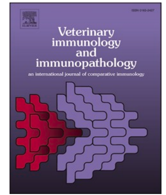
Veterinary Immunology and Immunopathology 271 (2024) 110753

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm



An isotonic protein solution favorably modulated the porcine intestinal immune response and cellular adhesion markers and reduced PEDV shedding *in vivo*

Dmytro M. Masiuk [a], Andrii V. Kokariev [a], Stefan G. Buzoianu [b], Ava M. Firth [b], Victor S. Nedzvetsky [a,*]

[a] *Dnipro State Agrarian and Economic University (DSAEU), Serhii Efremov Str., 25, Dnipro 49600, Ukraine*
[b] *Tonisity International Ltd., Tonisity International Ltd, 27-30 Merchants Quay, Dublin 8 D08 K3KD, Ireland*

A R T I C L E I N F O

Keywords:
Tonisity
PEDV
isotonic
cell adhesion
intestinal barrier

A B S T R A C T

Porcine epidemic diarrhea virus (PEDV) causes immensely large economic losses worldwide in the swine industry. PEDV attacks the intestine, disrupts intestinal epithelium morphology and barrier integrity, and results in profound diarrhea and high mortality. A commercially available isotonic protein solution (IPS) (Tonisity Px) has anecdotally been reported to be effective in supportive treatment of piglets with active PEDV infections. This study evaluated the effects of supplementing (or not) the drinking water of 14 day old PEDV-infected piglets with the IPS on the content of E-cadherin, fibronectin, interferon-alpha (IFN-α), and matrix metalloproteinase 9 (MMP-9) in duodenal tissue. The content of PEDV DNA in feces was also measured. Though both groups had similar PEDV shedding at day 1, IPS piglets had significantly lower PEDV shedding at day 5, 14 and 21. The IPS group also had a shorter duration of PEDV virus shedding.

Levels of E-cadherin and fibronectin, both of which are structural proteins in the intestine, remained unchanged from baseline in the IPS group, whereas the same molecules decreased significantly in the control group. IFN-α, an antiviral cytokine, and MMP-9, an enzyme that aids in tissue remodeling, were increased at days 5 and 14 post infection, and then decreased at day 21 post-infection in the IPS group compared to control.

Overall, the IPS used in this study enhanced epithelial intercellular adhesion (E-cadherin) and extracellular matrix structure (fibronectin), resulted in significantand favorable changes in MMP-9 activity, and favorably modulated IFN-α production.

This is the first report of this panel of biomarkers, especially MMP-9 and IFN-α, in the face of *in vivo* PEDV infection. This is also the first report to investigate a commercially available swine product that does not need to be administered in solid feed, and that is already registered for use throughout Asia, Europe, South America, and North America.

Overall, the results of this study serve to clarify the behavior of 4 key biomarkers in the presence of *in vivo* PEDV infection. The results also indicate that IPS (Tonisity Px) supplementation is a viable intervention to modulate the porcine intestinal immune response with favorable effects on the intestine.

1. Introduction

Porcine epidemic diarrhea virus (PEDV), an *Alphacoronavirus*, was first identified in England and Belgium (Pensaert and de Bouck, 1978; Wood, 1977). Porcine epidemic diarrhea (PED) has been reported in pig industries worldwide over the last 30 years with extremely high mortality rates across Asian, American, and European countries (Liu and Wang, 2021). The economic losses to the swine industry are significant.

Abbreviations: CoV, Coronavirus; DSAEU, Dnipro State Agrarian and Economic University; Dpi, days post infection; ECL, enhanced chemiluminescence; ECM, extracellular matrix; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN-α, interferon-alpha; IPS, isotonic protein solution; LS, least squares; NS, non statistically significant; MMP-9, matrix metalloproteinase 9; PAGE, polyacrylamide gel electrophoresis; PBST, phosphate buffer saline + Tween-20; PEDV, Porcine epidemic diarrhea virus; WB, Western Blot.

* Corresponding author.
E-mail address: nedzvetskyvictor@ukr.net (V.S. Nedzvetsky).

https://doi.org/10.1016/j.vetimm.2024.110753
Received 28 November 2023; Received in revised form 20 March 2024; Accepted 2 April 2024
Available online 6 April 2024
0165-2427/© 2024 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

PEDV causes a highly contagious enteric disease characterized by severe symptoms, including vomiting, watery diarrhea, and dehydration (Li et al., 2020). Both *in vivo* and *in vitro* results have shown that PEDV mainly targets the enterocytes of the small intestine and the infection is usually accompanied by a substantial atrophy of the intestinal epithelium (Shen et al., 2020; Wu et al., 2019; Zong et al., 2019). PEDV also disrupts the expression of cell adhesion molecules (Jung et al., 2015). The adhesion between enterocytes is maintained by several protein systems including tight junctions, adherens junctions, and desmosomes (Schlegel et al., 2010). Although the most critical effects of PEDV infection are the disruption of intestinal water and nutrient uptake, permeability, and barrier function (Shen et al., 2020), the molecular mechanisms of intercellular and cell-ECM adhesion by which PEDV exerts such effects are complex and not fully understood (Jung et al., 2015; Luo et al., 2024; Qin et al., 2023; Ruedas-Torres et al., 2024; Zhou et al., 2021; Zong et al., 2019).

Pigs of all ages can be infected with PEDV but piglets aged four weeks or less are extremely susceptible to PEDV infection (Thomas et al., 2015) with a mortality rate that can reach up to 100% (Kikuti et al., 2022). Multiple pathologies, including the destruction of intestinal villi, reduction of mucins, loss of epithelial and goblet cells, and unbalanced digestive enzymes have been observed in piglets infected with PEDV. This damage results in intestinal hemorrhage, malabsorption, and dyspepsia (Curry et al., 2017; Jung et al., 2020, 2015; Jung and Saif, 2015). Moreover, the observed morphological abnormalities lead to the disruption of intestinal barrier integrity, allowing for increased intestinal permeability to bacteria and toxins, which leads to co-infections that aggravate the symptoms of PEDV infection (Jung et al., 2020).

The prevention of PEDV infection relies mainly on vaccination and antiviral agents. Despite the massive application of attenuated vaccines, highly virulent PEDV strains are still present and causing significant economic losses to swine producers in many countries (Jung and Saif, 2015). Various antiviral agents, such as *Lactobacillus plantarum,* milk proteins, hypericin (an extract of the St. Johns wort plant) have all been studied *in vitro* (J. Zhang et al., 2021a; Y. Zhang et al., 2021b).

In vivo +/- *in vitro* studies have also been performed on N-acetyl cysteine, puerarin (an extract of the Chinese herb Gegen) 25-hydroxy vitamin D3, bis-benzylisoquinolone and aqueous extract of Aloe sp. (Dong et al., 2022; Wang et al., 2017; Wu et al., 2021; Xu et al., 2020; Yang et al., 2019). These have been shown to alleviate the pathophysiologic effects of PEDV. However, the use of these substances in large-scale commercial swine production has not been reported. To the best of the authors' knowledge, none of them except Vitamin D3 is widely approved as a feed additive for swine.

A widely available isotonic protein solution (IPS) known as Tonisity Px has been shown to reduce pre-weaning mortality in normal, healthy pigs under commercial conditions (Buzoianu and Firth, 2023), and also to improve the microbiome profile of the pre-weaning pig (Buzoianu et al., 2020). The manufacturer has received anecdotal reports of swine producers using the IPS to successfully support young pigs in a PEDV outbreak. Tonisity Px is unique in that it is delivered as a supplementary liquid drink that is consumed voluntarily, in a manner somewhat similar to milk replacer. This means that it can be used even in neonatal pigs who are still primarily dependent upon suckling the sow's milk.

