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Effect of direct-fed microbial *Bacillus subtilis* C-3102 on enteric health in nursery pigs after challenge with porcine epidemic diarrhea virus

P. Canning, DVM; C. Ruston; D. Madson, DVM, PhD, Diplomate ACVP; J. Bates, DVM, MS; K. Skoland; J. Davenport; S. Gaul; C. Wang, MS, PhD; Q. Chen, BVM, MS, PhD; J. Zhang, MD, MS, PhD; L. Karriker, DVM, MS, Diplomate ACVPM

Summary

Objective: To examine the effects of feeding *Bacillus subtilis* C-3102 at the target inclusion rates of 0 colony forming units (CFU) per g, 500,000 CFU per g, and 1 million CFU per g on intestinal health in weaned pigs after challenge with porcine epidemic diarrhea virus (PEDV).

Materials and methods: A two-by-three factorial design was conducted, composed of three experimental diets and PEDV or sham challenge. Sixty 14-day-old pigs, negative for PEDV by quantitative real-time reverse transcription polymerase chain reaction (PCR) and negative by PCR for porcine reproductive and respiratory syndrome virus, were randomly allocated into six treatment groups with 10 pigs per group. Pigs were housed in groups of five in solid-floor pens. Treatment diets were fed for a total of 23 days, including 19 days before and 4 days after PEDV challenge or sham challenge by oral gavage.

Results: Pathological changes associated with PEDV were significantly less severe in challenged treatment groups that received *B subtilis* C-3102 than in the group that received no *B subtilis* treatment. There were no significant differences in small intestinal length, ratio of small intestinal weight to body weight, colon dry matter content, average daily gain, or fecal scoring between any of the six treatment groups.

Implication: Under the conditions of this study, treatment with *B subtilis* C-3102 in nursery pigs challenged with PEDV is associated with better enteric health than in pigs not treated with *B subtilis* C-3102.

Keywords: swine, porcine epidemic diarrhea virus, direct-fed microbials, *Bacillus subtilis*

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Resumen - Efecto de la alimentación directa microbiana, *Bacillus subtilis* C-3102, en la salud entérica en cerdos de destete después del reto con el virus de la diarrea epidémica porcina

Objetivo: Examinar los efectos de la alimentación con el *Bacillus subtilis* C-3102 en la índices meta de inclusión de 0 unidades formadoras de colonia (CFU por sus siglas en inglés) por g, 500,000 CFU por g, y 1 millón de CFU por g en la salud intestinal en cerdos destetados después del reto con el virus de la diarrea epidémica porcina (PEDV).

Materiales y métodos: Se condujo un diseño factorial de dos por tres, compuesto de tres dietas experimentales y PEDV o reto falso. Sesenta cerdos de 14 días de edad, negativos al PEDV por medio de la reacción cuantitativa en tiempo real en cadena de la polimerasa con transcriptasa inversa cuantitativa en tiempo real (PCR por sus siglas en inglés) y negativa por medio de la PCR al virus del síndrome reproductivo y respiratorio porcino (PRRSV), se distribuyeron aleatoriamente en seis grupos de tratamiento con 10 cerdos por grupo. Los cerdos fueron alojados en grupos de cinco animales en corrales de piso sólido. Se alimentaron con las dietas tratamiento por un total de 23 días, incluyendo 19 días antes y 4 días después del reto de PEDV o reto falso por medio de alimentación oral forzada.

Resultados: Los cambios patológicos asociados con el PEDV fueron significativamente menos severos en los grupos retados y con tratamiento que recibieron *B subtilis* C-3102 que en el grupo que no recibió tratamiento con *B subtilis*. No hubo diferencias significativas en la longitud del intestino delgado, relación del peso del intestino delgado con el peso corporal, contenido de materia seca del colon, ganancia diaria promedio, o puntaje fecal entre cualquiera de los seis grupos de tratamiento.

Implicacione: Bajo las condiciones de este estudio, el tratamiento con *B subtilis* C-3102 en cerdos de destete retados con el PEDV se asocia con mejor salud entérica que en cerdos no tratados con *B subtilis* C-3102.

Résumé - Effet de l'administration orale de *Bacillus subtilis* C-3102 sur la santé entérique de porcelets en pouponnière suite à une infection avec le virus de la diarrhée épidémique porcine

Objectif: Examiner les effets de l'administration orale de *Bacillus subtilis* C-3102 à la dose cible d'inclusion de 0 unité

PC, CR, JB, KS, JD, LK: Swine Medicine Education Center, Iowa State University College of Veterinary Medicine, Ames, Iowa.

DM, QC, JZ: Iowa State University, Veterinary Diagnostic Laboratory, Ames, Iowa.

SG: Department of Animal Science, Iowa State University, Ames, Iowa.

CW: Veterinary Diagnostic and Production Animal Medicine, Iowa State University College of Veterinary Medicine, Ames, Iowa.

Corresponding author: Dr Locke Karriker, Swine Medicine Education Center, Iowa State University College of Veterinary Medicine, 2412 Lloyd Veterinary Medical Center, Ames, IA 50011; Tel: 515-294-2283; Fax: 515-294-1072; E-mail: karriker@iastate.edu.

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formatrice de colonie (UFC) par g, 500,000 UFC par g, et 1 million UFC par g sur la santé intestinale de porcelets sevrés suite à une infection défi avec le virus de la diarrhée épidémique porcin (VDEP).

