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Effect of formaldehyde-based additive against African swine fever virus in complete swine feed

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ABSTRACT

African swine fever (ASF) is currently considered the serious contagious disease of the swine industry worldwide. The feed and feed ingredients have been recognized as a potential risk factor for African swine fever virus (ASFV) transmission. No commercial vaccines and effective drugs against ASFV are available to date. This study aimed to examine the antimicrobial SALTEC™ 512, a formaldehyde-based additive, at an application rate of 1.0; 2.0; and 3.0 kg/t feed against ASFV in complete swine feed. The effect of SALTEC™ 512 was evaluated by a contaminated ASFV feed assay. Our study reveals that SALTEC™ 512 inactivated ASF/VN/Pig/Hue/1270 strain in complete swine feed at an inclusion rate of 1.0 kg/t feed on Day 1 post inoculation. The most effective action was noticed at an inclusion level of 3.0 kg/t feed on Day 7 post inoculation. The antiviral activity of SALTEC™ 512 against ASF/VN/Pig/Hue/1270 was dose and time-dependent. Overall, SALTEC™ 512 may be a potential additive to reduce the risk of ASFV transmission via feed contamination.

Keywords: African swine fever virus, Feed, Formaldehyde-based additive, SALTEC™ 512

INTRODUCTION

African swine fever (ASF), caused by the African swine fever virus (ASFV), is a highly contagious viral disease of the swine industry. It affects both domestic pigs and wild boars with mortality rates approaching 100% in infected animals. In 2007, a re-emergence of ASF was identified in Georgia that spread quickly to neighboring countries and further into Eastern Europe (Karger *et al*., 2019). Since 2016, the disease has been spreading widely in African, Europe, and most recently in Asian continents (Mulumba-Mfumu *et al*., 2019; FAO, 2020). The first outbreak of ASF in Vietnam was officially reported in February 2019. Importantly, the ASF outbreak seriously impacts global agricultural markets after the disease-spreading into China. The study of economic modeling of the global food system assumed that a reduction of pork production in China would lead to increases in world pork prices of 17–85% and prices of food types such as beef and poultry, whereas prices for maize and soybean used in feed decline (Mason-D' Croz *et al*., 2020). ASF is listed as notifiable by the World Organization for Animal Health (OIE) due to its infectivity, severity, and responsibility for serious economic and production losses.

ASFV is a large enveloped, cytoplasmic,

linear double-stranded (ds) DNA virus that belongs to the *Asfivirus* genus of the *Asfarviridae* family (Dixon *et al*., 2005; Galindo and Alonso, 2017). The risk of potential ASFV outbreaks mainly comes from poorly implemented biosecurity procedures. The continuous spread from farm to farm is driven by direct contact with excretions from infected pigs and indirect contact through contaminated materials, pork meat, or people (Beltran-Alcrudo *et al*., 2017; Cwynar *et al*., 2019). As already proven, contaminated feed can serve as a transmission vehicle for porcine epidemic diarrhea virus (PEDV) infection of naïve pigs (Dee *et al*., 2014b). ASFV can easily be transmitted orally. The median infectious dose of ASFV was $10^{6.8}$ $TCID_{50}$ (50% tissue culture infectious dose) for feed and $10^{1.0}$ $TCID_{50}$ for liquid (Niederwerder *et al*., 2019). Besides, feed ingredients promote the survival of ASFV, with the half-lives during exposure to 30 days shipment conditions ranging from 9.6–14.2 days (Stoian *et al*., 2019). Hence, the feed and feed ingredients have been identified as a potential risk factor for the ASF introduction to domestic pigs.

