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Inhibition of African swine fever virus replication by β-glucan

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Abstract
Background: African swine fever (ASF) is one of the most important diseases in pigs because of its effects on all ages and breeds. To date, commercial vaccines and drugs for the prevention of ASF are lacking in the market and the survival of African swine fever virus (ASFV) in various environmental, farm, and or feed matrices has allowed the virus to remain, causing new outbreaks in the pig population. Besides biosecurity and animal husbandry management practices, the improvement of the host immune responses is critical to control, managing, and preventing ASF.
Aim: In this study, we investigated the protective role of β-glucan against ASFV infection using a porcine alveolar macrophage (PAM) model.
Methods: The effects of β-glucan on cell proliferation were evaluated by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The potential effects of β-glucan against a field ASFV strain isolated in Vietnam were further examined by real-time PCR and hemadsorption assays. The interferon (IFN)-α and interleukin (IL)-6 protein production induced by β-glucan was determined using a sandwich enzyme-linked immunosorbent assay.
Results: Our results demonstrated that the β-glucan additive possessed an immune stimulus factor against ASFV. Specifically, protection of PAMs against ASFV infection *in vitro* was observed at 12 hours ($p < 0.05$) at the tested doses (30 and 50 µg/ml) as induced by incubation with β-glucan for 2 hours. These effects remained until 24 hours after post-infection. Additionally, at a high dose (50 µg/ml), pre-treatment with the β-glucan statistically increased the expression levels of IFN**α** and IL-6 when compared to untreated groups or only ASFV infection.
Conclusion: Together, these findings indicated that the β-glucan may protect the host against ASFV infection via the multiple cellular immune mechanisms.
Keywords: African swine fever virus, β-glucan, Cytokine.

Introduction

African swine fever (ASF) is one of the most important diseases in pigs because of its effects on all ages and breeds and is reported to the World Health Organization for Animal Health (OIE). In Asia, the first ASF outbreak was reported in China in 2018 and the ASF outbreaks were reported in Mongolia, Vietnam, Cambodia, North Korea, Laos, Myanmar, Philippines, South Korea, Timor-Leste, Indonesia, Papua New Guinea, India, and Malaysia (Vergne *et al*., 2020). African swine fever virus (ASFV) is highly virulent, and both the morbidity and mortality can be up to 100%, depending on the virulence of the virus, the host, and the transmission cycles (Kapoor *et al*., 2017). To date, the vaccines and drugs for the prevention of ASF are lacking in the market and the survival of ASFV in various environmental, farm, and or feed matrices has allowed the virus to remain to cause new outbreaks in the pig population (Cubillos *et al*., 2013; Kapoor *et al*., 2017). Besides biosecurity and animal husbandry management practices, the improvement of the host immune responses is critical to control, manage and prevent ASF.

β1,3-D-glucans (β-glucans) are naturally occurring glucose polymers forming structural components of cell walls of several species and are active biologically as immunomodulators or biological response modifiers (Moorlag *et al*., 2020; Iswarya *et al*., 2022; Wang *et al*., 2022). The function of glucans has been well identified and characterized *in vivo* and *in vitro* and includes antiviral activities, inhibition of cancer growth, induction of cell proliferation, and reduction of stress or cholesterol level (Anusuya and Sathiyabama, 2015; Dos Santos *et al*., 2019; Krishnan