Matériels et méthodes: Une étude avec un plan factoriel de 2 × 3 a été menée, composé de trois diètes expérimentales et une infection défi avec VDEP ou une infection simulée. Soixante porcs âgés de 14 jours, négatifs pour le VDEP par réaction d'amplification en chaîne quantitative (PCR) en temps réel avec la polymérase réverse et négatif par PCR pour le virus du syndrome reproducteur et respiratoire porcin (VSRRP), ont été répartis de manière aléatoire en six groupes de traitement de 10 porcs par groupe. Les porcs étaient logés en groupe de cinq porcs dans des parcs avec des planchers pleins. Les diètes ont été données aux groupes sous traitement pour un total de 23 jours, 19 jours avant et 4 jours après le challenge avec le VDEP ou l'infection simulée par gavage oral.

Résultats: Les changements pathologiques associés avec le VDEP étaient significativement moins sévères dans les groupes infectés ayant reçu *B subtilis* C-3102 comparativement aux groupes n'ayant pas reçu de traitement avec *B subtilis* C-3102. Il n'y avait aucune différence significative dans la longueur du petit intestin, le ratio du poids du petit intestin sur le poids corporel, le contenu en matière sèche du côlon, le gain moyen quotidien, ou le pointage fécal entre les six groupes de traitement.

Implication: Dans les conditions de la présente étude, le traitement avec *B subtilis* C-3102 de porcs en pouponnière infectés avec VDEP est associé avec une meilleure santé entérique que chez des porcs non traités avec *B subtilis* C-3102.

Porcine epidemic diarrhea virus (PEDV) was first detected in North America in April 2013 and since then has been diagnosed in over 30 US states, with more than 9000 confirmed cases.[1] It is well understood that PEDV affects intestinal health by producing atrophic enteritis through viral destruction of enterocytes.[1,2] Infected suckling and nursery pigs experience a maldigestive and malabsorptive diarrhea resulting in acute dehydration, and increased risk of septicemia due to decreased intestinal barrier functions. The severity of clinical signs is age dependent, with piglets less than 7 days of age demonstrating the most severe diarrhea and mortality rates.[1,2] Many swine production groups and stakeholders are interested in products that mitigate clinical signs and reduce intestinal pathology associated with PEDV. In addition to biologics and supportive fluid therapy, direct-fed microbials (DFMs) may present an alternative option to supportive therapy for PEDV-infected pigs. Direct-fed microbials, which are readily accessible to producers and easily incorporated into feed rations for adult and growing animals, have been investigated in the food-animal literature for their effects in improving intestinal health, feed efficiency, and meat quality, and modifying fecal consistency.[3,4] Targeted studies[5,6] have investigated the effects of DFMs on pathogen shedding and mitigating clinical signs caused by enteric pathogens, including *Salmonella* serovars and *Escherichia coli.* Common commercially available DFM products are strain specific and include yeasts and (or) bacteria, specifically *Bacillus* species and (or) *Lactobacillus* species. To date, we have been unable to find publically available peer-reviewed literature describing the effects of DFMs on intestinal health and pathology following challenge with or exposure to PEDV. As PEDV is a contemporary pathogen with several well developed challenge models, an assessment of the utility of DFMs in mitigating the effects of the disease was warranted and relevant to modern pig production.[3,7,8]

The focus of this study was on *Bacillus subtilis* C-3102 (Calsporin; Calpis Co Ltd, Japan and Quality Technology International, Huntley, Illinois), which is a gram-positive, catalase-positive, rod-shaped obligate aerobe that is stable in extreme environmental conditions.[9] It has a history of use in swine since 1981 and is manufactured as a commercial product available to the US swine industry.[10]

The objective of this study was to examine the effects on intestinal health and pathology of feeding *B subtilis* C-3102 prior to and during a PEDV challenge in weaned pigs. The targeted inclusion rates studied were 0 colony-forming units (CFUs) per g, 500,000 CFUs per g, and 1 million CFUs per g.

Material and methods

This study was approved by the Iowa State University Institutional Animal Care and Use Committee.

Animals and housing

Sixty 14-day old, high health status barrows (30) and gilts (30) were sourced from a private commercial provider in Iowa. Fourteen-day-old weaned piglets were selected to ensure that pigs would be sufficiently young at PEDV inoculation to experience clinical signs, and because suckling pigs would not have substantial feed intake while on the sow. Pigs and their dams had no history of PEDV or porcine deltacoronavirus exposure or clinical signs and were considered naive to these pathogens. Pigs were housed at the Iowa State University Laboratory Animal Resources Facility in 12 different rooms, five pigs per room, with two rooms for each treatment group. The rooms had solid floors, and each contained one water nipple and feeder. Pigs were fed ad libitum in a plastic pan-style feeder with 61 cm of tray length divided into three feeding spaces. Gruel feed was offered for the first 3 days of the study to ease the transition from milk to solid food. Floor space per animal met the requirements set in the Guide for the Care and Use of Agricultural Animals in Research and Teaching.[11]

Study design

Treatment allocation. Upon arrival, pigs were each weighed and given a uniquely numbered plastic livestock ear tag placed in the right ear (Allflex, Dallas, Texas). Pigs were randomized to treatment groups and to individual rooms using the random number generator function in Microsoft Excel (Microsoft, Redmond, Washington). The 60 pigs were each randomly allocated to one of the six treatment groups (Table 1), with 10 pigs per treatment group (five barrows and five gilts).

This was a 2 × 3 factorial design involving three experimental diets and PEDV or sham challenge. The experimental diets were standard, commercially prepared, antibiotic-free nursery pig diets containing *Bacillus subtilis* C-3102 (Calsporin at 0, 500,000, or 1 million CFUs per g). Groups were challenged with either PEDV-positive or PEDV-negative cell-culture fluids by oral gastric gavage. Table 1 displays the six treatment groups represented in this design. Pigs were acclimated to the research facilities and solid feed for 3 days. The study diets were then fed for 19 days and pigs were inoculated with PEDV-positive or PEDV-negative cell-culture fluid on day 22. On the basis of the manufacturer's recommendations, optimal gastrointestinal effect was anticipated after feeding for 14 to 21 days, which thus was selected as the targeted duration of feeding before inoculation with PEDV.