No commercial vaccines and effective drugs against ASFV are available to date (Revilla *et al*., 2018). The control of ASF outbreaks is focused on biosecurity standards to prevent the transmission of the virus. Formaldehyde influences both nucleic acids and proteins through the formation of either methylol adduct, Schiff bases, or methylene bridge (Metz *et al*., 2006; Delrue *et al*., 2012; Hoffman *et al*., 2015; Ricke *et al*., 2019). It is a disinfectant that is a bactericidal, sporicidal, and virucidal agent (McDonnell and Russell, 1999; Juszkiewicz *et al*., 2019). The use of formaldehyde for decontamination of feed has been documented (Carrique-Mas *et al*., 2006; Dee *et al*., 2014a; Sbardella *et al*., 2015). It has been reported that feed treated with a formaldehyde-based premix can reduce the risk of PEDV infection via contaminated feed (Dee *et al*., 2014a). SALTEC™ 512 is a formaldehyde-based additive that inhibits the growth of *Salmonella spp*. Nevertheless, the antiviral activity of SALTEC™ 512 has not been evaluated. This study aimed to determine the effect of SALTEC™ 512 against ASFV in complete swine feed by using real-time PCR and haemadsorption (HAD) assay. We hypothesized that the use of SALTEC™ 512 may alleviate ASFV transmission in pigs via feed consumption.

MATERIALS AND METHODS

Cell and Virus Strain

Primary porcine alveolar macrophages (PAMs) were collected from 10–20-day-old SPF pigs (Pig Research Centre of National Institute of Animal Science, Vietnam). The primary cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Scientific, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, USA) at 37°C with 5% CO_2. ASFV was isolated from dead pigs in Hue/1270 province in 2019. The virus was confirmed by real-time PCR according to the OIE-recommended procedure described by King *et al*. (2003), conventional PCR using p72U (5' GGCACAAGTTCGGACATGT 3')/p72D (5'

Table 1. Calculated energy and nutrient content of the commercial feed

Items	Amount
Crude protein (%)	18
Calcium (%)	0.7–1.2
Humidity (% max)	14
Non-phytate phosphorus [% (min–max)]	0.4–1.2
Cellular (% max)	6.0
Lysine (% min)	0.96
Methionine + Cystine (% min)	0.53
Metabolizable energy (kcal/kg)	3100

Ingredients: Cereal, soybean meal, fish oil, corn, broken rice, rice bran, vegetable oil, dicalcium phosphate, amino acids (L-Lysine, DL-Met, L-Threonine, L-Tryptophan), enzyme Bio-zeem™ (1,000 mg/kg), sodium chloride, sodium bicarbonate, copper sulfate, iron glycinate, phytase, premix vitamins, premix organic minerals.

GTACTGTAACGCAGCACAG 3') specific primer that amplifies 478 bp from the protein p72 of the ASFV strains (Bastos *et al*., 2003), and HAD assay as recommended by the OIE (2012). Sequence analysis of the ASFV isolate was performed based on genotyping primer p72U/p72D according to OIE protocol and phylogenetic analyses of nucleotide sequences of partial p72 were constructed by MEGA 7 (Kumar *et al*., 2016). The study was conducted in compliance with the institutional rules for care and use of laboratory animals and by using a protocol approved by the ethics committee of the Ministry of Agriculture and Rural Development (MARD) Vietnam (Approval no. TCVN 8402:2010).

HAD Assay

HAD assay was conducted as previously described (Malmquist and Hay, 1960). Briefly, primary PAMs cells were seeded in 96-well plates. The samples were then added and titrated in triplicate using ten times dilutions. HAD was observed for 4 days. The characteristic rosette formation representing haemadsorption of erythrocytes around infected cells was identified as HAD positive. 50% HAD doses (HAD_{50}) calculation followed the method of Reed and Muench (1938).

