



Salt inactivation of classical swine fever virus and African swine fever virus in porcine intestines confirms the existing *in vitro* casings model

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ARTICLE INFO

Keywords:

African swine fever
Classical swine fever
Intestine
Virus inactivation
3D collagen matrix model
D-values

ABSTRACT

Natural casings, to be used as sausage containers, are being traded worldwide and may be contaminated with contagious viruses. Standard processing of such natural casings is by salt treatment with a duration of 30 days before shipment. Since information is lacking about the efficacy of these virus inactivation procedures, an *in vitro* 3D collagen matrix model, mimicking natural casings, was developed previously to determine the efficacy of salt to inactivate specific viruses. To validate this model, a comparison *in vivo* experiment was performed using intestines of pigs experimentally infected with African swine fever virus (ASFV) and classical swine fever virus (CSFV). Decimal reduction (D) values, were determined at 4 °C, 12 °C, 20 °C and 25 °C. The standard salt processing procedure showed an efficient inactivation of ASFV and CSFV over time in a temperature dependent way. $D_{\text{intestine}}$ values of both viruses, treated with the standard salt treatment, were in line with the D_{collagen} values. It was concluded that these results underline the suitability of the 3D collagen matrix model to determine virus inactivation and to replace animal experiments. Furthermore, an increase in storage time for standard salt processed casings derived from CSFV endemic regions is highly recommended for an efficient inactivation of CSFV.

1. Introduction

Porcine intestines are used for the production of edible sausage containers (natural casings) and are derived from animals considered fit for human consumption after having passed ante- and post mortem inspection. In contrast to sheep casings (Koolmees et al., 2004), for which only the small intestines and caecum are used, the entire porcine intestinal tract is used to produce different kinds of natural casings (ENSCA, 2019). Due to a huge variety in qualities, calibres and their subsequent use for different sausages, natural casings are sourced around the globe and international trade plays a very important factor prior to reaching its final destination and consumer. As such, natural casings need to meet various international animal & public health and food safety requirements, similar to any other product of animal origin destined for human consumption.

Previous studies have focused on the inactivation of specific contagious animal diseases in casings, such as foot and mouth disease (FMDV) and classical swine fever (CSFV), which could be present in animals used as a source for the production of casings (Wijnker et al., 2008a, 2007, 2012, 2008b). In these studies standard operating

procedures for the production of casings, as implemented by the sausage casing industry, were applied and resulted in adequate viral inactivation. Specific treatments include salting using either sodium chloride (NaCl) or phosphate supplemented salt (P-salt) over a prolonged period of time (30 days) at specific temperatures. Some of the limitations encountered in these studies was the difficulty of generating a sufficiently high virus titre to reliably quantify the virus inactivation over time in the intestinal tract, specifically for FMDV (Wijnker et al., 2012).

To replace live animal experiments, an *in vitro* 3D collagen matrix model was developed to determine the inactivation kinetics of four animal viruses by either saturated NaCl or P-salt at four different temperatures (4 °C, 12 °C, 20 °C and 25 °C) (Wieringa-Jelsma et al., 2011). Based on this paper the OIE Terrestrial Animal Health Code article 15.1.24, "Procedures for the inactivation of ASFV in casings of pigs", was adopted. However, in 2012 the EFSA Panel on Animal Health and Welfare (AHAW) reviewed the treatments of animal casings in a "Scientific Opinion on animal health risk mitigation treatments as regards imports of animal casings" (EFSA, 2012). In this opinion, EFSA strongly recommended validation of the 3D collagen matrix model by

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<https://doi.org/10.1016/j.vetmic.2019.108424>

Received 26 July 2019; Received in revised form 20 September 2019; Accepted 20 September 2019

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actual animal studies.

In order to improve on the application and usability of the 3D collagen matrix model to study viral inactivation in casings a dual study design was developed. Pigs were infected with either classical swine fever virus (CSFV) or African swine fever virus (ASFV) and intestinal samples taken from these animals were subsequently treated for virus inactivation and reduction of infectivity over time was measured. The main goals of these experiments were to validate the original results from the 3D collagen matrix model, as published in 2011 and to show the efficacy of NaCl and P-salt for virus inactivation over time at different temperatures.

2. Materials and methods

2.1. Viruses and cell types

In this study, two virus strains were used: CSFV strain Paderborn 277, isolated in 1997 during the outbreak in the Paderborn area of Germany (Greiser-Wilke et al., 2000) and AFSV strain Georgia 2007/1, isolated in 2007 in the Caucasus region of Georgia, since then spreading to neighbouring countries (Rowlands et al., 2008). ASFV Georgia 2007/1 was kindly provided by Linda Dixon at the Pirbright Institute (Surrey, UK).

For virus titration of CSFV in the intestine samples, swine kidney cell line SK6 (Kasza et al., 1972) was used. Titration of ASFV was performed on porcine alveolar macrophages (PAMs) (Carrascosa et al., 1982), derived from 10-week-old conventional raised pigs with a high health status.

2.2. Preparation of saturated salt solutions

The routine salt solution consisted of a saturated Sodium Chloride (NaCl, molecular weight (Mw) 58) solution. The P-salt solution consisted of 86.5% Sodium Chloride (NaCl, Mw 58), 2.8% Trisodium Phosphate (TSP Na_3PO_4 , Mw 164) and 10.7% Disodium Hydrogen Phosphate (DSP, Na_2HPO_4 , Mw 142). Both saturated salt solutions were made in demineralised water and pH was measured, resulting in a pH of 6.5 and 9.3 for the NaCl and P-salt solutions, respectively.

2.3. Animal studies

Animal experiments were approved by the governmental Central Authority for Scientific Procedures on Animals (CCD) in the Netherlands under no. AVD401002016687.

A total number of six female pigs at slaughter age (~6 months, ~80–90 kg) were obtained from a conventional farm with a high health status in the Netherlands. Before arrival at the High Containment Unit (HCU) facilities of Wageningen Bioveterinary Research (WBVR) (Lelystad, The Netherlands), serum blood samples were taken and confirmed to be free of pestivirus antibodies by the Priocheck BVDV Ab ELISA (ThermoFisher Scientific).