The aim of this study was to examine the effect of the aforementioned IPS on the modulation of the immune response and the intestinal tissue adhesion of PEDV-infected piglets. This was achieved by analyzing the levels of structural proteins (E-cadherin and fibronectin), the production of the antiviral cytokine interferon-alpha (IFN-α), and the activity of matrix metalloproteinase 9 (MMP-9), an enzyme that aids in tissue remodeling and also participates in the initiation of pro-inflammatory responses. These four biomarkers are considered to be key indicators of intestinal integrity and intestinal immune response (Alvarado et al., 2008; Dalton and Lemmon, 2021; Gieryńska et al., 2022; González-Navajas et al., 2012; Jung and Saif, 2015; Li et al., 2019; Sumagin and Parkos, 2015).

2. Materials and methods

2.1. Ethical considerations

Animal studies were conducted within the framework of the "General Ethical Principles of Animal Experiments", which have been approved by the National Congress of Bioethics held in Kyiv in 2001, and in line with the provisions of the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (ETS No. 123; https://www.coe.int/en/web/cdcj/laboratory-animals). All experimental protocols were approved by the Institutional Animal Care and Use Committee of Dnipro State Agrarian and Economic University (DSAEU), Ukraine (Protocol No. 17–012022).

2.2. Animals and study design

Conventionally reared mixed-gender Landrace x Pietrain piglets were obtained from a commercial pig farm and transferred to the animal house of DSAEU at 14 days of age. All study procedures were carried out during 2022–2023 in the Biosafety Center of DSAEU. Two groups of 16 piglets each were established: a control and an experimental group. Piglets in each group were initially 14 days old and weighed 3.9 ± 0.38 kg on average. The piglets of both groups received milk replacer and standard pre-starter compound feed. Piglets of both groups were allocated to individual pens with a slatted floor, each equipped with a drinker, a feeder, and an infrared lamp. The piglets were acclimated for 2 days before the onset of the study. During the entire trial period, there was no direct contact between individual piglets. Treatment groups were in separate rooms. All 32 piglets were confirmed negative for the PEDV (using PCR, as outlined below in Section 2.6) and for the absence of specific PEDV immunoglobulin G in blood serum pre-inoculation (day 0). The detection of PEDV-specific IgG in the serum of piglets was carried out via ELISA using an ELx800 Absorbance Microplate Reader (BioTek, USA) and an «ID Screen® PEDV Indirect» kit («ID», France). A titer of 1:10 was used according to manufacturer's instructions.

A PEDV epizootic strain previously isolated from a Ukrainian swine farm (Masiuk et al., 2019) was used for inoculation. The virus preparation containing 500−1000 virions (Masiuk et al., 2019; Schumacher et al., 2016; Thomas et al., 2015) per animal was orally administered to piglets of both groups using a syringe connected to a 10 cm length orogastric tube immediately after the acclimation period. This was considered to be day 0 of the study period.

The piglets in the treatment group were provided with 120 mL/pig/day of a 3% IPS (Tonisity Px, Tonisity, Ireland) starting from day 0. The 3% concentration is the manufacturer's recommended concentration which ensures an isotonic solution of ~ 300 mOsm/l. The IPS was poured into a shallow, circular drinking pan once daily. The pan was placed in an easily accessible area of each pig's pen. Previous studies have shown that even neonatal piglets will consume the IPS voluntarily (Firth et al., 2017a) and that approach was used in this study. Tonisity Px is a patented formulation that contains a combination of dextrose, sodium chloride, potassium chloride, monosodium glutamate, monosodium phosphate, xanthan gum, glutamic acid, glycine, citric acid, and whey.

Piglets in both groups were randomly further subdivided into four subgroups of four piglets, each corresponding to the time at which they were euthanized: 1 (20 hours post-inoculation), 5, 14, and 21 days-post-inoculation (dpi) of PEDV. At each time point, duodenal tissue was collected from each piglet for western blot (WB) and zymography analyses. Quantitative real-time PCR (RT-qPCR) analysis was carried out on fecal samples taken from each euthanized piglet in each subgroup at each time point to detect PEDV.

2.3. Preparation of samples for protein content analyses

Freshly isolated duodenum tissue samples (1.5–2 cm from mid-

duodenum; n = 4 from each subgroup) were placed in liquid nitrogen and homogenized in 50 mM Tris–HCl (pH 7.6) containing 0.15 M NaCl, 1% sodium dodecyl sulphate, 2 mM ethyleneglycoltetraacetic acid, 2.5 mM ethylenediaminetetraacetic acid, 6.5 μM aprotinin, 1.5 μM pepstatin A, 22 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL soybean trypsin inhibitor, and 1 μM sodium orthovanadate (tissue:buffer, 1:10, w/v). The homogenates were sonicated three times for 30 s each using an ultrasonic disintegrator and then centrifuged at 20,000 × g for 30 min at 4 °C. The supernatants were mixed with Laemmli sample buffer (1:1), supplemented with 0.1 M dithiothreitol, and boiled for 5 min to obtain protein samples. Protein concentration in each duodenum tissue lysate was determined spectrophotometrically using the Bradford protein assay (Bradford, 1976). Briefly, protein extracts were diluted (1:5) in 50 mM Tris–HCl (pH 7.6) and vortexed. The diluted protein sample (2 μL) was then added to a tube containing 1 mL of Bradford dye reagent (Quick Start™ Bradford 1× Dye Reagent; Bio-Rad Laboratories, Hercules, CA, USA). The solution was vortexed and incubated for at least 5 min at room temperature before absorbance determination at 595 nm. A 50 mM Tris–HCl solution was used as the blank and bovine serum albumin was used to prepare the standard curve of absorbance from which protein was quantified. Protein samples were frozen and stored at −20 °C until further analyses as detailed below.

2.4. Western blots (E cadherin, fibronectin, IFN-α)

Protein samples (50 μg/track) were run in 5–20% denaturing polyacrylamide gel electrophoresis (PAGE) for 4 h and then transferred onto polyvinylidene fluoride membranes (GE Healthcare, Chicago, IL, USA) with a pore diameter of 0.45 μm. Then, membranes were blocked with 3% (w/v) non-fat dried milk for 90 min at 37 °C and probed with anti-E-cadherin (sc-8426, 1:1000), anti-fibronectin (sc-8422, 1:1000) (both Santa Cruz Biotechnology Inc., Dallas, TX, USA), anti-IFN-α (ab191903, 1:3000), or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ab8245, 1:5000) (both Abcam, Cambridge, UK) antibodies diluted in phosphate buffer saline containing 0.05% Tween-20 (v/v) (PBST), according to the manufacturers' recommendations. Primary antibodies were diluted in PBST and used at final concentrations ≥ 1 μg/mL.

After overnight incubation at 4 °C, membranes were washed five times with PBST and then incubated with anti-mouse (Abcam, ab6789) horseradish peroxidase-conjugated secondary antibody diluted in PBST for 60 min. Membranes were then washed in PBST three times. This was for the detection of E-cadherin, fibronectin and GAPDH. Using the same protocol, samples were incubated with anti-rabbit antibody (Abcam, ab205718) for the detection of IFN-α. The anti-mouse and anti-rabbit antibodies were internally validated to detect porcine E-cadherin, fibronectin, IFN-α and GAPDH (unpublished results).

