AMERICAN SOCIETY OF ANIMAL SCIENCE

Journal of Animal Science, 2020, 1–10

doi:10.1093/jas/skz382
Advance Access publication December 21, 2019
Received: 22 October 2019 and **Accepted:** 18 December 2019
Non Ruminant Nutrition

NON RUMINANT NUTRITION

Feed additive blends fed to nursery pigs challenged with *Salmonella*

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Abstract

Salmonella in pigs is a concern for human foodborne salmonellosis. Dietary fungal fermented products, coated butyrate, and organic acids (**OAs**) may be promising control strategies. The objectives of this study were (i) to evaluate in vitro binding affinity of *Salmonella enterica* serovar Typhimurium (S. Typh) and Enteritidis (S. Ent), and enterotoxigenic *Escherichia coli* (**ETEC**) F4 or F18 to mannan-rich hydrolyzed copra meal (**MCM**) and fermented rye (**FR**) with *Agaricus subrufescens*; and (ii) to assess MCM and FR efficacy to control in vivo S. Typh shedding when combined with OAs and compared with coated butyrate strategy. A 31-d study included 32 pigs [6.29 ± 0.76 kg BW] individually housed and distributed into four dietary treatments: control diet; OA.BU, 4 kg/t OA plus 6 kg/t coated butyrate; OA.MCM, 4 kg/t OA plus 1 kg/t MCM; and OA.FR, 4 kg/t OA plus 2 kg/t FR. All pigs were challenged for 7 d with 1 mL S. Typh (10^9 colony forming units daily) at 10 d postweaning. Temperature and fecal samples were collected before and after challenge, and fecal *Salmonella* shedding quantified. Diarrhea scores were monitored daily and growth performance was evaluated weekly. In vitro, culture with MCM and FR showed significant ($P < 0.01$) binding affinity for both S. Typh and S. Ent, but not for ETEC F4 and F18. In vivo, pigs fed OA.MCM and OA.FR had lower ($P < 0.05$) shedding and day 3 peak shedding of S. Typh after infections than pigs fed control and OA.BU diets. Pigs fed OA.FR diet tended to have an 18% increase ($P = 0.068$) in BW on day 14 post first inoculation compared with control and OA.BU, and 19% increased ($P = 0.093$) final BW at day 21 compared with control. Diarrhea frequency post infection was overall lower ($P = 0.006$) for OA.FR (18.9%) than OA.BU (44.8%) and OA.MCM (41.7%) while control (28.7%) was not different. In conclusion, FR and MCM show in vitro-binding affinity to *Salmonella enterica* serovars Typh and Ent. Feeding FR or MCM combined with OA to nursery pigs reduces the peak and averages S. Typh shedding compared with control. Fermented rye with OA tends to improve pig performance after S. Typh challenge.

Key words: Agaricus, fermented product, gut health, oligosaccharides, *Salmonella*, shedding

Introduction

Research for new strategies that promote gut health in pigs is key because there is an urgent need to reduce antibiotic usage in production animals and minimize risk of bacterial resistance against antibiotics. Relevant strategies may focus on reducing risk of pathogenic and zoonotic bacteria in the feed-to-food chain, mitigate postweaning diarrhea, and achieve profitable systems. Salmonellosis, although often asymptomatically in pigs (Andrés-Barranco et al., 2015), is a major human concern globally because contamination can occur at many different levels of the pig production chain [Regulation (EC) No. 2160/2003]. Probabilities of *Salmonella* shedding and contamination at

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slaughter are higher when pigs were already infected and(or) colonized at farm (Casanova-Higes et al., 2017). Prevent shedding load, infection, and colonization will contribute to reduce *Salmonella* contamination in the food chain. Other important pathogenic bacteria include enterotoxigenic *Escherichia coli* (**ETEC**), which cause major economic losses in the swine industry (Nataro and Kaper, 1998). Strains of ETEC are defined for producing heat labile and/or stable enterotoxins (Loos et al., 2012) and expressing adhesion factors such as fimbriae F4 linked to diarrhea and F18 linked to diarrhea and edema disease (Francis, 1999).

Fungal fermented products and their derivatives are described to contain several compounds that may play a role in gastrointestinal health and pathogenic bacteria control (Wisitrassameewong et al., 2012). Microbial enzymes produced by fungal during fermentation will degrade polysaccharides from feed material into indigestible and bioactive oligosaccharides (Ariandi and Meryandini, 2015). Adhesins from some pathogenic Enterobacteriaceae are known to show binding affinity to distinct indigestible oligosaccharides. Wang et al. (2015) demonstrated such affinity for suitable oligosaccharides, i.e., D-mannose showed between 20% and 60% inhibition of bacterial adhesion (*E. coli*, *Vibrio cholerae*, *Campylobacter jejuni*, and *Salmonella* Typhimurium) to host glycans from HT-29 cells. In vivo, β-1-4 mannobiose reduced *Salmonella* shedding after infection in broilers (Agunos et al., 2007), and β -galactomannan oligosaccharide reduced subclinical *Salmonella* infection in fattening pigs (Andrés-Barranco et al., 2015). Among raw materials, rye was found to have a strong binding affinity to pathogenic *E. coli* (Zhu et al., 2018).

Other bioactive components from fungal fermentation are β-glucans, which are a heterogeneous group of polysaccharides present in cereal grains, fungal cell walls, seaweed, and algae (Akramiene et al., 2007). The immunomodulatory properties of different β-glucans have been demonstrated in vitro (Smiderle et al., 2014; Choi et al., 2016) and in vivo (Samuelsen et al., 2014) to support intestinal health and microbiota balance in pigs (Kogan and Kocher, 2007; Kim et al., 2019). Other promising fungal examples are metabolites derived from edible mushrooms. Products from almond mushroom are reported to contain prophylactic and therapeutic properties including antimicrobial and immunomodulatory (Smiderle et al., 2011; Wisitrassameewong et al., 2012).