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et al., 2022). Recently, the major function of glucans has been reported as the inhibition of pathogens such as leptospira, mycobacterium, influenza infection, and cancer because it enhances the proliferation and phagocytotic capacity of macrophages, as well as the oxidative burst of monocytes, macrophages, and neutrophils (Hetland *et al*., 1998; Wang *et al*., 2019; Moorlag *et al*., 2020; Peymaeei *et al*., 2020). In addition, the role of glucans was reported to affect the immune system by induced cytokines expression which is primarily associated with host responses to infectious diseases (Nemoto *et al*., 1994; Soltys and Quinn, 1999). The research demonstrated that treatment with glucan induced the proliferation of dendritic and macrophages cells, and produced chemokine and cytokines such as interferons (IFNs), tumor necrosis factor (TNF)-α, interleukin (IL)-10, IL-6, IL-12, IL-1β, IL-12, CXCL2 via binding and activation of dectin-1 (a type II transmembrane protein receptor), activated the associated toll-like receptor (TLR) signaling, JAK-STAT signaling, and also NF-κβ signaling pathways (Soltys and Quinn, 1999; Wouters *et al*., 2002; Harada *et al*., 2006; Ikeda *et al*., 2007; Mochizuki *et al*., 2013; Su *et al*., 2020). Furthermore, glucan can activate the immune system to produce IFNs which induces the antimicrobial functions of macrophages by increasing pinocytosis and phagocytosis, and enhancing the host inhibition of microbial infections (Soltys and Quinn, 1999; Fan *et al*., 2006; Werner *et al*., 2009). In addition, the cell-surface major histocompatibility complex (MHC) class I, class II and related genes have been shown to be upregulated by IFN-γ and IFN-α induction, which increased the cytotoxic T-cell and activation of $CD4^+$ and $CD8^+$ T cells, which play important roles in the host response to infectious diseases (Konopski *et al*., 1994; Harada *et al*., 2006). In this study, we evaluated the ability of β-glucan to inhibit the ASFV and produce IFN-α and IL-6 in primary porcine alveolar macrophages (PAMs).

Materials and Methods

Experimental design

The β-glucan was provided by Kemin Industries, Singapore and the chemical structures of β-glucan are shown in Figure (1A). To evaluate the potential

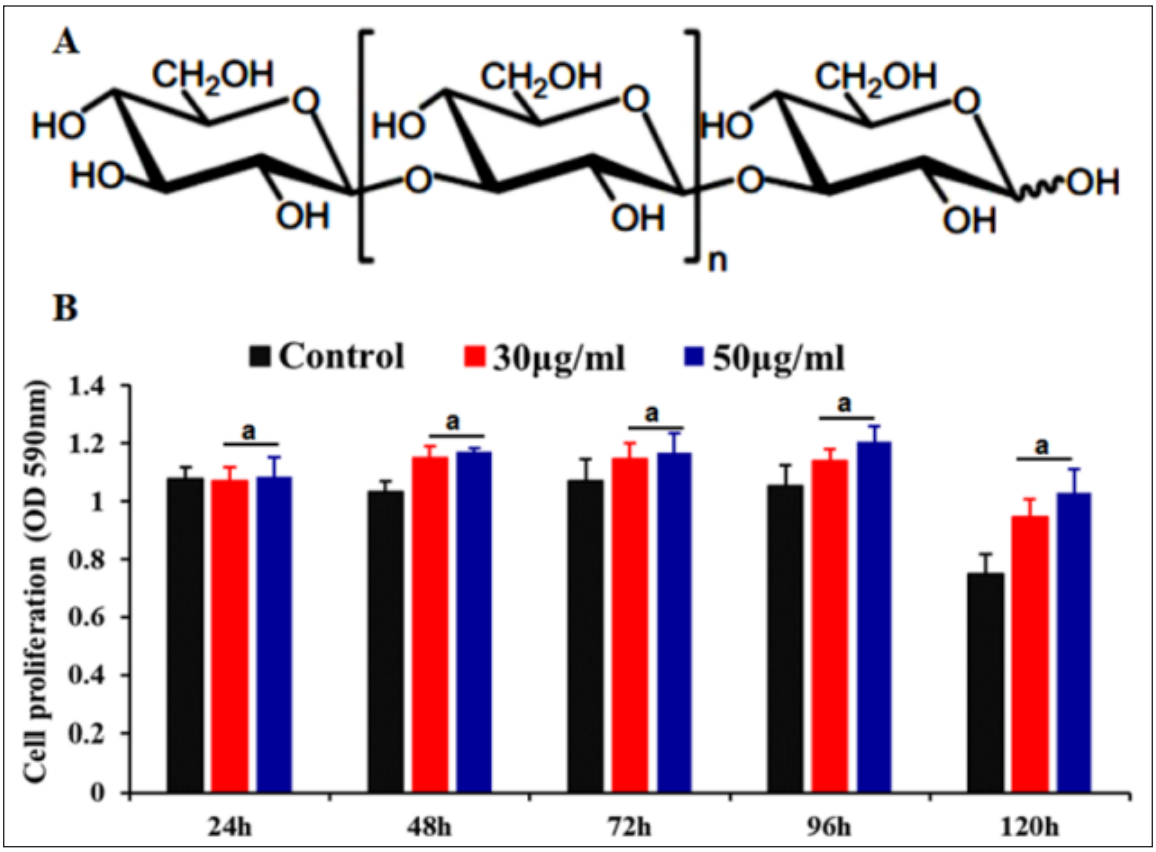

Fig. 1. (A) Chemical structures of β-glucan. (B) The effects of β-glucan on PAMs cell at the cell viability by MTT assay. The data are presented as the mean ± SEM of three independent experiments indicating the significant differences between the control and treatment groups were determined by a one-way analysis of variance. a: $p < 0.05$.