In each animal experiment, pigs were intranasally inoculated with either CSFV Paderborn ($n = 2$) or ASFV Georgia 2007/1 ($n = 4$; 2 experiments with 2 pigs) at a dose of 2 ml $10^{5.5}$ TCID₅₀/ml or $10^{5.5}$ TCID₅₀/ml, 1 ml per nostril, respectively. The first animal experiment was performed with CSFV, because knowledge about the viral load and virus distribution of CSFV in the intestines was already determined in a previous pilot study (data not shown). For ASFV, this information was not available, therefore two separate ASFV experiments were planned, creating the opportunity to modify the study design in case new insights were obtained. The health of the pigs was monitored daily by measuring the rectal temperatures and clinical observations as previously described (Mittelholzer et al., 2000). EDTA stabilized blood samples were taken on day 0 (D0; prior to inoculation), day 3 and daily from day 6 until the end of the animal study. Blood samples (1:1 diluted in phosphate buffered saline (PBS)) were analysed daily for relative virus

titres (viremia) by quantitative real-time PCR (qPCR) as described below. Based on clinical observations and body temperatures measured, all pigs were euthanized in the acute phase of the disease at the peak of viremia.

2.4. Experimental design intestine inactivation

2.4.1. Collection of samples

After euthanizing the pigs, the intestines were removed and approximately 1.5 to 2 m of both the jejunum and colon of each pig were excised from the start of the concerning section, approximately 0.5 m after the duodenum and caecum, respectively. Faeces was squeezed out of the jejunum by hand, faeces in the colon was removed by rinsing with water.

From the CSFV-infected pigs the following samples were collected:

- Jejunum, 109 consecutive samples, each 1–1.5 cm long and weight of ~2–3 g.
- Colon, also 109 consecutive samples, each 1–1.5 cm long and weight of ~3–4 g.

Each sample was weighed separately

From each section, 13 out of 109 samples served as non-treated controls (D0 samples) and were collected throughout the entire length of the section. D0 samples were immediately stored at -70 °C and used for the determination of the viral load present in each intestine section. Forty-eight samples from each section were designated for treatment with either NaCl or P-salt, as shown in Fig. 1A. Samples were placed into 6-wells plates and transported to the laboratory on ice.

From the ASFV-infected pigs the following samples were collected:

- Jejunum, 97 consecutive samples, 1–1.5 cm long each and weight of ~2–3 g.

Each sample was weighed separately

Seventeen samples served as non-treated controls (D0 samples), also collected throughout the entire length of the jejunum sections and immediately stored at -70 °C. Forty samples were designated for treatment with either NaCl, P-salt or medium, as shown in Fig. 1A. The samples were placed into 6-wells plates and transported to the laboratory on ice. The differences in sampling between CSFV and ASFV were caused by insights along the course of the experiments. Briefly, because CSFV results showed comparable results to the 3D collagen model, it was decided to exclude the incubation at 25 °C for the ASFV experiment. The ASFV inactivation experiments focussed on the jejunum sections due to its relevance for the casing industry and the availability of PAMs, which limited the possibility to test large amounts of samples. Besides that, a successful pilot experiment with non-infected intestines, prior to the start of the first ASFV experiment, made it feasible to incorporate the non-treated samples in the ASFV study design.

2.4.2. Incubation of samples

For CSFV, 48 jejunum samples and 48 colon samples per pig were incubated in either NaCl or P-salt (10 ml/well) at 4 °C, 12 °C, 20 °C or 25 °C. Duplicate samples were incubated for either 7, 14, 21, 30, 45 or 60 days (Fig. 1A and Supplementary Allotments).

For ASFV, 40 jejunum samples per pig were incubated in either NaCl or P-salt at 4 °C, 12 °C and 20 °C. Duplicate samples were incubated for either 2, 4, 7, 14, 21, 30, 45 or 60 days. Additionally at 4 °C, non-treated jejunum samples were incubated in medium (RPMI, Gibco) containing a 10% antibiotics mixture ABII (1000U/ml penicillin, 1 mg/ml streptomycin, 20 mg/ml fungizone, 500 mg/ml polymixin-Band10 mg/ml kanamycin) to prevent decay (Fig. 1A and Supplementary Allotments).