In the swine industry, short chain fatty acids or organic acids (**OAs**) and their salts are used in nursery pig diets to improve performance via effects on digestion and gastrointestinal health (Partanen and Mroz, 1999). Formic and lactic OAs increase stomach barrier function by reducing pH and destabilizing bacterial membranes (Van der Wolf et al., 2001; Zentek et al., 2013). Butyrate, the main source of energy for the epithelial cells (Roediger, 1980; Chapman et al., 1995), is well known to enhance epithelial morphology, homeostasis, and microbiota balance (Biagi et al., 2007; Fang et al., 2014). Additionally, coated butyrate appears promising to control *Salmonella*. Several studies report in vitro antimicrobial properties and in vivo decreased *Salmonella* shedding and intestinal colonization (Van Immerseel et al., 2005; Boyen et al., 2008; Guilloteau et al., 2010; De Ridder et al., 2013).

Therefore, fungal fermented products and their derivatives contain several compounds that may reduce pathogenic bacteria load and play a role in gastrointestinal health. OAs can enhance stomach barrier function and are used to manage risk against pathogenic bacteria. Additionally, butyrate may inhibit growth of *Salmonella* in the intestine. However, a strategic combination of OAs with fungal fermented products has not been previously studied to control *Salmonella* in pigs exposed for several days.

The objective of this study was to evaluate mannan-rich hydrolyzed copra meal (**MCM**) and fermented rye (**FR**) with *Agaricus subrufescens* to control specific *Salmonella* and *Escherichia coli* in vitro, and to assess their efficacy to control in vivo shedding of *Salmonella enterica* serovar Typhimurium when combined with OA and compared with coated butyrate.

Material and Methods

Diets and additives

A standard experimental diet was used and produced at the Research Feed Plant (Heijen, The Netherlands) without additional additives or medication (Table 1). All diets met or exceeded current nutrient requirement estimates for nursery pigs (NRC, 2012). Spray-dried plasma, antibiotics, and Zn oxide were not included in the diets. Experimental diets were pelleted using a 4-mm die and fed to pigs throughout the experiment. Selko (Trouw Nutrition, Tilburg, the Netherlands) provided all feed additives. The OA blend used in the present experiment was 88% formic acid and 12% lactic acid. Selko-SR Butyrate 30 was the coated butyrate used. The FR used contained ~40% mycelium of *A. subrufescens*. The MCM used contained 14% β-1,4-mannobiose.

Diets were analyzed for moisture (EC regulation 152/2009, appendix III A), CP (ISO 16634-1:2008), acid hydrolyzed ether extract (EC regulation 152/2009, appendix III H method A), and crude fiber (ISO 6865:2000), and ICP-OES spectrometry (Perkin-Elmer S10, model Avio 200; MA) was used to determine calcium, phosphorus, and sodium (ISO 15510:2008).

Bacterial strains

Salmonalla Typhimurium (**S. Typh**) strain DT12 (B; O1, 4, 5, 12; Van Winsen et al., 2001) obtained from De Gezondheidsdienst voor Dieren (Deventer, The Netherlands) was used for both the in vitro binding assay and in vivo study. Additionally, the following strains we used in the in vitro binding assays: *Salmonella* Enteritidis (**S. Ent**) isolated from infected broiler (Trouw Nutrition Poultry Research Centre, Spain), ETEC F4 (O149:K88acK91; from Wageningen Bioveterinary Research, the Netherlands), and ETEC F18 isolated from infected piglet (Trouw Nutrition Swine Research Centre, the Netherlands).

In vitro-binding affinity assay

Five in vitro binding assays (A, B, C, D, and E) were conducted using the same methodology to assess binding affinity of four different Enterobacteriaceae (S. Typh, S. Ent, ETEC F4, and ETEC F18) to MCM and FR, as described in Table 2. There were some design differences as study A did not test FR and did not include ETEC F18, whereas in study B binding to MCM was not tested. The binding affinities of the bacteria to the feed additives were measured by time required to reach OD_{600nm} of 0.5. Less time indicates that more bacteria adhered to the substrate resulting in less time to reach the OD_{600nm} cutoff.

For each binding assay, different wells of a 96-well microplate were coated with MCM, FR, or BSA as a control in which the binding of different bacteria were tested according to Becker et al. (2007) with some modifications. Briefly, MCM and FR (0.5 mm grinded) were suspended to a final concentration of a 1% (w/v) in PBS suspension. Subsequently, the suspensions were incubated 3 times 2 min in a sonication water bath (Branson

Table 1. Composition of the experimental diets (as fed basis)

Item	Control	OA.BU	OA.MCM	OA.FR
Ingredients, %				
Barley	23.3	23.3	23.3	23.3
Wheat	20.0	20.0	20.0	20.0
Corn	18.0	18.0	18.0	18.0
Wheat bran	3.0	3.0	3.0	3.0
Soybean meal (crude fiber < 50 g/kg)	17.3	17.3	17.3	17.3
Potato protein (as <1.0%)	2.25	2.25	2.25	2.25
DL-Methionine (99%)	0.2	0.2	0.2	0.2
L-Lysine HCl (98%)	0.56	0.56	0.56	0.56
L-Threonine (98%)	0.2	0.2	0.2	0.2
L-Tryptophan (98%)	0.05	0.05	0.05	0.05
Na bicarbonate	0.54	0.54	0.54	0.54
Ca carbonate	0.53	0.53	0.53	0.53
Monocalcium phosphate	0.96	0.96	0.96	0.96
Salt (NaCl)	0.37	0.37	0.37	0.37
Lactose	6.36	6.36	6.36	6.36
Sugar	2.5	2.5	2.5	2.5
Soybean oil	2.47	2.47	2.47	2.47
Vitamin E (50% adsorbate)	0.24	0.24	0.24	0.24
Vitamin–mineral premix	1.0	1.0	1.0	1.0
Phyzyme[1]	0.01	0.01	0.01	0.01
L-Valine (96.5%)	0.12	0.12	0.12	0.12
Choline chloride (50%)	0.03	0.03	0.03	0.03
Organic acid blend (82% formic, 12% lactic)	-	0.4	0.4	0.4
Coated butyrate	-	0.6	-	-
Hydrolyzed copra meal	-	-	0.2	-
Fermented rye	-	-	-	0.2
Calculated content, %				
DM[2]	89.6	90	89.3	89.5
NE[3], kcal	2,425	2,425	2,425	2,425
SID Lys[4]	1.21	1.21	1.21	1.21
SID Met[4]	0.45	0.45	0.45	0.45
SID Met + Cys[4]	0.7	0.7	0.7	0.7
SID Trp[4]	0.23	0.23	0.23	0.23
SID Thr[4]	0.75	0.75	0.75	0.75
CP[2]	17.9	17.7	17.7	17.2
Acid hydrolyzed ether extract[2]	4.3	5.1	4.7	4.6
Crude fiber[2]	3.0	3.1	3.0	3.1
Ash[2]	5.2	5.4	5.1	5.1
Neutral detergent finer	10.3	10.3	10.3	10.3
Acid detergent fiber	3.9	3.9	3.9	3.9
Nonstarch polysaccharides	14.5	14.5	14.5	14.5
Sodium[2]	0.33	0.38	0.35	0.35
Potassium	0.7	0.7	0.7	0.7
Chloride	0.39	0.39	0.39	0.39
Magnesium	0.17	0.17	0.17	0.17
Calcium[2]	0.65	0.67	0.66	0.68
Phosphorus[2]	0.59	0.59	0.60	0.59
Copper, mg/kg	166	166	166	166
Manganese, mg/kg	51	51	51	51
Zinc, mg/kg	126	126	126	126

