Veterinary Integrative Sciences 2026; 24(3): e2026077-1-18. DOI: 10.12982/VIS.2026.077

Veterinary Integrative Sciences
ISSN; 2629-9968 (online)

Research article

Efficacy of a bioactive protein on experimental oral infection by a highly virulent African swine fever virus

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Abstract

African swine fever (ASF), caused by the ASF virus (ASFV) infection, poses a huge threat to the pork industry due to its ineffective preventive and control measures. Currently, no effective commercial vaccines or antiviral therapies are available. This study evaluated the efficacy of CelluTEIN® X (CT_X), a bioactive protein feed additive, to delay and reduce ASFV infections in a pig challenge model. Sixteen 8-week-old crossbred pigs were divided into four groups: 500 ppm-Chall and 1000 ppm-Chall (n = 5 pigs in each group; fed with CT_X at 500 ppm and 1000 ppm, respectively), and Chall and Mock groups (n = 3 pigs in each group). Animals were orally challenged with ASFV genotype II (103HAD50, 3mL/each pig) and monitored for 21 days post-infection (dpi). Clinical outcomes, macroscopic pathology, histopathology, viral load (qPCR), and immunohistochemistry (IHC) were assessed. Pigs receiving the CT_X supplementation demonstrated markedly mild clinical outcomes, lower viral loads, and reduced tissue damage compared to those pigs without supplementation. CT_X at 500 ppm showed promising efficacy, achieving 40% survival and significantly higher survival rate than the challenged control (log-rank p = 0.049), whereas no survival was observed in the 1000 ppm–Chall or Chall groups. This group also displayed substantially lower clinical scores and milder gross and microscopic lesions, accompanied by markedly reduced blood and tissue viral loads; importantly, no ASFV antigen was detectable by qPCR or IHC in surviving 500ppm-Chall animals. In contrast, although 1000ppm-Chall pigs exhibited delayed disease progression, this did not translate into improved survival, suggesting the 500-ppm dose may be the more compelling candidate for further evaluation. This study highlights the potential of CT_X as a novel prophylactic substance in prevention of ASFV infection.

Keywords: African swine fever virus, CelluTEIN® X, delay, mTOR signaling pathway

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Article history; *received manuscript: 3 November 2025, revised manuscript: 7 December 2025, accepted manuscript: 19 May 2026, published online: 28 May 2026,*
Academic editor; *Korakot Nganvongpanit*

INTRODUCTION

African swine fever virus (ASFV), a devastating and contagious infectious disease, has up to 100% mortality (Galindo and Alonso, 2017). Over the past decade, African swine fever (ASF) has emerged as a global threat to both domestic and wild pig populations, leading to significant socio-economic impacts on the swine industry (Blome et al., 2020). Ongoing recombination and evolutions of ASFV have led to the emergence of genotype II with reduced virulence compared to its originally highly virulent counterparts. These strains demonstrated comparable transmissibility and were characterized by reduced mortality, frequently culminating in non-fatal, subacute, or chronically persistent infections (Sun et al., 2021). In contrast, highly virulent recombinant ASFV strains of genotypes I and II (rASFV I/II), cause more severe and fatal disease manifestations (Zhao et al., 2023). The concurrent circulation of both attenuated and highly virulent strains significantly complicates timely diagnosis and hinders the implementation of effective control measures for ASF. The development of a safe and effective vaccine remains the most desired strategy for controlling ASFV. However, this goal has remained the same for several decades, primarily due to a limited understanding of ASFV pathogenesis and its complex interactions with the host's immune responses. Consequently, biosecurity measures and large-scale culling of infected and at-risk animals continue to be the principal approaches for ASF prevention and control (Costard et al., 2009).

The absence of a fully developed, safe, and effective ASF vaccine further underscores the urgent need for alternative approaches, including the development of functional bio-products. In recent decades, various interventions demonstrating *in vitro* antiviral activity against African swine fever virus (ASFV) have been grouped into six major categories based on specific targets and mechanisms of action: nucleoside analogs, interferons, antibiotics, small interfering RNAs (siRNAs), CRISPR/Cas9-based approaches, and plant-derived natural products (Arabyan et al., 2019). Among these, plant-based natural compounds are particularly promising due to the rich diversity of bio-active molecules, favorable biocompatibility, low likelihood of promoting drug resistance, and strong potential for the development of novel antiviral agents (Owen et al., 2022).

While several natural compounds such as silver nanoparticles, medium-chain fatty acids, and β-glucan have exhibited inhibitory effects against ASFV *in vitro* (Dung et al., 2020; Tran et al., 2021; Tran et al., 2022). However, no bio-based therapeutics have yet reached a clinical application. Additionally, the inclusion of spray-dried porcine plasma in swine diets has been reported to delay ASFV transmission and reduce the viral load. The interpretation of these results may be confounded by potential experimental bias, particularly in relation to the unresolved issue of whether this intervention enhances antigen-specific T-cell priming following primary ASFV exposure (Blázquez et al., 2023).

The proprietary postbiotic was namely CelluTEIN® X (CT_X) derived from *Saccharomyces cerevisiae* yeast, enriched with key amino acids to enhance the biological value of protein products. CT_X activates the mTOR signaling pathway and elicits a range of physiological and immunological responses across various species (Duarte and Kim, 2024). When incorporated into swine diets, CT_X has been shown to consistently improve growth performance, feed efficiency, reproductive outcomes, and overall animal survival, particularly under conditions of stress, such as pathogen exposure (Vaughn and Gonzalez, 2022; Kim and Duarte, 2024). Duarte and Kim (2024) have demonstrated that the beneficial effects of CT_X are primarily due to its mode of action that facilitates an efficient immune response, stimulates crypt cell proliferation, and reduces the expression of genes associated with apoptosis, all without influencing inflammatory or oxidative stress markers in the jejunum of nursery pigs. As a result, CT_X enhances the integrity of the intestinal barrier, improves intestinal morphology, and promotes a balanced gut microbiota (Duarte and Kim, 2024). These improvements in intestinal health

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ultimately lead to a more effective immune system and enhanced growth performance (Peace et al., 2011). An ASFV infection induces varying levels of apoptosis in distinct immune cell subsets. While T-cell activation occurs early, T-cell functionality becomes impaired at the later stages of infection. The inability to generate an effective adaptive immune response to ASFV may be attributed to ASFV-induced immunosuppression or dysfunction of antigen-presenting cells (APCs), B cells, and T cells, resulting in a concurrent impairment of both humoral and cellular immune responses (Tian et al., 2024). This disruption of host immune functions is a primary strategy by which ASFV evades immune surveillance (Schäfer et al., 2022). Notably, dietary supplementation with CT_X has been shown to enhance various aspects of the immune response, including phenoloxidase and lysozyme activity, B cell function, and immunoglobulin production. Given these findings, we hypothesize that CT_X could serve as a potential nutritional intervention to mitigate ASFV infection and replication. This study provided evidence that CT_X supplementation delays the onset of ASFV infections and reduces the severity of clinical manifestations, pathological lesions, and viral load.