During the incubation period of 60 days, plates were frequently

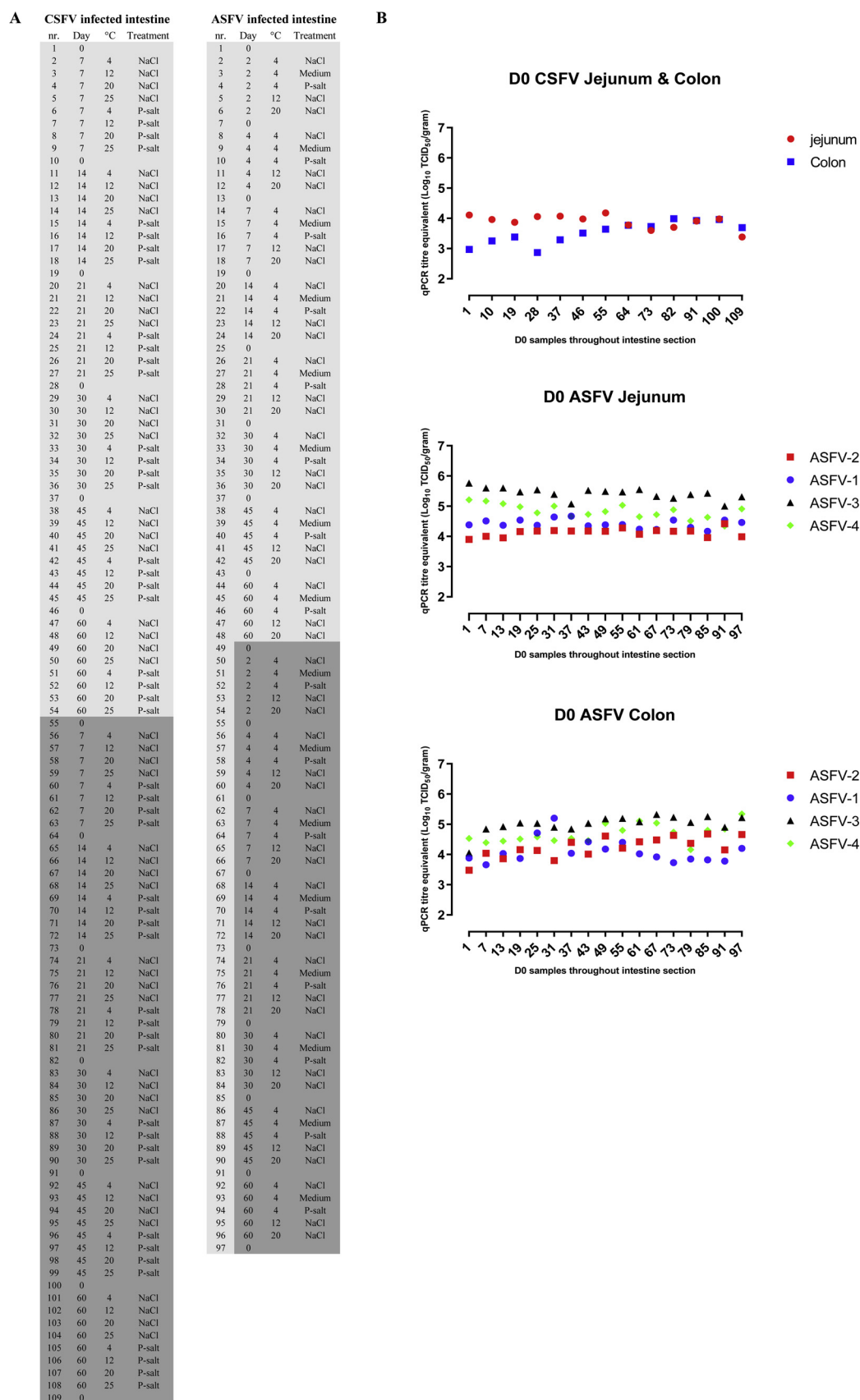


Fig. 1. A. Sampling schemes of the CSFV and ASFV infected intestines. Consecutive samples for each treatment were sampled per incubation time (days) along the intestine section, and duplicate samples were taken from the first and second half (highlighted in grey) of each intestine section (jejunum or colon) per animal. B. Viral loads distributed throughout the entire length of the CSFV and ASFV intestine sections, indicated by the qPCR results expressed as virus equivalent titres ($\log_{10}$ TCID₅₀/gram) of the CSFV D0 samples per intestine section (upper panel); jejunum (red dot) and colon (blue square) and 17 ASFV D0 samples per pig; ASFV-1 (blue dot), ASFV-2 (red square), ASFV-3 (black triangle), ASFV-4 (green diamond): jejunum (centre panel) and colon (lower panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

examined for evaporation and if needed, salt solution was added.

2.4.3. Harvesting of samples

For both CSFV and ASFV, after the designated incubation period, respective duplicate samples were harvested from the plates by rinsing twice in phosphate-buffered saline (PBS), followed by immediate storage at -70°C .

2.4.4. Homogenizing of samples

For CSFV, frozen samples were homogenized by grinding, using a cryogenic tissue lyser (ProFicocook). Whole samples were grinded in the presence of dry-ice, after sublimation of dry-ice, 4 volumes of the weight of the sample of Eagle's Minimum Essential Medium (EMEM) was added to obtain a 20% tissue homogenate, e.g. to 2 g of sample 8 ml of medium was added. The tissue lyser was cleaned after each sample with Decon 90 (Agar Scientific) to avoid cross-contamination between samples.

For ASFV, D0 samples were homogenized as described for CSFV above, only the type of medium (RPMI) differed. All other samples were grinded with Ultra-Turrax® tube drive (Ika), using cooled metal balls to grind frozen samples for 2 min at maximum speed. Both methods were compared before changing to the Turrax system and no differences were found. By using Turrax the possible risk of cross-contamination was reduced to zero, because grinding was performed in separate containers for each sample, therefore avoiding the time-consuming cleaning step. Likewise to above, samples were homogenized by adding the calculated amount of medium to obtain 20% homogenate suspensions.

All homogenates, CSFV and ASFV, were clarified by centrifuging at $1966 - 2570 \times g$ for 10 min at 4°C and stored at -70°C until testing in qPCR and/or virus titration.

2.4.5. Additional sampling

Besides jejunum samples, seventeen colon samples (similar to D0 jejunum) and duplicate samples were collected from the intestines of the ASFV infected pigs. This included the following sections: duodenum, ileum, caecum, after end (1 m after colon), fat end (1 m before rectum) and mucosa relevant for the casing industry were collected and stored immediately at -70°C (ENSCA, 2019). Homogenizing was performed with the Turrax system as described above. Clarified homogenates were stored at -70°C until testing by qPCR.

2.5. Quantitative CSFV RT-PCR and ASFV PCR

To determine whether the viral load and homogeneity of the virus distribution over each intestine section was sufficient for enrolment in the inactivation study, D0 samples were tested by quantitative RT-PCR, detecting CSFV (Weesendorp et al., 2014, 2011) or ASFV qPCR (de Carvalho Ferreira et al., 2012). The latter was also used to test the additional intestine sections of the ASFV infected pigs.

To quantify the amount of virus DNA/RNA present, a standard curve with known titres of either CSFV Paderborn or ASFV Georgia strain was used, therefore the results were expressed in TCID_{50} equivalents. Because PCR detects viral DNA or RNA, which can be derived from both infectious and non-infectious virus, qPCR results represent a relative measure for the viral load.

2.6. Virus titration

Ten-fold serial dilutions of each sample, either CSFV or ASFV infected, were titrated in four-fold on SK6 cells or on PAMs, respectively. Samples were mixed with cells and incubated for 4 days in 24-wells microtitre culture plates at 37°C in a humidified atmosphere with 5% CO_2 and examined for presence of virus by immunoperoxidase-monolayer-assay (IPMA) using either CSFV-specific monoclonal antibodies (V3/V4) or anti-ASFV hyper-immune serum (de Carvalho Ferreira et al.,

2012; Weesendorp et al., 2010). Plates were read macroscopically for CSFV, scoring coloured wells as positive, and microscopically for ASFV, scoring coloured cells as positive. Titres were calculated using Reed and Muench's method and expressed as TCID_{50} /gram of intestine.