[1]Phyzyme XP (5000 TPT; Danisco Animal Nutrition, Marlborough, UK).
[2]Analyzed composition.
[3]NE was calculated using CVB equations (2006).
[4]SID for AAs was calculated using CVB equations (2006).

5510) with intermediate vortexing and centrifuged at 460 × *g* for 5 min. The wells of the microtiterplate (Microlon F plate 655092 Greiner Bio_one B.V.) were coated overnight at 4 °C with 250 µL of supernatant from the different suspensions in duplo. The control wells were coated with 250 µL PBS. After coating, the wells were washed with 300 µL of PBS and subsequently blocked with 300 µL 1% (w/v) BSA [Sigma-Aldrich (A7906)] for 1 h at 4 °C. After blocking the wells, they were washed twice with 300 µL PBS. Consequently, the bacterial suspension was grown to end the logaritmic phase in Brain Heart Infusion broth (BHI; Oxiod). Then washed and resuspended in PBS adjusted to $OD_{600\ nm}$ 0.02 and 250 µL added to the wells of the microplate. Bacteria were allowed to adhere at room temperature for 30 min. After adhesion, the plate was washed 3 times with 300 µL of PBS and 250 µL of BHI was added to each well. The plate was incubated in the microplate reader (SpectraMax M2, Molecular Devices Corporation, Silicon Valley, CA) at 37 °C and growth was monitored by measuring OD_{600nm} every 5 min for 18 h with 5 s shaking before each measurement. Therefore, the binding affinities of the bacteria to the feed additives were measured by time required to reach OD_{600nm} of 0.5 cutoff. Blank culture controls for MCM and FR without adding bacteria were also included. These controls show no bacterial growth for >16 h. Bacterial growth was specific measured for the bacteria added.

In vivo study

The protocol for this experiment was reviewed and approved by the Animal Experiment Committee (DEC) of Utrecht and applied under project license permit number 2014.III.07.063.

Animals, housing, and experimental design

A total of 32 weaning male pigs (Topi × Hypor 24 d of age ± 3 d SD) with an average initial BW of 6.29 ± 0.76 kg were selected from the Swine Research Center of Trouw Nutrition R&D facilities (P.O. Box 220, 5830 AE Boxmeer, the Netherlands). After weaning, all pigs were individually housed (pen size: 1.60 × 1.60 m), and taking into account litter origin, were randomly assigned to 1 of 4 dietary treatments: (i) a control; (ii) 4 kg/t OAs plus 6 kg/t coated butyrate (OA.BU); (iii) 4 kg/t OAs plus 1 kg/t MCM (OA.MCM), and (iv) 4 kg/t OAs plus 2 kg/t FR (OA.FR). The experimental unit was pig and there were 8 replicate pigs per treatment. Each piglet received a feed matrix containing ~9.0 $\log_{10}$ cfus of S. Typh for 7 d consecutive at 10 d postweaning (described below). Pigs were under environmentally control unit for 31 d, as 10 d before (–10 d) and 21 d after the first S. Typh inoculation (day 0) with ad libitum access to feed and water. A summary of the infection model and sampling is presented in Figure 1. Environmental enrichment was provided for each pig. The room was 25 to 27 °C, and daily light was on at 0700 h and off at 1900 h throughout the experiment.

The inoculum matrix (ladyfinger biscuits) preparation and administration were based on the Litjens et al. (2017) and Van der Wolf et al. (2017) methodology. Briefly, the feed matrix was inoculated with 1 mL of an S. Typh culture of ~9.0 $\log_{10}$ CFU/mL. The piglets received the inoculated feed matrix for 7 d consecutive after 6 d training with non-infected matrix. The required inoculated biscuits were freshly prepared each day before challenge (in the morning), except for weekend days when biscuits were prepared on the Friday before and stored at 4 °C. *Salmonella* Typh was quantified in the feed matrix after storage for 24 and 48 h at 4 °C to ensure viability of the matrix inoculum (8.2 to 8.7 $\log_{10}$ CFU/piece of feed matrix). Initially, there were two surplus animals per treatment but the day before challenge, 8 piglets were selected for inclusion in the experiment on the basis of the following criteria: (i) no *Salmonella* detected in feces, (ii) >50 g/d weight gain, (iii) willingness to consume the feed

Table 2. Design of 5 in vitro assays to test binding affinity of different Enterobacteriaceae to feed additives[1] compared with the control[2].

	Bacteria											
Study	*Salmonella* Typhimurium			*Salmonella* Enteritidis			*Escherichia coli* F4			*Escherichia coli* F18		
A	Con		FR	Con		FR	Con		FR	Con		
B	Con	MCM		Con	MCM		Con	MCM		Con	MCM	
C	Con	MCM	FR	Con	MCM	FR	Con	MCM	FR	Con	MCM	FR
D	Con	MCM	FR	Con	MCM	FR	Con	MCM	FR	Con	MCM	FR
E	Con	MCM	FR	Con	MCM	FR	Con	MCM	FR	Con	MCM	FR

[1]Feed additives at 1% (w/v) were MCM, hydrolyzed copra meal; FR, fermented rye.
[2]Control, without feed additive.

matrix. When more pigs than required animals met all criteria, the BW average animals were selected. Consumption of the feed matrix was in the afternoon and monitored for each individual animal.