MATERIALS AND METHODS

Study design

Sixteen 8-week-old crossbred (Landrace x Yorkshire x Duroc) pigs, clinical healthy and negative for ASFV, Porcine circovirus type 2 (PCV2), Porcine reproductive and respiratory syndrome virus (PRRSV), and Classical swine fever virus (CSFV) were used in the experiment. The *in vivo* experiments were conducted under biosafety level 3 (BSL3) conditions at animal facilities at the Vietnam National University of Agriculture (VNUA) in Hanoi, Vietnam, in accordance with the institutional rules for the care and use of laboratory animals and using a protocol approved by the Ministry of Agriculture and Rural Development (MARD) Vietnam (TCVN 8402:2010). The feed and water for pigs were provided ad libitum. After an acclimatization period (7 days), the animals were block-randomized by initial body weight; observers recording clinical/pathology endpoints were blinded to group allocation via coded ear tags and pen IDs into four groups with a control group (Chall and Mock groups, n = 3 in each group) or the two treatment groups (fed with CT_X at 500ppm and 1000ppm, n = 5 in each group).

Pigs in the treatment groups were fed CT_X at concentrations of 500 ppm and 1000 ppm throughout the experimental period, including 14 days prior to the ASFV challenge and 21 days post-infection. The CT_X product (lot number PTB00322166CTB-NI1) is a dried yeast-based feed additive derived from *Saccharomyces cerevisiae* and formulated for animal nutrition. Pigs in the experimental groups were orally challenged by direct syringe administration into the oropharynx with 3 mL of cultured supernatant containing 10^3 HAD_{50} of ASFV. Pigs in the Mock group were not fed CT_X and challenged with PBS1X (Table 1 and Figure 1).

Table 1 Study design

Group	Eartag	Number of pigs	CelluTEIN X	ASFV Challenge
500ppm-Chall	A1, A2, A3, A4, A5	5	500ppm	10^3HAD_{50}, 3 mL
1000ppm-Chall	B6, B7, B8, B9, B10	5	1000ppm	10^3HAD_{50}, 3 mL
Chall	C11, C12, C13	3	-	10^3HAD_{50}, 3 mL
Mock	D14, D15, D16	3	-	-

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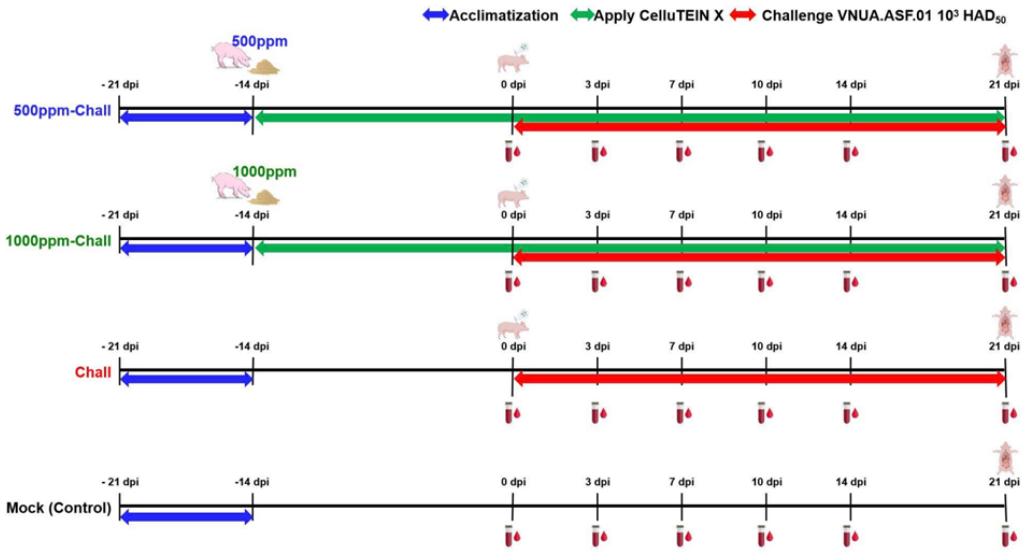

Figure 1 Schematic representation of the study design (celluTEIN X trial). d = day; dpi = day post infection; HAD = Hemadsorption doses; 500ppm = 500 mg of CT_X per kilogram of feed; 1000ppm = 1000 mg of CT_X per kilogram of feed.

Ethics statement

All animal procedures performed in this study were reviewed and approved by the Animal Ethics Committee for the Use of Animals in Research of Nong Lam University (AEC-NLU), under approval number NLU250702.

Clinical evaluation

Clinical signs were monitored daily to evaluate the disease progress and determine the humane endpoint. The severity of the disease was expressed with a quantitative clinical score (CS) obtained by adding the values for eight clinical signs recorded on a daily basis, as detailed by (Gallardo et al., 2015; Gallardo et al., 2017); fever, anorexia, recumbency, skin hemorrhage or cyanosis, joint swelling, respiratory distress, ocular discharge, and digestive findings were assigned points on a severity scale of 0 least severe to 3 most severe. The recorded clinical score was used to define humane endpoints. Meanwhile, the pigs were euthanized at the accumulative clinical score > 18 or pigs displaying severe clinical signs (level 3) of fever, anorexia, recumbence, respiratory or digestive symptoms for more than two consecutive days (Gallardo et al., 2018).

Sample collection

Whole blood and plasma samples were collected from pigs at 0, 3, 7, 10, 14, and 21 dpi (end of study). Following death or euthanasia, tissue samples from lymph nodes, heart, liver, spleen, tonsils, and lung were collected for ASFV detection. Tissue samples intended for histopathological analysis were fixed immediately in neutral buffered formalin at necropsy, whereas blood samples and fresh tissues were processed and subjected to nucleic acid extraction within 48 h after collection to ensure sample integrity. Extracted DNA was used for downstream analyses, and the remaining samples were stored at –20 °C until further analysis.

Macroscopic lesions

Macroscopic lesions were assessed during postmortem examinations following a standardized protocol previously described by Rodríguez-Bertos et al. (2020). A total of 30 lesion parameters were evaluated, corresponding to specific

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organs or tissues affected in each animal. Each lesion was graded semi-quantitatively using a four-point scale: 0 (normal), 1 (mild), 2 (moderate), and 3 (severe). The cumulative macroscopic lesion score for each pig was obtained by summing the individual scores across all parameters, yielding a total score ranging from 0 to 90.