2.7. Regression analysis

Linear regression analysis was performed with Graphpad Prism 8 (GraphPad Software, Inc., San Diego, California) for each virus at different temperatures, excluding data at the detection limit, with the exception of data reaching the detection limit immediately after day 0. From linear regression of virus titres *versus* time the decimal reductions (D-values) per temperature were calculated as the negative reciprocal of the slope. During regression analysis, the coefficient of correlation of the regression analysis (R^2) was included. The equations and result tables are included in the supplemental data section.

2.8. Comparison 3D collagen model

In order to validate the 3D collagen model (Wieringa-Jelsma et al., 2011), the $D_{\text{intestine}}$ and D_{collagen} values were compared per treatment and incubation temperature, if possible.

This model consisted of a bovine collagen type I gel matrix in which cells infected with either ASFV or CSFV were embedded. Collagen was pipetted into a 24-wells plate and left to solidify into a firm gel. By applying this process the actual submucosa layer of the intestinal tract is mimicked, with embedded virus resembling either a viremia or having infected host cells which are a normal part of the cellular structure of the intestinal wall. The collagen model was then subjected to storage for specific periods at certain temperatures and treated with either regular salt (NaCl) or P-salt. This entire procedure closely resembled the standard processing and storage of porcine (hog) casings under industrial conditions.

3. Results

3.1. Animal studies

CSFV; From 8 days post infection (dpi) both pigs (CSFV-1 and CSFV-2) were less active, showed less appetite and feed leftovers were present in the animal unit. No other clinical symptoms related to a CSFV infection were observed. No fever (rectal temperatures above 40.0°C) was observed in either pig during the entire study. Viremia was observed in both pigs, starting at 6 dpi. High blood titre equivalents determined by qPCR for pigs CSFV-1 and CSFV-2 were reached at 9 dpi ($3.1 \text{ Log}_{10} \text{ TCID}_{50}\text{-eq/ml}$) and 10 dpi ($4.4 \text{ Log}_{10} \text{ TCID}_{50}\text{-eq/ml}$), respectively. At 10 dpi both pigs were euthanized and intestines were collected and prepared for the inactivation study (Supplementary Fig. S1).

ASFV; Similar to the CSFV infected pigs, the ASFV infected pigs were less active from 4 to 5 dpi, showed less appetite and feed leftovers were present in the animal unit. No other clinical symptoms related to an ASFV infection were observed. Fever was observed during a short period (3 days), starting at 4 dpi (ASFV-3; 40.5°C), 5 dpi (ASFV-1; 40.6°C , ASFV-2; 41.0°C) and 6 dpi (ASFV-4 (40.3°C)). Viremia was observed in all pigs, starting at 4 dpi and high blood titre equivalents were reached at 7 dpi (day of euthanasia) for ASFV-1 (6.7), ASFV-2 (6.8) and ASFV-3 ($6.7 \text{ Log}_{10} \text{ TCID}_{50}\text{-eq/ml}$). The highest blood titre of $6.6 \text{ Log}_{10} \text{ TCID}_{50}\text{-eq/ml}$ of ASFV-4 was reached at 8 dpi and this pig was euthanized the next day (Supplementary Fig. S1).

3.2. Initial virus load in D0 samples (qPCR)

In the intestines of CSFV-1, no virus, except for one colon sample with a titre of $2.9 \text{ log}_{10} \text{ TCID}_{50}\text{-eq/gram}$, was detected by qPCR. Therefore, the intestines of pig CSFV-1 were not included in the

Table 1Summary of the mean $\pm$ SD qPCR results of the additional intestine sections ($\log_{10}$ TCID₅₀/gram).

Pig	Duodenum	Ileum	Caecum	After end	Fat end	Mucosa [*]
ASFV-1	4.9 $\pm$ 0.2	4.7 $\pm$ 0.1	4.8 $\pm$ 0.1	4.6 $\pm$ 0.1	5.0 $\pm$ 0.0	3.2
ASFV-2	4.7 $\pm$ 0.1	4.4 $\pm$ 0.0	4.5 $\pm$ 0.1	4.5 $\pm$ 0.1	4.6 $\pm$ 0.1	4.1
ASFV-3	6.0 $\pm$ 0.0	5.3 $\pm$ 0.1	5.1 $\pm$ 0.1	5.3 $\pm$ 0.1	5.4 $\pm$ 0.0	4.1
ASFV-4	5.2 $\pm$ 0.2	4.9 $\pm$ 0.2	4.7 $\pm$ 0.1	5.1 $\pm$ 0.4	5.3 $\pm$ 0.2	3.9

^{*} Single sample.

inactivation study. The mean $\pm$ SD viral load in the jejunum and colon D0 samples of pig CSFV-2 were 3.9 $\pm$ 0.2 and 3.6 $\pm$ 0.3 $\log_{10}$ TCID₅₀-eq/gram, respectively. CSF virus was evenly (within 1 $\log_{10}$ TCID₅₀-eq/gram) distributed throughout the intestines (Fig. 1B) and jejunum and colon samples from this pig were included in the inactivation study according to the study design (Fig. 1A).

For ASFV, the mean $\pm$ SD viral loads found in the jejunum D0 samples were 4.4 $\pm$ 0.1, 4.1 $\pm$ 0.1, 5.4 $\pm$ 0.2 and 4.8 $\pm$ 0.2 $\log_{10}$ TCID₅₀-eq/gram for ASFV-1, -2, -3 and -4, respectively. In the colon the mean viral loads were 4.1 $\pm$ 0.4, 4.2 $\pm$ 0.3, 5.0 $\pm$ 0.3 and 4.7 $\pm$ 0.3 $\log_{10}$ TCID₅₀ equivalents/gram for ASFV-1, -2, -3 and -4, respectively. Likewise to CSFV, the ASF virus was evenly (within 1 $\log_{10}$ TCID₅₀-eq/gram) distributed throughout both intestine sections (Fig. 1B) and therefore the jejunum samples of all pigs were included in the inactivation study according to the study design (Fig. 1A).