Clinical observations and sample collection

A summary of sample collection is presented in Figure 1. Clinical observations and diarrhea score were recorded daily throughout the experiment and rectal temperature before and after the challenge on days 0, 1, 2, 3, 4, 5, 6, 7, 14, and 21 postchallenge. The diarrhea score was visually assessed daily for each pig by a trained evaluator using a scoring system (Van der Wolf et al., 2017) ranging from 0 to 3 (0 = normal feces, 1 = shapeless or loose feces, 2 = diarrhea with thick liquid feces, 3= severe diarrhea as thin watery feces, and 9 = no score possible). Diarrhea incidence was calculated as the percentage of days with a fecal score of 2 and 3 per pen using the following different periods; −6 to 0 d, 1 to 7 d, 8 to 14 d, and 15 to 21 d post first contact to S. Typh. Pigs were weighed at weaning day (−10 d) and 0, 7, 14, and 21 d post challenge. ADG, ADFI, and feed efficiency (G:F) were calculated for each interval and overall. Fecal samples (5 g per pig) were collected at −6, 0, 1, 2, 3, 4, 7, 14, and 21 d post challenge and analyzed quantitatively for S. Typh fecal shedding.

Salmonella shedding

Fecal samples were collected and directly stored at 4 °C and processed within 24 h as described by Litjens et al. (2017). Briefly, 1 g of each fecal sample was diluted 1:10 in buffered peptone water (Oxoid) supplemented with 20 mg/L novobiocin (AppliChem GmbH, Darmstadt, Germany), homogenized using a stomacher, and 10-fold serial dilutions were made in sterile 0.1% peptone physiological salt solution (Tritium Microbiologie B.V.) up to 10^{-4}. Dilutions were surface plated (100 µL) onto Brilliant Green Agar plates with 20 mg/L novobiocin + 40 g/L potassium iodide (Tritium Microbiologie B.V.) and incubated for 21 ± 3 h at 37 °C ± 1 °C for colony counting. For analysis, individual *Salmonella* counts after challenge were converted into $\log_{10}$ values. The remaining 1:10 suspensions were pre-enriched for 16 to 20 h at 37 °C ± 1 °C. If no *Salmonella* were found on the counting plates, the pre-enriched samples were analyzed for the presence or absence of *Salmonella* according to ISO 6579:2002. This method was also used at day −6 before first S. Typh contact to confirm all pigs were *Salmonella* negative.

Presumptive *Salmonella* (red/pink) colonies were enumerated and confirmed as *Salmonella enterica* (spp.) and S. Typh-specific multiplex via quantitative PCR (**qPCR**) using two randomly selected colonies per sample. The real-time PCR reaction was performed in a CFX96 Real-Time PCR system on a C1000 thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA). The conditions for real-time PCR reaction and the reagent mixes, primers, and probes used were as in Litjens et al. (2017). Positive criterion was set at a cycle threshold smaller than 35. Samples remaining negative were presumed to contain <1 CFU/g feces. Samples were presumed to contain <100 CFU/g feces (as detection limit for quantification) and were included in the data set as "50." when *Salmonella* was detected after pre-enrichment.

Statistical analysis

The normality of data was checked on the basis of visual assessment of residual plots (SAS Inst. Inc., Cary, NC). In general, data were analyzed by the MIXED procedure of SAS unless otherwise stated. The time in hours to reach an OD_{600nm} cutoff of 0.5 was analyzed using treatment (control, MCM, and FR) as the main effect, the within study variation included as a random effect, and independent assays (A, B, C, D, and E) were included as a repeated effect for the in vitro assay data. Pig performance data were analyzed using the pig as the experimental unit and the model included treatment (control, OA.BU, OA.MCM, and OA.FR) as the main effect, BW block (pig) as the random effect, and time of measurements included as a repeated measurement. Treatment means were separated by using the LSMEANS statement, PDIFF option, and SIMULATE adjustment for comparison in PROC MIXED. A diarrhea score equal to 9 (no score possible) occurred only at weaning and on 3 d postweaning, and these data were excluded from analysis. Diarrhea incidence was not normally distributed and, therefore, were analyzed using PROC GLIMMIX which included treatment (control, OA.BU, OA.MCM, and OA.FR) as the main effect, BW block (pig) as the random effect, and time of frequency measurements included as a repeated effect. The dist = beta and link = logit functions were used to manage frequency data. Statistical significance and tendency were considered at $P \leq 0.05$ and $0.05 \leq P \leq 0.10$, respectively.

Results

In vitro-binding affinity

The in vitro results are presented in Figure 2. Culture substrate with additional MCM and FR showed less ($P < 0.01$) time to growth OD_{600nm} than control culture for both S. Typh and S. Ent. The time (hours) to grow to OD_{600nm} of 0.5 cutoff were 7.57 for control, 5.79 for MCM, and 6.12 for FR on S. Typh and for S. Ent the times were 6.92 for control, 5.88 for MCM, and 5.94 for FR on S. Ent. Less time to grow to OD_{600nm} indicated that more bacteria adhered to the substrate (control, MCM, or FR), which resulted in less time to reach the OD_{600nm} cutoff. There was no effect of culture substrate with treatment for ETEC F4 and F18

Figure 1. Summary of infection model with *Salmonella* Typhimurium for 7 d in nursery pigs and measurements collected.

Figure 2. In vitro assay to investigate binding affinity of pathogenic Enterobacteriaceae to culture substrate including feed additives[1]. Results read as time required to reach OD_{600nm} of 0.5. Less time indicates that more bacteria adhered to the substrate (control, MCM, or FR) resulting in it taking less time to reach the OD_{600nm} cutoff. [1]Feed additives at a 1% (w/v) were MCM, hydrolyzed copra meal; FR fermented rye. [a, b]Means without a common superscript are different ($P \leq 0.01$).

species. However, a study interaction was observed among the 5 independent assays (A–E). This interaction indicated that study A had a greater ($P < 0.05$) and study E tended to have a greater ($P < 0.10$) binding affinity of FR with ETEC F4 compared with the control. The time to grow to OD_{600nm} of 0.5 cutoff was 7.27 h for FR and 9.17 h for the control in study A (SE = 0.805, $P = 0.045$), and 5.67 h for FR and 7.11 h for control in study E (SE = 0.922, $P = 0.101$). The overall variance in the mixed model for ETEC F4 was high and this effect was not consistent across studies.