Feed Assay

Five g of commercial swine feed samples (Table 1) were treated with SALTEC™ 512 (33% formaldehyde) (LINQ Technology Corporation, Chachoengsao, Thailand), at an inclusion rate of 1.0, 2.0, and 3.0 kg/t feed. The inclusion rates in this study based on the recommendation of the US Food and Drug Administration (FDA: CFR 573.460): food additive formaldehyde (37% solution) is allowed in feed and drinking water of animals with a dosage of 2.7 kg/t feed; equivalent to 3 kg/t of 33% formaldehyde in the feed. The samples were further inoculated with 100 µL of MEM (Thermo Scientific, USA) containing 1×10^5 HAD_{50} ASFV. Additional 5 g of sample were inoculated with PBS (Sigma-Aldrich, USA) served as negative controls. The positive controls included 5 g of feed samples inoculated with 1×10^5 HAD_{50} ASFV. At Days 1, 3, and 7 post inoculation, the samples were re-suspended in 20 mL

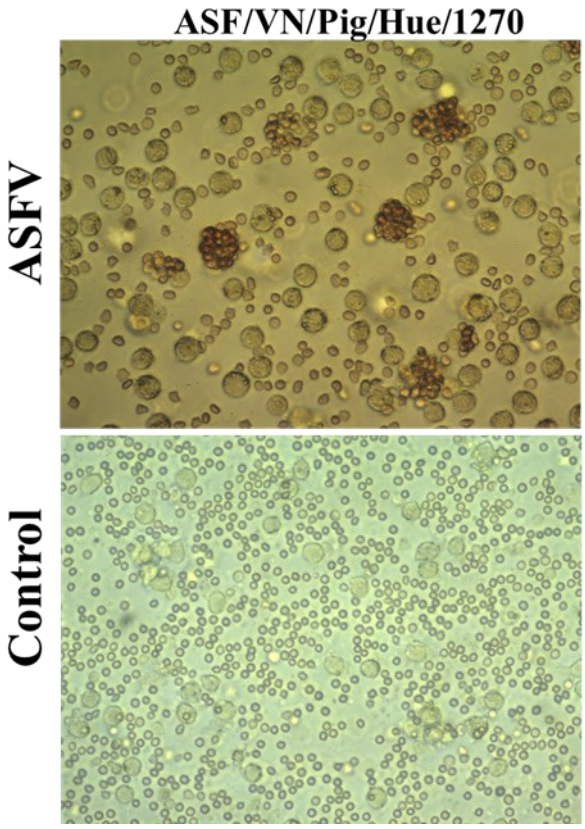

Figure 1. ASFV detection from the dead pig samples in Hue/1270 province, Vietnam by HAD assay (×200).