Four days after inoculation (day 26), pigs were euthanized and necropsied. The pigs remained on the treatment diets up to and

Table 1: Pigs fed *Bacillus subtilis* C-3102 at one of three concentrations in the feed for 19 days were then administered, by gavage, cell culture either positive (three groups) or negative (three groups) for porcine epidemic diarrhea virus (PEDV)*

Treatment group	Inoculum†	Diet treatment
1	PEDV-negative cell culture	None
2	PEDV-negative cell culture	*B subtilis* C-3102, 500,000 CFU/g
3	PEDV-negative cell culture	*B subtilis* C-3102, 1,000,000 CFU/g
4	PEDV-positive cell culture	None
5	PEDV-positive cell culture	*B subtilis* C-3102, 500,000 CFU/g
6	PEDV-positive cell culture	*B subtilis* C-3102, 1,000,000 CFU/g

* 2 × 3 factorial design with 10 pigs randomly allocated to each dietary treatment group for a total of 60 pigs. Pigs weaned at 14 days of age were each fed *B subtilis* C-3102 (Calsporin; Calpis Co Ltd, Japan and Quality Technology International, Huntley, Illinois) for 19 days at one of three concentrations in the feed. On day 20 of feeding treatment diets, pigs received cell culture either positive or negative for PEDV by oral gavage. Four days after inoculation, pigs were euthanized by captive bolt and intestinal samples were collected.

† PEDV in cell culture was confirmed by quantitative real-time reverse transcription polymerase chain reaction testing.
CFUs = colony-forming units.

including the day of necropsy, totaling 23 days on the experimental diets.

Pre-trial processing

On day 1 of the study, blood and fecal swabs were collected from each pig. Whole blood was collected by jugular venipuncture into a serum separator tube. Whole blood was centrifuged at 2000*g* for 10 minutes and serum was poured into plastic snap cap 5-mL serum tubes. Polyester-tip swabs were used to collect fecal samples. Swabs were then placed in 1 mL of sterile phosphate buffered saline (PBS) in a 5-mL snap cap tube. Serum and fecal samples were submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL). The following assays were performed: PRRSV polymerase chain reaction (PCR)[12] was performed on serum samples pooled in groups of five. Additionally, PEDV[13] and PRRSV[12] enzyme-linked immunosorbent assays (ELISAs) were performed individually on serum samples. Fecal swabs were pooled in groups of five, and PEDV PCR[1,8] was performed on each pool. Pigs included in the trial did not consume water or feed with antibiotics for at least 7 days prior to the beginning of the trial, and had no prior injectable antibiotic treatments before the trial start date.

Feed and water

Heartland Co-op (Waukee, Iowa) provided three custom grind and mix diets for the trial (*B subtilis* C-3102 at 0, 500,000, and 1 million CFU per g). The diets contained no antibiotics and met the National Research Council nutrition requirement for pigs.[14] The diets were produced at the mill in 453-kg batches. One 22.7-kg bag of *B subtilis* C-3102 (Calsporin 2.0B lot 190588) containing 412.3 billion CFU per kg of *B subtilis* C-3102, guaranteed analysis, was supplied to the feed mill and incorporated into the rations as per label instructions for each given concentration. The three rations were then processed into 22.7-kg bags labeled with the corresponding *B subtilis* C-3102 concentration. The feed was stored in bins with locking lids in each room. Correct diet allocation to each study room was verified by two people, independently, each day. Commercial nursery phase-one starter pellets without *B subtilis* C-3102 or antibiotics were fed during the first 3 days of the acclimation period. After the acclimation period, the pigs were started on the *B subtilis* C-3102 diets ad libitum and continued on these diets for the remainder of the trial. Daily feed and water samples were collected from each room and stored at -20°C for further analysis if needed.

Porcine epidemic diarrhea virus (PEDV) challenge

On day 22 of the trial, the sham challenge (groups 1, 2, 3) and the PEDV challenge (groups 4, 5, 6) were performed by orogastric gavage using an 18 French rubber catheter. Each pig in treatment groups 1, 2, and 3 was inoculated with 10 mL of a PEDV-negative virus culture medium. Each pig in treatment groups 4, 5, and 6 was challenged with 10 mL of PEDV-positive cell culture containing 10^4 median tissue culture infective doses ($TCID_{50}$) of PEDV per mL. Ten-mL aliquots of PEDV-positive and PEDV-negative inoculum were collected at the time of inoculation and submitted for PEDV PCR to confirm status.

Biological sample collection

Pig weights and fecal swabs. Pig weights were obtained on arrival (day 0), at commencement of study diets (day 3), on day of inoculation (day 22), and on day of necropsy (day 26). At each weigh point, the scale was calibrated with a 20-kg calibration weight. Average daily gain (ADG) was calculated for the 23 days of the study period and reflected the time in which pigs were fed the treatment diets (day 3 to day 26).

Fecal swabs were collected on arrival (day 0), at commencement of feeding study diets (day 3), after 11 days (day 14) and 18 days (day 21) on the study diet, on day of inoculation (day 22), at 2 and 3 days post inoculation (dpi), and on the day of necropsy (day 26). Fecal swabs collected at necropsy (4 dpi) were tested individually for PEDV by quantitative real-time reverse transcription PCR. The remaining swabs were stored at -80°C for further analysis if needed.