In vivo study

Growth performance, rectal temperature, and diarrhea score

Pigs fed OA.FR at 14 d post challenge (14.7 kg) tended to be heavier ($P = 0.068$) than those fed OA.BU (11.9 kg) and control (12.0 kg), while OA.MCM was not different (13.4 kg; Figure 3). Similarly, pigs fed the OA.FR (19.8 kg) tended to be heavier ($P = 0.093$) on 21 d post challenge compared with control (16.0 kg) fed pigs, while pigs fed OA.BU (16.6 kg) and OA.MCM (17.7 kg) were not different. Pigs fed the diet containing OA.FR showed a near tendency ($P = 0.101$) for higher ADG (641 g) compared with pigs fed control (484 g) and OA.BU (436 g) between days 7 and 14 post first S. Typh contact (Table 3). Rectal temperature was not different amongst pigs fed dietary treatments (Figure 4). All pigs had a higher ($P < 0.01$) rectal temperature on day 4 post S. Typh challenge.

Diarrhea was already present before the challenge as indicated by 57.4 and 21.4% of pigs had a score greater than or equal to 2 and a score of 3, respectively. The diarrhea incidence was maintained during the S. Typh infection week as indicated by 54.9% of pigs had a score ≥2 and 19.7% of pigs had a score equal to 3. Diarrhea incidence, defined as mild and severe diarrhea (score ≥ 2), tended to be lower ($P = 0.100$) prior to challenge in pigs fed OA.FR (43.3%) compared with pigs fed OA.MCM (74.0%; Table 4). During the challenge week, diarrhea incidence tended to be lower ($P = 0.056$) in pigs fed OA.FR (43.3%) or control (43.3%) compared with pigs fed OA.MCM (72.7%). Between days 8 and 14 after first S. Typh inoculation, diarrhea incidence tended to be lower ($P = 0.054$) in pigs fed OA.FR (21.2%) than pigs fed OA.BU (61.9%). Overall, diarrhea incidence post challenge was lower ($P = 0.006$) for pigs fed OA.FR (18.9%) than OA.BU (44.8%) and OA.MCM (41.7%), while diarrhea incidence of pigs fed the control (28.7%) was intermediate and not different. No difference in a diarrhea score of 3 was observed for pigs fed different feed additives post first S. Typh inoculation. However, a

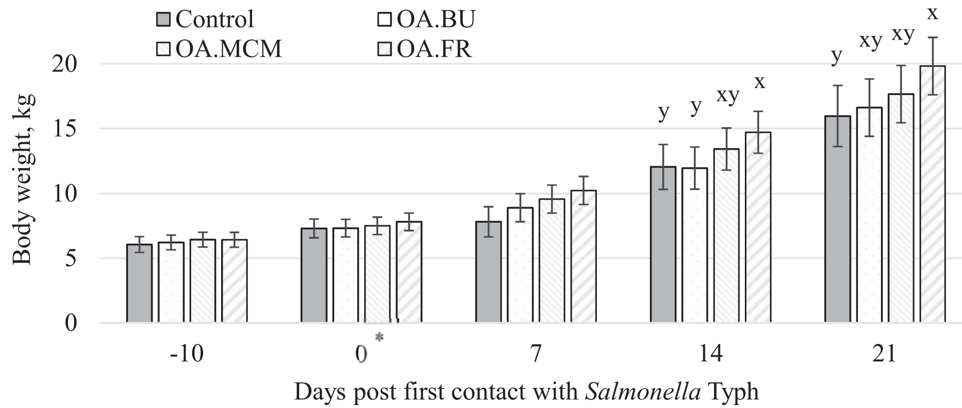

Figure 3. BW of weaned pigs (n = 32) challenged (*) at 34 ± 3 d of age (10 d postweaning) with *Salmonella* Typhimurium (Typh; oral 8.2 to 8.7 $\log_{10}$ CFU per day) for 7 d consecutive (0 to 6 d) and fed four blend dietary treatments. [x,y]Means without common superscript showed a tendency for difference at time point ($P \leq 0.10$). OA.BU, 4 kg/t organic acids plus coated 6 kg/t butyrate; OA.MCM, 4 kg/t organic acids plus 1 kg/t hydrolyzed copra meal; OA.FR, 4 kg/t organic acids plus 2 kg/t fermented rye.

Table 3. Weekly performance of weaned pigs (n = 32) challenged (*) at 34 ± d of age 3 d SD (10 d postweaning) with *Salmonella* Typhimurium (oral 8.2 to 8.7 $\log_{10}$ CFU per day) for 7 d consecutive (0 to 6 d) and distributed in 4 blend dietary treatments

		Control	OA.BU[1]	OA.MCM[2]	OA.FR[3]	SEM[4]	P-value
ADG, g	−10 to 0*	124	111	106	139	16.3	0.451
	1 to 7	194	225	295	345	56.7	0.220
	7 to 14	484	436	552	641	63.7	0.101
	14 to 21	561	666	606	730	61.2	0.221
ADFI, g	−10 to 0*	171	155	164	181	17.4	0.714
	1 to 7	268	287	313	354	41.0	0.453
	7 to 14	547	606	654	742	65.8	0.191
	14 to 21	779	828	874	997	74.0	0.183
FE[4] g/g	−10 to 0*	0.75	0.72	0.66	0.77	0.073	0.691
	1 to 7	0.74	0.72	0.91	0.97	0.103	0.162
	7 to 14	0.88	0.70	0.84	0.88	0.067	0.203
	14 to 21	0.70^{xy}	0.83^{x}	0.69^{y}	0.73^{xy}	0.046	0.086

[1]OA.BU, additional 4 kg/t organic acids plus 6 kg/t coated butyrate.
[2]OA.MCM, additional 4 kg/t organic acids plus 1 kg/t hydrolyzed copra meal.
[3]OA.FR, additional 4 kg/t organic acids plus 2 kg/t fermented rye.
[4]FE, feed efficiency as gram of weight gain divided by gram of feed intake.
[x,y]Means without common superscript showed a tendency for difference at time frame ($P \leq 0.10$).

diarrhea score equal to 3 was lower (P = 0.042) for OA.FR (8.27%) fed pigs compared with OA.BU (15.5%) fed pigs while OA.MCM (13.4%) and control (11.3%) fed pigs were not different over the entire experimental period (i.e., including pre-infection time).

