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The risk and mitigation of foot-and-mouth disease virus infection of pigs through consumption of contaminated feed

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Abstract

Transboundary movement of animal feed and feed ingredients has been identified as a route for pathogen incursions. While imports of animals and animal-derived products are highly regulated for the purpose of infectious disease prevention, there has been less consideration of the viability of infectious agents in inanimate products, such as feed. This study investigated the ability of foot-and-mouth disease virus (FMDV) to remain infectious as a contaminant of commercial whole pig feed and select pig feed ingredients, and to establish the minimum infectious dose (MID_F) required to cause foot-and-mouth disease (FMD) in pigs that consumed contaminated feed. FMDV viability in vitro varied depending on virus strain, feed product, and storage temperature, with increased duration of infectivity in soybean meal compared to pelleted whole feed. Specifically, both strains of FMDV evaluated remained viable through to the end of the 37 day observation period in experimentally contaminated soybean meal stored at 4 or 20°C . The MID_F for pigs consuming contaminated feed varied across virus strains and exposure duration in the range of $10^{6.2}$ to 10^7 $TCID_{50}$. The ability of FMDV to cause infection in exposed pigs was mitigated by pre-treatment of feed with two commercially available feed additives, based on either formaldehyde (SalCURB®) or lactic acid (Guardian™). Our findings demonstrate that FMDV may remain infectious in pig feed ingredients for durations compatible with transoceanic transport. Although the observed MID_F was relatively high, variations in feeding conditions and biophysical characteristics of different virus strains may alter the probability of infection. These findings may be used to parameterize modelling of the risk of FMDV incursions and to regulate feed importation to minimize the risk of inadvertent importation.

KEYWORDS
contamination, feed, foot-and-mouth disease, foot-and-mouth disease virus, pig

1 | INTRODUCTION

Foot-and-mouth disease (FMD) is a viral disease of cloven-hooved animals that is caused by FMD virus (FMDV; genus: *Aphthovirus*, family Picornaviridae), and is considered one of the most socio-economically important diseases of livestock (Arzt et al., 2011; Grubman & Baxt, 2004). Despite its generally low mortality (Arzt et al., 2011), FMD has the potential to cause substantial economic losses through decreased production, culled animals, and loss of export markets (Knight-Jones & Rushton, 2013). Pigs have been implicated as the index cases of multiple major FMD outbreaks (Gibbens et al., 2001; Huang et al., 2000; Park et al., 2018), and their ability to spread the virus is potentiated by shedding high levels of aerosolized virus during the infectious period compared to ruminants (Donaldson et al., 2001; Sellers, 1971). The susceptibility of pigs to FMDV infection via the upper gastrointestinal tract (oropharyngeal mucosa) (Fukai et al., 2015; Stenfeldt et al., 2016) makes them potentially vulnerable to infection through contaminated feed. This vulnerability to FMDV infection via the oral route, combined with the potential to disseminate virus efficiently via aerosol, underscores the importance of determining the risk of, and appropriate mitigation strategies for potential FMDV incursions via contaminated pig feed.

Although it has traditionally been conjectured that a greater dose of FMDV is required to establish infection in pigs compared to ruminants, there is scant quantitative data on the minimum infectious dose (MID) of FMDV by natural routes. The most recent treatment of the subject is a review published by Sellers (1971) that summarized the knowledge of the time, and concluded that although pigs could be infected by as little as 10^1 infectious doses (ID) by intradermal injection, as much as 10^5 ID was required to cause FMD by ingestion of infected material (offal). Furthermore, the original work referenced by Sellers describes an experimental study in which only 2 of the 30 pigs were confirmed to have been infected from ingestion of the infectious material (Henderson & Brooksby, 1948).

Experimental studies have shown that primary FMDV infection in pigs occurs in the oropharynx, within tonsil crypt epithelium, directly adjacent to mucosa-associated lymphoid tissue (MALT), where the submucosal lymphoid follicles are closely associated with the epithelium (Stenfeldt, Diaz-San Segundo, et al., 2016). This is a distinction from cattle which have been shown to be infected via the nasopharynx in the upper respiratory tract (Arzt et al., 2010; Stenfeldt et al., 2015; Stenfeldt et al., 2018). The time elapsed between virus exposure and onset of clinical disease varies with virus strain, experimental design, and exposure dose but has been shown to be as little as 24 h (Arzt et al., 2011). Clinical signs include vesicles on the coronary bands, oral cavity, and snout, increased body temperature, and mild to severe lameness. Onset of clinical signs is also associated with viremia and substantial shedding of virus in oral and nasal secretions (Stenfeldt, Diaz-San Segundo, et al., 2016).

Due to the well-established risk of pathogen incursion through feeding swill (food waste), which sometimes includes raw animal products, the practice is legal in only 28 US states where law requires that the swill is heat-processed by licensed producers ("USDA Swine Health Protection Rule,", 2017). Less is known about the potential risks associated with processed commercial feeds or feed ingredients, much of which is imported, introducing the risk of viral contamination before or during transport (Jones et al., 2020). It is theorized that Porcine Epidemic Diarrhea Virus (PEDV), an alpha-coronavirus, was introduced to the United States in 2013 through imported contaminated feed or feed ingredients (A. Scott et al., 2016), causing an outbreak that led to an estimated $900 million to $1.8 billion decline in US economic welfare (Paarlberg, 2014). The suspected association between the PEDV incursion and legal importation of commercial pig feed products has raised concerns about the possible introduction and spread of other viral pig diseases, including FMD, via this route. Recent studies have investigated the ability of various pathogens to remain infectious in feed ingredients for longer durations that simulate transit from sites of origin in Asia or Europe (Dee et al., 2018; Niederwerder et al., 2020; Niederwerder et al., 2019). One study investigated the viability of various viral pig pathogens in feed matrices under simulated transoceanic transport conditions, demonstrating that 7 of the 11 viruses studied remained infectious through transport conditions in at least one feed ingredient, but were inactivated during similar simulation in the absence of a feed matrix (Dee et al., 2018). This finding demonstrates the ability of feed ingredients to provide a suitable environment for extended viral viability and some degree of protection from the environment, reiterating the need to consider feed contamination when implementing effective biosecurity measures.

Due to the requirement of high containment laboratory facilities for the study of FMDV, this pathogen has been excluded from previous studies investigating the viability of high-consequence viral pathogens of pigs in feed products. As a surrogate for FMDV, *Senecavirus* A (SVA), another picornavirus, has been utilized in prior studies with a demonstrated viability of up to 37 days in 10 of the 12 investigated products including conventional soybean meal, distillers' dried grains with solubles (DDGS), and complete pig feed (Dee et al., 2018).

The objective of the current study was to investigate the risk of FMDV infection in pigs through natural consumption of contaminated feed. The viability of two distinct strains of FMDV representing serotypes O and A was evaluated in vitro in pelleted whole pig feed, soybean meal, and DDGS, and the MID_F of both virus strains was established in pigs exposed to virus by natural feeding. Additionally, the ability of select commercially available feed additives (mitigants) to inactivate FMDV and prevent infection was investigated through in vitro and in vivo experimentation.

2 | METHODS

2.1 | Viruses

Two FMDV strains were used for the studies described herein: FMDV A24 Cruzeiro and FMDV O/SKR/2010. Detailed information regarding the generation of the original virus stocks has been published previously (Pacheco & Mason, 2010; Pacheco et al., 2016). In brief, both viruses were originally cattle derived. The FMDV A24 virus had been

passaged in pigs for adaptation to this host species, whereas the FMDV O/SKR/2010 had been amplified in cattle. For in vitro experiments, the animal-derived viruses were further amplified through one passage in LFBK-$\alpha v\beta 6$ cells (LaRocco et al., 2013, 2015). The titers of the virus stocks (animal derived as well as cell culture passaged) were determined through end-point titrations using LFBK-$\alpha v\beta 6$ cells (LaRocco et al., 2013, 2015).