Microscopic lesions

Histopathological evaluations were conducted on tissue samples (lungs, heart, liver, spleen, kidneys, and lymph nodes) collected from pigs in four experimental groups. Tissue sections were processed and stained with hematoxylin and eosin (HE) using the routine histological procedures. The evaluation protocol was based on the guidelines established by Galindo-Cardiel et al. (2013), with modifications incorporating additional histopathological findings reported in recent studies (Porras et al., 2024).

Immunohistochemistry

Immunohistochemistry (IHC) for the detection of the ASFV antigen in the tissues was performed using a monoclonal antibody against the virus protein p30/CP204L (Alpha Diagnostic International, USA). The Histofine Simple Stain MAX PO (Multi; Nichirei Bioscience, Tokyo, Japan) was used as the secondary antibody. Tissue samples of the organs of sixteen recovered pigs (lungs, heart, liver, spleen, kidneys, and lymph nodes) were processed on a paraffin block and carried out immunohistochemistry (IHC) according to Oh et al. (2021) that was performed as described previously.

Viral load

DNA was extracted from whole blood and tissue using a commercial kit (GeneJET™ Viral DNA and RNA Purification Kit, Thermo Scientific, Lithuania), according to the manufacturer's instructions. Briefly, 10% w/v clarified homogenized tissue suspensions were prepared in phosphatebuffered saline (PBS1X). The p30-Realtime PCR was performed as described previously (Tram et al., 2024). Primers and probe targeting the CP204L gene were designed based on the ASFV Georgia strain sequence (GenBank accession no. FR682468.2). The forward primer sequence was 5′-ATGAAAATGGAGGTCATCTTCAAAAC-3′, the reverse primer was 5′-AAGTTTAATGACCATGAGTCTTACC-3′, and the probe was 5′-FAM-TGAGCAAGAGCCCTCATCGGAGGCC-BHQ1-3′ (Ngo et al., 2024). Each 20 µL qPCR reaction contained 10 µL SensiFAST™ Probe No-ROX Master Mix (Bioline, UK), 0.5 µL of each primer (5 µM), 0.5 µL probe (5 µM), 2 µL DNA template, and nuclease-free water to volume. Amplification was carried out on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) under the following cycling conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 15s and 60°C for 40s (Ngo et al., 2024).

Antibody Detection

Plasma samples were tested for the presence of ASFV-specific antibodies with the commercially available enzyme-linked immunosorbent assay (ELISA) kit (INgezim® PPA Compac R.11.PPA.K3), following the manufacturer's protocol. Briefly, pre-coated plates were equilibrated to room temperature for at least 30 min. Control and test sera were diluted 1:2 using the provided diluent, and 100 µL of each diluted sample was added to the plate in duplicate. Plates were sealed and incubated for 1 h at 37°C, followed by four washes with the supplied wash solution. A peroxidase-conjugated monoclonal antibody specific to ASFV p72, diluted 1:100, was then added (100 µL/well) and incubated for 30 min at 37°C. After five additional washes, 100 µL/well of TMB substrate was added and the reaction was developed for 15 min in the dark. The reaction was stopped by adding 100 µL/well of stop solution, and absorbance was measured at 450 nm within 5 min. The percentage inhibition (blocking percentage) was calculated as: IP (%) = [(NC – Sample OD)/(NC

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– PC)] × 100, where NC is the negative control OD and PC is the positive control OD. Results were interpreted as positive when IP ≥ 50%, negative when IP < 40%, and suspicious when 40% ≤ IP < 50%.

Statistical Analysis

Kaplan–Meier survival curves were used to estimate cumulative survival, and differences in survival distributions among groups were assessed using the log-rank test. Cox proportional hazards regression was performed to identify the factors associated with the hazard of all-cause mortality, and hazard ratios (HRs) with 95% confidence intervals (CIs) were reported. For repeated measurements within the same animals over time, the Friedman test, a non-parametric alternative to repeated-measures one-way ANOVA, was applied when appropriate. Between-group comparisons at predefined time points or study endpoints were performed using the Kruskal–Wallis test, followed by Dunn's post hoc test for pairwise comparisons with multiplicity adjustment when applicable. Additionally, the one-way ANOVA was estimated to identify the differences in the distribution of macroscopic scores. Chi-square test was performed to assess the differences in positive rates following Real-time PCR. Statistical analyses were conducted using R software (version 4.4.1; r-project.org).

RESULTS

Clinical signs

Supplementation with CT_X at 500 ppm prior to ASFV challenge improved survival outcomes in pigs. Kaplan–Meier analysis showed that 40% (2/5) of pigs in the 500 ppm–Chall group survived to day 21, whereas the Chall and 1000 ppm–Chall groups exhibited 100% mortality (Figure 2). Overall survival distributions among challenged groups did not differ significantly, but showed a trend toward a difference (log-rank p = 0.054). In pairwise comparisons, survival in the 500 ppm–Chall group was higher than in the Chall group (log-rank p = 0.049). Consistently, Cox proportional hazards regression indicated a lower hazard of mortality in the 500 ppm–Chall group compared with the Chall group (HR = 0.114, 95% CI: 0.013–0.99), whereas the 1000 ppm–Chall group did not differ significantly from the Chall group (HR = 0.168, 95% CI: 0.02–1.33, p = 0.149). Although pigs in the 1000 ppm–Chall group also experienced 100% mortality, the onset of clinical signs and time to death were delayed by several days relative to the challenged control. All pigs in the Mock group remained clinically healthy throughout the duration of the study.

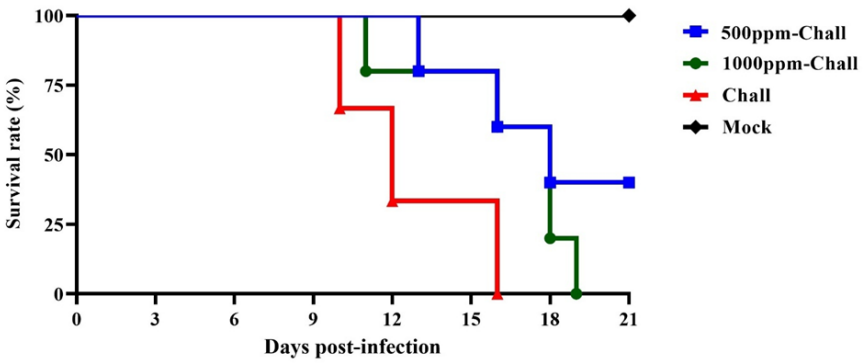

Figure 2 Kaplan–Meier survival curves of pig groups treated with CT_X following ASFV challenge. Differences among groups were analyzed using the Friedman test.