Salmonella shedding

All pigs were negative to *Salmonella* on days −6 and 0. *Salmonella* shedding in feces of pigs was detected postchallenge and had a higher ($P < 0.05$) peak on days 2, 3, and 4 after first inoculation compared with day 7, which was still higher ($P < 0.05$) than days 14 and 21 (Figure 5). *Salmonella* Typh fecal shedding counts ($\log_{10}$ CFU/g) for pigs fed OA.MCM (3.62) and OA.FR (3.79) were lower ($P < 0.05$) than for pigs fed control (5.39) and OA.BUT (5.05) on day 3 post first challenge day. Over the 21-d period post challenge, pigs fed OA.MCM (3.19) and OA.FR (3.11) had reduced ($P < 0.05$) S. Typh shedding ($\log_{10}$ CFU/g) compared with control (3.88) and OA.BUT (3.87) fed pigs.

Discussion

Current measures to prevent pig-related pathogenic zoonotic bacteria, such as *Salmonella*, in the feed-to-food chain are not sufficient (Baptista et al., 2010; Savall et al., 2016). Furthermore, new and practical strategies for pig producers are required due to the urgent need to reduce antibiotic usage. The present study provided insight for feed additives using in vitro-binding affinity to 2 *Salmonella* pathogenic species and this insight was used to demonstrate in vivo reduction of S. Typh fecal shedding in nursery pigs.

Oral administration of a matrix containing 8.2- to 8.7-$\log_{10}$ CFU S. Typh (DT12 field strain) to pigs for 7 d consecutive resulted in a detectable and quantifiable fecal *Salmonella* shedding. Also, fever was detected through an increased rectal temperature in pigs up to day 4 postchallenge, at which point rectal temperature slowly lowered back to normal. Thereafter, the temperature was increasing again to age physiological levels (Sipos et al., 2013). *Salmonella* shedding during peak days was ~4.5 $\log_{10}$ CFU/g, which is similar to our previous experiments (Litjens et al., 2017; Van der Wolf et al., 2017). Fecal shedding of *Salmonella* was reduced in pigs fed OA in combination with MCM or FR. This effect was most evident during acute infection and peak of shedding (2 to 4 d post), but not thereafter (7, 14, and 21 d post infection), which suggests a limitation to reduce colonization above a certain threshold (i.e., ~3.5 $\log_{10}$ CFU/g).

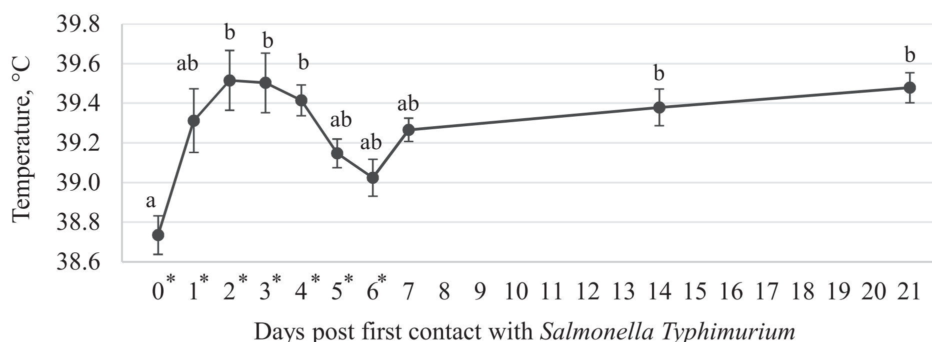

Figure 4. Rectal temperature of weaned pigs (*n* = 32) challenged at 34 ± 3 d of age (10 d postweaning) with (*) *Salmonella* Typhimurium (Typh; oral 8.2 to 8.7 log_{10} CFU per day) for 7 d consecutive (*). [a,b]Means without a common superscript are different ($P \leq 0.05$).

Table 4. Weekly frequency of days with diarrhea scores[1] in weaned pigs (*n* = 32) challenged (*) at 34 d of age ± 3 d SD (10 d postweaning) with *Salmonella* Typhimurium (oral 8.2 to 8.7 log_{10} CFU per day) for 7 d consecutive (0 to 6 d) and distributed in four blend dietary treatments

	Control	OA.BU[2]	OA.MCM[3]	OA.FR[4]	SEM	P-value
SCORE ≥ 2						
−6 to 0*	50.6[xy]	61.7[xy]	74.0[y]	43.3[x]	8.734	0.100
1 to 7	43.3[x]	60.3[xy]	72.7[y]	43.3[x]	7.751	0.036
8 to 14	46.0[xy]	61.9[y]	56.6[xy]	21.2[x]	10.14	0.054
15 to 21	17.7	33.6	17.7	0.0	10.22	0.557
Post infection[5]	28.7[ab]	44.8[b]	41.7[b]	18.9[a]	6.393	0.003
Overall[5]	32.8[ab]	47.3[b]	47.2[b]	22.2[a]	5.553	0.006
SCORE 3						
−6 to 0	27.2	24.3	26.3	7.7	7.791	0.253
1 to 7	21.7	23.2	26.3	7.7	6.791	0.209
8 to 14	17.7	14.2	1.77	0.0	5.944	0.267
15 to 21	5.31	12.4	0.0	0.0	4.555	0.572
Post infection[5]	11.2	12.8	8.69	7.04	2.945	0.152
Overall[5]	11.3[ab]	15.5[b]	13.4[ab]	8.27[a]	2.938	0.042

[1]Diarrhea score was assessed as 0 = normal feces, 1 = shapeless or loose feces, 2 = thick soft feces as mild diarrhea; and 3 = thin liquid feces as watery severe diarrhea (Wolf et al., 2017).
[2]OA.BU, additional 4 kg/t organic acids plus 6 kg/t coated butyrate.
[3]OA.MCM, additional 4 kg/t organic acids plus 1 kg/t hydrolyzed copra meal.
[4]OA.FR, additional 4 kg/t organic acids plus 2 kg/t fermented rye.
[5]Overall and regardless of time point.
[x,y]Means without common superscript showed a tendency for difference at time frame ($P \leq 0.10$).
[a,b]Means without a common superscript are different at time frame ($P \leq 0.05$).