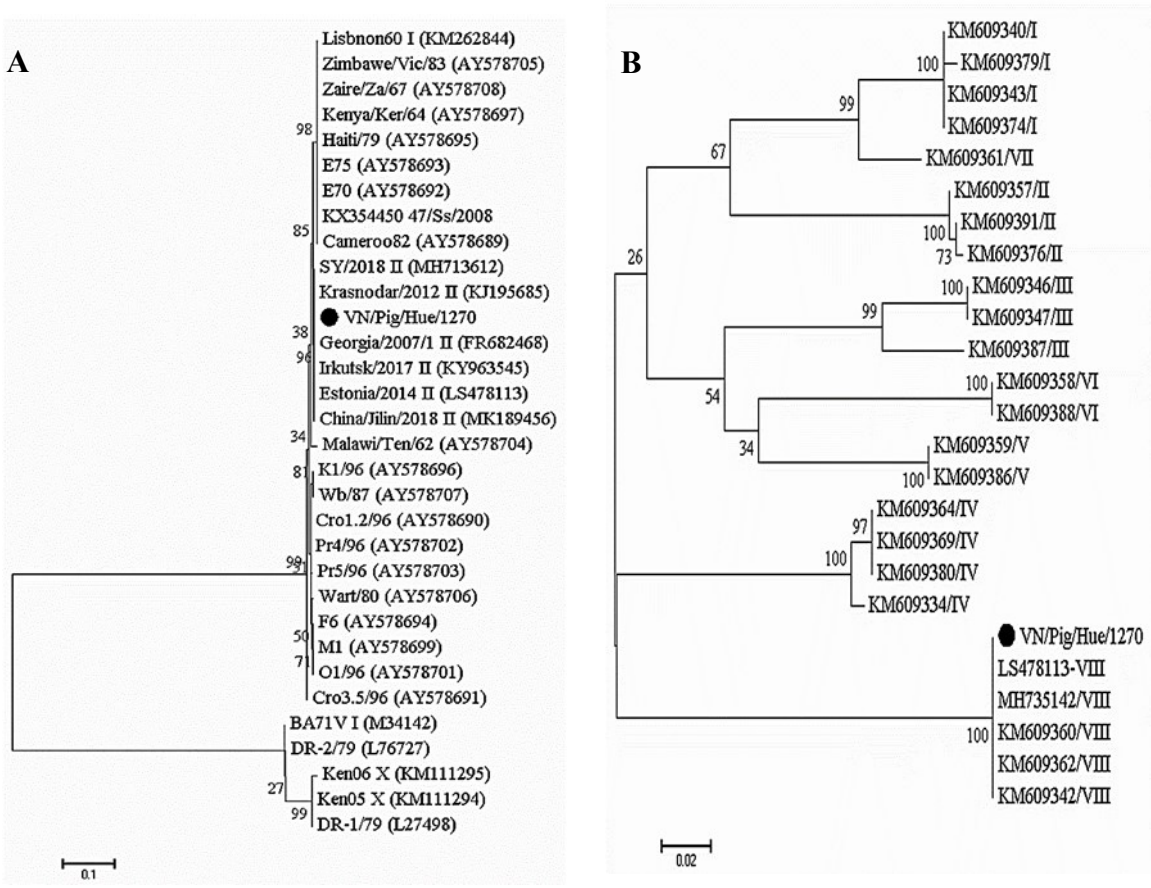

Figure 2. Phylogenic analysis of VN/Pig/Hue/1270. The black circle indicates the ASFV isolate used in the present study. (A) Genotype group (B) Serotype group. Scale bars indicate nucleotide substitutions per site.

sterile PBS and vortexed for 30 seconds. The supernatant was aliquoted, centrifuged at 10,000 × *g* for 10 minutes, filtered by 0.45 µm filter, and then subjected to the determination of capsid protein p72. The genomic DNA was extracted by using the QIAamp DNA Mini Kit (QIAgen, Germany). Specific steps were carried out following the manufacturer's protocols. Real-time PCR was conducted using an Agilent AriaMx Real-Time PCR System (Agilent, SC, CA, USA), according to the OIE-recommended procedure as described by King *et al*. (2003). The supernatant of the treatment group or control group were collected on Days 1, 3, and 7 post inoculation and subject to the virus titration by HAD.

Statistical Analysis

Differences among the groups were tested by Duncan's multiple comparison methods. All statistical analyses used IBM SPSS software (SPSS 23.0 for Windows; IBM, Chicago, IL, USA), and $P<0.05$ was considered significant.

RESULTS

ASFV Isolation

The ASFV was successfully isolated from the dead pig samples in Hue/1270 province, Vietnam. HAD positive clearly detected in the samples when compared to control samples (Figure 1). Positive HADs were assured by OIE real-time PCR method (data not shown). The results demonstrated that dead pig samples from Hue/1270 province, Vietnam contained infectious ASFV. This virus isolate was identified as VN/Pig/Hue/1270. Besides, the phylogenic analysis suggested that VN/Pig/Hue/1270 strain belongs to genotype II and serotype 8 (Figure 2). The VN/Pig/Hue/1270 strain was used for the experiment in the present study.

