock (vector only) transformant, respectively. Arrows indicate the corresponding capsid VP3 protein of VLPs of FMDV. (B) The Coomassie Brilliant Blue-stained twin gel was used as a control.

ACCEPTED

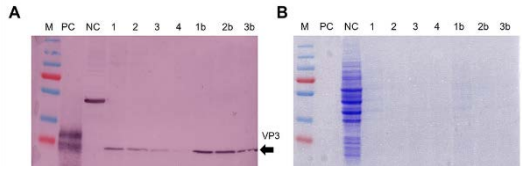


Fig. 4. Western blot analysis of purified VLPs. (A) Western blot analysis using the anti-VP3 antibody was performed with or without boiling the protein samples prior to loading the gel. Lanes 1–3 contain samples from the selected fractions after ultracentrifugation without boiling. Lanes 1b-3b contain samples from the corresponding selected fraction with boiling. PC and NC contain protein preparations from *E. coli* M15 transformed with VP3 and a mock (vector only) transformant, respectively. Arrows indicate the capsid VP3 protein of VLPs of FMDV. (B) The Coomassie Brilliant Blue-stained twin gel was used as a control. Note that lane 4 contains a sample from the selected fraction after ultracentrifugation, but it was not included in the boiling treatment due to the low concentration

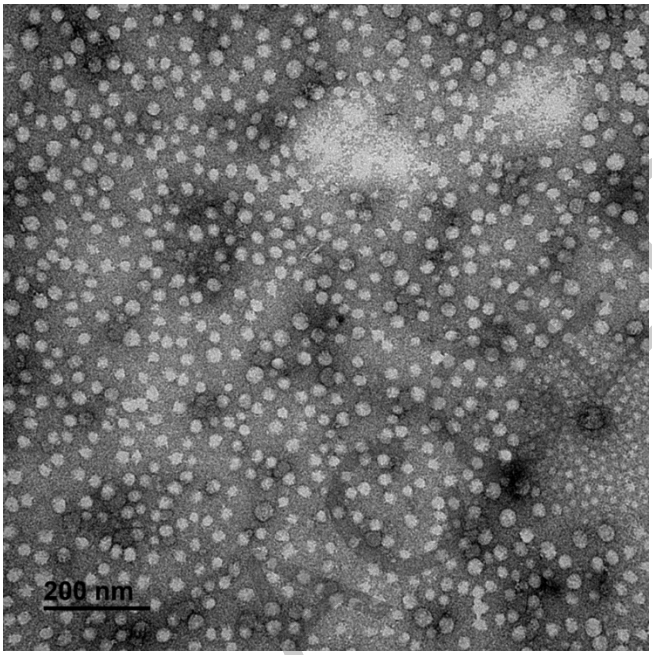


Fig. 5. Transmission electron microscopy (TEM) image of a purified FMDV virus-like particle (VLPs). Purified VLPs were negatively stained with 2% uranyl acetate and visualized by TEM. Particles approximately 30 nm in diameter were observed.