The titres of the additional ASFV intestine sections: duodenum, ileum, caecum, after end, and fat end, ranged from 4.4 to 6.0 TCID₅₀-eq/gram. In the mucosa, lower titres were observed, ranging from 3.2 to 4.1 TCID₅₀-eq/gram (Table 1).

3.3. Inactivation of CSFV and ASFV (virus titration)

Virus titration was performed to determine virus titres in treated intestine samples, including day zero samples.

For CSFV, the mean $\pm$ SD titre results of the D0 jejunum and colon were 3.7 $\pm$ 0.5 and 3.7 $\pm$ 0.4 $\log_{10}$ TCID₅₀/gram, respectively and in agreement with the qPCR results (Supplementary Table S1). After 7 days of incubation, no viable CSFV (detection limit: 1.7 $\log_{10}$ TCID₅₀/gram) was detected in either intestine sections after treatment with P-salt at all temperatures. The NaCl treatment results showed inactivation in a temperature dependent way (Supplementary Fig. S2 and Tables S3 and S4).

For ASFV, the mean $\pm$ SD viral load results of the D0 ASFV infected jejunum sections of pigs ASFV-2/-3/-4 were 4.3 $\pm$ 0.6 $\log_{10}$ TCID₅₀/gram, confirming the qPCR results (mean 4.8 $\pm$ 0.6 TCID₅₀-eq/gram) (Supplementary Table S2). Unfortunately, for unknown reasons no virus titres were obtained with the jejunum samples of pig ASFV-1 and was therefore excluded from this analysis. After 7 days of treatment with P-salt at 4 °C, no viable ASF virus (detection limit: 1.7 $\log_{10}$ TCID₅₀/gram) was detected. Similar to the CSFV inactivation, the ASFV inactivation by NaCl was found to be temperature dependent (Supplementary Fig. S3 and Table S5). After 14 days of incubation at 4 °C no viable virus could be detected in the non-treated samples, which were incubated in medium.

3.4. Inactivation of CSFV and ASFV (D-values)

D-value is the time required in days to reduce the virus titre by one $\log_{10}$.

For CSFV, treatment with NaCl resulted in D-values ranging from 18.3 days (4 °C) to 4 days (25 °C) for jejunum and 18.1 days (4 °C) to 3 days (25 °C) for colon (Table 2). After treatment with P-salt, the detection limit was reached at all temperatures at 7 days of incubation. For regression analysis, the detection limit of 1.7 $\log_{10}$ TCID₅₀/gram was therefore used as the maximum titre at that time point. This

resulted in worst case (maximum) D-values of 3.5 and 3.4 days (all temperatures) for jejunum and colon respectively (Table 2).

Overall, inactivation in the colon was somewhat faster achieved than in the jejunum (Table 2).

For ASFV, treatment of all jejunum samples with NaCl resulted in mean D-values ranging from 5.5 $\pm$ 2.4 days at 4 °C to 0.8 $\pm$ 0.1 days at 20 °C (Table 2). At 4 °C, mean D-values of 1.9 $\pm$ 1.2 and 2.7 $\pm$ 1.1 days were found after treatment with P-salt and medium, respectively (Table 2). Regression equations are indicated in the Supplementary Table S6.

3.5. Comparison 3D collagen matrix model

For CSFV, the D-values found for the NaCl and P-salt treatments in the 3D collagen matrix model were in general comparable or higher than in the intestines obtained from the animal experiment for all tested temperatures (Table 2 and Fig. 2).

Because the CSFV infected intestines were not tested with medium, no comparison could be made with the non-treated results of the 3D collagen matrix model.

Also for ASFV, in general, comparable or higher D-values were obtained for both salt treatments in the 3D collagen matrix model than the intestines at all temperatures (Table 2 and Fig. 3). Non-treated intestine samples, tested only at 4 °C, showed a substantially lower D-value than obtained with the 3D collagen matrix model. Regression equations of the 3D collagen matrix model are indicated in the supplementary Table S6.

4. Discussion

Casings represent a highly valued and globally used food commodity and are considered fit for human consumption. In 2011, the 3D collagen matrix model, mimicking natural casings, was developed to study the inactivation kinetics of contagious animal viruses *in vitro* (Wieringa-Jelsma et al., 2011). The motivation to develop this model was not just driven by ethical or economic reasons, but also by being able to establish a consistent experimental model that would allow for the comparison of the inactivation of different animal viruses possibly present in casings. However, the available methods to achieve the intended inactivation are limited and must comply with food safety requirements. By replacing the varying intra / inter animal response to a clinical infection with a consistent *in vitro* model, allowing high-titre worst-case scenarios, suitable and effective treatments can become possible for casings.

In 2012, in a scientific opinion, EFSA recommended to validate this model by *in vivo* experiments, leading to the current study (EFSA, 2012). Our results demonstrated an efficient inactivation of CSFV and ASFV present in porcine intestines by NaCl and P-salt in a temperature dependent way, showing a faster inactivation at the higher temperatures. The obtained D_{intestine} values of both viruses were in line with the previous results obtained with the 3D collagen matrix model, which demonstrates the suitability of this model to study the inactivation kinetics of multiple viruses without the need of animal experiments. As a result, information obtained from this model can be used for evaluating risk mitigation measures to control the animal health risks posed by the

Table 2

Decimal reduction values determined by regression analysis of the results of the CSFV and ASFV inactivation by NaCl, P-salt and medium at different temperatures.