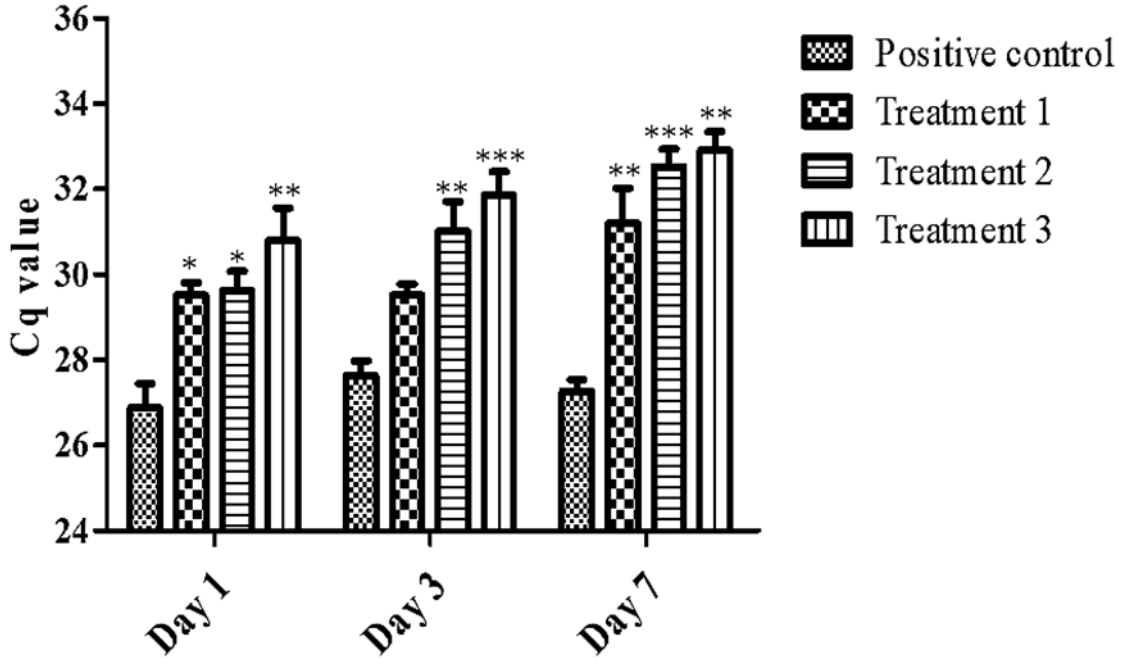

Figure 3. The mean Cq values of ASF/VN/Pig/Hue/1270 DNA in feed samples on Days 1, 3, and 7 post inoculations evaluated by real-time PCR. Positive control included complete swine feed containing 1 × 10^5 HAD_{50} of ASFV; Treatment 1 was positive control treated with SALTEC™ 512 at an inclusion rate of 1.0 kg/t feed; Treatment 2 was positive control treated with SALTEC™ 512 at an inclusion rate of 2.0 kg/t feed; Treatment 3 was positive control treated with SALTEC™ 512 at an inclusion rate of 3.0 kg/t feed. Data represent the mean of three independent experiments and error bars indicate ± SD. Significant differences compared to control are denoted by *P<0.05, **P<0.01, and ***P<0.001.

Effect of SALTEC™ 512 on ASFV DNA Replication

The presence of ASF/VN/Pig/Hue/1270 strain in the feed samples was detected by using real-time PCR (Figure 3). All samples from the negative controls were negative for the presence of the ASF/VN/Pig/Hue/1270 DNA (Cq≥40), whereas mean Cq values of the positive controls were 26.88 (range, 26.07–27.28), 27.64 (range, 27.20–28.02), and 27.26 (range, 26.88–27.46) on Days 1, 3, and 7 post inoculation, respectively. SALTEC™ 512 showed antiviral activity against ASF/VN/Pig/Hue/1270 strain in the feed samples. At an inclusion rate of 1 kg/t feed, the presence of ASF/VN/Pig/Hue/1270 DNA was significantly decreased on Day 1 post inoculation (P<0.05). The most effective action of SALTEC™ 512 against ASF/VN/Pig/Hue/1270 strain was markedly noticed at an inclusion rate of 3.0 kg/t feed, with a mean Cq value on Day 1 of 30.08 (range, 29.72–31.34), mean Cq value on Day 3 of 31.86 (range, 31.17–32.53), and mean Cq value on Day 7 of 32.90 (range, 32.59–33.53). Our findings demonstrated that SALTEC™ 512 significantly inactivated the ASF/VN/Pig/Hue/1270 DNA in the feed samples. The effect of SALTEC™ 512 against ASF/VN/Pig/Hue/1270 strain was dose and time-dependent.