The WB results were visualized using enhanced chemiluminescence (ECL). Densitometric analysis of the developed films was performed with TotalLab TL120 (https://totallab.com/) and normalized to the intensity of the respective bands obtained for the house-keeping marker GAPDH. Each trace was corrected for background noise by subtracting the non-reactive area trace on the blot. Antigens of various molecular weights were identified by extrapolation of plots to pre-stained protein markers of known molecular weight (PageRuler™ Prestained Protein Ladder; Fermentas, Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Zymography (MMP-9)

The gelatinolytic activity of MMP-9 was estimated by substrate gelatin zymography. Protein extract samples were first separated by sodium dodecyl sulfate (SDS)-PAGE (0.5 % SDS, 7.5 % PAGE, and 0.1 % gelatin) for 3 h. The gel was then washed with 2.5 % Triton X-100 buffer to remove the SDS. To develop the MMP-9 enzymatic activity, the gel was incubated in a buffer (pH 7.5) containing 50 mM Tris–HCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, and 200 mM NaCl at 37 °C for 20−24 h. Then, the gel was stained with 0.05% Coomassie brilliant blue R-250 dissolved in 40% methanol and 7.5 % acetic acid. Gel de-staining was carried out in a 20 % methanol and 7.5 % acetic acid solution to visualize the clear zones corresponding to local gelatin lysis, which indicated the presence of MMP-9 activity (Hanifeh et al., 2014). MMP-9 activity was estimated through the polypeptide bands detection for pro-MMP-9 and active MMP-9 with MW 95 kDa and 80 kDa respectively.

2.6. Quantitative RT-PCR for PEDV quantification

Feces collected from each euthanized piglet at each timepoint on rectal swabs were homogenized for 5 min (FastPrep-24™; MP Biomedicals, Irvine, CA, USA) using quartz beads and a lysis buffer (ATL Buffer; Biosellal, Dardilly, France). Two hundred microliters of each sample were used to extract nucleic acids with the BioExtract® Premium Mag (Biosellal) kit and an automatic extractor (KingFisher™ Duo, USA). The amount of cDNA in each sample was detected using the oneMIX & PEDV kit (Exopol, Zaragoza, Spain), and the qRT-PCR was carried out in the CFX 96 system (Bio-Rad Laboratories, USA) following the instructions of the oneMIX & PEDV kit. Briefly, the reaction started with reverse transcription (15 min at 45 °C), followed by reverse transcriptase inactivation and polymerase activation (95 °C for 5 min). This was followed by amplification, which consisted of 42 cycles of gradual temperature changes between denaturation (95 ° C for 15 seconds) and hybridization/elongation (60 ° C for 1 min). Every step was performed according to the manufacturer's recommendation.

All samples, including negative controls, were analyzed in triplicate. PCR efficiency (E) and correlation coefficients of standard curves were assessed in the 89.20–111.00 % range. A relatively high index of linear dependence was observed (0.988−0.999). The sensitivity limit for the qRT-PCR was 10–100 cDNA copies. Standard curves were constructed by 10-fold serial dilutions of a known copy number of PEDV cDNA positive control to verify the linearity and dynamics of the qRT-PCR. Results were analyzed using the CFX Manager software (Bio-Rad Laboratories), and E was estimated as $E = (10^{-1}/slope) - 1$.

2.7. Statistical analyses

The data were analyzed using a Generalized Linear Mixed Model within SAS 9.4 (SAS Inst. Inc., Cary, NC), as recommended for non-normally distributed data (Stroup, 2015). Treatment, day, and their interaction were included as main effects in the model. The SLICE option was used to test for treatment effects at each time point. Data were transformed within the model using a link function to facilitate analysis and were transformed back to the original data scale for presentation of the results. P-values were corrected for multiple comparisons using the Tukey-Kramer adjustment. Data are presented as least squares means and their 95% confidence intervals. Significance was reported for $P \leq 0.05$. For all response criteria, the individual pig was the experimental unit.

3. Results

3.1. Clinical symptoms

All pigs developed diarrhea after PEDV inoculation. Blood was not grossly observed in the feces.

3.2. IPS consumption

All 16 piglets in the IPS group consumed the product voluntarily and quickly each day, verified by visual confirmation of the empty drinking pans.

3.3. Western blot results

3.3.1. E-cadherin

Compared to the control group, the E-cadherin content was significantly higher in the IPS group on days 14 and 21 (Fig. 1; P<0.05).

During the study period, E-cadherin did not change significantly over time in the IPS group (Fig. 1). However, the E-cadherin content was significantly lower for the control group on days 14 and 21 post-infection compared to day 1 (Fig. 1).

3.3.2. Fibronectin

Compared to the control group, the fibronectin content was significantly higher in the IPS group on days 14 and 21 (Fig. 2; P<0.05).

During the study period, fibronectin did not change significantly over time in the IPS group (Fig. 2) and never reached lower than 90 % of the baseline level. However, the fibronectin content was significantly lower for the control group on days 5, 14 and 21 post-infection compared to day 1 (Fig. 2).

3.3.3. IFN-α

Compared to the control group, the IFN-α content was significantly higher in the IPS group at day 5 and 14 (P<0.05) and significantly lower (P<0.05) on day 21 (Fig. 3).

During the study period, IFN-α increased significantly from baseline at day 5 and then again at day 14. IFN-α then significantly decreased from day 14 to day 21 in both groups (P<0.05; Fig. 3).

3.3.4. Western Blots

The images of the Western Blots are presented in Fig. 4 below.

3.4. Zymography - MMP-9 activity

Compared to controls, the active form of MMP-9 (80 kD protein) was significantly higher on days 5 and 14 and significantly lower on day 21 in the IPS group (P<0.05) (Fig. 5).

During the study period, MMP-9 significantly increased from baseline at day 5 in both groups. At day 14, MMP-9 levels were slightly decreased but were still significantly increased from baseline (P<0.05). By day 21, MMP-9 levels had decreased to baseline in the IPS group but remained significantly elevated in the control group (P<0.05; Fig. 5).

3.5. Quantitative RT-PCR for PEDV quantification

Both groups had statistically similar levels of PEDV at day 1. IPS pigs had a significantly lower PEDV load at all subsequent time points, with

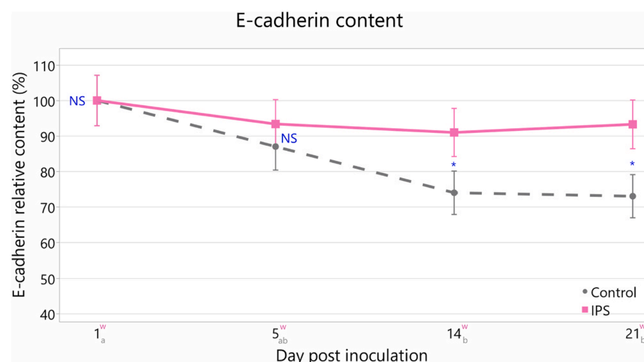


Fig. 1. Relative content (%; LS means and 95 % confidence interval) of **E-cadherin** in the duodenum of PEDV-infected piglets (n = 4 piglets/treatment/sampling day). *(P≤0.05); NS (P>0.05) indicate differences between treatment groups at the same time point; Different superscripts indicate differences between dpi, separately for each of the treatment groups (^{a,b,c,d} Control; ^{w,x,y,z} IPS).