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Clinical observations revealed distinct differences in disease progression among the experimental groups. Continuous monitoring of body temperature (Figure 3) showed that the earliest onset response occurred in Pig C12 of the Chall group at 5 days post-infection (dpi), followed by rapid clinical course and death at 9 dpi. The remaining two pigs in this group developed fever at 6 dpi and 12 dpi, respectively, and both reached humane endpoints by 16 dpi (Figure 4). Pigs in the 1000 ppm-Chall group exhibited similar patterns of high fever and succumbed to infection within 18 dpi. However, pigs in the 500 ppm-Chall group experienced only mild and transient fluctuations in body temperature during the observation period, which correlated with improved survival outcomes. The surviving pigs in this group displayed only mild clinical signs. Meanwhile, pigs in the Chall groups presented typical signs of acute ASFV infection, including persistent high fever, anorexia, lethargy, locomotor difficulties, widespread skin erythema, and ocular discharge.

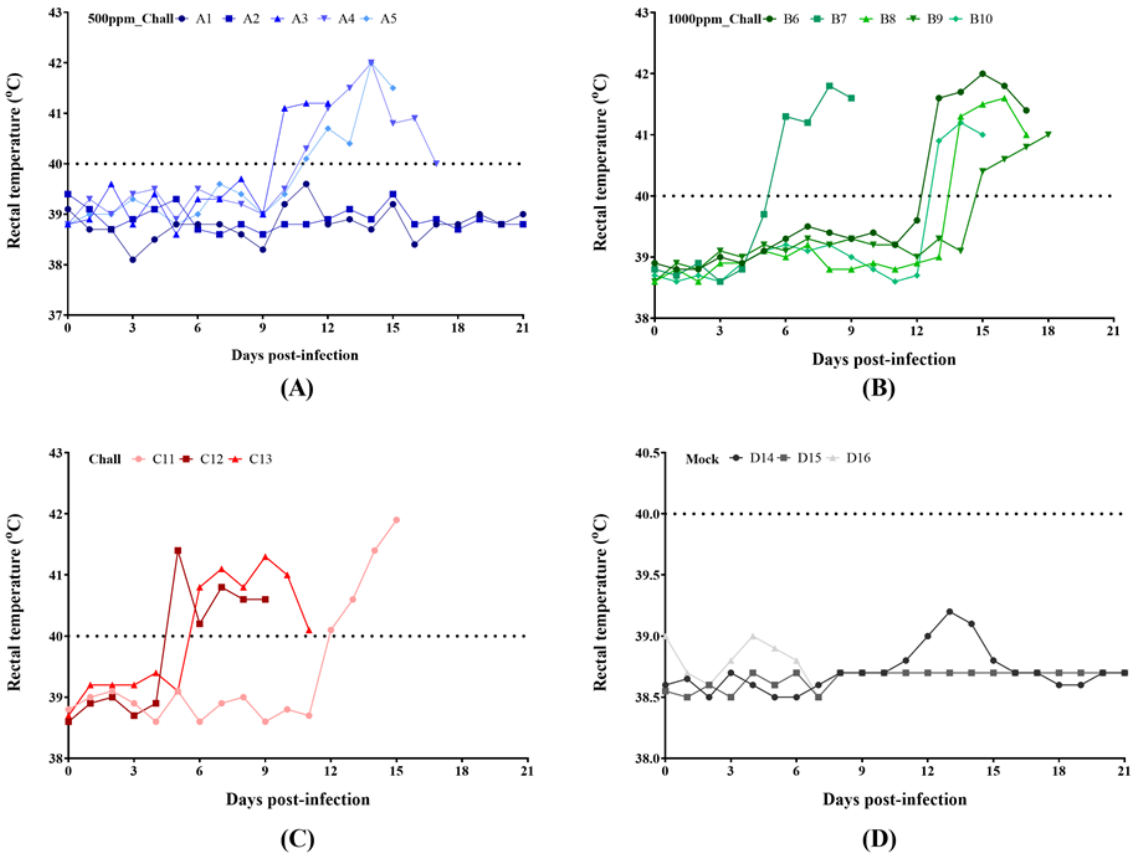

Figure 3 Daily rectal temperature of each individual pig during the period of the study, (A) 500 ppm treatment group, (B) 1000 ppm treatment group, (C) challenge control group, and (D) mock-inoculated control group. Each line represents one pig. The dotted horizontal line indicates the fever threshold (40.0°C). Differences among groups were analyzed using the Friedman test.

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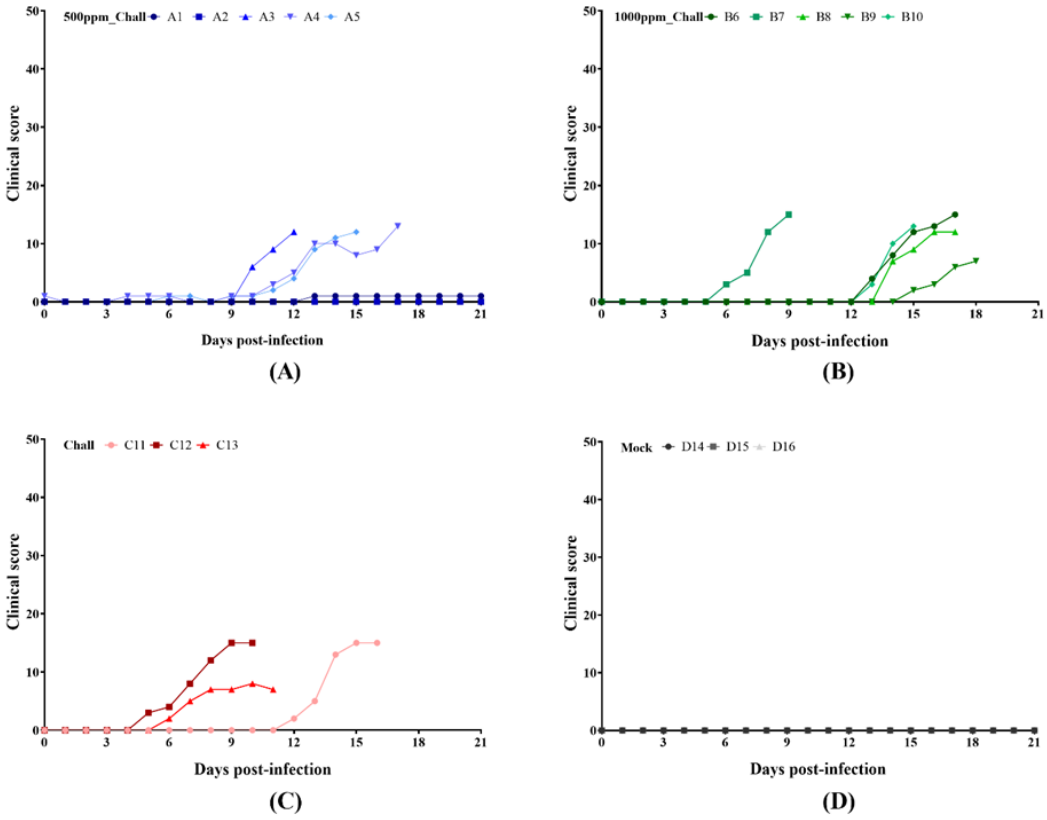

Figure 4 The clinical evaluation of each individual pig during the period of the study, (A) 500 ppm treatment group, (B) 1000 ppm treatment group, (C) challenge control group, and (D) mock-inoculated control group. Cumulative clinical score (y-axis) was assessed on different days after the challenge of pigs (x-axis). Differences among groups were analyzed using the Friedman test.