Effect of SALTEC™ 512 on ASFV Infection

The results of HAD assay are shown in Figure 4. No viral titer of ASF/VN/Pig/Hue/1270 strain in PAMs cells was observed in the negative controls, whereas the viral titer did not change in positive control samples and the samples treated with SALTEC™ 512 at an inclusion rate of 1.0 kg/t feed on Days 1, 3, and 7 post inoculation. In contrast, the SALTEC™ 512 signif-

icantly decreased the viral titer from 4.7 logs HAD_{50}/mL to 4.5 logs HAD_{50}/mL and 4.3 logs HAD_{50}/mL at an inclusion rate of 3.0 kg/t feed on Days 1, 3, and 7 post inoculation, respectively ($P<0.05$). The antiviral activity of SALTEC™ 512 was markedly observed when administered over Day 7 post inoculation. Our findings demonstrated that SALTEC™ 512 reduced ASF/VN/Pig/Hue/1270 infection via contaminated feed.

DISCUSSION

Feed and feed ingredients have been recently recognized as a potential risk factor for the ASF introduction to domestic pigs. No commercial vaccines and effective drugs against ASFV are available to date. The control of ASF spreads is focused on the biosecurity procedure. In the present study, we proposed the antiviral activity of SALTEC™ 512, a formaldehyde-based additive, against AFSV in a complete swine feed. Our results demonstrated that SALTEC™ 512 inactivated ASFV DNA and decreased virus infectivity in contaminated feed.

In this study, the ASF/VN/Pig/Hue/1270 strain was successfully isolated from the dead pig samples in Hue/1270 province, Vietnam. The virus isolated was characterized by HAD assay, real-time PCR, and sequence analysis. The genetic characterization of the ASF/VN/Pig/Hue/1270 strain is close to Georgia 2007/1 that spread into the Russian Federation and Eastern Europe (Sánchez-Cordón *et al*., 2018). The ASF/VN/Pig/Hue/1270 strain belongs to genotype II and serotype 8.

Feed additives with antimicrobial activity have been considered to inhibit ASFV *in vitro* including aqueous formaldehyde, medium-chain fatty acids, short-chain fatty acids, organic acids, and essential oils. Essential oil blend deactivated the lethal dose of 10^5 HAD50 of ASFV in PAMs cells at a concentration of 0.01–25% (Truong *et al*., 2021). At 0.6% inclusion, a blend of three