2.2 | FMDV viability in contaminated feed and feed components

FMDV viability in vitro was determined for two virus strains: FMDV A24 and FMDV O/SKR/2010, in three substances: pelleted whole pig feed, soybean meal, and distiller's DDGS. The whole feed blend used through the experiments was a commercial low-energy, high-fibre feed blend optimized for laboratory pigs (Lab Diet catalog #5081, St Lois, MO, USA). The feed blend was based on ground oats, dehydrated alfalfa meal, wheat middlings, and soybean meal, and did not contain any animal-derived products. To avoid microbial contamination of the cell culture used for virus titrations, all feed substances were treated by gamma irradiation (25–40 kGy) by a certified vendor prior to the experiments. Aliquots (5 g) of each substance were placed in 50 mL conical tubes and contaminated with 10^5 $TCID_{50}$ FMDV diluted in 1 mL of media (Dulbecco's minimal essential media with 25 mM HEPES, antibiotics and antimycotics (Sigma-Aldrich A5955) and Na pyruvate) at time point 0. Conical tubes were stored at 4 or 20°C and FMDV viability was measured in duplicate aliquots (two from each storage temperature) at 1 h, as well as 1, 3, 7, 14, 21, and 37 days post contamination (dpc). Experimental controls consisted of 5 mL aliquots of media contaminated and stored under similar conditions as the evaluated feed substances. At the time of "harvest," the remaining virus was recovered by addition of 15 mL media to conical tubes with feed substances followed by mixing by vortexing and two rounds of centrifugation (10 min at 1000G and 10 min at 2000G) with transfer of retrieved media to clean conical tubes in between centrifugations. Virus titers in the recovered media were determined by end-point titration using LFBK-$\alpha v\beta 6$ cells. Media controls were titrated without further processing. Due to toxicity to cells at lower dilutions of feed substances, titers lower than $10^{2.6}$ $TCID_{50}$ could not be measured. To confirm the presence or absence of infectious virus, samples without measurable titers were subjected to virus isolation (Dee et al.) on LFBK-$\alpha v\beta 6$ grown in T25 flasks for which the lower feed substance to cell ratio abrogated toxicity.

2.3 | Evaluation of select feed additives (mitigants) for reduction of FMDV viability in pig feed

Additional in vitro experiments were performed to evaluate the effect of select commercially available feed additives in reducing infectivity of FMDV in whole pig feed under controlled experimental conditions. Due to poor viability of FMDV O/SKR/2010 in whole pig feed, the evaluation of mitigants was performed using FMDV A24 only. The experiments were performed following a similar approach as the in vitro FMDV viability experiments, with the exception that batches of pig feed were pre-treated with the mitigants 24 h prior to virus contamination. Evaluated mitigants were: Sal CURB® ASF liquid (Kemin industries Inc. Des Moines, IA, USA); a formaldehyde-containing mixture approved for mitigation of Salmonella and mould growth in pig feed for up to 21 days, CaptiSURE™ (Kemin industries Inc. Des Moines, IA, USA); a proprietary medium chain fatty acid mixture, and Guardian™ (Alltech Inc, Nicholasville, KY, USA); a lactic acid-based acidifying feed additive. The mitigants were added to batches of 500 g gamma-irradiated pig feed at inclusion rates recommended by the producers: 0.33% for Sal CURB®, 1% for CaptiSURE™, and 0.44% for Guardian™ (by weight). Liquid additives (Sal CURB® and CaptiSURE™) were added to the feed by use of small fingertip spray bottles to ensure an even distribution. The spray bottles were weighed before and after application, and the treated feed was thoroughly mixed during and after application. Guardian™, which is in powder form, was added by weight and similarly mixed with the pelleted feed. Treated feed was stored at room temperature for 24 h and was subsequently divided into 5 g aliquots that were contaminated with FMDV A24 and stored and analyzed as described above.

2.4 | Half-life calculations

Viral half-life was calculated for all in vitro samples (with and without mitigants) using a previously published approach (Stoian et al., 2019). A linear regression was fitted to each graph of log10 of viral titer versus time using GraphPad Prism. The slope of this line was used to calculate half-life using the equation: $-\log(2)/\text{slope}$. The standard error (SE) of each half-life was calculated using the SE of the slope with the equation: $SE_{slope} * \log(2)/Slope^2$. The upper and lower bounds of a 95% confidence interval (Niederwerder et al.) were calculated as half-life $\pm$ SE*t where t is the critical value of Student's t-distribution with a 0.025 quantile and $n-2$ degrees of freedom, with n being the number of observations. For data sets that had multiple timepoints equivalent to the lowest limit of detection (feed ingredient/mitigant groups) or at zero (controls), only the first of these timepoints was included in the analysis.

2.5 | Animals and animal experiments

Animal experiments were carried out within BSL3Ag research facilities at the Plum Island Animal Disease Center, New York. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) that functions to ensure ethical and humane treatment of animals (protocol 259.02-18-R). The animals used were castrated male Yorkshire pigs, approximately 12 weeks old (20–25 kg) at delivery. The pigs were allowed 2 weeks of acclimation in the facility prior to the start of experiments.

TABLE 1 Summary of experimental designs and outcomes of in vivo MID_F studies

Virus	Estimated dose† ($TCID_{50}$)	Dose added to feed or inoculum‡ ($TCID_{50}$)	Route	Outcome
FMDV A24				
	10^4	$10^{4.44}$	Single feeding (100 g)	No FMD
	10^5	$10^{5.44}$	Single feeding (100 g)	No FMD
	10^6	$10^{6.19}$	Single feeding (100 g)	No FMD
	10^7	$10^{7.19}$	Single feeding (100 g)	No FMD
	10^6	$10^{5.98}$	Three feedings (100 g each)	No FMD
	10^7	$10^{6.98}$	Three feedings (100 g each)	FMD
	$10^{8.5}$	$10^{8.1}$	Three feedings (100 g each)	FMD
FMDV O/SKR/2010				
	10^3	$10^{4.23}$	Three feedings (100 g each)	No FMD
	10^4	$10^{5.10}$	Three feedings (100 g each)	No FMD
	10^5	$10^{5.98}$	Three feedings (100 g each)	No FMD
	10^5	$10^{6.23}$	Three feedings (100 g each)	FMD
	10^6	$10^{7.23}$	Three feedings (100 g each)	FMD
	10^6	$10^{7.23}$	Three feedings (100 g each)	FMD
	10^7	$10^{8.1}$	Three feedings (100 g each)	FMD§
	10^7	$10^{8.35}$	Three feedings (100 g each)	FMD
	10^8	$10^{8.6}$	Three feedings (100 g each)	FMD

†Estimated dose represents the target dose per pig that was used to calculate dilution of the original virus stock.
‡Dose added to feed or inoculum represents the total dose per pig based on titration of the diluted inoculum.
§This group consisted on only two pigs.

2.6 | Minimum infectious dose experiments

Five in vivo experiments were performed to determine the MID_F of FMDV A24 and O by feeding; two experiments using FMDV A24, and three with FMDV O/SKR/2010 (Table 1). Each experiment contained three or four study groups with four pigs per group with the exception of one FMDV O/SKR/2010 group that contained only two pigs. All remaining study groups were exposed to a predetermined dose of FMDV mixed into a pre-measured portion of pig feed. In experiment 1 (FMDV A24), the pigs received the predetermined virus dose mixed into one portion of 100 g feed per pig. In all subsequent experiments, the virus dose was divided across three separate 100 g portions of feed that were fed consecutively through a period of approximately 2 h, with approximately 20–30 min between each feeding. At the time of challenge, the pigs had not been fed for 18–24 h. After consumption of the FMDV-spiked feed portions, the pigs received the remainder of their normal daily feed ration. Water was available ad libitum throughout the experiments. Virus stocks were diluted in minimum essential media (MEM) with 25 mM HEPES based on the initially determined titers of the stocks. The titers of the diluted virus inoculums were subsequently confirmed through additional titrations on LFBK-$\alpha v\beta 6$ cells. The virus doses reported for the study groups represent the confirmed titers of the diluted virus inoculums that were subsequently added to the feed. Based on this, the exposure doses ranged from $10^{4.4}$ to $10^{8.1}$ $TCID_{50}$ for FMDV A24, and $10^{4.2}$ to $10^{8.6}$ for FMDV O/SKR/2010, with an estimated 10-fold difference in virus doses between study groups (Table 1).

The pigs were individually fed their ration(s) from a clean plastic dish. Each portion consisted of 100 g pelleted feed, with 10 mL of diluted virus added to each portion. Thus, for experiment 1, the total virus dose was diluted in 10 mL per pig, whereas for subsequent experiments, the virus dose was diluted in 30 mL per pig and split into 10 mL aliquots that were added to the feed portions directly prior to feeding.

Each study group was housed in a separate isolation room, and free contact was allowed between individuals within the same group. Due to the high likelihood of contact transmission from an infected individual to the remaining pigs in the group, only the pigs from which FMDV-positive samples were obtained at the earliest detection of infection within the group could be conclusively determined to have been infected by contaminated feed, and were thus designated as "primary cases." Infections that were detected at later time points were designated "secondary cases," because infection from contact with the primary case(s) could not be ruled out. For the purposes of this study, the earliest detection of infection was defined as the first OP swab sample that contained detectable FMDV RNA, provided that individual also became viremic and/or developed clinical FMD at a subsequent timepoint. Because of the variable timing of infections, some pigs were considered infected on the basis of antemortem sampling but did not develop clinical disease before euthanasia. Pigs were euthanized 1 or 2 days after any individual in the group developed signs of

clinical FMD due to the inability to discriminate between primary infection caused by feed exposure versus within-group contact transmission after that point. Lack of infection in pigs from which no positive samples were obtained was confirmed by virus neutralization assays using serum samples obtained at 14 days post exposure (dpe).