Viral load

Dynamic analysis of viral load in whole blood revealed the potential antiviral effect of CT_X supplementation, with greater efficacy observed at the 500ppm-Chall group. Whole blood samples were collected from all surviving pigs at 0, 3, 7, 10, 14, and 21 dpi. At 7 dpi, ASFV DNA was detected in only one pig from the 1000ppm-Chall group (1/5) and one from the Chall group (1/3). By 14 dpi, the detection rate increased to 75% (3/4) in the 1000ppm-Chall group and 100% (1/1) in the Chall group. In contrast, no viremia was detected in any of the surviving pigs in the 500ppm-Chall group throughout the observation period. The findings are summarized in Table 2.

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Table 2 Proportion of positive blood sample and mean of Ct value in experimental pigs.

Dpi	500ppm-Chall[a]		1000ppm-Chall[b]		Chall[c]		Mock[d]	
	n/N	Ct-value	n/N	Ct-value	n/N	Ct-value	n/N	Ct-value
0	0/5	nd	0/5	nd	0/3	nd	0/3	nd
3	0/5	nd	0/5	nd	0/3	nd	0/3	nd
7	1/5	36.15±0.00[a]	1/5	17.54±0.00[b]	1/3	16.27±0.00[c]	0/3	nd
10	1/5	23.55±0.00[a]	1/5	16.77±0.00[b]	2/3	16.085±0.00[c]	0/3	nd
11	na	na	na	na	1/2	18.68±0.00	na	na
12	1/5	18.55±0.00	na	na	na	na	na	na
14	2/4	23.54±0.00[a]	3/4	19.42±0.94[b]	1/1	20.45±0.00[ab]	0/3	nd
16	1/4	21.1±0.00	1/4	18.7±0.00	1/1	18.3±0.00	na	na
17	1/3	21.5±0.00	na	na	na	na	na	na
18	na	na	2/3	17.45±0.00	na	na	na	na
19	na	na	1/1	18.3±0.00	na	na	na	na
21	0/2	nd	0/0	nd	0/0	nd	0/3	nd

Different letters in the same row mean statistical significance ($p < 0.05$); nd: not detected; na: not available. Positive rates were compared among groups using the Chi-square test, whereas differences in Ct-values were assessed by the Kruskal–Wallis test followed by Dunn's post-hoc pairwise comparisons.

The protective effects of CT_X against ASFV-induced tissue damage and viral replication were evaluated across major tissues, including the heart, liver, spleen, lungs, kidneys, tonsils, and submaxillary lymph nodes. As indicated in Table 3, surviving pigs in the 500ppm-Chall group exhibited low levels of viral load, suggesting partial viral suppression. Pig A1 tested positive in the heart, spleen, and lymph nodes, with Ct-values of 34.24, 35.09, and 32.67, respectively, corresponding to low viral DNA levels. Similarly, a Ct value of 34.6 was obtained from a lung sample of Pig A2, consistent with minimal ASFV DNA. No viral DNA was detected in the remaining tissues, implying that CT_X may have limited ASFV dissemination and contributed to improved tissue protection in surviving animals.

Table 3 Proportion of tissue sample and mean of Ct-value in experimental pigs.

Tissue	500ppm-Chall		1000ppm-Chall		Chall		Mock		
	n/N	Ct-value	n/N	Ct-value	n/N	Ct-value	n/N	Ct-value	*p-value*
Lungs	4/5	25.53±6.92[b]	5/5	17.33±1.09[c]	3/3	18.71±2.78[bc]	0/3	nd[a]	0.000
Heart	4/5	27.4±4.57[b]	5/5	21.8±2.18[b]	3/3	23.29±1.93[b]	0/3	nd[a]	0.000
Liver	3/5	18.92±1.62[b]	5/5	15.75±2.96[b]	3/3	18.43±2.44[b]	0/3	nd[a]	0.000
Spleen	4/5	22.63±8.53[b]	5/5	14.36±1.57[b]	3/3	16.53±2.52[b]	0/3	nd[a]	0.000
Kidney	3/5	22.74±1.34[b]	5/5	18.35±2.49[b]	3/3	20.33±2.92[b]	0/3	nd[a]	0.000
Lymph nodes	4/5	22.5±6.89[b]	5/5	17.71±5.77[b]	3/3	21.73±1.71[b]	0/3	nd[a]	0.001
Tonsil	3/5	21.25±2.26[b]	5/5	18.05±4.96[b]	3/3	18.47±5.6[b]	0/3	nd[a]	0.000

Different letters a, b in the same row mean statistical significance ($p < 0.05$); nd: not detected. Differences in positive rates were analyzed using the Chi-square test, while Ct-values were compared among groups using the Kruskal–Wallis test followed by Dunn's post-hoc pairwise comparisons ($p < 0.05$).

ASFV antibody

No antibodies were detected in the sera at blood sample collection day (0, 3, 7, 10, 14, and 21 dpi).

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Pathology

Macroscopic lesions

Comparative analysis of macroscopic lesions revealed distinct pathological patterns among experimental groups. As shown in Figure 5, the median cumulative macroscopic lesion scores were significantly lower in the 500ppm-Chall group compared to the Chall group ($p < 0.05$), while no significant differences were observed among the other groups.

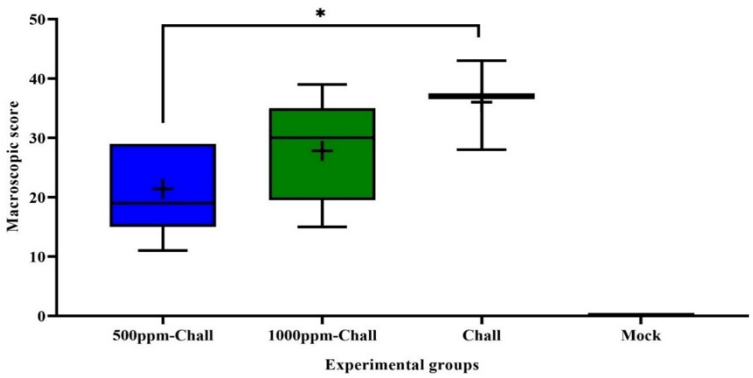

Figure 5 Median of cumulative macroscopic lesion scores (y-axis) in each experimental group (x-axis). Asterisks indicate statistically significant differences among experimental groups of pigs (*p= 0.0075). Differences among groups were analyzed using one-way ANOVA followed by post-hoc pairwise comparisons. Asterisks indicate statistically significant differences ($p < 0.05$).