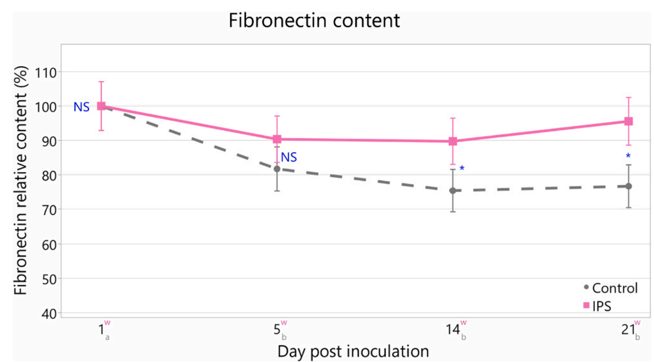


Fig. 2. Relative content (%; LS means and 95 % confidence interval) of **fibronectin** in the duodenum of PEDV-infected piglets (n = 4 piglets/treatment/sampling day). *(P≤0.05); NS (P>0.05) indicate differences between treatment groups at the same time point; Different superscripts indicate differences between dpi, separately for each of the treatment groups (^{a,b,c,d} Control; ^{w,x,y,z} IPS).

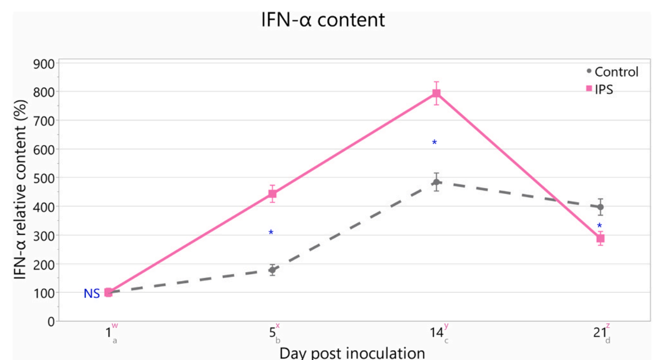


Fig. 3. Relative content (%; LS means and 95 % confidence interval) of **IFN-α** in the duodenum of PEDV-infected piglets (n = 4 piglets/treatment/sampling day). *(P≤0.05); NS (P>0.05) indicate differences between treatment groups at the same time point; Different superscripts indicate differences between dpi, separately for each of the treatment groups (^{a,b,c,d} Control; ^{w,x,y,z} IPS).

no detectable PEDV at day 21 (P<0.05; Fig. 7).

During the study period, the PEDV load significantly decreased from baseline by day 14 and decreased further at day 21 in both treatment groups (Fig. 7).

4. Discussion

4.1. Overview

This study evaluated the effects of PEDV infection on the levels of E-cadherin, fibronectin, IFN-α, and MMP-9. E-cadherin and fibronectin are structural proteins, while IFN-α and MMP-9 are immune mediators. All four of them are involved in intestinal barrier maintenance and the immune response to viral infections. To the best of the authors' knowledge, this is the first report of this panel of biomarkers' activity in the face of *in vivo* PEDV infection.

This study also evaluated the effect of a commercially available IPS on the same parameters. As mentioned in the introduction, though there are various studies of individual substances on immunomodulation in PEDV, this is the first report to investigate a commercially available swine product that is already registered for use throughout Asia, Europe, South America, and North America.

The dose of PEDV (500–1000 virions per pig) used in this study was based on previous reports in the literature (Masiuk et al., 2018; Schumacher et al., 2016; Thomas et al., 2015). The dose will reliably create

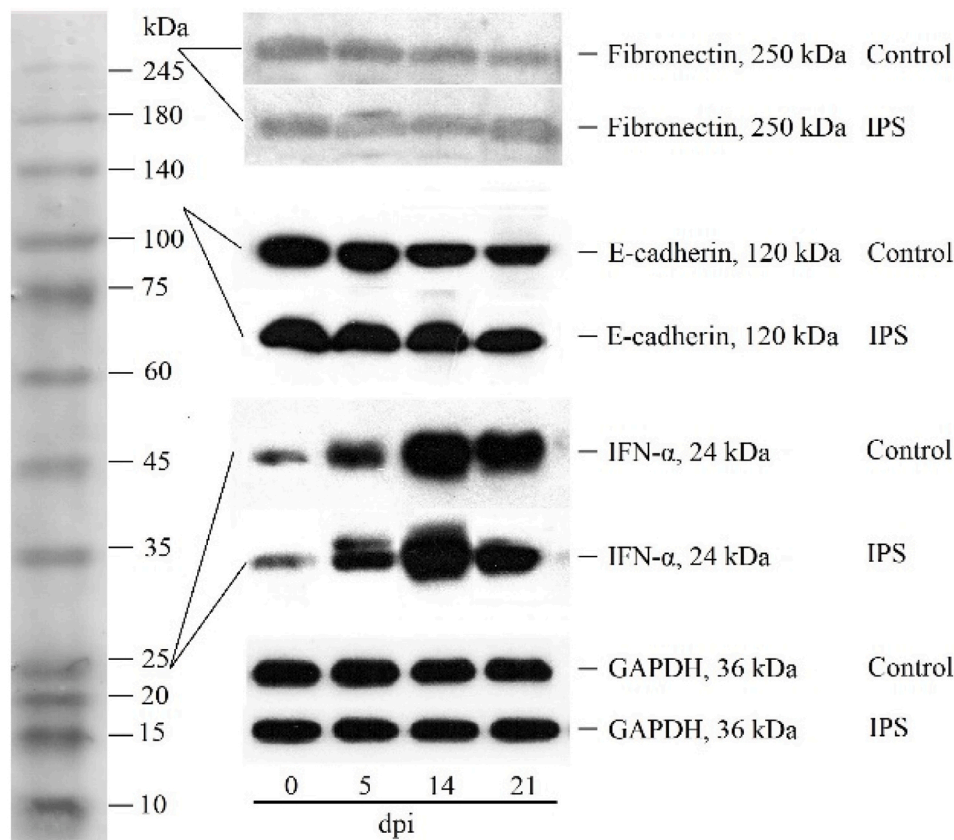


Fig. 4. Western Blot Images.

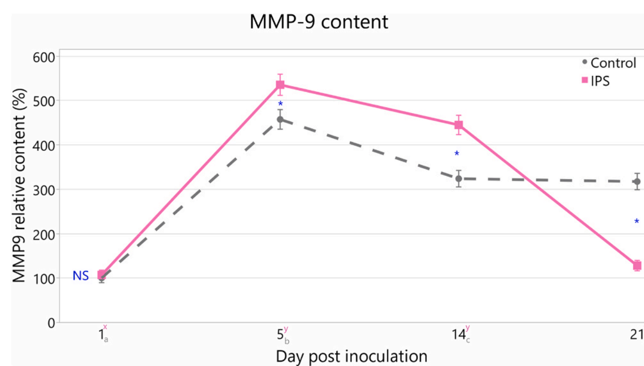


Fig. 5. Relative content (%; LS means and 95% confidence interval) of **MMP-9** in the duodenum of PEDV-infected piglets (n = 4 piglets/treatment/sampling day). *(P≤0.05); NS (P>0.05) indicate differences between treatment groups at the same time point; Different superscripts indicate differences between dpi, separately for each of the treatment groups ([a,b,c,d] Control; [w,x,y,z] IPS).

PEDV clinical signs without causing death of the study subjects. All piglets in this study developed moderate symptoms of PEDV, namely diarrhea. While our results were obtained on a small sample size, the results clearly show that PEDV was indeed present, active and impacting the biomarkers. Measurable and statistically significant changes were seen over time and between the IPS group and controls.

4.2. Biomarkers

4.2.1. E-cadherin

E-cadherin is a component of adherens junctions in epithelial layers. It contributes to intercellular adhesion by interacting with the cytoskeleton structures inside cells (Coopman and Djiane, 2016). Levels of E-cadherin have been reported to decrease when intestinal integrity is compromised (Sumagin and Parkos, 2015). Previous studies have investigated the modulation of specific adhesion proteins in intestinal cells infected with PEDV (Chen et al., 2022; Jung et al., 2015; Liu et al., 2020).