Sample collection at necropsy. Four days after inoculation, all pigs were euthanized by captive bolt and exsanguination and necropsied. If gross pathology was observed at necropsy, observations were recorded and fresh and fixed tissues of affected organs were collected for diagnostic testing. Each pig's gastrointestinal tract was then removed. Zip ties were placed 1 cm distally from the junction of the stomach and duodenum and 1 cm proximal to the ileocecal junction to standardize removal between pigs. The intestines were separated from body wall attachments and laid out on a necropsy table. The large intestine was then removed.

Milking out the contents of the intestines was possible, but was not performed due to concern about disrupting intestinal mucosa for fixation and histopathologic examination. The entire length of the small intestine was measured and recorded. As the small intestine was susceptible to elongation from manual manipulation, the tension applied to the intestine might have greatly affected the measured length. To mitigate variation, two people were solely responsible for measuring small intestinal length (SIL) and were trained to apply equal tension to facilitate consistency between measurers. The weight of the gastrointestinal tract was recorded on a digital 500-g × 0.01-g scale that was calibrated with a 100-g calibration weight. Small intestine weight-body weight (SIWB) is the weight of the small intestine divided by body weight to create a standardized weight for each pig, which was then used in the statistical models.

The small intestinal tract was folded on itself to create four segments of equal length in a W formation. From the proximal end, the sample sites were duodenum (W1), proximal jejunum (W2), mid-jejunum (W3), distal jejunum (W4), and ileum (W5). Two 1-cm sections were collected at each of the five sites for fresh and fixed tissues.

Immunohistochemistry

Histopathology slides were prepared for PEDV immunohistochemistry (IHC) for each of the five small intestinal sections of all pigs as described in Madson et al.[1] Viral antigens (PEDV) were detected and semi-quantitatively scored[1] on the basis of the following criteria: 0 = no signal; 1 = 1% to 10% of villous enterocytes within section showing a positive signal; 2 = 11% to 50% of villous enterocytes showing a positive signal; and 3 = 51% or more of villus enterocytes showing a positive signal.[1] All scoring was performed by a veterinary pathologist blinded to treatment allocation.

Histopathology and atrophic enteritis scoring

A veterinary pathologist, blinded to treatment allocation, measured three perceived full-length villi and crypts per section using a computerized image system (Olympus DP72 camera, cellSens digital imaging software, Olympus, Waltham, Massachusetts). The mean villus length and crypt depth were calculated for each segment and used to determine villus height-to-crypt-depth ratios (VCR).[1] Atrophic enteritis (AE) scoring was performed for each of the five small intestinal sections of each pig. Scores were based on the following criteria: 0 = no enteritis observed; 1 = mild atrophic enteritis; 2 = moderate atrophic enteritis; and 3 = severe atrophic enteritis. Scoring was performed by a single veterinary pathologist blinded to treatment allocation.

Inoculum preparation

To generate the inoculum, PEDV US prototype strain isolate USA/IA19338/2013 was propagated and virus concentration was determined on Vero cells (ATCC CCL-81).[15] The eighth passage of the virus on cell culture was diluted with post-inoculation medium to a final concentration of 10^4 $TCID_{50}$ per mL and stored at -80°C upon use.[15]

Porcine epidemic diarrhea virus PCR and quantitation

A previously described PEDV nucleocapsid (N) gene-based qualitative real-time reverse transcription PCR (qRT-PCR) was used in this study for the detection of PEDV RNA.[1,8,15,16] Polymerase chain reaction amplification was performed using Path-ID Multiplex One-Step RT PCR kit (Thermo Fisher Scientific, Carlsbad, California) on an Applied Biosystem 7500 fast instrument (Thermo Fisher Scientific) and analyzed by system software. Serial 10-fold dilutions of in vitro transcribed RNA standards were used in the PEDV N gene-based qRT-PCR to generate standard curves and quantify viral loads (genome copies per mL) in test samples.[8] Results of PCR testing were reported as cycle threshold (Ct) values, with Ct values ≥ 36 considered negative.

Cecum and colon dry matter (DM)

Cecum and colon contents were milked out into separate sterile sample containers. These specimens were frozen at -20°C and then submitted for DM analysis. Digital photographs of the colon contents were taken for fecal consistency scoring.

Determination of cecum and colon DM was performed under standard operating protocols and supervision of one of the authors (SG). Cecum and colon contents were thawed over a 24-hour period, and 15 g of each sample was weighed out on a 500-g × 0.01-g scale. A volume of 20 mL of 1:32 accelerated hydrogen peroxide (Accel disinfectant; Virox Technologies Inc, Oakville, Ontario, Canada) was added to each sample. Aluminum pans were used to weigh out two 5.00-g aliquots of cecal contents on a 200-g × 0.01-g scale. This was repeated for colon contents of each pig. All samples were placed in a large convection oven (Yamato Mechanical Convection Oven, model DKN810, Santa Clara, California) at 20.6°C. At 48 hours, samples were removed and placed into a plastic desiccator to cool, and then weighed again on the 200-g × 0.01-g scale. The following equation to determine DM percentage equals

$$\frac{\text{dry weight} - \text{pan weight}}{\text{wet weight} - \text{pan weight}} \times 100$$

The mean DM of the two aliquots for each pig for both cecum and colon was calculated.

Fecal scoring methods

Photographs of colon contents were taken for each pig and assigned a fecal consistency score by a veterinarian using the photographs and blinded to treatment allocation. Fecal consistency was scored on a scale of 1 to 4, where 1 = normal; 2 = mild diarrhea; 3 = moderate diarrhea; and 4 = severe watery diarrhea.