In the Chall group, pigs consistently exhibited severe and widespread lesions characteristic of acute ASFV infections. These included mild to moderate hydropericardium with endocardial hemorrhages, pronounced erythema and necrosis of the tonsils, intense hepatomegaly with severe congestion and coalescent punctate hemorrhages, and marked splenomegaly. In contrast, pigs in the Mock group remained clinically and pathologically normal, with no observable gross lesions throughout the study period.

Pigs in the 1000ppm-Chall group displayed intermediate lesion severity. Four out of five pigs showed typical ASF-associated lesions, including mild hydropericardium, moderate multifocal hemorrhages in the myocardium, and congested, hemorrhagic, non-collapsed lungs with interstitial and alveolar edema. Additional findings included mild hepatic congestion, multifocal to diffuse renal hemorrhages in both the cortex and medulla, and moderate to severe hyperemic splenomegaly. Hemorrhagic lymphadenitis was also consistently detected in all examined lymph nodes (Figure 6).

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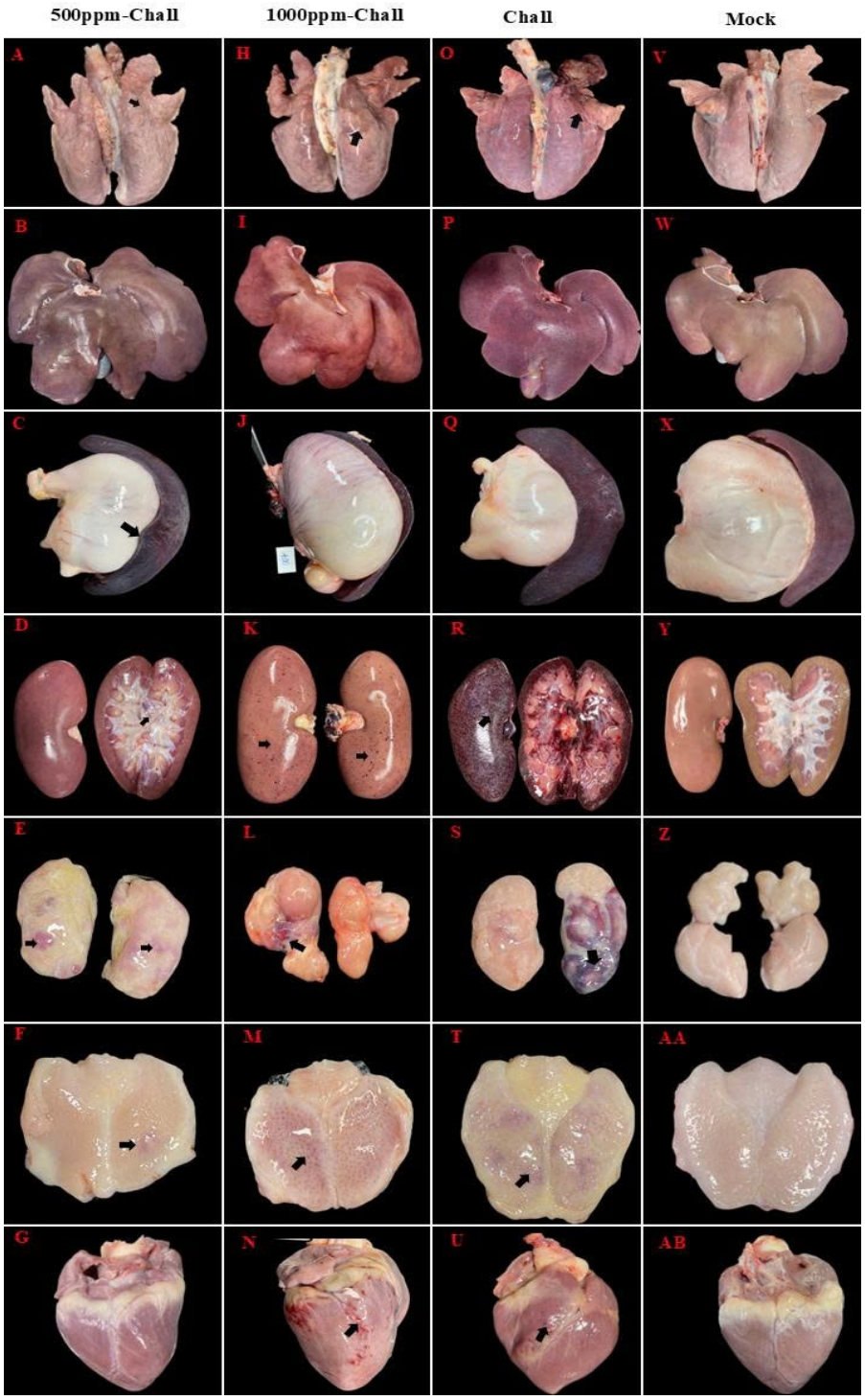

Figure 6 Representative figures for macroscopic lesions in four groups. Panels A–G show representative images from the 500ppm-Chall group, including lung (A), liver (B), spleen (C), kidney (D), lymph node (E), tonsil (F), and heart (G). Panels H–N correspond to the 1000ppm-Chall group, panels O–U to the Chall group, and panels V–AB to the Mock group, following the corresponding organs. Arrows indicate representative sites where characteristic lesions were observed

Pigs in the 500ppm-Chall group exhibited the mildest macroscopic lesions among the challenged groups. These lesions were limited to mild

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hydropericardium, mild pneumonia, mild hepatic congestion, hyperemic (but not markedly enlarged) spleens, and mild lymphadenitis.

Microscopic lesions

In the 500ppm-Chall group, histological lesions were minimal and largely non-specific. The two surviving pigs displayed mild vascular changes, including congestion and limited erythrocyte extravasation, primarily in lymph nodes. Additionally, mild inflammatory cell infiltrates were observed in the lungs, liver, and lymphoid tissues, suggesting low-grade immune activation without severe tissue damage. In contrast, pigs in the 1000ppm-Chall group exhibited more pronounced and widespread histopathological alterations. Notable findings included pulmonary hyperemia, edema, and focal hemorrhages, hepatic vascular congestion accompanied by lymphoplasmacytic infiltrates, and renal lesions characterized by hemorrhages and edema. The spleen and lymph nodes showed lymphoid depletion, lymphocytolysis, and vascular dilation, reflecting significant immune tissue disruption. Pigs in the Chall group demonstrated the most severe histopathological damage. Lung tissues exhibited diffuse alveolar edema and hemorrhages, and mild myocardial inflammation was also present. The liver showed marked congestion with inflammatory infiltrates, while the kidneys revealed severe lesions, including glomerulonephritis and tubular necrosis. The spleen and lymph nodes were profoundly affected, showing extensive lymphoid depletion, hemorrhages, and infiltration of erythrocytes and macrophages containing cellular debris, indicative of severe immune tissue destruction and systemic pathology (Figure 7).