Being a structural protein, E-cadherin levels will change rather slowly, in days rather than hours. After an insult, E-cadherin levels will decrease. The time to measured response is expected to be at least 3–5 days. In this study, E-cadherin in the control group was significantly decreased from baseline by day 14 and stayed decreased to day 21. This result is in broad agreement with that of Jung et al. (2015) who showed a downregulation of E-cadherin in the jejunum of piglets at 10 days after infection with PEDV.

In contrast, the E-cadherin content in the IPS group did not significantly decrease from baseline over the 21-day study period. Furthermore, E-cadherin levels in the IPS group were also significantly higher than control on days 14 and 21. This result suggests that the IPS has a favorable influence on the preservation of E-cadherin.

4.2.2. Fibronectin

Fibronectin is another structural protein involved in the adherence of intestinal cells to the basement membrane and ECM (Dalton and Lemmon, 2021). Fibronectin also participates in cell proliferation and migration (Gagné et al., 2010) and in the protective mechanisms against gut injury by inhibiting apoptosis (Niederlechner et al., 2012). Like E-cadherin, increased fibronectin levels indicate less tissue damage and faster repair.

Similar to the results seen for E-cadherin, fibronectin levels in the IPS group did not significantly change from baseline over the 21-day study period. This was in contrast to the control group, in which fibronectin levels had decreased significantly from baseline by day 5 and remained

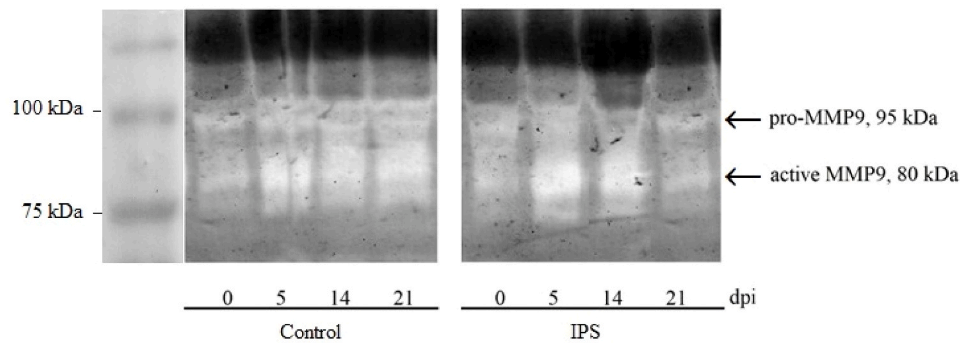


Fig. 6. Zymography Images.

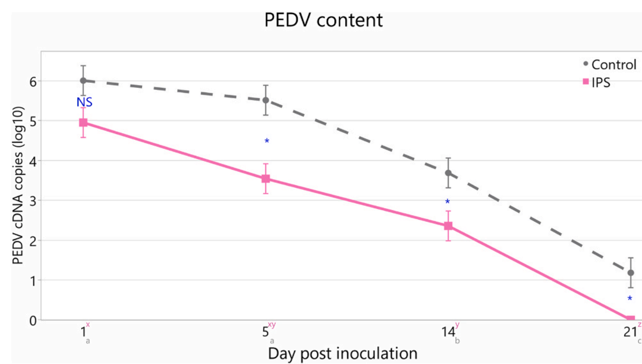


Fig. 7. PEDV content dynamics in the feces of PEDV-infected piglets (n = 4 piglets/treatment /sampling day). *(P≤0.05); NS (P>0.05) indicate differences between treatment groups at the same time point; Different superscripts indicate differences between dpi, separately for each of the treatment groups ([a,b,c,d] Control; [w,x,y,z] IPS).

significantly decreased for the entire study period.

The downregulation of fibronectin content observed in the duodenum of control piglets (Fig. 2) appears to be the direct result of virus-induced disturbances but could also have been caused by the significantly enhanced activity of MMP-9 (Fig. 5) as discussed in the next section.

4.2.3. MMP-9

MMP-9 is one of a family of zinc-dependent endopeptidases. MMPs as a family have a wide variety of roles, including proteolysis and cytokine activation, both of which impact intestinal permeability and activate immune responses (Van Spaendonk et al., 2017; Xiao et al., 2022). They are upregulated and secreted from a wide variety of immune cells, including neutrophils, macrophages and fibroblasts in inflammation (Yabluchanskiy et al., 2013). MMP-9 degrades the ECM and stimulates cell migration, thus aiding in tissue repair and remodeling (Bonnans et al., 2014; Cui et al., 2017; Garg et al., 2009). As tissue structure normalizes, MMP-9 levels will decrease.

In this study, both groups had peak MMP-9 levels at day 5, with the IPS group having significantly higher levels than controls. This could be interpreted as evidence of ongoing tissue remodeling and repair. By day 14, MMP-9 levels had decreased significantly from day 5 levels in the Control group, but not the IPS group. The MMP-9 levels in IPS piglets had returned to baseline by day 21. Conversely, MMP-9 levels in the control piglets were still significantly increased from baseline at day 21, indicating that some tissue remodeling was still occurring in control pigs. This suggests that tissue remodeling and a return to a "normal" homeostatic state occurred more quickly in the IPS group.

One possible limitation of this study was the fact that levels of MMP-2, another important enzyme in tissue remodeling, were not reported. MMP-2 has been studied *in vitro* in various porcine tissues such as mesenchymal stem cells (Almalki et al., 2017) and fibroblasts (Lafuma et al., 1994). It has also been studied *in vivo* in porcine follicular cysts (Grzesiak et al., 2022) and myocardium (Danielsen et al., 1998). MMP-2 activity in the intestinal cells could be investigated in future studies. However, to the best of the authors' knowledge, this is the first report of MMP-9 modulation in the presence of *in vivo* PEDV infection.

4.2.4. IFN-α

IFN-α is a cytokine which can have both pro-inflammatory and anti-inflammatory effects, depending upon the interplay of other mediators. In the initial stages of inflammation, IFN-α has a pro-inflammatory effect, but then exerts anti-inflammatory effects during the repair phase. IFN-α is used as a biomarker of immune activation and has a rather volatile response time, within hours.

The present study clearly showed upregulation of intestinal IFN-α production in the presence of *in vivo* PEDV infection, starting within the first 5 days post-infection. Peak levels of IFN-α were reached at day 14 in both study groups. The IPS group showed a markedly more rapid acceleration and then deceleration of IFN-α levels (Fig. 3). IFN-α levels in the IPS group were also significantly higher than control group at day 5 and day 14, but significantly lower at day 21. The day 21 result could be interpreted that the IPS group returned to a state of reduced inflammation by that time.

Although the antiviral activities of porcine IFN-lambda 3 (λ3) have been reported in PEDV-infected cells (Li et al., 2019) and for other enteropathogenic viruses (Van Winkle et al., 2022), this is the first report of the activity of porcine IFN-α in the presence of *in vivo* PEDV infection.

4.3. PEDV shedding and correlation with immune response

The results presented here show that the PEDV excretion period was significantly shorter in the IPS group. By day 21, PEDV was no longer detected in the feces of the IPS piglets but was still present in the control group. The mechanism by which this occurred certainly warrants further investigation.