Statistical analysis

Responses were analyzed using linear mixed models for each variable of interest. For analyses of intestinal variables, IHC, AE, VCR, PEDV genome copies per mL, treatment group, intestinal segment, and their interactions were used as fixed effects, whereas room and animal were used as random effects. A natural log transformation for the response variable PEDV genome copies per mL was performed to meet normality assumptions for its generalized linear mixed model. For the analyses of the following variables (ADG, cecum dry matter, colon dry matter, small intestine length, small intestine weight per kg body weight, and fecal score), treatment group was the fixed effect and room was the random effect. Comparisons among groups were assessed using F-tests followed by Tukey's *t* tests for multiple comparisons. Differences were considered significant at $P < .05$. All analysis was performed on SAS 9.4 (SAS institute Inc, Cary, North Carolina).

Results

Pre-trial contamination screening

Samples collected from all pigs on the day

of arrival were negative by PRRSV pooled PCR and individual PRRSV ELISAs on serum. Porcine epidemic diarrhea virus PCR tests on pooled fecal swabs and PEDV ELISA tests on serum were also all negative. For samples collected on the day of inoculation, pools of fecal swabs from all groups and PEDV-negative cell culture inoculum were PEDV-negative on PCR.

Clinical assessment

Pigs were generally healthy and active and there was no mortality during the trial. Fecal staining of pen walls or pigs, acute dehydration, vomiting, and lethargy were not observed in any treatment group after inoculation with PEDV.

Gross pathology

For PEDV-positive groups and at all diet inclusion levels of *B subtilis* C-3102, the small intestines were thin, dilated, and filled with watery ingesta. In the PEDV-negative groups, no gross lesions were noted for the small intestine at necropsy.

Immunohistochemistry (IHC) score

Results for pairwise comparisons of IHC scores between treatment groups are presented in Table 2 for each intestinal segment. There were significant differences in IHC scores between groups 4 and 5 and 4 and 6 for several segments. There were no significant differences in the IHC scores for any intestinal segments between groups 1 and 2, 1 and 3, 1 and 5, 2 and 3, 2 and 5, 3 and 5, and 5 and 6.

Villus-to-crypt (VCR) ratio

Table 3 presents comparisons of VCRs between treatment groups for each segment. Villus-to-crypt ratios were significantly higher in groups 1, 2, and 3 (all PEDV-negative) than in Group 4 (PEDV-positive), and were significantly lower in Group 4 than in Group 5.

Atrophic enteritis (AE)

Table 4 reports significant pairwise comparisons of AE score for each segment between treatment groups. Atrophic enteritis score was significantly higher in group 4 than in groups 5 and 6 for several segments within each comparison.

Quantitative real-time reverse transcription PCR for porcine epidemic diarrhea virus

The PEDV-positive cell-culture inoculum was PEDV-positive (cycle threshold value = 17.5). For fecal swabs collected on day 4 post inoculation, treatment groups 1, 2, and 3 tested PEDV-negative by PCR. Treatment groups 4, 5, and 6 were PEDV PCR-positive and further quantification was performed on each individual swab. Mean log PEDV genome copies per mL and standard error (in parenthesis) for groups 4, 5, and 6 were 19.18 (3.35), 13.29 (3.35), and 15.66 (3.35). None of the differences in pairwise comparisons between treatment groups were significant at $P < .05$ for mean log genome copies per mL of feces.

Table 2: Mean differences* and *P* values† for pairwise comparisons of porcine epidemic diarrhea virus (PEDV) immunohistochemistry (IHC) scores between treatment groups for each of the five intestinal segments assessed (W1 to W5)

		W1	W2	W3	W4	W5
Groups		**Difference (*P*)**	**Difference (*P*)**	**Difference (*P*)**	**Difference (*P*)**	**Difference (*P*)**
1	2	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
1	3	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
1	4	**-1 (< .001)**	**-2.4 (< .001)**	**-2.3 (< .001)**	**-2.3 (< .001)**	**-2 (< .001)**
1	5	-0.2 (.64)	-0.5 (.24)	-0.5 (.24)	-0.5 (.24)	-0.4 (.35)
1	6	-0.4 (.35)	**-0.9 (.04)**	**-0.9 (.04)**	**-0.9 (.04)**	**-0.9 (.04)**
2	3	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
2	4	**-1 (< .001)**	**-2.4 (< .001)**	**-2.3 (< .001)**	**-2.3 (< .001)**	**-2 (< .001)**
2	5	-0.2 (.64)	-0.5 (.24)	-0.5 (.24)	-0.5 (.24)	-0.4 (.35)
2	6	-0.4 (.35)	**-0.9 (.04)**	**-0.9 (.04)**	**-0.9 (.04)**	**-0.9 (.04)**
3	4	**-1 (< .001)**	**-2.4 (< .001)**	**-2.3 (< .001)**	**-2.3 (< .001)**	**-2 (< .001)**
3	5	-0.2 (.64)	-0.5 (.24)	-0.5 (.24)	-0.5 (.24)	-0.4 (.35)
3	6	-0.4 (.35)	**-0.9 (.04)**	**-0.9 (.04)**	**-0.9 (.04)**	**-0.9 (.04)**
4	5	0.8 (.06)	**1.9 (< .001)**	**1.8 (< .001)**	**1.8 (< .001)**	**1.6 (.002)**
4	6	0.6 (.16)	**1.5 (< .001)**	**1.4 (< .01)**	**1.4 (< .01)**	**1.1 (.01)**
5	6	-0.2 (.64)	-0.4 (.35)	-0.4 (.35)	-0.4 (.35)	-0.5 (.24)

* Treatment groups described in Table 1. Mean IHC scores were compared between treatment groups listed in the first two columns for each of the five intestinal segments assessed (W1 to W5). Intestinal segments examined: duodenum (W1), proximal jejunum (W2), mid-jejunum (W3), distal jejunum (W4), and ileum (W5).

† *P* refers to the *P* value for the difference in mean IHC scores between the two groups listed on each row, generated from Tukey's adjustment for multiple comparisons. Pooled standard error, 0.4. *P* values < .05 (bold print) were considered statistically significant.