ACCEPTED

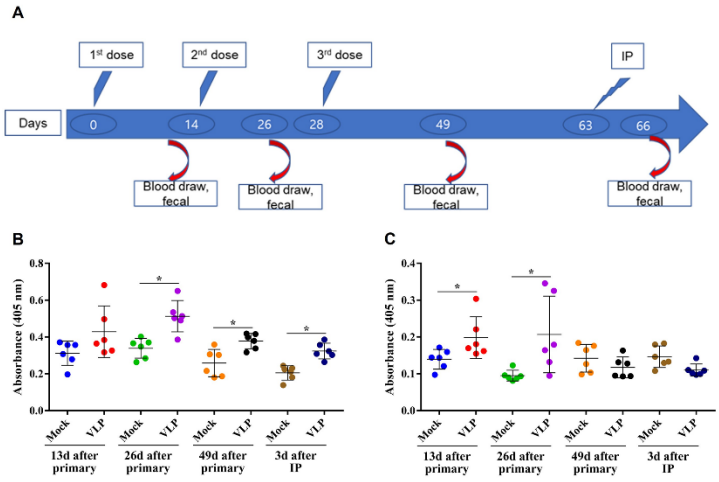
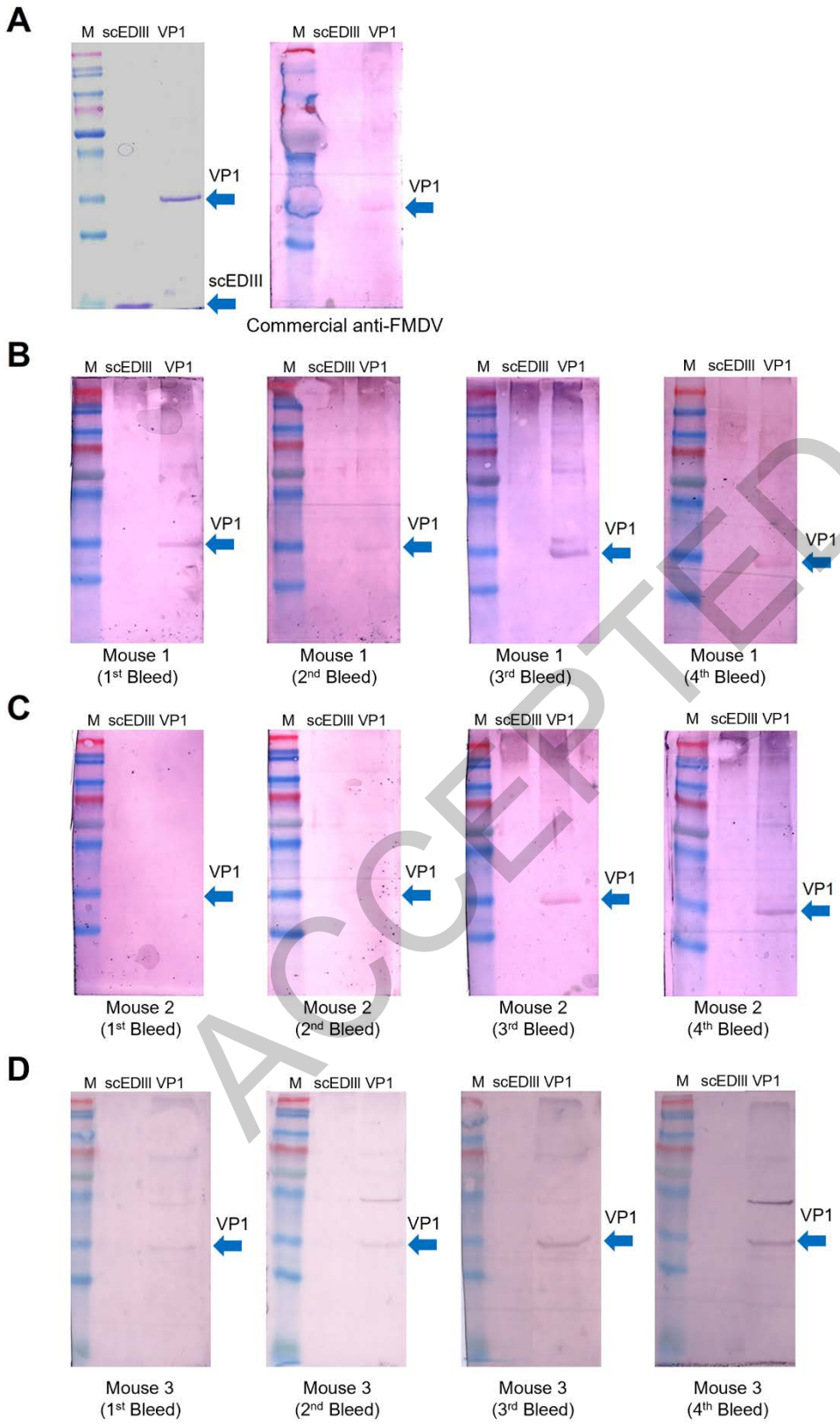


Fig. 6. Immune responses to oral administration of recombinant yeast. (A) Schematic experimental design for oral immunization and sample collection. Mice were orally immunized on days 0, 14, and 28, followed by an intraperitoneal (IP) injection on day 63. Blood and fecal samples were collected at the indicated time points. Humoral IgG (B) and mucosal IgA (C) responses in orally immunized mice. * indicates significant differences between samples, as determined using the independent samples *t*-test; *$p < 0.05$ indicates significant differences between the groups.

ACCEPTED



ACCEPTED

Fig. 7. Antigen-antibody responses of sera from purified VLP-injected mice against *E. coli*-expressed VP1. (A) SDS-PAGE analysis of purified *E. coli*-expressed VP1. The synthetic consensus sequence of the envelope protein domain

III from dengue virus (scEDIII), obtained from our previous study [26], was included as a control. Immunoblots showing antigen-antibody activity of a commercially available anti-FMDV antibody against *E. coli*-expressed VP1. Antigen-antibody responses of sera from individual mouse 1 (B) and 2 (C) against *E. coli*-expressed VP1. Tested antigens are indicated at the top of each lane, while specific sera (mouse identification and bleed numbers) are indicated at the bottom. The commercial anti-FMDV (Foot and Mouth Disease Virus Type O) monoclonal antibody used was MBS122094 (MyBioSource).

ACCEPTED