Temp	CSFV Jejunum		CSFV Colon		CSFV j&c		CSFV 3 D Collagen		ASFV-2		ASFV-3		ASFV-4		ASFV all		ASFV 3D Collagen	
	D (d)	R ²	D (d)	R ²	mean D (d)	SD	D (d)	R ²	D (d)	R ²	D (d)	R ²	D (d)	R ²	mean D (d)	SD	D (d)	R ²
NaCl																		
4°C	18.3	0.9	18.0	0.7	18.2	0.2	36.1	0.7	8.2	0.7	4.4	0.9	3.8	0.9	5.5	2.4	10.1	0.4
12°C	20.9	0.5	12	0.6	16.5	6.3	10.0	0.9	1.9	0.9	1.3	NA	0.9	NA	1.4	0.5	0.9	NA
20°C	7.6	0.7	3.4	NA	5.5	2.9	5.1	0.9	0.9	NA	0.7	NA	0.8	NA	0.8	0.1	0.9	NA
25°C	3.5	NA	3.4	NA	3.5	0.0	2.7	0.9	nd	nd	nd	nd	nd	nd	nd	nd	0.9	NA
P-salt																		
4°C	3.5	NA	3.4	NA	3.5	0.0	8.1	0.9	1.9	0.7	0.7	NA	3.1	0.7	1.9	1.2	0.9	NA
12°C	3.5	NA	3.4	NA	3.5	0.0	6.0	0.7	nd	nd	nd	nd	nd	nd	nd	nd	0.9	NA
20°C	3.5	NA	3.4	NA	3.5	0.0	2.7	0.7	nd	nd	nd	nd	nd	nd	nd	nd	0.9	NA
25°C	3.5	NA	3.4	NA	3.5	0.0	1.2	NA	nd	nd	nd	nd	nd	nd	nd	nd	0.9	NA
non-treated																		
4°C	nd		nd						3.7	0.7	1.5	0.9	3.0	0.7	2.7	1.1	44	0.3

D (d), D value in days; R², correlation coefficient; j&c, jejunum & colon; NA, not applicable; nd, not done.

spread of contagious viruses through the international trade of natural sausage casings.

The choice to use a particular CSFV or ASFV strain in this study was based on several criteria, involving availability and technical experience within the institute, a balance between higher and lower virulence of a particular strain, allowing sufficient time to develop clear disease symptoms and viremia, and epidemic relevance. The CSFV Paderborn 277 strain was well documented (Greiser-Wilke et al., 2000) and already included in the 3D collagen model study (Wieringa-Jelsma et al., 2011). The strain was chosen for this study to enable a direct comparison between the results of both studies mentioned above. The ASFV Georgia 2007/1 strain was chosen because it is similar to the strain currently infecting housed pigs and wild boar in Europe and Asia. It is confirmed as being a highly virulent strain of ASFV (Chapman et al., 2011; EFSA, 2010).

To measure inactivation of virus in the intestines, two important inclusion criteria were set at the start of the study; 1) virus titres should be high enough to measure a virus reduction of at least 2 log₁₀ TCID₅₀/gram, and 2) samples taken throughout the intestine section (D0 samples) should contain similar virus loads (within 1 log₁₀). In most cases, the results did meet both criteria, with adequate viral loads evenly distributed over the entire length of the intestines. In literature, hardly any studies describing viral loads in intestines are available. In one study, a CSFV titre of 6.8 log₁₀ TCID₅₀/gram was found in the ileum of an infected pig 25 days post infection (Wood et al., 1988). While in another study, the titres found in ileum, duodenum and colon ranged between 2.9 and 3.8 log₁₀ TCID₅₀/ml (Kamolsiriprichaiporn et al., 1992). Other studies have quantified viral loads of CSFV present in either of the following intestine sections; duodenum, jejunum, ileum and colon. However, due to the different methods of quantification (qPCR: viral genetic load or virus load ratios; Antigen-ELISA: S/N ratio's) these results were difficult to compare with the results obtained in present study where virus titres were expressed in TCID₅₀ (Liu et al., 2011; Ophuis et al., 2006; Shannon et al., 1993). Maximum virus titres of ASFV, ranging from 5.9 to 6.2 log₁₀ HAD₅₀/gram, were found in the mucosa of the ileum, caecum and colon of infected pigs, six days after infection (Plowright et al., 1968). Although these previous studies indicated that CSFV and ASFV were present in the porcine intestines, a homogenous distribution of the viral load of both viruses throughout the intestines, necessary to measure virus inactivation, has not been demonstrated before.

After regression analysis, most R² values were high enough to confirm that the data were fit for linear regression. The lowest R² value (0.5) was obtained with the NaCl-treated CSFV jejunum samples incubated at 12 °C, and the residual plot showed a slight under-prediction at days 7 and 14 and over-prediction at day 30 (data not shown). This might explain the differences in D-values between the jejunum (20.9) and 3D collagen matrix model (10.0) at this temperature. Performing non-linear regression (one phase decay) on these particular data resulted in a D-value of 8.3, calculated from the obtained half-life value. However, in order to make the comparison with the previous obtained D-values, which were all obtained by linear regression, the data were

found fit enough for linear regression.

Variation was observed between the individual ASFV infected pigs, with D-values ranging from 8.4 to 3.8 days for three pigs and non-detectable virus titres in the intestines of one pig. Of the CSFV-infected pigs, only one out of two pigs showed virus titres in the intestines above the detection limit, despite the time point of euthanasia at the peak of viremia. Variation between individual animals was also shown in a previous study, investigating the efficacy of NaCl and P-salt on FMDV infected porcine and sheep intestines (Wijnker et al., 2007). Although the *in vitro* model was tested only once, these factors show that testing virus inactivation by *in vivo* experiments, were more prone to variation. These findings emphasize the advantages of 3D collagen matrix model, e.g. the possibility to start with a known virus titre high enough to measure at least 2–3 log₁₀ reduction.

Very limited information is available about the CSFV and ASFV inactivation by NaCl in natural casings. Salted and standard processed casings infected with CSFV were inactivated within 17 days (Helwig and Keast, 1966). In contrast, in another study using infected natural casings treated with saturated NaCl brine and storage at 4 °C, CSFV and ASFV survived for at least 147 days and 97 days, respectively and were able to successfully infect pigs after feeding (McKercher et al., 1980). Unfortunately, no initial titres were shown in both publications and therefore, it was not possible to estimate D-values. In order to mimic a worst-case scenario and achieving the highest titres possible, samples in this study contained the full thickness of the intestinal tract, including all tissue layers present. Based on the upper limit of the mean D-values (mean ± SD) after NaCl treatment, an infected casing with a starting titre of 4 log₁₀ TCID₅₀/gram stored at 4 °C would be predicted to contain viable CSFV and ASFV for a period of 73 (4 × 18.3) and 30 (4 × 7.6) days, respectively. While at 20 °C, this would be predicted at 34 (4 × 8.4) and 4 (4 × 0.9) days for the CSFV and ASFV infected intestines, respectively. These results are more or less in line with the previous study in 1980, showing a faster inactivation by salt of the ASFV infected casings compared to the ones infected with CSFV (McKercher et al., 1980).