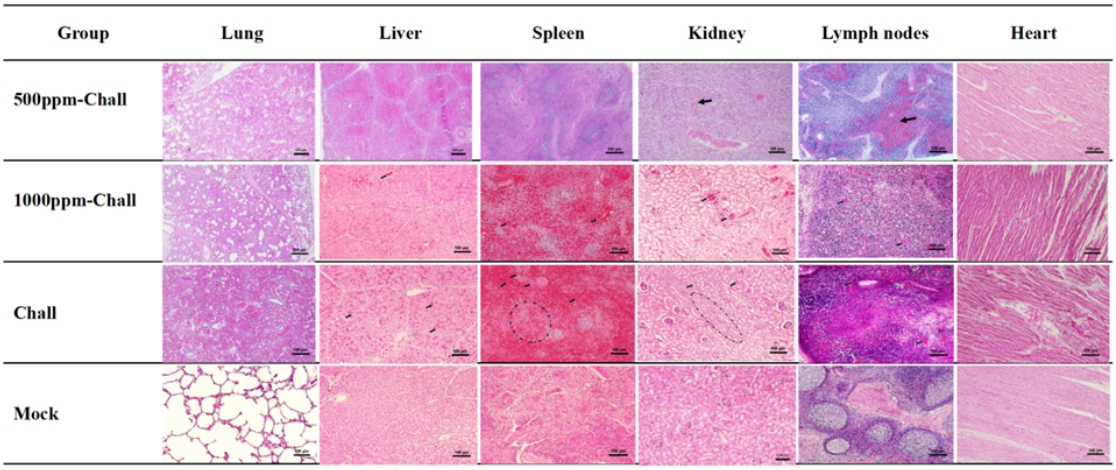

Figure 7 Hematoxylin and eosin staining (100X) showing pathological changes in the lung, liver, spleen, kidney, lymph node, and heart of pigs. Arrow and black dashed line indicate representative sites for key lesions.

ASFV antigen distribution

All pigs that succumbed to infection before reaching the humane endpoint tested positive for ASFV antigen in multiple tissues, confirming systemic viral dissemination. However, no ASFV antigen was detected in any of the examined tissues from the two surviving pigs in the 500ppm-Chall group or from pigs in the Mock group (Figure 8).

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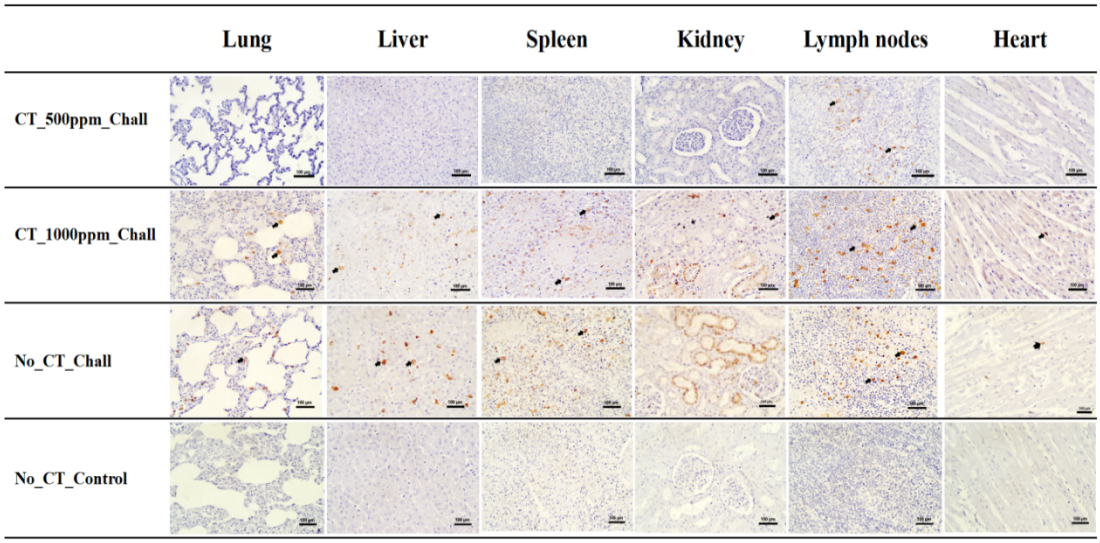

Figure 8 Immunohistochemistry (400X) using antibodies to ASFV p30 proteins. Arrows indicate representative sites for antigen-positive cells.

DISCUSSION

Since its incursion into Europe in 2007, African swine fever virus (ASFV) has continued to spread across continents, reaching Asia and causing severe socio-economic disruptions in the swine industry (Costard et al., 2009; Blome et al., 2020; Sun et al., 2021; Zhao et al., 2023). In the absence of a licensed vaccine, the development of effective therapeutic agents and viral inhibitors is critical to mitigating the global threat posed by ASFV. In the present study, we evaluated the *in vivo* antiviral beneficial of CT.

Five hundred ppm (500ppm-Chall) group exhibited significantly fewer clinical signs and milder macroscopic and histopathological lesions compared to 1000ppm-Chall and Chall group. No ASFV antigen was detected in the tissues of two pigs surviving 500ppm-Chall, and Realtime-PCR analysis confirmed no viral in the blood in these pigs. This observation is consistent with limited viral dissemination in these animals; however, given the small number of survivors, these findings should be interpreted with caution. Importantly, ASFV-specific antibodies were absent in all serum samples, suggesting a lack of seroconversion during the study period. The absence of detectable ASFV-specific antibodies up to 21 dpi is consistent with acute ASFV infection, in which rapid disease progression and severe virus-induced immunosuppression. Similar observations of weak or absent seroconversion have been reported in pigs infected with virulent ASFV strains during the early phase of infection. The failure to develop an effective adaptive immunity to ASFV may be related to ASFV-induced immunosuppression or the impaired function of APCs, B cells, and T cells, leading to a dual impairment of humoral and cellular immune response (Tian et al., 2024).

These findings indicate that CT_X may serve as a promising candidate for mitigating ASFV infection and its pathological consequences.

ASFV has continued to pose a major threat to global swine health, with highly virulent strains such as VNUA/HY/ASF1 causing consistent fatal outcomes in experimentally infected pigs. Nguyen et al. (2023) demonstrated that oral inoculation of domestic pigs with 10^3 HAD_{50} of this strain led to characteristic gross lesions, including hydropericardium, splenomegaly with infarction, hemorrhagic lymphadenitis, gastric mucosal hemorrhages, and erythematous tonsils. Additional findings included hepatic congestion with pale foci, interstitial pneumonia, and

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multifocal renal hemorrhages. Intramuscular inoculation at 103.5 HAD_{50} yielded comparable pathological features (Lee et al., 2021). Similarly, Cho et al. (2024) reported that oral administration of 10^6 HAD_{50} induced pronounced lesions such as lymphadenopathy, renal petechiae, hydropericardium, and severe splenic pathology. The pathological changes observed in Groups B and C in the present study were consistent with those described in the literature, although Group B displayed relatively milder lesions.