Reason for such limitation is unknown and could be speculated that may be related to a high load of *Salmonalla* exposure for several days. These in vivo findings are in agreement with the in vitro experiment that demonstrated binding affinity of both *Salmonella* strains to culture substrate including MCM and FR. Altogether, blocking adhesion and prevention of colonization during high *Salmonella* load exposition may be the mode of action of these feed additives.

Distinct, commonly used feed ingredients and their bran fractions are known to also possess an affinity to bind pathogenic bacteria. However, this may not always result in a biological benefit to the animal. Zhu et al. (2018) demonstrated that wheat, corn, oats, barley, rye, soybean meal, and sweet whey powder have affinity to bind ETEC F4, while only rye, oats, and wheat reduced ETEC F4 adhesion to IPEC-J2 cells. Thus, binding affinity does not always translate to reduced risk of pathogenic adhesion, whereas in vivo bacterial shedding and colonization explain the actual susceptibility to disease (De Ridder et al., 2013). The blocking of adhesion was not evaluated in the present study but shedding after infection was assessed in vivo.

Less fecal *Salmonella* shedding is indicative of less *Salmonella* colonization in the intestine and reduced severity of infection (Knetter et al., 2015; Casanova-Higes et al., 2017). However, lymph node tissue was not measured in this study to confirm colonization. Nonetheless, MCM and FR binding affinity taken together with a lower shedding suggest bioactivity to reduce *Salmonella* infection. More research is needed to elucidate the effect of MCM and FR on actual colonization of tissues. Adhesion of ETEC to FR was unclear and only observed for 1 study and a near tendency for another 1 out of 5 assays. This was unexpected since Zhu et al. (2018) reported ETEC F4 having 17.3% binding affinity and 9.02% blocking adhesion to nonfermented rye. Data from ETEC F4 assays showed greater variance than for *Salmonella* without a clear explanation, hence, ETEC binding affinity to FR remains unclear.

From the present results, MCM and FR effects on *Salmonella* shedding should be attributed only when combined with the OA blend; however, it is noteworthy that OA combined with coated butyrate (OA.BU) did not reduce *Salmonella* shedding. The lack of intervention from OA.BU was partly expected since additional OA showed only numerical reduction of shedding (Van der Wolf

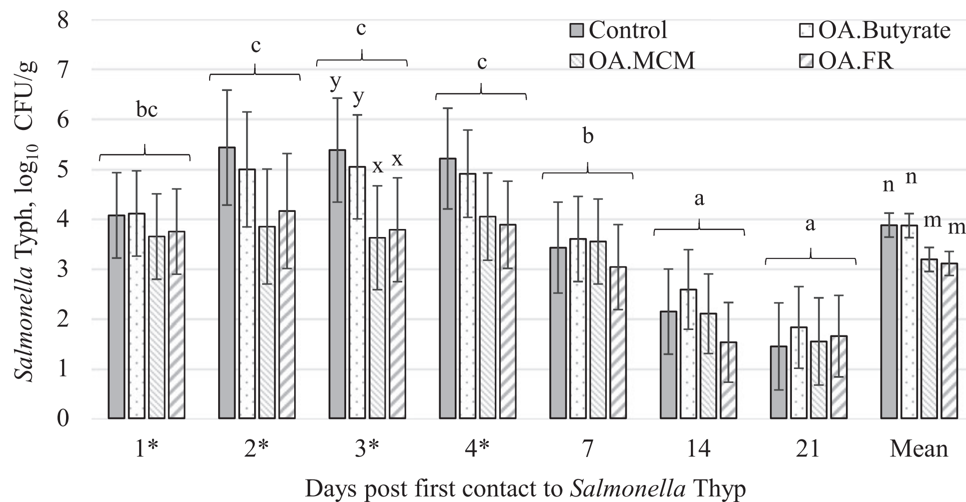

Figure 5. Shedding of *Salmonella* Typhimurium (presented as least squares means ± SE) in weaned pigs (n = 32) challenged at 34 ± 3 d of age (10 d post weaning) with (*) S. Typhimurium (Typh; oral 8.2 to 8.7 $\log_{10}$ CFU per day) for 7 d consecutive (0 to 6 d) and supplemented with different blend dietary treatments. OA.BU, 4 kg/t organic acids plus 6 kg/t coated butyrate; OA.MCM, 4 kg/t organic acids plus 1 kg/t hydrolyzed copra meal; OA.FR, 4 kg/t organic acids plus 2 kg/t fermented rye. [a–c]Means without a common superscript are different among time point ($P \leq 0.05$). [x,y]Means without a common superscript are different within time point ($P \leq 0.05$). [m,n]Means without a common superscript are different ($P \leq 0.05$).

et al., 2017). However, OA.BU results contrasts with Boyen et al. (2008), who reported a decreased colonization and shedding in challenged pigs with S. Typh when supplemented with 2 g/kg coated butyrate. Furthermore, De Ridder et al. (2013) observed a reduction of positive pigs as *Salmonella* shedding or having positive intestinal tissues feeding 3 g/kg coated butyrate. These experiments used a single day challenge model inoculating all animals with 10^7 CFU/mL or 2 out of 8 pigs per pen inoculated with 10^9 CFU/mL. Whereas the present study used a 7-d challenge with 10^9 CFU/mL per day resulting in higher infection pressure compared with the abovementioned experiments, thus, comparison across challenge studies is difficult.