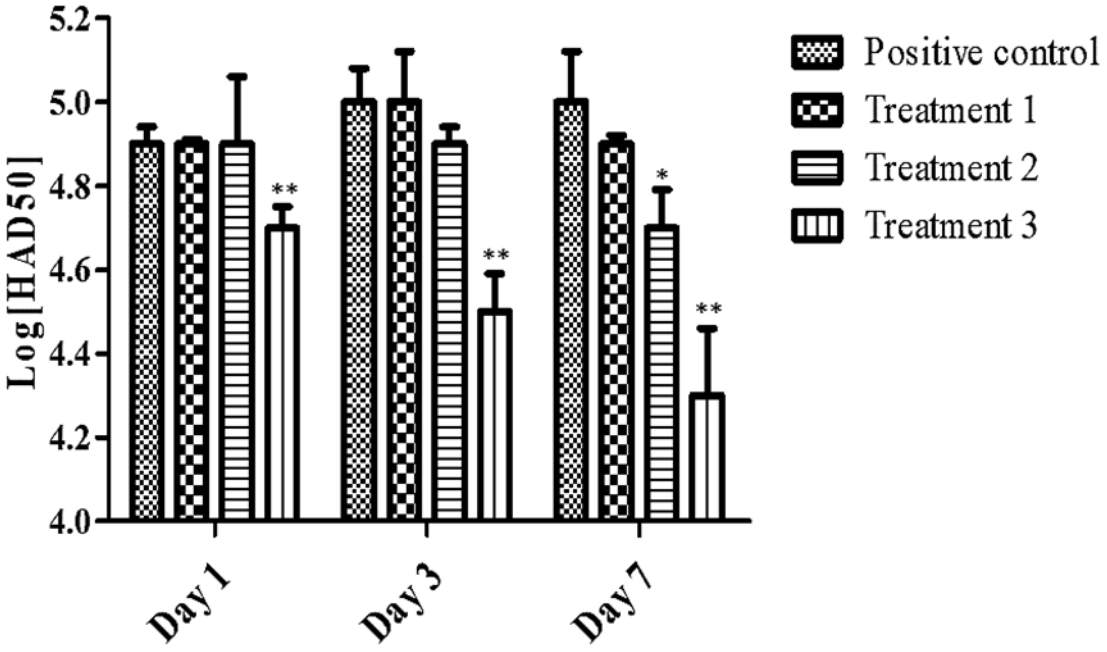

Figure 4**.** The viral titer of ASF/VN/Pig/Hue/1270 in feed samples on Days 1, 3, and 7 post inoculations evaluated by HAD assay. Positive control included complete swine feed containing 1×10^5 HAD_{50} of ASFV; Treatment 1 was positive control treated with SALTEC™ 512 at an inclusion rate of 1.0 kg/t feed; Treatment 2 was positive control treated with SALTEC™ 512 at an inclusion rate of 2.0 kg/t feed; Treatment 3 was positive control treated with SALTEC™ 512 at an inclusion rate of 3.0 kg/t feed. Data represent the mean of three independent experiments and error bars indicate ± SD. Significant differences compared to control are denoted by *P<0.05, **P<0.01, and ***P<0.001.

medium-chain fatty acids (MCFA) reduced ASFV titer with a greater than 99.9% reduction in virus concentration. All feed ingredients treated with MCFA had no infectious ASFV detected at 30-day post-contamination (Niederwerder *et al*., 2020). Among antimicrobial additives, formaldehyde-based products showed a strong effect against ASFV infection. At 3.0 kg/t feed, a liquid formaldehyde-based additive was greater decreased ASFV replication and the viral titer than an organic acid blend product (Tran *et al*., 2020). Similarly, our finding showed that the SALTEC™ 512 decreased ASFV replication in contaminated feed. The antiviral effect on the virus replication was clearly observed at an inclusion rate of 1.0 kg/t feed on Day 1 post inoculation, whereas the most effective activity of SALTEC™ 512 was markedly noticed at an inclusion rate of 3.0 kg/t feed on Day 7 post inoculation. The SALTEC™ 512 inactivated ASFV in feed in dose and time-dependent manners. Overall, SALTEC™ 512 may have the potential to reduce the risk of ASFV transmission via feed contamination.

The adverse effects of formaldehyde on animal performances have been examined. Broiler chicks fed with formaldehyde-treated soybean meal at the inclusion rate of 3 kg/t (37% formaldehyde) showed no adverse effects on body weight gain, feed consumption, or feed conversion (Spears *et al.*, 1980; Ricke *et al*., 2019). Likewise, a plasma-containing diet treated with Sal CURB® product containing 37% formaldehyde at an inclusion level of 3 kg/t feed did not affect pig growth performance when compared to the untreated spray-dried plasma diet (Campbell *et al*., 2019). Nevertheless, further studies on the use of the SALTEC™ 512 *in vivo* are desired.

CONCLUSION

The SALTEC™ 512 antimicrobial decreased ASFV replication in contaminated feed. The antiviral activity of SALTEC™ 512 against ASFV was dose and time-dependent. SALTEC™ 512 may be the potential additive to decrease the risk of ASFV transmission via feed contamination. Nevertheless, further studies on the use of the SALTEC™ 512 *in vivo* are desired.

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