Table 3: Mean differences* and *P* values† for pairwise comparisons of villus height-to-crypt-depth ratios (VCRs) between treatment groups for each of the five intestinal segments assessed (W1 to W5)

		W1	W2	W3	W4	W5
Groups		**Difference (*P*)**	**Difference (*P*)**	**Difference (*P*)**	**Difference (*P*)**	**Difference (*P*)**
1	2	-0.011 (.96)	**-0.580 (.01)**	-0.334 (.15)	-0.345 (.13)	-0.146 (.53)
1	3	**0.492 (.03)**	-0.294 (.20)	-0.100 (.66)	-0.112 (.62)	-0.0554 (.81)
1	4	0.395 (. 09)	**0.715 (.002)**	**0.624 (.01)**	**0.500 (.03)**	0.358 (.12)
1	5	-0.027 (.91)	0.261 (.26)	0.0351 (.88)	-0.0860 (.71)	-0.139 (.54)
1	6	0.123 (.59)	**0.474 (.04)**	**0.482 (.04)**	0.322 (.16)	0.270 (.24)
2	3	**0.503 (.03)**	0.285 (.21)	0.235 (.31)	0.233 (.31)	0.091 (.69)
2	4	0.406 (.08)	**1.30 (< .001)**	**0.958 (< .001)**	**0.845 (< .001)**	**0.504 (.03)**
2	5	-0.0161 (.94)	**0.841 (< .001)**	0.369 (.11)	0.259 (.26)	0.0066 (.98)
2	6	0.134 (.56)	**1.05 (< .001)**	**0.816 (.001)**	**0.667 (< .01)**	0.416 (.07)
3	4	-0.0974 (.67)	**1.01 (< .001)**	**0.723 (< .01)**	**0.612 (.01)**	0.414 (.07)
3	5	**-0.519 (.02)**	**0.555 (.02)**	0.135 (.56)	0.0263 (.91)	-0.084 (.71)
3	6	-0.370 (.11)	**0.768 (< .001)**	**0.581 (.01)**	0.434 (.06)	0.325 (.16)
4	5	-0.422 (.07)	**-0.454 (.04)**	**-0.588 (.01)**	**-0.586 (.01)**	**-0.498 (.03)**
4	6	-.272 (.23)	-0.242 (.29)	-0.142 (.54)	-0.178 (.44)	-0.0883 (.70)
5	6	0.150 (.51)	0.213 (.35)	0.446 (.05)	0.408 (.08)	0.409 (.08)

* Treatment groups are described in Table 1. Mean VCRs were compared between treatment groups listed in the first two columns for each of the five intestinal segments assessed. Intestinal segments examined: duodenum (W1), proximal jejunum (W2), mid-jejunum (W3), distal jejunum (W4), and ileum (W5).

† *P* refers to the *P* value for the difference in mean VCRs between the two groups listed on each row, generated from Tukey's adjustment for multiple comparisons. Pooled standard error, 0.229. *P* values < .05 (bold print) were considered statistically significant.

Macroparameters of gut health

Cecum and colon dry matter. Mean cecum and colon DM percentages were determined and pairwise comparisons of DM percentages for both cecum and colon samples revealed several statistically significant differences reported in Table 5.

Fecal score. Mean fecal score was recorded; however, there were no significant differences in fecal consistency for all pairwise comparisons of the six treatment groups ($P \geq .05$).

Small intestine weight by body weight (SIWB) and SIL. There were no significant differences in least squares mean SIWB or SIL between pairwise comparisons of all groups.

Average daily gain. Pairwise comparisons between treatment groups for 23-day ADG using Tukey's least squares means adjustment for multiple comparisons were not significantly different for any of the treatment groups.

Discussion

The literature available on the effects of DFMs on enteric disease or on reducing shedding of pathogens is limited. Trials assessing the effect of *Lactobacillus* species on fecal shedding of enteric pathogens and ADG in *Salmonella* Typhimurium and rotavirus challenge models have shown limited positive effects on study outcomes.[5,6] There is also currently a patent (WO 2005007834 A1) submitted for a *Lactobacillus* species with the claim that this strain can inhibit growth of enteric coronavirus in vitro. Sow fecal shedding, piglet mortality, and piglet diarrhea were assessed in sows and gilts fed *B subtilis* C-3102 for 3 weeks at 10^7 CFUs per gram of feed.[17] Sows receiving *B subtilis* C-3102 experienced a reduction in *Clostridium perfringens* fecal shedding and an increase in lactobacilli fecal shedding after 21 days of continuous exposure to the diet. *Bacillus subtilis* C-3102 is also reported to have a positive effect on sow feed consumption, wean-to-estrus interval, piglet birth weight, and fecal shedding of *Escherichia coli* and *Clostridium* species.[18] Upon review of the literature, comparable studies examining associations between *B subtilis* C-3102 and intestinal histopathology and macroparameters of gut health following PEDV infection were not found.