The biomarker results in this study suggest that IPS supplementation reduced the tissue damage, modulated the immune response, and accelerated tissue recovery, as evidenced by the trajectories of E-cadherin, fibronectin, IFN-α and MMP-9. PEDV levels were highest on day 1, and only reached a significant decrease from that peak on day 14 in both groups. The period between day 14-day 21 is also when the immune mediators IFN-α and MMP-9 decreased significantly. These results are congruent with each other.

4.4. Possible mechanisms

The positive effect of the IPS on the intestinal structure biomarkers and immune mediators as seen in this study is congruent with the *in vivo*

effects obtained in healthy pigs fed the same IPS. Previous work has reported improvements in the microbiome, intestinal architecture and production gains (reduced mortality and improved weights) (Buzoianu et al., 2020; Buzoianu and Firth, 2023; Cortyl et al., 2019; Firth et al., 2017b).

The precise mechanism(s) by which IPS contributed to the effects seen in this *in vivo* study of PEDV infection are an intriguing area for further investigation. The effects on intestinal cell adhesion markers could be the result of an enhancement of enterocyte function, since the IPS used in this study has been specifically formulated to support enterocyte metabolism. The IPS may also contribute to improved epithelial structure, since it has been demonstrated previously that IPS feeding improves villus height in pigs (Firth et al., 2017b). It is also possible that indirect effects via the intestinal microbiome could have a role. The same IPS that was used in this study showed a beneficial effect on intestinal microbiota abundance in piglets aged 9–30 days (Buzoianu et al., 2020). The protective effect of the IPS is probably multi-factorial and warrants further investigation.

4.5. What lies ahead?

The main challenges in the control and treatment of PEDV include the high morbidity and mortality rates in naïve herds, the persistence of the virus, and the lack of effective vaccines. Measures to alleviate the impact of PEDV on swine production are generic and indirect, such as improved biosecurity, diagnostics, surveillance, and management practices. As mentioned in the introduction, numerous substances have been investigated for their ability to reduce the pathology or severity of PEDV infection. The efficacy of all aforementioned compounds is not fully understood, and neither are the precise molecular mechanisms by which they suppress the pathophysiology of enteric CoVs such as PEDV. That in itself is not an issue – what matters is efficacy *in vivo* and availability to swine producers around the world. Any proposed substances need to be evaluated under commercial conditions for dosage, taste, and economic viability in large-scale systems. Also, for a proposed ingredient or substance to be used in commercial swine production, it must be registered as an approved swine feed additive or supplement in each country or regulatory jurisdiction.

This study used a commercial, widely available IPS product that could be useful in active infection scenarios. The product appeared to be highly palatable and was readily drunk by the piglets, even though they were only 14 days old at the beginning of the study. The dose of IPS used in this study was 120 mL/day for a 4 kg pig. This dose was extrapolated from the manufacturer's recommendations to use approximately 40 mL/day for a healthy neonatal pig weighing about 1.5 kg. Swine producer reports from Taiwan, Thailand and China (unpublished results) have used much higher volumes of the IPS in outbreaks of PEDV in commercial production. It should also be noted that this particular IPS is formulated to be administered in drinking water, instead of being mixed with solid feed. This feature is particularly relevant to very young piglets who may not yet be accustomed to solid feed.

5. Conclusion

Our study shows that IPS supplementation appears to be a valid strategy to support intestinal integrity, modulate the immune response, accelerate epithelial tissue repair, and potentially decrease the duration of PEDV virus shedding. There is certainly opportunity for further investigation of the cellular mechanisms by which such effects occur. The IPS used in this study is a swine feed product registered for use in over 40 countries and is therefore potentially useful to commercial swine producers. Further large-scale *in vivo* studies would be helpful in establishing the impact of IPS supplementation on morbidity and mortality in the face of PEDV outbreaks.

Funding

The work was supported by the Ministry of Education and Science of Ukraine [grant no. 0122U001318, ID:188187 05.11.2021].

Acknowledgments

The authors would like to thank the personnel at Biosafety Center for their involvement in the laboratory studies, and Mathieu Cortyl for his contribution.

Declaration of competing interest

S.G. Buzoianu and A. M. Firth are employees of Tonisity International. They had no participation in the field or laboratory phases of the study, or compilation of raw data. SGB performed the statistical analysis based on the data supplied and generated the results graphs. AMF served as the senior editor, given that the primary authors are not native-English speakers. The authors declare that they have no other known competing financial interests or personal relationships that could have influenced the work reported in this paper.

References

Almalki, S.G., Valle, Y.L., Agrawal, D.K., 2017. MMP-2 and MMP-14 silencing inhibits VEGFR2 cleavage and induces the differentiation of porcine adipose-derived mesenchymal stem cells to endothelial cells. Stem Cells Transl. Med. 6, 1385–1398. https://doi.org/10.1002/sctm.16-0329.

Alvarado, J., del Castillo, J.R., Thomas, L.E., 2008. Modulation of membrane type 1 matrix metalloproteinase by LPS and gamma interferon bound to extracellular matrix in intestinal crypt cells. Cytokine 41, 155–161. https://doi.org/10.1016/j.cyto.2007.11.006.

Bonnans, C., Chou, J., Werb, Z., 2014. Remodelling the extracellular matrix in development and disease, 2014 1512 Nat. Rev. Mol. Cell Biol. 15, 786–801. https://doi.org/10.1038/nrm3904.

Buzoianu, S.G., Firth, A.M., 2023. Distribution of pre-weaning mortality in 20 920 pigs and effectiveness of an isotonic protein drink intervention. Anim. - Sci. Proc. 14, 769–770. https://doi.org/10.1016/J.ANSCIP.2023.08.050.

Buzoianu, S.G., Firth, A.M., Putrino, C., Vannucci, F., 2020. Early-life intake of an isotonic protein drink improves the gut microbial profile of piglets. Anim 10, 879. https://doi.org/10.3390/ani10050879.

Chen, J., Xu, W., Li, P., Song, L., Jiang, Y., Hao, P., Gao, Z., Zou, W., Jin, N., Li, C., 2022. Antiviral effect of pIFNLs against PEDV and VSV infection in different cells. Int. J. Mol. Sci. 23 https://doi.org/10.3390/ijms23179661.

Coopman, P., Djiane, A., 2016. Adherens junction and E-cadherin complex regulation by epithelial polarity. Cell. Mol. Life Sci. 73, 3535–3553. https://doi.org/10.1007/s00018-016-2260-8.

Cortyl, M., Carlson, A., Eisenhart, M., Buzoianu, S.G., Bretey, K., Firth, A., 2019. Effect of supplemental nutrition through an isotonic protein solution provided days 2 to 8 of life on pre-weaning mortality of piglets and their lifetime performance. ASIAN PIG VETERINARY Soc. Congr. 82.

Cui, N., Hu, M., Khalil, R.A., 2017. Biochemical and Biological Attributes of Matrix Metalloproteinases. Prog. Mol. Biol. Transl. Sci. 147, 1–73. https://doi.org/10.1016/BS.PMBTS.2017.02.005.

Curry, S.M., Schwartz, K.J., Yoon, K.-J., Gabler, N.K., Burrough, E.R., 2017. Effects of porcine epidemic diarrhea virus infection on nursery pig intestinal function and barrier integrity. Vet. Microbiol. 211, 58–66. https://doi.org/10.1016/j.vetmic.2017.09.021.

Dalton, C.J., Lemmon, C.A., 2021. Fibronectin: molecular structure, fibrillar structure and mechanochemical signaling. Cells 10. https://doi.org/10.3390/cells10092443.

Danielsen, C.C., Wiggers, H., Andersen, H.R., 1998. Increased amounts of collagenase and gelatinase in porcine myocardium following ischemia and reperfusion. J. Mol. Cell. Cardiol. 30 https://doi.org/10.1006/jmcc.1998.0711.