2.7 | Feed additive experiment

One in vivo experiment was performed to investigate the ability of three feed additives, consisting of two commercial mitigants (Guardian™, Sal CURB®) and one common feed ingredient (DDGS), to reduce infectivity of FMDV in feed. Each of the three study groups was administered FMDV-spiked feed that had been pre-treated with each feed additive, and a fourth group received FMDV-contaminated feed with no additive to serve as a positive control. The amount of each commercial mitigant added to the feed was similar as had been evaluated in vitro, whereas the DDGS was included at 30% by weight. FMDV A24 was the only virus strain evaluated in this experiment due to the poor viability of FMDV O/SKR/2010 in whole feed in the in vitro experiments. The two mitigants were added to batches of 1350 g gamma-irradiated pig feed and incubated at 20°C for 24 h. After the incubation with mitigant, the feed was aliquoted into "pig portions" of 300 g. Similarly, aliquots for the DDGS study group consisted of 210 g irradiated whole feed with 90 g added DDGS, and aliquots for the pigs in the control group consisted of 300 g irradiated whole feed. A total dose of $10^{8.3}$ $TCID_{50}$ of FMDV A24 diluted in 5 mL of media was added to each "pig portion," which were subsequently incubated at 20°C for another 24 h. The prepared feed was fed to the four study groups as described for the MID experiments; that is, each 300 g pig portion was divided into 3 × 100 g portions that were consecutively fed to individual pigs with 20–30 min in between feedings.

2.8 | Clinical evaluation and sampling

A standardized protocol for sampling and clinical evaluation was followed in all experiments. Blood samples were collected from the jugular vein and oropharyngeal (OP) swabs were obtained through direct targeting of the tonsil of the soft palate using a large cotton swab. Swabs were immersed in 2 mL minimal essential media containing 25 mM HEPES directly upon collection. Blood samples were separated through centrifugation, and tonsil swabs were also centrifuged to extract the fluid absorbed by the cotton swab. All samples were frozen at −70°C until further processing.

Samples were collected prior to virus exposure or inoculation. After that, sampling was performed every other day for 10 days (days 2, 4, 6, 8, and 10) after exposure and again at 14 dpe. The progression of the clinical infection (lesion distribution) was quantitated using a previously described scoring system (Pacheco & Mason, 2010). In brief, each of 16 digits showing a characteristic FMDV lesion contributed one point towards a cumulative score, with additional single points counted for lesions within the oral cavity, on the snout, on the lower lip, and on carpal/tarsal skin for a maximum score of 20. Clinical examinations were performed daily until 8 dpe, and again at 10 and 14 dpe.

2.9 | FMDV RNA detection

Serum and swab samples were analyzed using quantitative real-time RT-PCR (rRT-PCR), targeting the 3D region of the FMDV genome (Callahan et al., 2002) with forwards and reverse primers adapted from (Rasmussen et al., 2003) following a previously described protocol (Stenfeldt, Pacheco, Smoliga, et al., 2016). To convert cycle threshold values to RNA genome copies per millilitre, serial 10-fold dilutions of strain-specific in vitro synthesized FMDV RNA of known concentration were analyzed by the same rRT-PCR protocol. The equation of the curve of RNA copy versus cycle threshold value was used for subsequent conversions.

2.10 | FMDV serology

FMDV-neutralizing antibody titers against FMDV A24 or FMDV O/SKR/2010 (depending on the challenge virus) were determined in serum samples obtained on the last study days for pigs that did not develop clinical FMD. Serum samples were heat-inactivated for 30 min at 56°C and analyzed in a microtiter neutralization assay. Serial four-fold dilutions of serum (in MEM with 25 mM HEPES) on 96-well plates were incubated with 100 $TCID_{50}$ of FMDV A24 or FMDV O/SKR/2010 for 1 h at 37°C and 5% CO_2. Freshly trypsinized LFBK-$\alpha V\beta 6$ cells were re-suspended in MEM with 25 mM HEPES, 4×10^4 cells/well were added to the plates, and the plates were incubated for another 72 h at 37°C and 5% CO_2. After microscopic evaluation of cell monolayers, the plates were treated with crystal violet dissolved in tissue fixative (HistoChoice®; AMRESCO, Solon, OH, USA), then washed and air-dried before cytopathic effect was again evaluated visually. Titers were calculated as the reciprocal of the highest dilution of serum that fully neutralized the virus in 50% of replicate wells.

3 | RESULTS

3.1 | FMDV viability in contaminated feed

The viability of FMDV A24 and FMDV O/SKR/2010 was evaluated in experimentally contaminated whole pig feed, soybean meal, and DDGS stored at 4 or 20°C for up to 37 days. FMDV viability varied greatly across substances and virus strains (Figure 1). For both virus strains, infectious titers remained unchanged for the longest duration in soybean meal stored at 4°C. Specifically, under these conditions, FMDV A24 titers remained stable until 14 days, and did not decline to the limit of detection by 37 days, while FMDV O/SKR/2010 titers remained largely unchanged through 37 days (Figure 1). The estimated half-life of FMDV A24 in soybean meal at 4°C was 199.4 ± 34.5 h (Table 2) but could not be appropriately estimated for FMDV O/SKR/2010 under

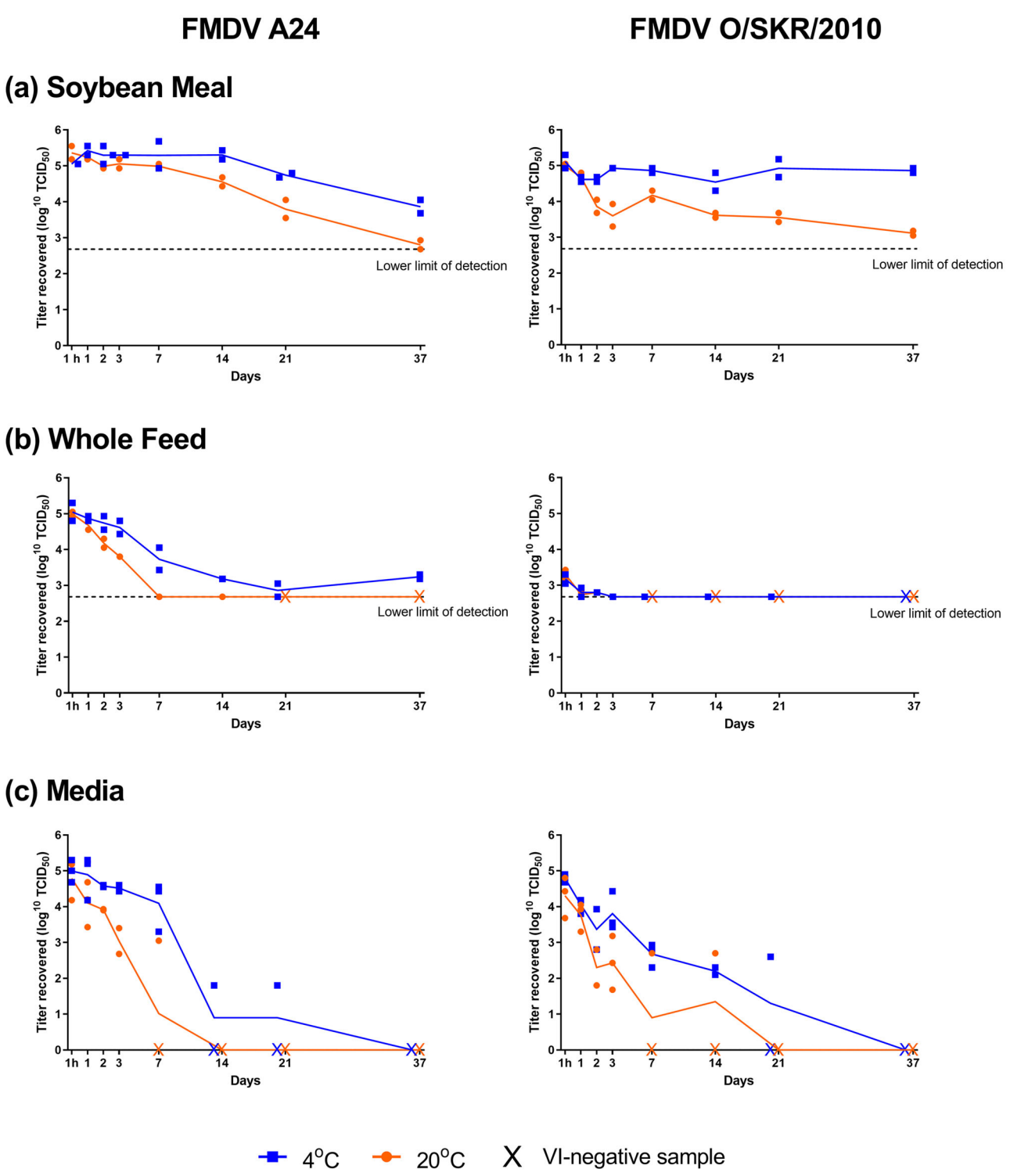

FIGURE 1 Duration of infectiousness of FMDV recovered from whole pig feed and soybean meal. Recovery of FMDV A24 (left) and FMDV O/SKR/2010 (right) from 5 g aliquots of (a) soybean meal, (b) pelleted whole feed, or (c) media, stored at 4°C (blue) or 20°C (orange) from 1 h to 37 days. Samples were contaminated with 10^5 $TCID_{50}$ of FMDV at time point 0. Data points represent titers recovered from duplicate samples for each time point, matrix, and temperature. The lower limit of detection ($10^{2.6}$ $TCID_{50}$) for recovery of virus from whole feed and soybean meal was a consequence of cell toxicity at lower dilutions of these samples. Samples that were at the lower limit of detection were analyzed for the presence or absence of FMDV by virus isolation (Dee et al.). Solid squares or circles indicate VI-positive samples; whereas VI-negative samples are marked by X