effects of β-glucan on ASFV infection and cytokine production, the cells were pre-treated with 30 and 50 µg/ml of glucan for 2 hours and then spiked with the ASFV/Vietnam/Pig/HN02 virus strain (p72 genotype II, CD2v serotype 8, I73R/I329L variant I and A179L/A137R variant 2 (Tran *et al*., 2021). The final concentration of ASFV was 10^3 hemadsorption $(HAD)_{50}$/ml. Negative controls were inoculated with phosphate-buffered saline (Sigma-Aldrich), while positive controls were inoculated with 10^3 HAD_{50} of ASFV. The samples from the experimental and control groups were collected at 12, 24, or 36 hours post-inoculation (hpi).

Cell proliferation was measured by MTT assay: briefly, PAMs were harvested, counted by hemocytometer, and diluted with medium, yielding a concentration of 10^6 cells/ml. From this cell suspension, 100 µl was pipetted into 96-well microtiter plates (Nunc, Denmark) and incubated for 24 hours in a 5% CO_2 incubator at 37°C. Cells were then treated with 30 and 50 µg/ml of glucan. After adding the glucan, a new medium was added to make the final volume of 200 µl per well. The plate was then incubated in a 5% CO_2 incubator at 37°C for 24, 48, 72, 96, and 120 hours for determining the cell proliferation by MTT kit (Abcam, Waltham, MA) according to the manufacturer's protocols.

The HAD assay was performed as described previously (Thanh *et al*., 2021). The HAD assay was observed for 5 days, and 50% HAD doses (HAD_{50}) were calculated by using the method of Reed and Muench (1938). The genomic DNA of ASFV in the supernatant was detected by the real-time PCR targeting the ASFV p72 gene as recommended by OIE (2012).

The IFN-**α** and IL-6 production induced by β-glucan was also assessed in this study. After 12- or 24-hour treatment, the supernatant was collected, and the cytokine production was determined using a sandwich enzyme-linked immunosorbent assay (ELISA). The cytokines were further analyzed using the porcine IFN-**α** and IL-6 ELISA kit according to the manufacturer's instructions (Invitrogen, Waltham, MA).

Statistical analysis

Statistical analysis was performed using IBM SPSS software (SPSS 23.0 for Windows; IBM, Chicago, IL). A p-value < 0.05 was considered to be statistically significant. Differences among the groups were tested by Duncan's multiple comparison methods.

Ethical approval

The study was conducted in compliance 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).

Results and Discussion

β-glucan has been demonstrated to enhance the cell proliferation and differentiation of various cells such as macrophage cells (RAW 264.7 cell, THP-1 cell), or T cells (Xiao *et al*., 2004; Muramatsu *et al*., 2014; Dos Santos *et al*., 2019). Several research studies have demonstrated that various forms of β-glucan can be antiviral, antitumor, or antibacterial activities (Xiao *et al*., 2004; Maity *et al*., 2019; Wang *et al*., 2019, 2022; Liu *et al*., 2021; Krishnan *et al*., 2022). Furthermore, β-glucan can be used as an immunomodulator, particularly acting as a stimulator of cytokine production in vitro and in vivo (Werner *et al*., 2009; Maity *et al*., 2019; Wang *et al*., 2019). We investigated if the β-glucan would or could affect ASFV growth and the production of important immune system cytokines such as IFN**α** and IL-6 in PAMs. In this study, β-glucan can enhance PAM cell proliferation as evidenced in two different doses of β-glucan when compared with the untreated control group (Fig. 1B). The rate of survival of PAMs treatment with β-glucan was highest at 50 µg/ml β-glucan, especially at 120-hour post-treatment, compared to un-treatment and 30 µg/ml β-glucan. Our results demonstrated that 30 or 50 µg/ml β-glucan can enhance cell survival in a dose-dependent manner and these effects remained until 120 hours compared with the control.