CT_X has been shown to enhance gut barrier integrity, modulate immune responses, and influence microbial composition (Peace et al., 2011; Kim and Duarte, 2024). Improved intestinal health may influence ASFV susceptibility, as previous studies have linked gut microbiota modulation to enhanced resistance to ASFV (Lacasta et al., 2014; Zhang et al., 2020). The precise mechanisms by which CT_X contributes to ASFV inhibition remain under investigation. However, *in vitro* studies conducted at the University of Minnesota and Iowa State University have shown that CT_X enhances both aerobic and anaerobic metabolism in activated B cells via the mTOR pathway. ASFV infection is known to induce apoptosis in various immune cells and compromise adaptive immunity by impairing antigen-presenting cells (APCs), B cells, and T cells (Tian et al., 2024). Immune evasion through disruption of both humoral and cellular responses is a well-documented viral strategy (Schäfer et al., 2022). In this context, the current study provides preliminary evidence supporting the immunomodulatory and antiviral potential of CT_X against ASFV.

Interestingly, the 500ppm-Chall group demonstrated better protective effects compared to the 1000ppm-Chall group, which exhibited more severe clinical signs and histopathological lesions. One possible explanation is that excessive CT_X levels may disrupt the host's physiological or immunological balance, potentially leading to impaired immune modulation or mucosal irritation. Moreover, high concentrations of CT_X might interfere with microbiota homeostasis or nutrient absorption, indirectly weakening the host's ability to mount an effective immune response. Alternatively, a hormetic effect may be at play, in which moderate CT_X levels activate protective signaling pathways (e.g., mTOR, antioxidant responses), while higher levels induce cellular stress and dysfunction (Xu et al., 2012; Smolinska et al., 2025; Vallejos et al., 2025).

This study may not have achieved a 100% infection rate through oral administration in both groups. However, it seems not to be a major problem that has developed closely replicates the natural exposure model of African swine fever (ASF) observed in field conditions. While this may appear to limit the study's power, it reflects the complexity of modeling natural ASFV exposure, which varies in dose and transmission dynamics. A study according to Cho et al. (2024) indicated that the intramuscular (IM) route is not suitable for investigating the mechanisms of ASFV infection, suggesting that intraoral (IO) challenges at specified doses provide a viable alternative to IM inoculation in ASFV challenge trials. Early studies indicated a minimum dose of 105 HAD50 of ASFV KWH/12 was required to cause infection (3/8 pigs) when administered orally (Greig, 1972). Later, Howey et al. (2013) determined the infectious potential of 3 doses of ASFV Malawi 1983 delivered intraorally to commercial pigs. Although a low dose of 102 HAD50 did not induce infection (0/2 pigs), moderate (104 HAD50) and high (106 HAD50) doses were sufficient to cause infection in 100% of the pigs (4/4 pigs). In a recent study, the infection probability of ASFV Georgia 2007 via the oral route following a minimum infectious dose of 10 TCID50 was calculated to be around 37.5% (3/8 pigs) (Niederwerder et al., 2019).

Several factors may explain the variable infection rates in the present study. These include the method of virus administration, limited exposure of the virus to tonsillar lymphoid tissue (a primary replication site), and the pig's physiological condition. For instance, the use of a rapid oral gavage method may reduce virus contact with the tonsils (Raymond et al., 2017). Additionally, although ASFV is stable across a wide pH range (4–10), its ability to survive passage through the

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gastric environment remains limited (Zhao et al., 2015). Other factors such as infection dose, viral strain virulence, route of administration, and host immune status further modulate infection dynamics (Pietschmann et al., 2015; Sánchez-Vizcaíno et al., 2015; Gallardo et al., 2017; Sánchez-Cordón et al., 2018; Li et al., 2022). Furthermore, the systemic bioavailability and tissue distribution of CT_X were not assessed, precluding definitive conclusions regarding its pharmacokinetics and mode of action. In addition, since this was a pilot study and the significant cost associated with BSL3 work, animal numbers were restricted for this study. Accordingly, the findings should be interpreted as exploratory and require confirmation in larger cohorts.

CONCLUSIONS

In conclusion, the present study provides promising preliminary evidence that CT_X supplementation may contribute to mitigate and delay clinical severity, pathological damage, and ASFV replication in orally challenged pigs, likely through modulation of immune and intestinal health parameters. While these findings suggest that CT_X could represent a supportive component within integrated ASFV control strategies, further research is warranted to elucidate the mechanistic basis of CT_X's antiviral properties and to validate its protective potential in larger-scale trials under field conditions.

ACKNOWLEDGEMENTS

The authors thank the Animal Biomedical Research Laboratories, Nong Lam University HCMC, Vietnam, for the support in providing testing facilities. We also thank the BSL-3 Laboratory, Vietnam National University of Agriculture, for providing biosafety facilities and technical assistance during this research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Tram Thi Ngoc Ngo: Investigation (lead); Formal analysis (lead); Data curation (lead); Writing -original draft (lead); Writing - review & editing (equal).
Hai Minh Nguyen: Methodology (equal); Investigation (equal); Resources (supporting); Writing - review & editing (supporting).
Trieu Duc Hai Du: Investigation (supporting); Data curation (supporting); Formal analysis (supporting); Writing - review & editing (supporting).
Lua Thi Nguyen: Investigation (supporting); Histopathological analysis (lead); Validation (supporting); Writing - review & editing (supporting).
Linh Ngoc My Tran: Formal analysis (supporting); Visualization (lead); Writing - review & editing (equal).
Thang Toan Lai: Investigation (supporting); Resources (supporting); Writing - review & editing (supporting).
Bui Thi To Nga: Methodology (equal); Validation (equal); Project administration (supporting); Writing - review & editing (equal).
Duy Tien Do: Conceptualization (lead); Methodology (lead); Validation (lead); Project administration (lead); Supervision (lead); Funding acquisition (lead); Writing - review & editing (lead).

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How to cite this article;

Thi Ngoc Ngo, Hai Minh Nguyen, Trieu Duc Hai Du, Lua Thi Nguyen, Linh Ngoc My Tran, Thang Toan Lai, Bui Thi To Nga and Duy Tien Do. Efficacy of a bioactive protein on experimental oral infection by a highly virulent African swine fever virus. Veterinary Integrative Sciences. 2026; 24(3): e2026077-1-18.

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