TABLE 2 Calculated half-lives of FMDV in feed components with and without added mitigants

Virus	Feed Component	Temperature (°C)	Half-life ± SE (h)	95% confidence interval (h)	Latest time point of FMDV detection[†] (days)
FMDV A24	Soybean meal	20	107.5 ± 6.4	93.8–121.3	37
	Soybean meal	4	199.4 ± 34.5	125.4–273.3	37
	Whole feed	20	21.8 ± 1.2	19.0–24.6	14
	Whole feed	4	131.5 ± 27.2	73.1–189.8	37
	DDGS	20	<1	N/A	N/A
	DDGS	4	<1	N/A	N/A
	Media (control)	20	13.4 ± 2.3	8.4–18.4	7
	Media (control)	4	45.9 ± 6.5	32.1–59.6	21
FMDV O/SKR/2010	Soybean meal	20	196.8 ± 49.0	91.7v301.8	37
	Soybean meal	4	N/A[‡]	N/A	37
	Whole feed	20	35.6 ± 15.2	−6.0–77.8	3
	Whole feed	4	44.7 ± 18.3	−6.1–95.4	21
	DDGS	20	<1	N/A	N/A
	DDGS	4	<1	N/A	N/A
	Media (control)	20	41.1 ± 12.7	13.9–68.2	14
	Media (control)	4	53.6 ± 8.1	36.6–70.6	21
FMDV A24	Whole Feed with Sal CURB®	20	4.0[§]	N/A	7
	Whole feed with Sal CURB®	4	9.4 ± 1.3	3.7–15.1	14
	Whole feed with Guardian™	20	14.3 ± 0.1	14.0–14.5	14
	Whole feed with Guardian™	4	13.9 ± 2.6	2.5–25.2	37
	Whole feed with CaptiSURE™	20	45.8 ± 12.4	11.4–80.3	21
	Whole feed with CaptiSURE™	4	223.0 ± 55.0	88.4–357.6	37

[†]FMDV detection by virus isolation (Dee et al.); latest time point tested: 37 days.
[‡]Half-life not calculated due to positive slope of line of best fit.
[§]Standard error and confidence interval not calculated because data set contained only two observations.

these conditions due to the positive slope of the line of best fit. The decrease in infectivity started earlier and declined faster for both viruses when soybean meal was stored at 20°C. The estimated half-lives at 20°C were 107.5 ± 6.4 h for FMDV A24 and 196.8 ± 49 h for O/SKR/2010.

There was a substantially greater difference between the virus strains when viability was evaluated in whole pig feed. Specifically, there was a 2–2.5 log10 reduction in O/SKR/2010 titers within 1 h of virus contamination of whole pig feed at both 4 and 20°C, and the titers for all subsequent time points were at the lower limit of detection. It was, however, possible to detect infectious FMDV O/SKR/2010 by virus isolation (Dee et al.) up to 3 days in samples stored at 20°C, and up to 21 days when samples were stored at 4°. The half-life, which varied little with temperature for this virus in whole feed, was estimated to be 35.6 ± 15.2 h at 20°C and 44.7 ± 18.3 h at 4°C (Table 2). The titers of FMDV A24 in whole pig feed declined gradually through the first 3 days, and steeper thereafter (Figure 1). Samples stored at 20°C reached the lower limit of detection for titers by 7 days, with infectious virus detected by VI for up to 14 days for an estimated half-life of 21.8 ± 1.2 h (Table 2). The titers for the corresponding samples stored at 4°C were reduced by approximately 2 log10 by 14 days but remained above the lower limit of titer detection through 37 days resulting in an estimated half-life of 131.5 ± 27.2 h (Table 2).

The third feed component evaluated was DDGS. However, this substance proved to be highly toxic to the cell cultures, which prohibited titration of samples. The cytotoxic effect of the DDGS was overcome when virus isolation was attempted in T25 flasks, but all samples evaluated were FMDV negative by VI. The half-life for all DDGS experiments was therefore assumed to be less than 1 h. Based on these findings, continued in vitro evaluation of FMDV viability in DDGS was aborted and DDGS was instead evaluated for its ability to reduce FMDV infectivity as a feed component in subsequent in vivo experiments.

3.2 | The ability of select feed additives to reduce FMDV viability in pig feed in vitro

In order to evaluate the potential ability of select feed additives to mitigate FMDV infectivity, the in vitro evaluation of FMDV viability was repeated in the presence of additives; Sal CURB®, Guardian™,

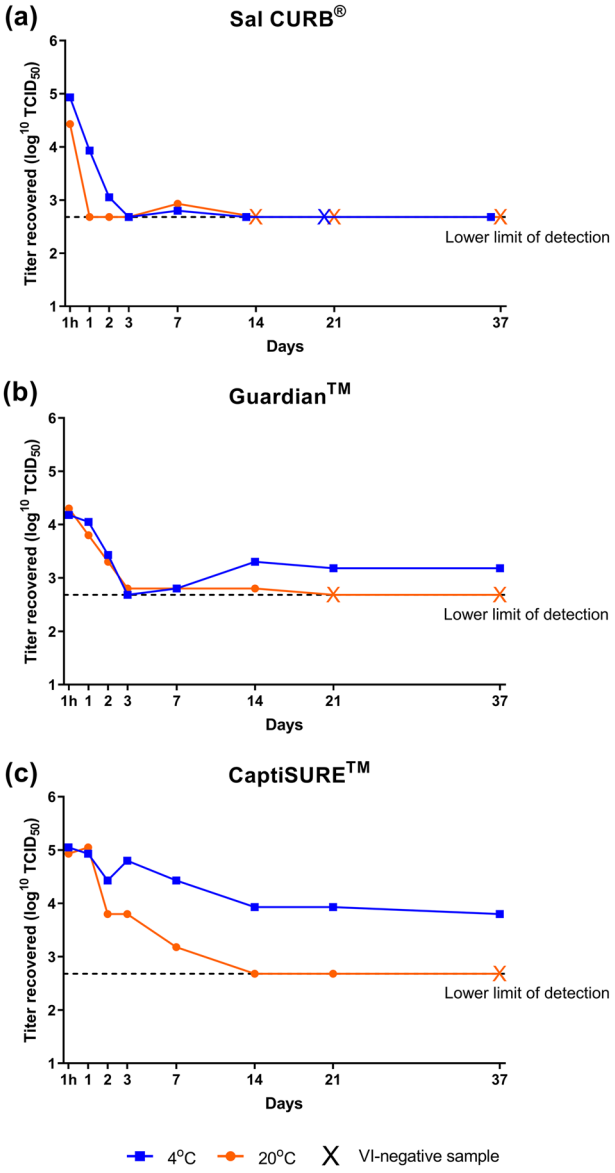

FIGURE 2 Effect of select mitigants on the duration of infectiousness of FMDV A24 in whole pig feed. Recovery of FMDV A24 from samples of whole pig feed that was pre-treated with (a) SalCURB®, (b) Guardian™, or (c) CaptiSURE™. Batches of feed were treated with mitigants 24 h prior to contamination with 10^5 $TCID_{50}$ of FMDV A24 at time point 0 and stored at 4°C (blue) or 20°C (orange) from 1 h to 37 days. The lower limit of detection ($10^{2.6}$ $TCID_{50}$) for recovery of virus from whole feed was a consequence of cell toxicity at lower dilutions of these samples. Samples that were at the lower limit of detection were analyzed for presence or absence of FMDV by virus isolation (Dee et al.). Solid squares or circles indicate VI-positive samples whereas VI-negative samples are marked by X

CaptiSURE™. This part of the investigation utilized only FMDV A24 due to the poor viability of FMDV O/SKR/2010 in whole pig feed. Pre-treating feed with the formaldehyde-based Sal CURB® reduced FMDV titers to the lower limit of detection by 1 day when stored at 20°C (estimated half-life 4.0 h), and 3 days when stored at 4°C (estimated half-life 9.4 ± 1.3 h) (Table 2, Figure 2). The latest time points at which infectious FMDV could be isolated were 7 days for samples stored at 20°C, and 37 days for samples stored at 4°C, although the 21 day/4°C sample was VI negative. The FMDV titers in whole feed treated with the lactic acid-based Guardian™ reached the lower limit for titration by 3 days for both storage temperatures, although there was some variability in later time points stored at 4°C, which may have been a consequence of uneven distribution of this powdered additive within the relatively small aliquots of pelleted feed (Figure 2). The estimated half-life of FMDV in Guardian™-treated feed was 14.3 ± 0.1 h at 20°C and 13.9 ± 13.9 h at 4°C (Table 2). For this mitigant, infectious virus could be isolated up to 14 days at 20°C, and 37 days at 4°C. The medium chain fatty acid-based additive CaptiSURE™ did not reduce FMDV titers compared to the untreated control samples. Samples stored at 4°C had measurable titers through 37 days (approximately 1 log10 reduction compared to starting titer), while samples stored at 20°C reached the lower limit of titration by 14 days, with infectious virus isolated as late as 21 days. This compound was not further evaluated in the in vivo experiments.