Dong, S., Yu, R., Wang, X., Chen, B., Si, F., Zhou, J., Xie, C., Li, Z., Zhang, D., 2022. Bisbenzylisoquinoline alkaloids inhibit Porcine Epidemic Diarrhea Virus in vitro and in vivo. Viruses 14. https://doi.org/10.3390/v14061231.

Firth, A.M., Lopez, Cano, G., Morillo Alujas, A., 2017a. Effect of gruel and Tonisity Px on feed intake and weight gain at weaning. 48th Meet. Am. Assoc. Swine Vet. 134–137.

Firth, A.M., Lopez, Cano, G., Morillo Alujas, A., 2017b. Effect of Tonisity Px administration on intestinal morphology. 48th Meet. Am. Assoc. Swine Vet. 310.

Gagné, D., Groulx, J.-F., Benoit, Y.D., Basora, N., Herring, E., Vachon, P.H., Beaulieu, J.-F., 2010. Integrin-linked kinase regulates migration and proliferation of human intestinal cells under a fibronectin-dependent mechanism. J. Cell. Physiol. 222, 387–400. https://doi.org/10.1002/jcp.21963.

Garg, P., Vijay-Kumar, M., Wang, L., Gewirtz, A.T., Merlin, D., Sitaraman, S.V., 2009. Matrix metalloproteinase-9-mediated tissue injury overrides the protective effect of matrix metalloproteinase-2 during colitis. Am. J. Physiol. - Gastrointest. Liver Physiol. 296, 175–184. https://doi.org/10.1152/AJPGI.90454.2008/ASSET/IMAGES/LARGE/ZH30110852380004.JPEG.

Gieryńska, M., Szulc-Dąbrowska, L., Struzik, J., Mielcarska, M.B., Gregorczyk-Zboroch, K.P., 2022. Integrity of the intestinal barrier: the involvement of epithelial cells and microbiota-a mutual relationship. Anim. Open Access J. MDPI 12. https://doi.org/10.3390/ANI12020145.

González-Navajas, J.M., Lee, J., David, M., Raz, E., 2012. Immunomodulatory functions of type I interferons. Nat. Rev. Immunol. 12, 125–135. https://doi.org/10.1038/NRI3133.

Grzesiak, M., Kaminska, K., Knapczyk-Stwora, K., Hrabia, A., 2022. The expression and localization of selected matrix metalloproteinases (MMP-2, -7 and -9) and their tissue inhibitors (TIMP-2 and -3) in follicular cysts of sows. Theriogenology 185. https://doi.org/10.1016/j.theriogenology.2022.03.029.

Jung, K., Eyerly, B., Annamalai, T., Lu, Z., Saif, L.J., 2015. Structural alteration of tight and adherens junctions in villous and crypt epithelium of the small and large intestine of conventional nursing piglets infected with porcine epidemic diarrhea virus. Vet. Microbiol. 177, 373–378. https://doi.org/10.1016/j.vetmic.2015.03.022.

Jung, K., Saif, L.J., 2015. Porcine epidemic diarrhea virus infection: Etiology, epidemiology, pathogenesis and immunoprophylaxis. Vet. J. 204, 134–143. https://doi.org/10.1016/j.tvjl.2015.02.017.

Jung, K., Saif, L.J., Wang, Q., 2020. Porcine epidemic diarrhea virus (PEDV): An update on etiology, transmission, pathogenesis, and prevention and control. Virus Res 286, 198045. https://doi.org/10.1016/j.virusres.2020.198045.

Kikuti, M., Drebes, D., Robbins, R., Dufresne, L., Sanhueza, J.M., Corzo, C.A., 2022. Growing pig incidence rate, control and prevention of porcine epidemic diarrhea virus in a large pig production system in the United States. Porc. Heal. Manag. 8, 1–7. https://doi.org/10.1186/S40813-022-00268-9/TABLES/2.

Lafuma, C., Azzi El Nabout, R., Crechet, F., Hovnanian, A., Martin, M., 1994. Expression of 72-kDa gelatinase (MMP-2), collagenase (MMP-1), and tissue metalloproteinase inhibitor (TIMP) in primary pig skin fibroblast cultures derived from radiation-induced skin fibrosis. J. Invest. Dermatol. 102 https://doi.org/10.1111/1523-1747.ep12384118.

Li, L., Xue, M., Fu, F., Yin, L., Feng, L., Liu, P., 2019. IFN-lambda 3 mediates antiviral protection against Porcine Epidemic Diarrhea Virus by inducing a distinct antiviral transcript profile in porcine intestinal epithelia. Front. Immunol. 10, 2394. https://doi.org/10.3389/fimmu.2019.02394.

Li, Z., Ma, Z., Li, Y., Gao, S., Xiao, S., 2020. Porcine epidemic diarrhea virus: Molecular mechanisms of attenuation and vaccines. Microb. Pathog. 149, 104553 https://doi.org/10.1016/j.micpath.2020.104553.

Liu, Q., Wang, H.-Y., 2021. Porcine enteric coronaviruses: an updated overview of the pathogenesis, prevalence, and diagnosis. Vet. Res. Commun. 45, 75–86. https://doi.org/10.1007/s11259-021-09808-0.

Liu, Y.-S., Liu, Q., Jiang, Y.-L., Yang, W.-T., Huang, H.-B., Shi, C.-W., Yang, G.-L., Wang, C.-F., 2020. Surface-displayed porcine IFN-lambda3 in Lactobacillus plantarum inhibits porcine enteric coronavirus infection of porcine intestinal epithelial cells. J. Microbiol. Biotechnol. 30, 515–525. https://doi.org/10.4014/jmb.1909.09041.

Luo, L., Gu, Z., Pu, J., Chen, D., Tian, G., He, J., Zheng, P., Mao, X., Yu, B., 2024. Synbiotics improves growth performance and nutrient digestibility, inhibits {PEDV} infection and prevents intestinal barrier dysfunction by mediating innate antivirus immune response in weaned piglets challenged with {PEDV}. J. Anim. Sci. 25, skae023. https://doi.org/10.1093/jas/skae023.

Masiuk, D.N., Hlebeniuk, V.V., Kokariev, A.V., Vasylenko, T.A., 2019. Molecular characteristics of the Porcine Epidemic Diarrhea Virus strains isolated in different regions of Ukraine. Biopolym. Cell 35, 486–494. https://doi.org/10.7124/bc.000A1B.

Masiuk, D.N., Nedzvetsky, V.S., Sosnitskiy, A.I., Kokarev, A.V., Koliada, S.G., 2018. The characteristics, emergent properties and manner of spread in Ukraine of the Porcine Epidemic Diarrhea Virus. Regul. Mech. Biosyst. 9, 401–408. https://doi.org/10.15421/021860.

Niederlechner, S., Klawitter, J., Baird, C., Kallweit, A.-R., Christians, U., Wischmeyer, P. E., 2012. Fibronectin-integrin signaling is required for L-glutamine's protection against gut injury. PLoS One 7, e50185. https://doi.org/10.1371/journal.pone.0050185.

Pensaert, M.B., de Bouck, P., 1978. A new coronavirus-like particle associated with diarrhea in swine. Arch Virol. 58, 243–247. https://doi.org/10.1007/BF01317606.

Qin, W., Qi, X., Xie, Y., Wang, H., Wu, S., Sun, M.-A., Bao, W., 2023. LncRNA446 regulates tight junctions by inhibiting the ubiquitinated degradation of alix after Porcine Epidemic Diarrhea Virus infection. J. Virol. 97, e0188422 https://doi.org/10.1128/jvi.01884-22.