The mode of action of most short chain fatty acids in the gastrointestinal tract is linked to pH-lowering and antimicrobial anion toxicity properties (Van der Wolf et al., 2001). Indeed, *Salmonella* seroprevalence can be controlled with acidifiers used in water or feed under field conditions (Van der Wolf et al., 2001; Van der Heijden et al., 2005). For coated butyrate, bioactivity is more complex and includes downregulation of *Salmonella* virulence, renewal of intestine necrotic areas, and a reduced inflammatory response (Van Immerseel et al., 2005; Boyen et al., 2008; Hamer et al., 2008). Although unknown intestinal tissue morphplogy, inflammation, and virulence of *Salmonella* herein, growth performance and shedding were not influenced in the present use of OA.BUT (including formic and lactic acids plus coated butyrate). Our 7-d oral administration of *Salmonella* is the main difference compared with a single inoculum dose in the abovementioned literature, which may explain the varying outcome. Differently, fermented or enzymatically hydrolyzed product as FR and MCM, respectively, did show a reduction of *Salmonella* shedding during the 1-wk inoculation. These findings are important since under field conditions carrier pigs can shed and expose pen mates to high *Salmonella* loads for extended periods (Griffith et al., 2006).

The MCM and FR include indigestible polysaccharides and oligosaccharides from fungal enzyme hydrolysis of copra meal and rye, which may be promoting the bioactivity against *Salmonella*. Wang et al. (2015) demonstrated blocking adhesion of S. Typh to HT-29 cells for fucoidan (71.4%, β-1, 4-mannose, GlcUA, Gal, and α-1,3-fucose), tara gum (62.1%, β-1, 4-mannose, α-1, and 6-galactose), and guar gum (54.5%, β-1, 4-mannose, and α-1,6-galactose) oligosaccharides. Copra meal includes ~40% to 45% of mannan-polysaccharides from a total 61% carbohydrate content (Saittagaroon et al., 1983). Industrial hydrolysis into mannan-oligosaccharides (i.e., mannose, mannobiose, mannotriose, etc.) is feasible with fungal β-mannanases (i.e., Actinomycetes from the Streptomycetes group; Ademark et al., 1998; Ariandi and Meryandini, 2015). The oligosaccharide content is ~14% β-1,4-mannobiose in MCM. Hydrolyzed copra meal which contained 11.4% β-1,4-mannobiose and was supplemented at 1 g/kg feed in broilers reduced *Salmonella* colonization, caecal carriage, and fecal shedding after infection (Agunos et al., 2007). Such effects were accompanied with immunomodulatory properties including IgA production. Furthermore, β-1,4-mannobiose increased in vitro Salmonella-killing activity in chicken macrophages (Ibuki et al., 2011). In a colitis pig model, it was reported that β-1,4-mannobiose downregulated innate T helper pro-inflammatory pathways which maintained intestinal permeability and histological morphology (Ibuki et al., 2014). Altogether, this suggests a mode of action more complex than bacterial-binding affinity. Rye fermented with *A. subrufescens* poly-oligosaccharide composition is not reported; however, use of nonfermented rye already shows promising binding affinity to pathogenic *E. coli* (Zhu et al., 2018). Further investigation is needed to better elucidate the binding affinity, shedding reduction, and other health promoting potential associated with FR.

The observed diarrhea before *Salmonella* challenge increased variance and might interfere with dietary treatment intervention and caution must be used when interpreting the results. It was confirmed, however, that Salmonellosis was not the cause of diarrhea prechallenge because pigs tested negative for S. Typh pre challenge. Dietary treatment did influence diarrhea outcome. Furthermore, whether mild diarrhea may interact with *Salmonella* shedding outcome is not clear. Higher intestinal transit may reduce chances of *Salmonella* colonization or derived inflammation may increase *Salmonella* colonization. In fact,

research of *Salmonella* challenge interaction with postweaning diarrhea is lacking. Because all treatments had between 43% and 74% postweaning and prechallenge diarrhea, none included mortality, and performance differences were not observed, the outcome reported herein is relevant.

Although the diarrhea incidence was altered, results are worthy of discussion since postweaning diarrhea is a common commercial problem that is difficult to fully explain because it is multifactorial. Pigs fed OA.FR diets tended to have a low frequency of diarrhea before *Salmonella* challenge, and had lower diarrhea postchallenge and overall compared with pigs fed OA.BU. Additionally, OA.FR fed pigs tended to have a greater final BW. This fungal itself (also known as almond mushroom or sun mushroom) and extracts of it are reported to have several bioactive properties, i.e., tumor suppressor, immune modulatory, antimicrobial, antiviral, antioxidant, and anti-allergy (Wisitrassameewong et al., 2012) and these bioactive components within FR may have improved intestinal health of these pigs and this may have led to an improved BW. Furthermore, ~40% of FR is mycelium of *A. subrufescens* that grow during the fermentation of the rye, of which β -glucan polysaccharides are important active components in *A. subrufescens* (Kogan and Kocher, 2007; Ohno et al., 2011). β -Glucans are recognized by distinct cell receptors (i.e., dectin, TLR-2, TLR-4, and CR3) which are known to promote gut health via immunomodulatory properties demonstrated in vitro (Smiderle et al., 2014; Choi et al., 2016) and in vivo (Samuelsen et al., 2014; Kim et al., 2019). Phenolic compounds (i.e., as gallic acid, syringic acid, and pyrogallol) in the mycelia from FR may also include valuable antioxidant properties (Carvajal et al., 2012). Nonetheless, to our knowledge, this is the first time that a derivate product of *A. subrufescens* rye fermentation was used in a *Salmonella* challenge and in a pig model. Therefore, further studies to evaluate FR composition and bioactivity are needed to elucidate its mode of action and potential effects on the gastrointestinal health of pigs.

Conclusion

Hydrolyzed copra meal and fermented rye feed additives showed in vitro binding affinity to *S.* Typh and *S.* Ent. Feed additive blends including MCM or FR combined with OA reduced peak shedding and mean shedding of *S.* Typh in nursery pigs under a 7-d challenge evaluated for 21 d. Shedding was not influenced by the use of coated butyrate with OA blend. Fermented rye combined with OA fed to pigs tended to improve BW compared with control and coated butyrate with OA (to 14 d post first inoculation) and to control (as final BW at 21 d post first inoculation). In addition, FR combined with OA shows the greatest potential to reduce frequency of postweaning diarrhea and 7 d post *Salmonella* infection.

Acknowledgments

Research was subsidized by the European Union and European Regional Development Fund. Authors would like to thank Scelta Mushrooms BV (Venlo, The Netherlands) providing fermented rye.

Conflict of interest statement

All authors declare no conflict of interest.

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