3.3 | Minimum infectious dose of FMDV by exposure of pigs to contaminated feed (MID_F)

3.3.1 | FMDV A24

Two experiments were performed to establish the MID_F of FMDV A24 in pigs through natural feeding for a total of seven experimental groups (28 pigs) (Figure 3). Four groups received the full virus dose in a single feeding, and three groups received the dose spread across three consecutive feedings offered within approximately 2 h. No pigs became infected in the first experiment in which the full virus dose (ranging from $10^{4.4}$ to $10^{7.2}$ $TCID_{50}$) was consumed in a single feeding.

There were, similarly, no FMD cases in the study group that received $10^{6.0}$ $TCID_{50}$ split across three feed portions. There was one FMDV-positive OP swab sample at 2 dpe in the $10^{6.0}$ $TCID_{50}$ study group (Figure 3). However, there was no detection of neutralizing anti-FMDV antibodies in serum samples from any individuals within the group, and the FMDV RNA-positive swab sample was therefore considered to represent residual inoculum detection or environmental contamination rather than actual infection. For FMDV A24, the minimum dose that caused infection when separated across three feedings was $10^{7.0}$ $TCID_{50}$ (Figure 3). Within this study group, there were two presumed primary cases and one secondary case. The two primary cases had FMDV RNA-positive OP swabs from 2 dpe. One of these pigs was viremic (defined by FMDV RNA detection in serum) at 2 dpe and developed vesicular lesions at 4 dpe. The other primary case was viremic at 6 dpe but did not develop clinical FMD prior to euthanasia. A third individual had FMDV RNA-positive OP swab and serum at 4 dpe, but was considered a secondary case due to a negative OP swab at 2 dpe. All pigs in the $10^{7.0}$ $TCID_{50}$ study group were euthanized at 6 dpe. All four pigs that consumed $10^{8.1}$ $TCID_{50}$ of virus over three feedings were considered primary cases based on detectable FMDV RNA in OPF at 2 dpe (Figure 3). Three of the four pigs were also viremic at 2 dpe and developed vesicular lesions at 3 dpe. All four pigs were euthanized at 4 dpe.

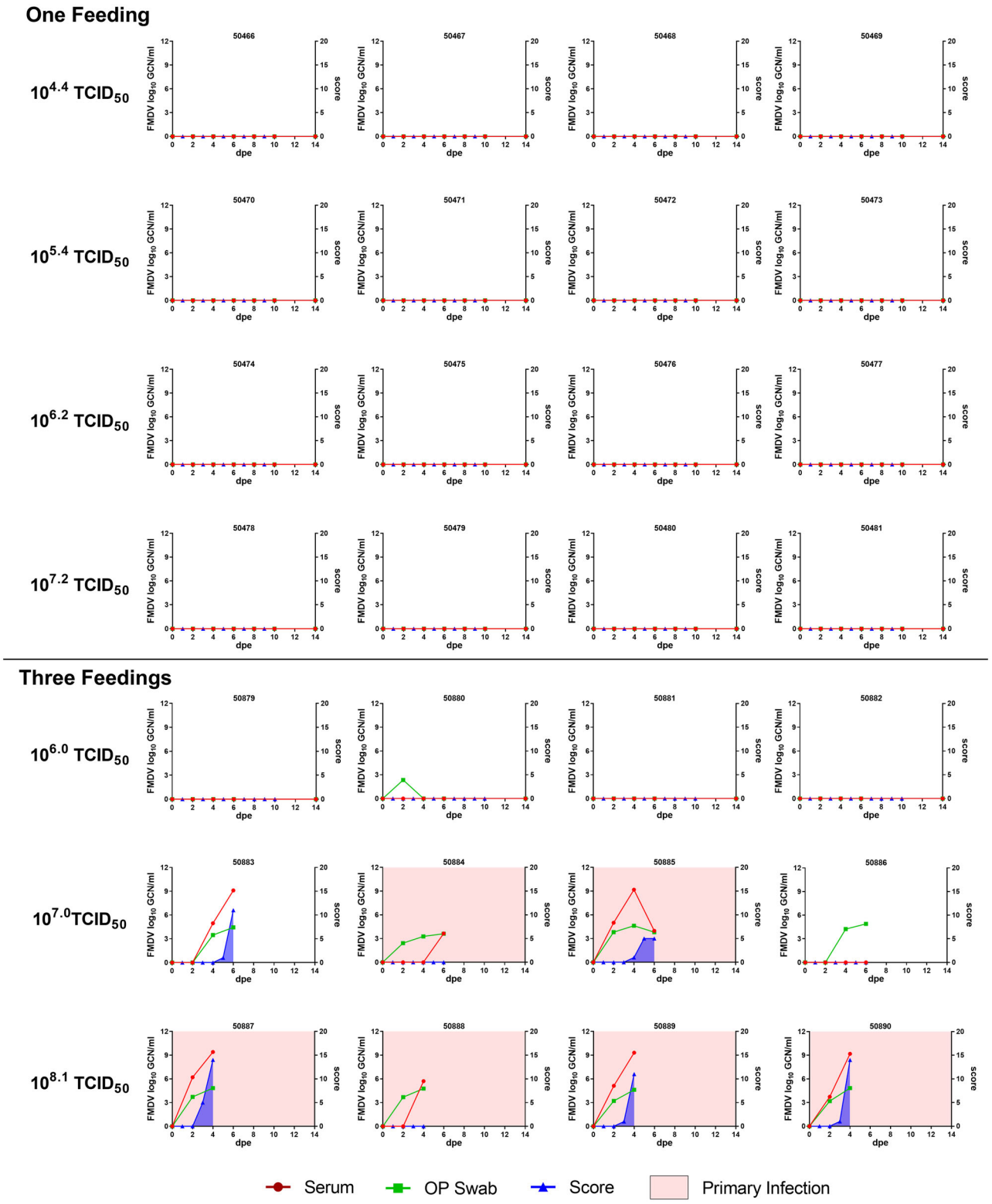

FIGURE 3 FMDV infection dynamics in pigs exposed to FMDV A24 through contaminated feed. Seven groups of four pigs were fed experimentally contaminated feed containing $10^{4.4}$ to $10^{8.1}$ $TCID_{50}$ FMDV A24 (total dose per animal). The first three groups received the full virus dose in one feed portion (100 g), whereas remaining groups were fed the full virus dose divided across three consecutively fed portions (3 × 100 g) over approximately 1 h. Graphs show detection of FMDV RNA in oropharyngeal (OP) swabs (green), and serum (red), as well as cumulative FMD lesion scores (blue line, purple shading). A pink background indicates pigs that were considered primary cases based on having FMDV RNA-positive OP swabs at the first time point of FMDV detection within the group. Pigs that did not develop FMD were monitored for 14 days. Groups in which FMD was confirmed were euthanized within 2 days of confirmed detection

3.3.2 | FMDV O/SKR/2010

Three experiments were performed to establish the MID_F of FMDV O/SKR/2010 in pigs through natural feeding for a total of nine experimental groups (34 pigs) (Figure 4). All experimental groups received the full dose of virus spread across three consecutive feedings. There was no clinical FMD in the study groups that received $10^{4.2}$, $10^{5.1}$, and $10^{6.0}$ $TCID_{50}$ of FMDV O/SKR/2010, and all obtained samples were FMDV RNA negative (Figure 4). Similarly, there was no detection of neutralizing anti-FMDV antibodies in serum samples collected at 14 dpe from any pigs in these groups.

The minimum dose of FMDV O/SKR/2010 that caused infection was $10^{6.2}$ $TCID_{50}$. Of the four pigs that were exposed to this dose, there was one primary case, with an FMDV RNA-positive OP swab at 2 dpe, viremia at 4 dpe and vesicular lesions at 5 dpe (Figure 4). The remaining three pigs in this study group developed synchronous secondary infections, with all three having FMDV RNA-positive OP swabs at 4 dpe, and viremia at 6 dpe. Two of these pigs developed vesicular lesions at 6 dpe, at which point the group was euthanized.