Over the years, the global trade in porcine natural casings has only received limited attention from policy makers or risk managers, in terms of concrete trade requirements. As a result no specific requirements were ever developed or included for casings in regard to ASFV and CSFV by competent authorities until recent in the OIE Terrestrial Animal Health Code (Article 15.1.24). The growing concern due to the recent outbreaks around the globe and the theoretical possibility of involvement of casings in spreading either disease, prompted this study in an attempt to quantify the actual risk involved in global casing trade. However, the standard practice applied by the international casing industry of salting casings with NaCl for at least 30 days has been in place for many years and its efficacy against bacterial agents and other specific viruses has been well documented (ISWG, 2014). This minimum salting period has been included as a standard requirement in various casing trade certificate (European Commission, 2003). The 30-day period is considered sufficient to reduce any contamination present to an acceptable low level as part of a public / animal health risk

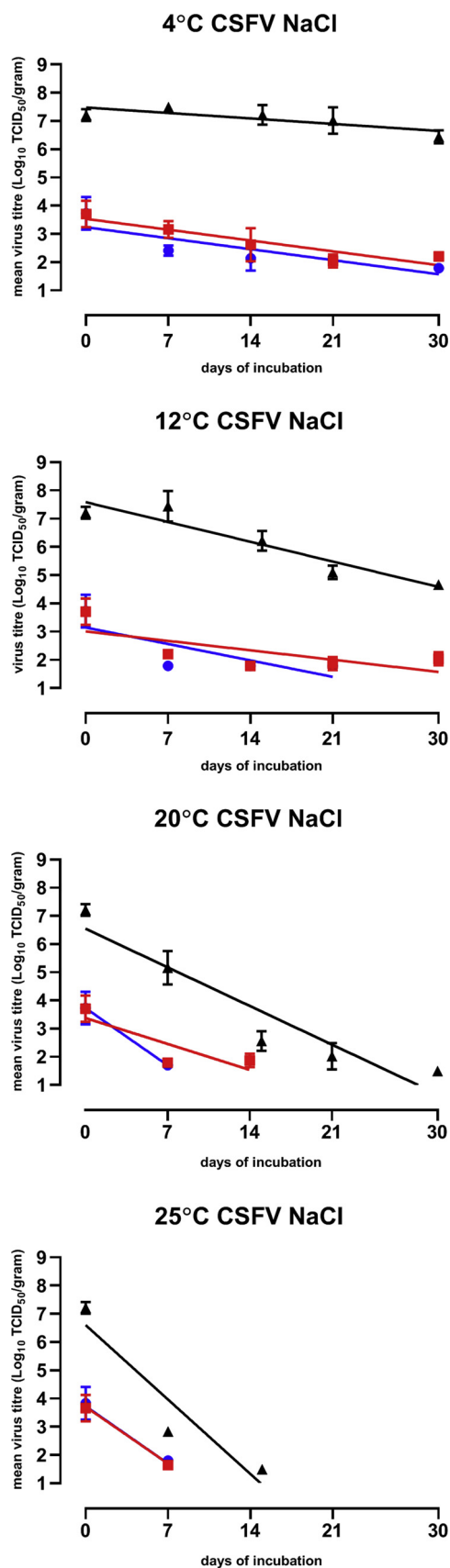


Fig. 2. Comparison of mean titres and trend lines of CSFV jejunum (red square) and colon (blue dot) versus the collagen model (black triangle). Error bars indicate SD values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

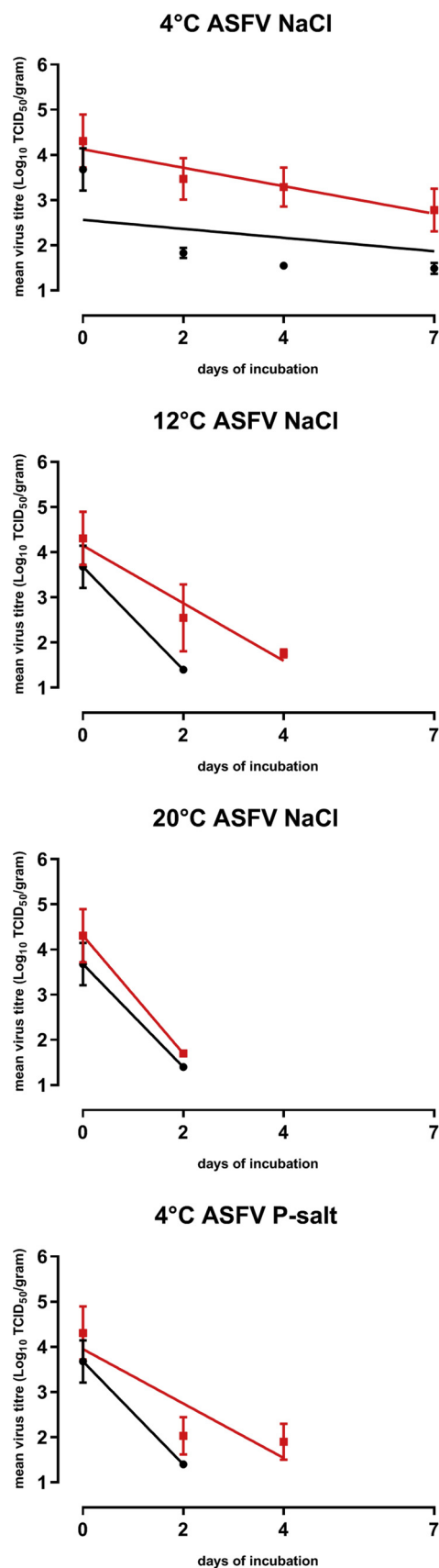


Fig. 3. Comparison of the mean titres and trend lines of ASFV jejunum (red square) versus the collagen model (black dot). Error bars indicate SD values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

reduction. Any time spent in transit of a shipment, via land, sea or air, should not be considered to be part of this 30-day period.