Significant differences in IHC score between Group 1 and Group 4 on control diets indicated that the PEDV challenge did cause enteric infection with PEDV in the study pigs. Mean IHC scores for Group 4 at 4 dpi were comparable to those reported for 3-week-old pigs.[1] For PED-positive groups, those fed *B subtilis* C-3102 (groups 5 and 6) had lower IHC scores than those fed control diets (Group 4), indicating that there was a lower percentage of enterocytes infected with PEDV in groups 5 and 6. The mechanism for this difference in IHC scores is unknown and pigs were not followed beyond 4 dpi to evaluate the duration and magnitude of this difference over a longer time period. There was no statistical difference in IHC scores between Group 5 and Group 6, although both were significantly lower than in Group 4. This suggests that the effect on IHC may not be dependent on dietary

Table 4: Mean differences* and *P* values† for pairwise comparisons of atrophic enteritis (AE) scores between treatment groups for each of the five intestinal segments assessed

Groups		W1	W2	W3	W4	W5
		Difference (*P*)	Difference (*P*)	Difference (*P*)	Difference (*P*)	Difference (*P*)
1	2	0 (1)	0 (1)	0 (1)	0 (1)	0 (1)
1	3	-0.5 (.16)	0 (1)	-0.1 (.78)	0 (1)	0 (1)
1	4	**-1.2 (< .001)**	**-2 (< .001)**	**-1.6 (< .001)**	**-1.6 (< .001)**	**-1.4 (< .001)**
1	5	-0.4 (.26)	-0.4 (.26)	-0.2 (.57)	-0.1 (.78)	-0.2 (.57)
1	6	**-0.7 (.049)**	**-1.1 (< .01)**	**-0.8 (.02)**	**-0.9 (.01)**	**-0.9 (.01)**
2	3	-0.5 (.16)	0 (1)	-0.1 (.78)	0 (1)	0 (1)
2	4	**-1.2 (< .001)**	**-2 (< .001)**	**-1.6 (< .001)**	**-1.6 (< .001)**	**-1.4 (< .001)**
2	5	-0.4 (.26)	-0.4 (.26)	-0.2 (.57)	-0.1 (.78)	-0.2 (.57)
2	6	**-0.7 (.049)**	**-1.1 (.002)**	**-0.8 (.02)**	**-0.9 (.01)**	**-0.9 (.01)**
3	4	**-0.7 (.049)**	**-2 (< .001)**	**-1.5 (< .001)**	**-1.6 (< .001)**	**-1.4 (< .001)**
3	5	0.1 (.78)	-0.4 (.26)	-0.1 (.78)	-0.1 (.78)	-0.2 (.57)
3	6	-0.2 (.57)	**-1.1 (.002)**	**-0.7 (.049)**	**-0.9 (.01)**	**-0.9 (.01)**
4	5	**0.8 (.03)**	**1.6 (< .001)**	**1.4 (< .001)**	**1.5 (< .001)**	**1.2 (< .001)**
4	6	0.5 (.16)	**1.6 (< .001)**	**0.8 (.03)**	0.7 (.49)	0.5 (.16)
5	6	-0.3 (.40)	**-0.7 (.049)**	-0.6 (.09)	**-0.8 (.02)**	**-0.7 (.049)**

* Treatment groups are described in Table 1. Mean AE scores were compared between treatment groups listed in the first two columns for each of the five intestinal segments assessed. Sample sites were duodenum (W1), proximal jejunum (W2), mid jejunum (W3), distal jejunum (W4), and ileum (W5).

† *P* refers to the *P* value for the difference in mean AE scores between the two groups listed on each row, generated from Tukey's adjustment for multiple comparisons. Pooled standard error was 0.4. *P* values < .05 (bold print) were considered statistically significant.

concentration of *B subtilis* C-3102. The sample size of this study was small and the study had insufficient power to elucidate and quantify a dose-dependent relationship between Group 4 and Group 5 compared to Group 4 and Group 6. This represents an area for future studies to investigate further.

The findings described in the comparisons between treatment groups for IHC scores were also identified in the atrophic enteritis scores. As atrophic enteritis is a pathologic lesion association with enteric viral pathogens, and IHC is a quantitative measure of PEDV infection of the enterocytes, it is logical that these scores would mirror each other. In treatment Group 3, there was mild atrophic enteritis in the W1 and W3 segments. As these segments were IHC-negative for PEDV, and the fecal swabs from these pigs were PEDV-negative on PCR, this mild enteritis could be attributed to other enteric viruses such as rotavirus, or to enteric inflammation related to diet or to other bacterial pathogens.

In swine, *B subtilis* increases the prevalence of bacterial colonization with *Streptococcus* species, *Bifidobacterium* species, and *Lactobacillus* species, which may act to competitively exclude pathogens from colonizing the mucosal surface.[3,10,19] Lactobacilli produce lactic acid, which lowers gut pH and optimizes the gut environment for commensal bacteria.[3,6] *Bacillus* species also produce catalases and proteases that may serve as exogenous digestive enzymes. These enzymes can alter the protein content of gut ingesta and create optimal conditions for *Lactobacillus* colonization and growth.[20] The resultant decrease in intestinal pH and increase of commensal bacteria may impact the ability of PEDV to infect enterocytes and may explain the lower IHC scores in Group 4 than in Group 5 or Group 6.

Values for VCR for each intestinal region were uniformly smaller, across all six treatment groups, than those reported for 3-week-old pigs.[1,21] Pigs in this study were approximately 5 weeks old at the time of necropsy. Villus-crypt ratio is dependent on age, diet, and genetic factors,[1] thus the lower VCR observed here for all treatment groups (1, 2, 3, 4, 5, and 6) may reflect a difference in pig age between the studies.

There was a significantly lower VCR for PEDV-challenged pigs in Group 4 compared to those that were sham challenged (groups 1, 2, and 3). Villus length and crypt depth are highly dynamic and reflect local insult and subsequent regeneration. The VCR provides a snapshot of current status of the villi and crypts at the time of necropsy, and the VCR is expected to increase post inoculation as the pig recovers.[1,7] Although VCRs were significantly higher in PEDV-positive pigs fed *B subtilis* C-3102 than in controls, the clinical significance of this finding in terms of ADG and morbidity is unclear. It would be necessary to stagger necropsies over several dpi and extend the duration of the study to assess the long-term effect of *B subtilis* C-3102 on VCR after PEDV challenge.