Due to the need of repeated experiments to establish the MID_F (FMDV O/SKR/2010 experiments were performed in reverse order, starting with the higher doses), there were two study groups that received $10^{7.2}$ $TCID_{50}$, with slight variation in outcomes. In the first group exposed to this dose there was one primary case defined by an FMDV RNA-positive OP swab at 2 dpe and three secondary cases with positive FMDV RNA detection in OP swabs at 4–5 dpe (Figure 4). The primary case was the only individual that developed clinical FMD, whereas viremia was also detected in one of the secondary cases. All four pigs were euthanized at 5 dpe. All four pigs in the second group exposed to $10^{7.2}$ $TCID_{50}$ were defined as primary cases with positive FMDV RNA-detection in OP swabs from 2 dpe. One of these individuals was viremic at 2 dpe and had vesicular lesions at 3 dpe. The remaining three pigs were viremic at 3 dpe, at which point the group was euthanized.

All pigs in the groups that received doses equal to or higher than $10^{8.1}$ $TCID_{50}$ FMDV O/SKR/2010 were considered primary cases, with detectable FMDV RNA in OPF and serum at 2 dpe. All pigs developed vesicular lesions at 3–4 dpe with the exception of two pigs in the $10^{8.3}$ $TCID_{50}$ group, which had not yet developed lesions by the time the group was euthanized at 3 dpe.

3.4 | In vivo evaluation of the reduction of FMDV infectivity by select feed additives

One in vivo experiment was performed to measure the ability of select feed additives to reduce infectivity of FMDV A24 in feed for a total of three experimental groups of 4 pigs each (12 pigs; Figure 5). The exposure dose for this experiment was determined based on a combination of the outcome of the MID_F experiments and the in vitro viability experiments. FMDV O/SKR/2010 was excluded from this part of the investigation due to the poor viability in whole pig feed.

For the in vivo experiment, FMDV A24 ($10^{8.3}$ $TCID_{50}$) was added to feed aliquots that had been pre-treated with a commercially available mitigant for 24 h (Sal CURB®or Guardian™) or contained 30% DDGS (by weight). A control group that received untreated feed was also included in the study. For all groups, FMDV was added to the feed 24 h prior to exposure of the pigs. The virus dose added to feed was thus determined based on a target of maintaining a virus level above the MID of FMDV A24 in the untreated feed, when allowing for 24 h storage at room temperature.

In the group for which the feed was pre-treated with Sal CURB®, there was no clinical FMD and all antemortem samples were FMDV RNA negative (Figure 5). Serum samples obtained at 14 dpe did not contain any neutralizing anti-FMDV antibodies. Pre-treating whole pig feed with Guardian™ also prevented clinical FMD. However, there was one pig from which three consecutive OP-swab samples were FMDV RNA positive at 2–6 dpe. Serum samples obtained at 14 dpe from this individual had a low neutralizing antibody titer (1.2) against FMDV, and this pig was thus considered to have been subclinically infected. There were no other FMDV RNA-positive or FMDV antibody-positive samples from the group.

In the group that received whole feed with 30% DDGS, there was one primary case with FMDV RNA-positive OP swabs from 6 dpe, and vesicular lesions at 7 dpe (Figure 5). The remaining three pigs all had FMDV RNA-positive OP swab and serum samples at 8 dpe and were thus considered secondary infections. All pigs in the DDGS group were euthanized at 8 dpe.

In the control group which received untreated feed, three pigs were considered primary cases with FMDV RNA-positive OP swab samples at 2 dpe. The fourth individual in this group had positive OP swabs from 4 dpe and was thus considered a secondary infection. The pigs in the control group were euthanized at 5 dpe.

4 | DISCUSSION

The objective of this study was to evaluate the potential risk of incursion of FMDV into naïve pig herds through contamination of feed. This goal was pursued by assessing the infectiousness (viability) of FMDV in commercial whole pig feed and pig feed ingredients, determining the dose required to infect pigs through natural feeding behaviour, and the ability of select commercially available feed additives to reduce infectivity of contaminated feed. While comparable research investigating the potential biosecurity risks of imported feed exists for other viral pig pathogens (Dee et al., 2018; Niederwerder et al., 2020; Niederwerder et al., 2019), this is the first comprehensive evaluation of the risk of FMDV infection of pigs through ingestion of contaminated feed under controlled experimental conditions.

Many conditions must be met for transmission of viral diseases through pig feed to occur, and understanding these conditions is critical to quantitate the risk of such an event and consider preventative measures. The feed must first become contaminated with the virus. The virus in the feed must then remain viable until it is fed, and the

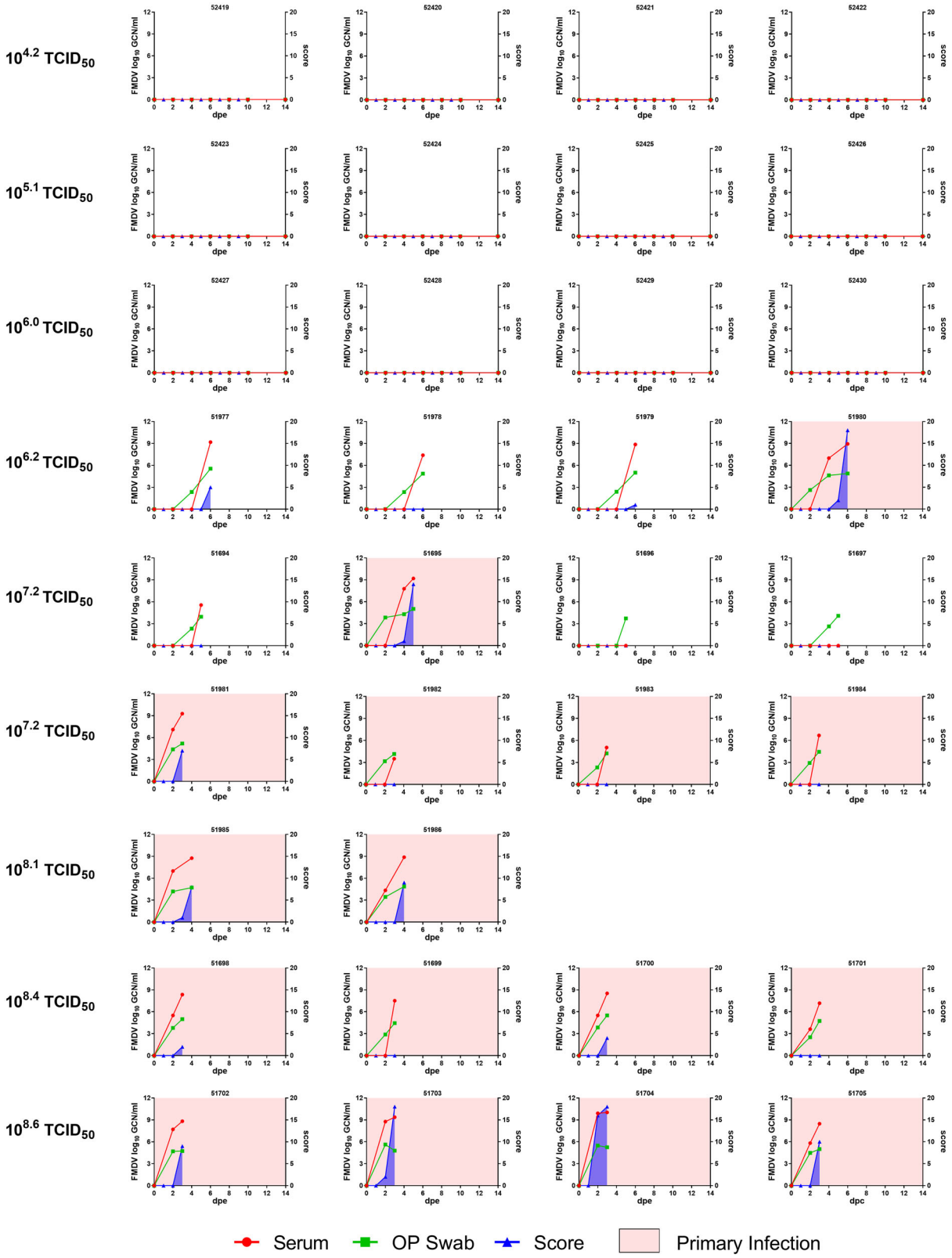

FIGURE 4 FMDV infection dynamics in pigs exposed to FMDV O/SKR/2010 through contaminated feed. Nine groups of 2–4 pigs were fed experimentally contaminated feed containing $10^{4.6}$ to $10^{8.6}$ $TCID_{50}$ FMDV O/SKR/2010 (total dose per animal). The full virus dose was divided across three consecutively fed portions (3 × 100 g) over approximately 1 h. Graphs show detection of FMDV RNA in oropharyngeal (OP) swabs (green), and serum (red), as well as cumulative FMD lesion scores (blue lines, purple shading). A pink background indicates pigs that were considered primary cases based on having FMDV RNA-positive OP swabs at the first time point of FMDV detection within the group. Pigs that did not develop FMD were monitored for 14 days. Groups in which FMD was confirmed were euthanized within 2 days of confirmed detection