Recently, several reports demonstrated that β-glucan can inhibit the growth of several pathogens such as leptospira, influenza, mycobacteria, and tuberculosis (Hetland *et al*., 1998; Wang *et al*., 2019; Moorlag *et al*., 2020). However, the potential effects of β-glucan against ASFV infection in the host remained unclear. To evaluate the role of β-glucan against ASFV infection, two doses of β-glucan (30 and 50 µg/ml) were used to show efficacy against a field ASFV strain isolated in Vietnam at 10^3 HAD_{50}/ml. As shown in Figure 2A, PAMs pre-incubated with β-glucan for 2 hours reduced significantly ASFV invasion at 12 hours post-infection compared with the control group. These effects remained statistically until 24 hours and then reached normal levels at 36 hours post-infection. Moreover, the titer of ASFV confirmed by HAD assay showed that the titer of ASFV was significantly reduced at 12 hours post-infection when compared to the positive control. Specifically, the titer of the virus at 12 hours post-infection was $10^{2.16}$ HAD_{50}/ml for 50 µg/ml of β-glucan and $10^{2.40}$ HAD_{50}/ml for 30 µg/ml of β-glucan, when compared to a positive control ($10^{2.67}$ HAD_{50}/ml) with $p < 0.05$ (Fig. 2B). A modest effect at living viral levels was noted at 24 hours post-infection in this study. Similarly, the antiviral activity of β-glucan inhibiting the pathogens on macrophages has been demonstrated for influenza A virus, mycobacteria, tuberculosis, and herpes simplex virus 1 and indicated that β-glucan significantly decreased the pathogen growth and induced the cytokines expression through activating the JAK-STAT and TLR signaling pathways,