Ruedas-Torres, I., Puente, H., Fristikova, K., Argüello, H., Salguero, F.J., Carvajal, A., Gómez-Laguna, J., 2024. Pathological and immunohistochemical assessment of the impact of three different strains of swine enteric coronaviruses in the intestinal barrier. Vet. Microbiol 290, 109956. https://doi.org/10.1016/j.vetmic.2023.109956.

Schlegel, N., Meir, M., Heupel, W.-M., Holthöfer, B., Leube, R.E., Waschke, J., 2010. Desmoglein 2-mediated adhesion is required for intestinal epithelial barrier integrity. Am. J. Physiol. Liver Physiol. 298, G774–G783. https://doi.org/10.1152/ajpgi.00239.2009.

Schumacher, L.L., Woodworth, J.C., Jones, C.K., Chen, Q., Zhang, J., Gauger, P.C., Stark, C.R., Main, R.G., Hesse, R.A., Tokach, M.D., Dritz, S.S., 2016. Evaluation of the minimum infectious dose of porcine epidemic diarrhea virus in virus-inoculated feed. Am. J. Vet. Res. 77, 1108–1113. https://doi.org/10.2460/ajvr.77.10.1108.

Shen, X., Yin, L., Pan, X., Zhao, R., Zhang, D., 2020. Porcine epidemic diarrhea virus infection blocks cell cycle and induces apoptosis in pig intestinal epithelial cells. Microb. Pathog. 147, 104378 https://doi.org/10.1016/j.micpath.2020.104378.

Stroup, W.W., 2015. Rethinking the analysis of non-normal data in plant and soil science. Agron. J. 107, 811–827. https://doi.org/10.2134/AGRONJ2013.0342.

Sumagin, R., Parkos, C.A., 2015. Epithelial adhesion molecules and the regulation of intestinal homeostasis during neutrophil transepithelial migration. Tissue Barriers 3, e969100. https://doi.org/10.4161/21688362.2014.969100.

Thomas, J.T., Chen, Q., Gauger, P.C., Giménez-Lirola, L.G., Sinha, A., Harmon, K.M., Madson, D.M., Burrough, E.R., Magstadt, D.R., Salzbrenner, H.M., Welch, M.W., Yoon, K.-J., Zimmerman, J.J., Zhang, J., 2015. Effect of Porcine Epidemic Diarrhea Virus infectious doses on infection outcomes in naïve conventional neonatal and weaned pigs. PLoS One 10, e0139266. https://doi.org/10.1371/journal.pone.0139266.

Van Spaendonk, H., Ceuleers, H., Witters, L., Patteet, E., Joossens, J., Augustyns, K., Lambeir, A.-M., De Meester, I., De Man, J.G., De Winter, B.Y., 2017. Regulation of intestinal permeability: The role of proteases. World J. Gastroenterol. 23, 2106–2123. https://doi.org/10.3748/wjg.v23.i12.2106.

Van Winkle, J.A., Peterson, S.T., Kennedy, E.A., Wheadon, M.J., Ingle, H., Desai, C., Rodgers, R., Constant, D.A., Wright, A.P., Li, L., Artyomov, M.N., Lee, S., Baldridge, M.T., Nice, T.J., 2022. Homeostatic interferon-lambda response to bacterial microbiota stimulates preemptive antiviral defense within discrete pockets of intestinal epithelium. Elife 11. https://doi.org/10.7554/eLife.74072.

Wang, L., Zhou, J., Hou, Y., Yi, D., Ding, B., Xie, J., Zhang, Y., Chen, H., Wu, T., Zhao, D., Hu, C.-A.A., Wu, G., 2017. N-Acetylcysteine supplementation alleviates intestinal injury in piglets infected by porcine epidemic diarrhea virus. Amino Acids 49, 1931–1943. https://doi.org/10.1007/s00726-017-2397-2.

Wood, E.N., 1977. An apparently new syndrome of porcine epidemic diarrhoea. Vet. Rec. 100, 243–244. https://doi.org/10.1136/vr.100.12.243.

Wu, M., Yi, D., Zhang, Q., Wu, T., Yu, K., Peng, M., Wang, L., Zhao, D., Hou, Y., Wu, G., 2021. Puerarin enhances intestinal function in piglets infected with porcine epidemic diarrhea virus. Sci. Rep. 11, 6552. https://doi.org/10.1038/s41598-021-85880-5.

Wu, Y., Li, W., Zhou, Q., Li, Q., Xu, Z., Shen, H., Chen, F., 2019. Characterization and pathogenicity of Vero cell-attenuated porcine epidemic diarrhea virus CT strain. Virol. J. 16, 121. https://doi.org/10.1186/s12985-019-1232-7.

Xiao, Y., Lian, H., Zhong, X.S., Krishnachaitanya, S.S., Cong, Y., Dashwood, R.H., Savidge, T.C., Powell, D.W., Liu, X., Li, Q., 2022. Matrix metalloproteinase 7 contributes to intestinal barrier dysfunction by degrading tight junction protein Claudin-7. Front. Immunol. 13, 1020902 https://doi.org/10.3389/fimmu.2022.1020902.

Xu, Z., Liu, Yuan, Peng, P., Liu, Yufang, Huang, M., Ma, Y., Xue, C., Cao, Y., 2020. Aloe extract inhibits porcine epidemic diarrhea virus in vitro and in vivo. Vet. Microbiol. 249, 108849 https://doi.org/10.1016/j.vetmic.2020.108849.

Yabluchanskiy, A., Ma, Y., Iyer, R.P., Hall, M.E., Lindsey, M.L., 2013. Matrix Metalloproteinase-9: many shades of function in cardiovascular disease. Physiology 28, 391. https://doi.org/10.1152/PHYSIOL.00029.2013.

Yang, J., Tian, G., Chen, D., Zheng, P., Yu, J., Mao, X., He, J., Luo, Y., Luo, J., Huang, Z., Wu, A., Yu, B., 2019. Dietary 25-hydroxyvitamin D3 supplementation alleviates Porcine Epidemic Diarrhea Virus infection by improving intestinal structure and immune response in weaned pigs. Anim 9. https://doi.org/10.3390/ani9090627.

Zhang, J., Li, M., Qi, X., Liu, Y., Liu, Q., Xie, M.-Y., Xie, Y.-L., Liu, J., Wei, P., Cui, W., et al., 2021a. Novel small peptides derived from milk proteins are protective against porcine epidemic diarrhea virus (PEDV) infection in vitro. J. Dairy Sci. 104, 5712–5726. https://doi.org/10.3168/jds.2020-19654.

Zhang, Y., Chen, H., Zou, M., Oerlemans, R., Shao, C., Ren, Y., Zhang, R., Huang, X., Li, G., Cong, Y., 2021b. Hypericin inhibit alpha-coronavirus replication by targeting 3CL protease. Viruses 13, 1825.

Zhou, Y., Ren, Z., Zhang, S., Wang, H., Wu, S., Bao, W., 2021. Analysis of intestinal mucosa integrity and GLP-2 gene functions upon Porcine Epidemic Diarrhea Virus infection in pigs. Anim 11, 644. https://doi.org/10.3390/ani11030644.

Zong, Q.F., Huang, Y.J., Wu, L.S., Wu, Z.C., Wu, S.L., Bao, W.B., 2019. Effects of porcine epidemic diarrhea virus infection on tight junction protein gene expression and morphology of the intestinal mucosa in pigs. Pol. J. Vet. Sci. 22, 345–353. https://doi.org/10.24425/pjvs.2019.129226.