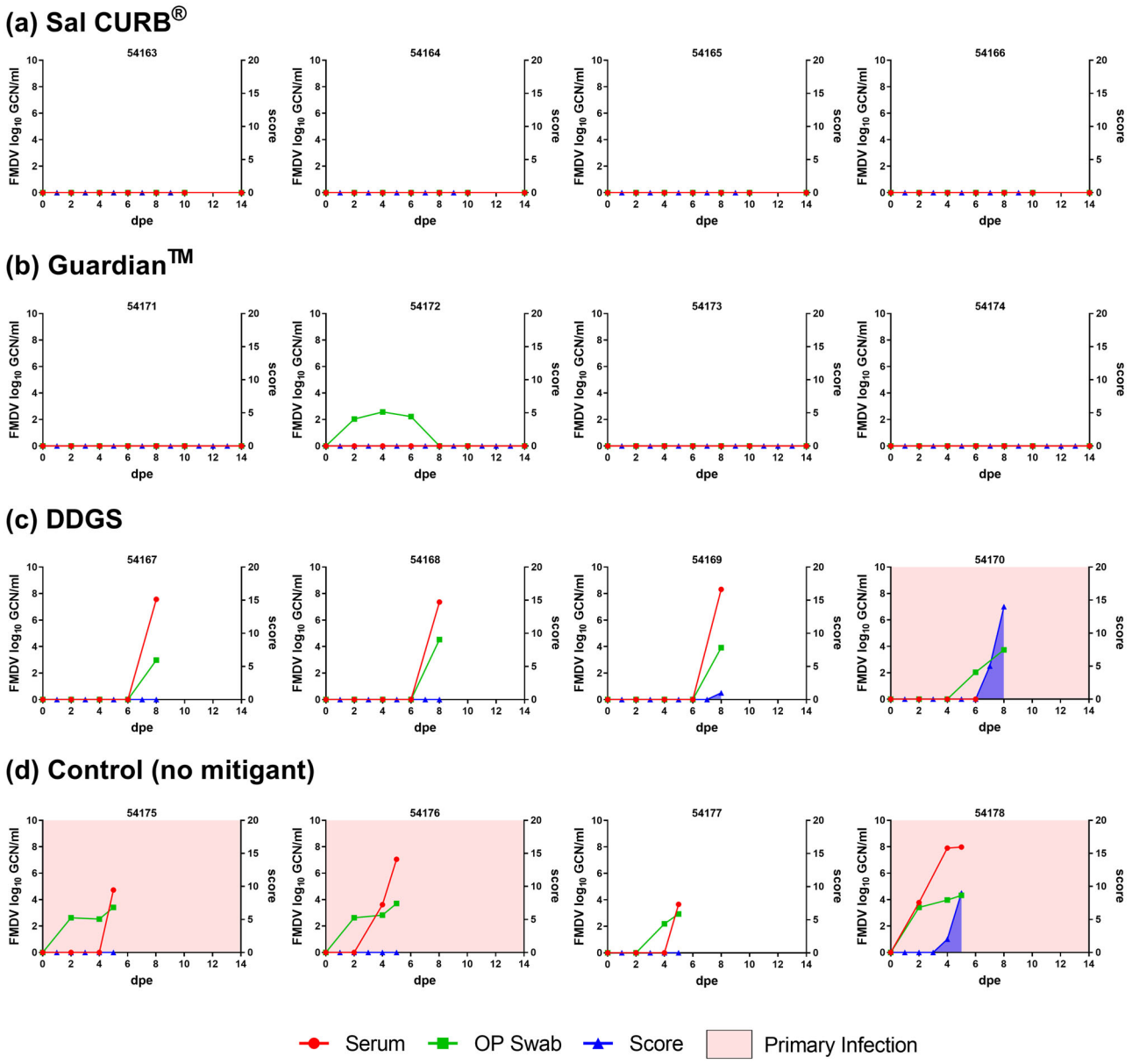

FIGURE 5 FMDV infection dynamics in pigs exposed to FMDV A24 in feed treated with select mitigants. Four groups of 4 pigs were fed whole feed that had been pre-treated for 24 h with (a) SalCURB®, (b) Guardian™, (c) 30% distiller's dried grains with solubles (DDGS), or was untreated (d; control). The mitigant-treated feed was contaminated with $10^{8.3}$ $TCID_{50}$ FMDV A24 (total dose per animal) and incubated at 20°C for 24 h prior to feeding. Each pig consumed 3 portions of 100 g feed over approximately 1 h. Graphs show detection of FMDV RNA in oropharyngeal (OP) swabs (green), and serum (red), as well as cumulative FMD lesion scores (blue lines, purple shading). A pink background indicates pigs that were considered primary cases based on having FMDV RNA-positive OP swabs at the first time point of FMDV detection within the group. Pigs that did not develop FMD were monitored for 14 days. Groups in which FMD was confirmed were euthanized within 2 days of confirmed detection

quantity of infectious virus must be sufficient to surpass the MID_F. Finally, at least one pig must consume enough virus to become infected over one or multiple feedings. Within this study, these requirements were considered in the context of pathogen incursion occurring before or during transoceanic transport (Dee et al., 2018). The combined output from the current investigation demonstrated that the likelihood of all of these conditions being met will depend on the specific feed ingredient (and any added mitigants), the strain of the virus, and the environmental conditions during storage and transport of the feed.

The duration of FMDV infectiousness in vitro varied across virus strains, feed products, and storage temperatures. While FMDV A24 titers were still measurable after 37 days of storage of contaminated whole feed at 4°C, FMDV O/SKR/2010 titers were below the limit of detection within 1 day of storage in this matrix, which is consistent with a more stable capsid of FMDV serotype A versus O (Doel & Baccarini, 1981). Viability of both virus strains was greatly improved when the experiment was repeated using soybean meal as the matrix. While FMDV A24 titers started to decline after 7 or 14 days of

storage at 20 and 4°C, respectively, titers were still measurable after 37 days, and the half-lives were estimated to be 108 h at 20°C and 199 h at 4°C. By contrast, it was not possible to estimate a half-life for FMDV O/SKR/2010 in soybean meal stored at 4°C as the titers did not decline within the 37 day observational period. The half-life of FMDV O/SKR/2010 in soybean meal stored at 20°C was estimated to be 197 h.

With the exception of FMDV O/SKR/2010 in whole feed, the half-lives of both viruses were higher in the selected feed products than in media alone, demonstrating that these matrices extend viral viability and provide some degree of protection from environmental degradation. Conventional soybean meal has been shown to extend viability for several pathogens previously studied including PEDV, porcine deltacoronavirus (PDCoV), porcine reproductive and respiratory syndrome virus (PRRSV), and African swine fever virus (ASFV) (Dee et al., 2018; Trudeau et al., 2017). It has been hypothesized that the high moisture content of soybean meal contributes to this effect (Trudeau et al., 2017). Because previous studies have found that conventional soybean meal is a good matrix for viral stability and 8.5 million metric tons of soybean meal was fed to US swine in 2019 (ASA, 2020), importation of this feed ingredient is a sensible target for enhanced biosecurity measures.

A previous investigation utilized SVA as a surrogate for FMDV in a study that simulated transpacific transport conditions to estimate viability of pathogens as contaminants of pig feed and feed ingredients (Dee et al., 2018). In that study, SVA titers were measurable in 10 of the 12 tested ingredients after 37 days. Although both FMDV and SVA are picornaviruses, the two viruses belong to different genera: *Aphthovirus* versus *Senecavirus*. While the stability of the FMDV capsid is known to be highly sensitive to pH fluctuations (Curry et al., 1995; Grubman & Baxt, 2004; Yuan et al., 2017), there is less information available regarding the biophysical stability of the relatively recently discovered SVA. However, given the demonstrated pH sensitivity of the FMDV when compared to other picornaviruses (Newman et al., 1973), as well as the known variability in stability between different strains of FMDV (Martin-Acebes et al., 2011 ; K. A. Scott et al., 2019), it is not surprising that the viability of FMDV was lower than that of SVA.

In the current study, whole feed and all feed ingredients had a toxic effect to the LFBK-$\alpha v\beta 6$ cells used for virus titrations, which determined the "lower limit of detection" for the titers. It was not possible to accurately quantify viral titers below this level, which limited our ability to characterize the in vitro activity and calculate half-lives for some virus-feed ingredient combinations. To overcome this limitation and gain some information about virus viability in those samples, all samples for which the resulting titers were at the lower limit of detection were further analyzed by virus isolation in T25 flasks to generate binary (positive/negative) results for the presence of infectious FMDV.