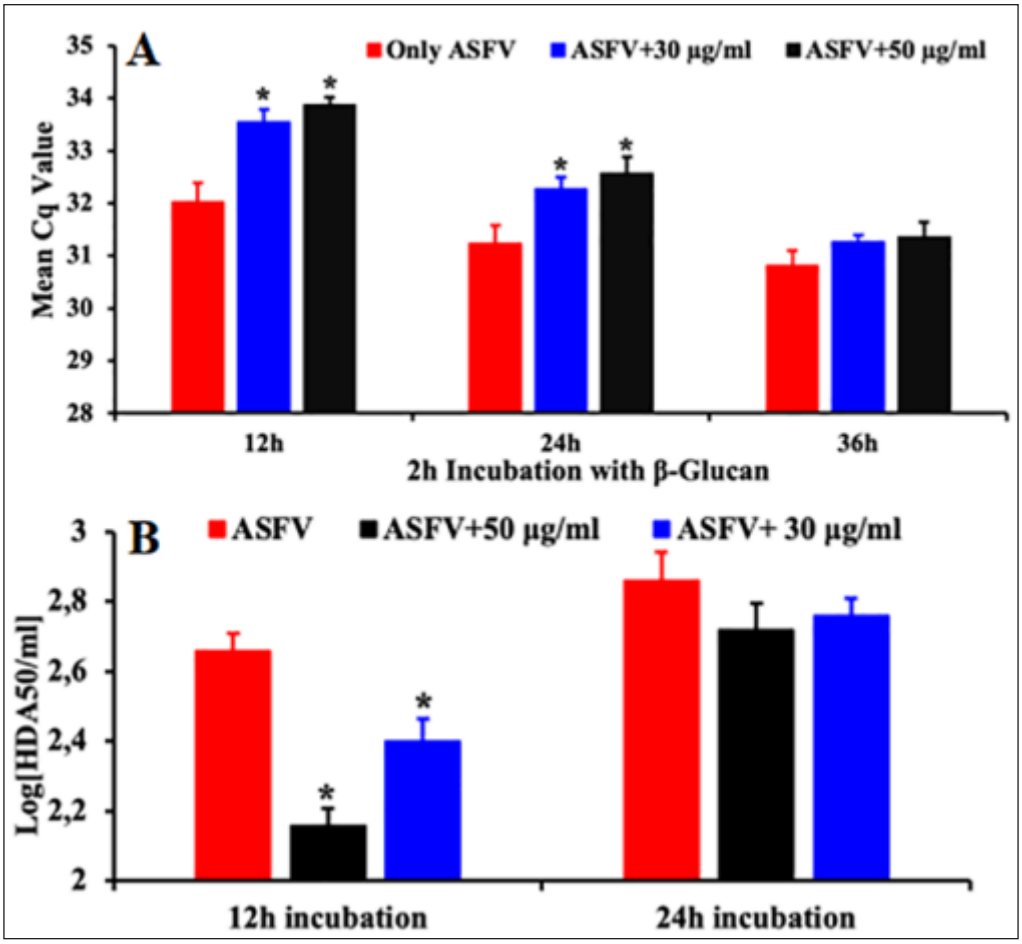

Fig. 2. The antiviral activity of β-glucan against PAMs. PAMs were pre-treated with 30 µg/ml and 50 µg/ml β-glucan, and exposed to 10^3 HAD_{50}/ml ASFV for 12, 24, or 36 hours. The viral DNA of ASFV in the supernatants was collected and then real-time PCR amplification of the p72 gene was performed to detect the presence of viral DNA in the PAMs treated with β-glucan. (A) Real-time PCR amplification of the p72 gene was performed to detect the presence of viral DNA at 12, 24, and 36 hours post-infection in the PAMs treated with β-glucan for 2 hours and then infected with 10^3 HAD_{50}/ml ASFV; and (B) Titer of ASFV in the PAMs pre-treated with 30 or 50 µg/ml β-glucan in 2 hours and then exposed to 10^3 HAD_{50}/ml ASFV at 12 and 24 hours post-treatment. The data were presented as the mean ± SEM of three independent experiments. *: $p < 0.05$.

especially produce type I IFN genes (Harada *et al*., 2006; Wang *et al*., 2019; Moorlag *et al*., 2020; Lopes *et al*., 2021; Perveen *et al*., 2021; Krishnan *et al*., 2022; Pan *et al*., 2022). The findings from our study have shown that β-glucan protected the host against the ASFV invasion in PAMs.

It has been indicated previously that β-glucans are active as an immunomodulator and regulate both innate and adaptive immunity (Ishibashi *et al*., 2005; Amphan *et al*., 2019; Dos Santos *et al*., 2019). The ability of β-glucans in the innate immune system to recognize and respond to pathogen infection plays an important role in controlling the infection (Hetland *et al*., 2000; Guselle *et al*., 2007; Bayrak *et al*., 2008; Chen *et al*., 2020; Lee *et al*., 2020; Moorlag *et al*., 2020; Krishnan *et al*., 2022). The ability of β-glucans in the innate immunity system was inducement of cytokines and immune-related genes against pathogen infection such as IFNs, IL-1β, IL-12, IL-6, TNF-α, IL-10, and TLR2/6 through activated and associated signaling pathways such as NF-κB, MAPK, TLR, JAK-STAT (Muramatsu *et al*., 2014; Udayangani *et al*., 2017; Wang *et al*., 2019; Moorlag *et al*., 2020; Peymaeei *et al*., 2020; Liu *et al*., 2021; Pan *et al*., 2022). The research suggests that β-glucans may involve activation pathways and influence the innate immune system in response to pathogen infection. Additionally, IFNα and IL-6 play an important role in fighting viral infections. IL-6 is a regulatory cytokine that activates natural killer, T helper 1 (Th1), and Th17 cells to produce IFN and reduce apoptosis and cytolysis (Ikeda *et al*., 2007). Therefore, IFNs plays an important role in activating the process of phagocytosis and improving protection