In two recent studies, survival of ASFV in natural pork casings in a transboundary shipping model was studied (Stoian et al., 2019 in Press; Dee et al., 2019, 2018). Commercially available pork casings were spiked with ASFV and half-life estimates between 4.4 and 13.1 days were determined after incubation under conditions simulating transoceanic shipment. Here it should be noted that regular natural casings will always be treated with NaCl for at least 30 days before shipment (ENSCA, 2019). Because, in this study casings were spiked without the standard salt treatment it was not possible to look at these results as if they were obtained from a standard salt-treated infected casing. Recently, survival of ASFV in dry-cured meat products was described. Here salami was prepared with salted and spiced minced meat derived from ASFV infected pigs with at titre of approx. 4–4.5 Log₁₀ HAD₅₀/gram (= equal to TCID₅₀/gram) packed in non-edible casings and processed according to the standard procedures at temperatures not below 10 °C (Petrini et al., 2019). When 50 g was orally administered to susceptible pigs, none of the pigs became infected after 26 days of processing the salami, which observation is in agreement with the storage time of 30 days in NaCl at temperatures of ≥ 4 °C for natural casings.

Swill feeding is considered to be one of the major causes of the ASFV spreading in Eastern Europe and Asia (China and Vietnam) (Dixon et al., 2019). In addition, also CSFV can be spread by swill feeding pigs held in back-yard farms (Blome et al., 2017). Because of this relevant transmission route, it is important to determine the minimal viral load, in contaminated pork products, needed to infect pigs by ingestion. After feeding blister packs containing CSFV doses ranging from 10¹ to 10⁶ TCID₅₀, the lowest infectious doses of two CSFV strains UK2002/7.1 and Brescia to infect 50% of pigs via the oral route were determined at 10^{3.6} and 10^{5.1} TCID₅₀, respectively (Cowan et al., 2015). Recently, the lowest median infectious dose to infect 50% of pigs orally with the of ASFV strain Georgia 2007/1 was estimated to be 10^{4.6} TCID₅₀ in plant-based feed (Niederwerder et al., 2019). Using the obtained upper limit of the mean D-values (mean plus SD) a dose of 100 g of standard processed casings with an initial CSFV viral load of 4 log₁₀ TCID₅₀/gram, as found in the present study, would be predicted to contain 4.4 log₁₀, 4.7 log₁₀, 2.4 log₁₀ TCID₅₀ and zero when stored at 4 °C, 12 °C, 20 °C and 25 °C for 30 days, respectively. Likewise, the predicted doses of 100 g of ASFV infected casings with an initial titre of 5 log₁₀ TCID₅₀/gram would be 3.2 log₁₀ TCID₅₀ at 4 °C and zero at temperatures ≥ 12 °C, due to the predicted 16 log₁₀ and 33 log₁₀ reductions after storage for 30 days at 12 °C and 20 °C, respectively. With the exception of CSFV intestines stored at 4 °C and 12 °C, all predicted doses for CSFV and ASFV showed titres below the previous found infectious doses to initiate infection in 50% of the pigs after ingestion. As mentioned, the above doses were predicted for the consumption of 100 g of natural casings, however, in general, when a sausage is consumed, the casing contributes to only 2–5% of the total weight. Nevertheless, regarding the CSFV results, for standard processed casings originating from countries where CSFV is endemic and stored at temperatures below 20 °C in NaCl, an increase in storage time is highly recommended. Prolonged (> 3 months) exposure of porcine casings to temperatures higher than 20 °C has a detrimental effect on the quality and usability of these casings for sausage production (Bakker et al., 1999). As casings are produced, shipped and used in sausage production on a global scale instead of regional or national, a lower storage temperature (< 20 °C) is highly recommended. An extended, uninterrupted, mandatory storage period at temperatures below 20 °C could be considered to allow for the export of porcine casings from CSFV endemic areas, whilst applying regular sodium chloride (NaCl) as main preservative. This would prevent trade disruption for a commodity with a global demand.

The efficacy of P-salt was determined in a previous study where P-salt treated CSFV infected natural casings were fed to piglets. None of the piglets became infected after receiving CSFV infected casings

treated with P-salt at 4 °C or 20 °C for 30 days (Wijnker et al., 2008a). In the 3D collagen matrix model, the efficacies of NaCl and P-salt solutions were compared, resulting in a faster virus reduction with the latter treatment (Wieringa-Jelsma et al., 2011), consistent with the results in the present study using CSFV infected intestines. Besides the results of the 3D collagen matrix model, no previous studies concerning the inactivation of ASFV by P-salt exist. Comparable D-values at 4 °C were obtained, and both the ASFV intestines and 3D model results showed a faster inactivation with P-salt than with NaCl. However, because P-salt is quite difficult to acquire due to a limited availability, problematic logistics and additional costs, treatment with NaCl remains the standard procedure for preserving natural casings.

Although most D-values were comparable, the non-treated ASFV intestine samples at 4 °C showed a much lower D-value than the D-value obtained with the collagen model. Most likely, the ongoing decomposition process within the intestine affected the virus reduction in these intestine samples, despite the added antibiotics and anti-fungal ingredients. Most likely, enzymatic reactions, induced by lipases, may have disrupted the viral envelope causing a rapid inactivation of ASFV, as has been postulated for the survival of CSFV in fat (Cowan et al., 2015). This decomposition process is not present in the 3D collagen matrix model, therefore the survival of ASFV in the non-treated samples obtained in the collagen model represented a worst case scenario.

In conclusion, this study provided information about the virus titres and virus distribution in the intestines of CSFV and ASFV infected pigs. Virus inactivation by salt, routinely used when processing natural casings, resulted in the estimation of decimal virus reduction values at different temperatures. These findings enabled a validation of the previous developed 3D collagen matrix model, showing comparable D-values. Therefore, this study showed the suitability of the 3D collagen matrix model to investigate the inactivation of viruses relevant for the natural casing industry without the need of animal experiments. To further validate this model, experiments will be planned to test reproducibility.

Acknowledgements

The authors would like to thank the International Scientific Working Group (ISWG) of the International Natural Sausage Casings Association (INSCA) for funding this study. The authors are also very grateful to Mieke Maris-Veldhuis, Sophie van Oort and Nora Gerhards for assisting during the post-mortem examinations and laboratory testing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetmic.2019.108424>.

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