The findings from the in vivo experiments demonstrate that pigs can become infected with FMDV through consumption of contaminated feed and that the probability of infection varies with exposure dose, virus strain, and feeding pattern. Overall, the exposure dose required to cause FMD in pigs through feeding (MID_F) is at least 100-fold higher than the standardized dose used for FMD vaccine testing when virus challenge is done by intra-epithelial injection. However, MID_F was more similar to doses which have been demonstrated to cause FMD in pigs by simulated natural oropharyngeal exposure (Stenfeldt et al., 2014, 2014a b). Specifically, for FMDV A24, 50% of pigs that consumed $10^{7.0}$ $TCID_{50}$ over three feedings were infected within 2 days. The percentage of pigs infected by 2 dpe increased to 100% when that dose was increased to $10^{8.1}$ $TCID_{50.}$ However, pigs that consumed as much as $10^{7.2}$ $TCID_{50}$ of FMDV A24 in one single feeding did not become infected at any point during the 14-day study period. For FMDV O/SKR/2010, the MID was 10-fold lower, as at least one pig that consumed a dose of $10^{6.2}$ $TCID_{50}$ across three feedings was infected within 2 dpe, efficiently spreading infection within the group. Interestingly, when $10^{7.2}$ $TCID_{50}$ of FMDV O/SKR/2010 was administered using the same experimental protocol to two groups of pigs as a part of two different experiments, the proportions of primary infections within the groups varied. In the first group that received this dose, only one of four pigs was infected at 2 dpe. When this dose was repeated in a second group, all four pigs were confirmed to have been infected by 2 dpe. These results demonstrate the variability of this route of virus exposure compared to standardized experimental routes, which are more likely to produce synchronous, repeatable infection of pigs across individuals and experiments (Stenfeldt et al., 2014b). The MID_F for FMDV reported herein are 10–100 times higher than previously reported for exposure of pigs to FMDV through feeding (Henderson & Brooksby, 1948; Sellers, 1971). This difference may be a consequence of the more sensitive system for virus titrations used in the current study. While previous reports were based on titrations performed in tongue epithelium of live cattle, the current study utilized a highly sensitive cell line transfected with the bovine $\alpha v\beta 6$-integrin to facilitate FMDV entry (LaRocco et al., 2013, 2015). Additional sources of variation in the current work compared to previous studies may be related to study design choices and intrinsic characteristics of virus strains used. Thus, direct comparison across studies should be done with some caution.

FMDV infection in pigs initiates in the epithelium overlying the oropharyngeal tonsils (Stenfeldt et al., 2014a), and contaminated feed likely contacts these sites only briefly during mastication and deglutition before entering the acidic environment of the lower gastrointestinal system, where FMDV is readily inactivated (Alexandersen et al., 2003 ; Bachrach et al., 1957). The increased duration of contact between contaminated feed and susceptible tonsil epithelium that resulted when the same dose of virus was divided across three feedings rather than one, likely explains the increased probability of infection associated with multiple feedings in the current study. A similar effect has also been demonstrated in a study of MID of ASFV through ingestion, where statistical modelling showed that a lower titer of virus had a higher probability of causing infection when multiple exposures occurred over time rather than a single feeding (Niederwerder et al., 2019).

The animals in all study groups were co-housed and allowed unlimited contact with each other out of consideration for animal welfare. Swab and serum sampling were limited to every other day based on similar ethical concerns. For groups in which the timing of

infection was non-synchronous, this design precluded, in some cases, our ability to discern between infection from consuming contaminated feed versus through contact transmission. Contact transmission can occur from an infected pig starting at 24 to 32 h following inoculation (Stenfeldt, Pacheco, Brito, et al., 2016), so while there is a high degree of confidence that the primary infections in each group were infected by feed ingestion, the route of infection for pigs that became infected on subsequent days may have been due to primary exposure (feed ingestion), contact transmission, or a combination of the two types of exposure. While this limitation prevented calculation of a precise attack rate of each dose, this design is relevant to a typical commercial swine farm, where viral exposure sufficient to infect just one individual would likely lead to an outbreak affecting all animals within the housing unit. However, previous experimental studies with these same virus strains demonstrated that a relatively low exposure dose may result in a higher degree of variability in the timing of onset of FMD within groups compared to higher exposure doses (Moreno-Torres et al., 2018; Stenfeldt et al., 2014b). Thus, the definition of primary cases based on synchronous detection of FMDV RNA in OP swabs used in the current study likely represents a conservative estimate of the number of individuals that were infected by the contaminated feed.

Both formaldehyde-based and acidifying feed additives have been shown to have antiviral properties against consequential viral swine pathogens such as ASFV and PEDV (Dee et al., 2016; Niederwerder et al., 2020), but their efficacy has not previously been demonstrated against FMDV. Our findings demonstrate that Sal CURB®, a formaldehyde-based feed additive, effectively reduced the infectivity of FMDV in contaminated whole feed within 24 h at room temperature. None of the pigs in the group that consumed Sal CURB®-treated food became infected, compared to three out of four pigs that were infected by 2 dpe in the positive control group (no feed additive). We also demonstrated that Guardian, a lactic acid-based feed additive, reduces FMDV infectivity in feed, despite questionable reduction in viral viability in the in vitro study. This product is added to the feed in powder form, which may account for the differences observed in vitro versus in vivo as the overall acidifying effect may be enhanced by salivation and deglutination during eating. While none of the pigs in the Guardian group had clinical signs of FMD, one of four pigs had three consecutive OP swab samples that were positive for FMDV RNA (2, 4, and 6 dpe) as well as detectable neutralizing anti-FMDV antibodies at 14 dpe, suggesting a subclinical infection that did not transmit within the group. In the current investigation, mitigants were added to feed 24 h prior to FMDV contamination, and the contaminated feed was allowed another 24 h incubation at room temperature prior to feeding to the pigs. This approach was based on recommendations from the manufacturers of the mitigants, and it is not known if or how variation in the timing and formulation of treating feed with mitigants in relation to virus contamination may affect the outcomes of the mitigation. DDGS, which is considered a feed ration ingredient rather than a feed additive, was found to rapidly inactivate FMDV in vitro. On this basis, this compound was evaluated at 30% inclusion, as a potential FMDV mitigant in our in vivo experiment. DDGS did not effectively prevent FMDV infection at the tested inclusion level, though it did delay onset of the single primary infection in the DDGS group compared to all other infected groups in this study, suggesting some mitigation effect. In the current study, DDGS was mixed with pelleted whole feed for the in vivo experiment. The overall findings suggest that although the inclusion of DDGS did not mitigate FMDV contamination of the whole feed, the observed anti-FMDV properties of this compound may still be relevant if used in a different formulation. The in vitro evaluation of feed additives also included evaluation of a medium chain fatty acid-based product (MCFA; CaptiSURE™). MCFA additives have proven antiviral activity against enveloped viruses (Thormar & Hilmarsson, 2007; Thormar et al., 1987), but there is little evidence for their efficacy against non-enveloped viruses like FMDV. Our findings suggested that this MCFA-based additive had little to no anti-FMDV activity in vitro, and the compound was therefore not further evaluated in vivo.

5 | CONCLUSION

The combined output of this investigation demonstrated that FMDV can remain viable as a contaminant of pig feed products through 37 days. In addition to expected variation associated with storage temperature, there was also substantial variability in viability of different FMDV strains in different feed matrices. FMDV exposure by feeding of experimentally contaminated feed to pigs caused FMD with dose dependency. The minimum dose required to cause FMD varied between virus strains and with experimental design and the probability of infection increased when a given dose of virus was divided across three consecutive feedings, likely due to increased exposure time. Overall, these findings demonstrate that FMDV introductions through import of contaminated feed products is plausible, that addition of additives to feed may mitigate this risk, and that the risk of infection varies depending on the contaminated product, the viral strain, and the feeding conditions.

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AUTHORS' CONTRIBUTIONS

CS led study design and execution, coordinated and executed the animal experiments, oversaw laboratory analyses, and drafted the manuscript. MRB contributed to study design and execution of the animal experiments. HCM contributed to execution of the animal

experiments, drafting of the manuscript, and performed statistical analyses. EJH performed laboratory analyses and interpretation of data. GRS performed laboratory analyses and interpretation of data. MCN, DGD, and SAD contributed to study design and scientific content. JA conceived and coordinated the work, contributed to study design, data interpretation, and writing the manuscript. All authors have critically reviewed and revised the manuscript and approved the final product.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. All experimental procedures involving animals were approved by the Plum Island Animal Disease Center Institutional Animal Care and Use Committee (IACUC) that functions to ensure ethical and humane treatment of animals (protocol 259.02-18-R).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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