In this study, quantitation of the PEDV challenge was used to verify that the challenge dose was homologous between groups. Groups 4, 5, and 6 were all PEDV-positive at 4 dpi and there were no significant differences in genome copies per mL shed in the feces. This indicates that continuously feeding pigs

Table 5: Pairwise comparison of mean cecum and colon DM percent between treatment groups*

Groups		Difference in mean cecum DM (%)	*P* value for cecum DM comparison between groups†	Difference in mean colon DM (%)	*P* value for colon DM comparison between groups†
1	2	0.28	1.00	1.25	.74
1	3	-0.23	1.00	0.83	.93
1	4	3.37	**.01**	2.71	.15
1	5	1.77	.16	5.89	**< .01**
1	6	1.61	.22	1.55	.57
2	3	-0.51	.95	-0.42	1.00
2	4	3.09	**.02**	1.46	.62
2	5	1.49	.27	4.65	**.02**
2	6	1.33	.36	0.30	1.00
3	4	3.60	**< .01**	1.88	.40
3	5	2.00	.11	5.06	**.01**
3	6	1.84	.14	0.72	.96
4	5	-1.60	.22	3.19	.08
4	6	-1.76	.16	-1.16	.79
5	6	-0.16	1.00	-4.35	**.02**

* Treatment groups are described in Table 1. Mean differences in colon and cecum percent DM calculated by subtracting the mean cecal or colon dry matter percentage in column two from the percentage in column one.

† *P* values were generated from Tukey's adjustment for multiple comparisons. Pooled standard error of cecum DM content was 0.61% and of colon DM was 0.91%. *P* values < .05 (bold print) were considered statistically significant.

DM = dry matter.

with *B subtilis* C-3102 after PEDV challenge did not alter fecal virus shedding. Groups 1, 2, and 3 remained PEDV-negative throughout the trial, thus the PEDV-positive and PEDV-negative cell culture inoculum was successfully administered without cross-contamination at or after inoculation.

The 23-day ADG was lower than industry standards of 250 to 350 g per day for nursery pigs for the first 3 weeks post weaning.[22] Conventionally, pigs are weaned at 21 days, but pigs were weaned at 14 days of age for this study. Clinical signs of PEDV (diarrhea, dehydration, mortality, and depression) are age dependent.[1,2] As the goal of the study was to produce a successful PEDV infection with enteric lesions, 14-day-old pigs, in lieu of 21-day-old pigs, were utilized in this study. Technical representatives for Calpis Co Ltd and Quality Technology International recommended feeding *B subtilis* C-3102 continuously for approximately 3 weeks prior to PEDV challenge to provide optimal opportunity for the product to impact the gut. This extended feeding period could negatively impact clinical severity of PEDV challenge by increasing pig age at challenge, thus it was elected to start the trial with a younger pig, at the age of 14 days.

There were no differences in SIWB or SIL between the groups. Small intestinal weight-body weight was impacted by the volume and composition of intestinal ingesta. Despite the large potential for variability, the mean SIWB did not differ between groups, and there was also no difference between groups for SIL. The SILs determined in this study are comparable to SILs in 35- and 39-day-old pigs.[23,24] The functional significance of SIL is related to digestion capacity to maximize growth and is strongly related to pig age and growth rate.[24] As ADG increases with days on feed in the growing period,[24] a difference in SIL between groups might have been appreciated if the duration of the study had been extended.

Fecal scores for all groups did reflect mild looseness in the stool; however, there was no difference in fecal scores between groups. This finding indicates that PEDV status is not correlated with visual fecal scores in weaned pigs under the conditions of this study. As PEDV status is critical to personnel, equipment, trucking logistics, and biosecurity, the results of the fecal score analysis highlight the importance of conducting diagnostic testing to ascertain the PEDV status of weaned pigs.

The cecum and colon DM were measured to provide an objective assessment for fecal consistency. Keeping PEDV status constant, cecum DM did not differ significantly between diet groups. Disease status did impact cecum DM, but not colon DM in this study, as Group 1 and Group 4 did not differ significantly in colon DM. For future PEDV studies, cecum DM appears to have more utility than colon DM in reflecting disease-associated changes in fecal consistency.

The findings of this study support an association between feeding *B subtilis* C-3102 and mitigating severity of PEDV lesions, including lower IHC scores, lower AE scores, and higher VCRs at 4 dpi in nursery pigs challenged with PEDV, compared to cohorts that did not receive *B subtilis* C-3102. However, amount of virus shedding at 4 dpi did not differ with *B subtilis* C-3102 feeding. The results did appear to show that Group 5 animals benefited more than Group 6 animals

from treatment with *B subtilis* C-3102. The study was not designed to measure dose-dependent effects of *B subtilis* C-3102, and an additional study with this purpose would be needed to ascertain if differences between Group 5 and Group 6 were truly due to a dose-dependent or random effect. The impact of these parameters on morbidity, mortality, amount and duration of virus shedding, ADG, and feed efficiency during the entire growing period is unknown and should be assessed with an additional study of longer duration and larger sample size. In a field scenario, it would be rare for DFM administration to precede an enteric disease outbreak by at least 2 weeks. If exposure to *B subtilis* C-3102 before PEDV exposure is critical to impact enteric lesions, then application in a field scenario may be challenging, as it would require administration to all groups or the identification of "at risk" groups for pre-emptive feeding.

Implications

- Under the conditions of this study, there is no grossly detectable difference in fecal consistency between PEDV-positive and PEDV-negative pigs fed the direct-fed microbial *B subtilis* C-3102.
- Pigs that receive *B subtilis* C-3102 prior to PEDV challenge have lower PEDV IHC scores and better histopathology scores and villus-to-crypt ratios after PEDV challenge, compared to control cohorts.

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Conflict of interest

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