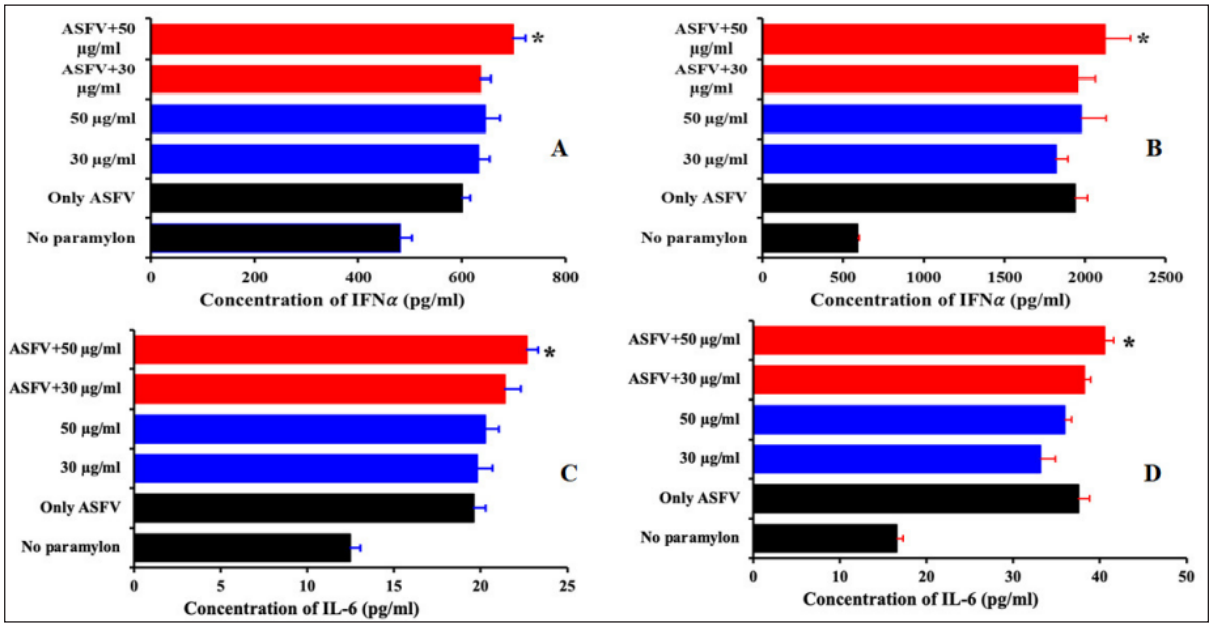

Fig. 3. Cytokine production by the PAMs infected with ASFV. PAMs were pre-treated with 30 or 50 µg/ml β-glucan for 2 hours and then exposed to 10^3 HAD_{50}/ml ASFV for 12 (A, C) and 24 hours (B, D). The data are presented as the mean ± SEM of three independent experiments. *: $p < 0.05$.

against intracellular viral infections (Mochizuki *et al*., 2013; Udayangani *et al*., 2017; Dos Santos *et al*., 2019; Wang *et al*., 2019; Liu *et al*., 2021; Lopes *et al*., 2021; Pan *et al*., 2022). A recent study showed that ASFV infection at the doses of 1 and 0.1 MOI for 24 hours significantly increased IFNα production in PAMs when compared to control in PAMs (Li *et al*., 2021; Wang *et al*., 2021), suggesting the protective role of type I IFN in response to ASFV infection. Additionally, the activation of this IFN expression may inhibit ASFV infection through the cGAS/STING signal pathway (Li *et al*., 2021; Wang *et al*., 2021). Our results showed that the production of IFNα and IL-6 were significantly increased in both treated groups (30 µg/ml or 50 µg/ml) at 12 hours and remained until 24 hours when compared with the control groups ($p < 0.05$) (Fig. 3). As expected, ASFV infection at 10^3 HAD_{50}/ml induced a significant increase in the production of these cytokines. Interestingly, at the high dose of this chemical (50 µg/ml), a statistical enhancement of IFN-α and IL-6 secretion induced by β-glucan was observed when compared to only ASFV infection in these experiments ($p < 0.05$). The results indicated that the high dose of β-glucan enhanced significantly the IFN-α and IL6 production in PAMs in response to ASFV infection. The findings suggested the potential protective application of β-glucan in which this additive may exert its preventive effects against ASFV invasion by activating IFNα and IL6.

Conclusion

Our results pose an interesting question about the protective effect and mode actions of β-glucan in response to ASFV infection. The results from this study showed that β-glucan may exert its effects to reduce/inhibit ASFV invasion *in vitro* via the cellular immune mechanisms in a dose-dependent manner. Additionally, these findings suggest that β-glucan is a good candidate to improve the host immune systems against ASFV circulating in Vietnam.

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Authors' contribution

HTTT, ADT, and HVD conceived and designed the experiments. HTTT, ADT, NTC, HTN, HNV, and ADT performed the experiments. HTTT, ADT, TN, FS, HL, AL, AGY, and HVD analyzed the data. HVD and KL contributed the reagents, materials, and analytical tools. HTTT, ADT, TN, FS, HL, AL, AGY, and HVD wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no competing interests.

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