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 (10⁹ 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
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 fungal fermented products and their derivatives. The immunomodulatory properties of OAs with fungal fermented products has not been previously studied to control Salmonella in pigs exposed for several days. 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

Salmonella 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 OD600nm of 0.5. Less time indicates that more bacteria adhered to the substrate resulting in less time to reach the OD600nm 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 1% (w/v) in PBS suspension. Subsequently, the suspensions were incubated 3 times 2 min in a sonication water bath (Branson

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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  |
| ni-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  |
| Sodium (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 coropa meal                 | -       | -0.2  | -      | -     |
| Fermented rye                          | -       | -     | 0.2    | -     |
| DM, %                                  | 89.6    | 90    | 89.3   | 89.5  |
| NE, kcal                               | 2,425   | 2,425 | 2,425  | 2,425 |
| SID Lys                                 | 1.21    | 1.21  | 1.21   | 1.21  |
| SID Met                                 | 0.45    | 0.45  | 0.45   | 0.45  |
| SID Met + Cys                           | 0.7     | 0.7   | 0.7    | 0.7   |
| SID Trp                                 | 0.23    | 0.23  | 0.23   | 0.23  |
| SID Thr                                 | 0.75    | 0.75  | 0.75   | 0.75  |
| CP                                      | 17.9    | 17.7  | 17.7   | 17.2  |
| Acid hydrolyzed ether extract          | 4.3     | 5.1   | 4.7    | 4.6   |
| Crude fiber                            | 3.0     | 3.1   | 3.0    | 3.1   |
| AAT                                    | 5.4     | 5.1   | 5.1    | 5.1   |
| Neutral detergent fiber                 | 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                                 | 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                                 | 0.65    | 0.67  | 0.66   | 0.68  |
| Phosphorus                              | 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   |

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

5S510 with intermediate vortexing and centrifuged at 460 × g for 5 min. The wells of the microtiterplate (Micronol 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 logarithmic phase in Brain Heart Infusion broth (BHI; Oxoid). Then washed and resuspended in PBS adjusted to OD600 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 OD600nm 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 OD600nm 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.MCM); (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 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 log10 cfus of S. Typh for 7 d consecutive after 6 d training with non-infected matrix. The 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 (ladypfinger 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 log10 cfu 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.
A Con FR Con FR Con FR Con FR
iodide (Tritium Microbiologie B.V.) and incubated for 21 ± 3 h
Green Agar plates with 20 mg/L novobiocin + 40 g/L potassium
Salmonella at 37 °C ± 1 °C for colony counting. For analysis, individual
analyzed quantitatively for
S. collected at −6, 0, 1, 2, 3, 4, 7, 14, and 21 d post challenge and
for each interval and overall. Fecal samples (5 g per pig) were
challenge. ADG, ADFI, and feed efficiency (G:F) were calculated
were weighed at weaning day (−10 d) and 0, 7, 14, and 21 d post
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 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.)
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
ycler (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 ≤ 0.05 and 0.05 ≤ P ≤ 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, 5.79 for MCM, and 6.12 for FR
on S. Typh. 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

Table 2. Design of 5 in vitro assays to test binding affinity of different Enterobacteriaceae to feed additives1 compared with the control2.

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

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

Table 2. Design of 5 in vitro assays to test binding affinity of different Enterobacteriaceae to feed additives1 compared with the control2.
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 ($P = 0.010$) prior to challenge 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
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 Typhimurium* (DT12 field strain) to pigs for 7 d consecutive (0 to 6 d) 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 log10 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 log10 CFU/g).

**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 *Salmonella* pathogenic species and this insight was used to demonstrate in vivo reduction of *S. Typhimurium* fecal shedding in nursery pigs.

*Oral administration of a matrix containing 8.2- to 8.7-log10 CFU/day for 7 d consecutive* (0 to 6 d) *Salmonella Typhimurium* (oral 8.2 to 8.7 log10 CFU per day) for 7 d consecutive (0 to 6 d) and distributed in 4 blend dietary treatments

**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 log10 CFU per day) for 7 d consecutive (0 to 6 d) and fed four blend dietary treatments: 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 ± 3 d of age 3 d SD (10 d postweaning) with *Salmonella Typhimurium* oral 8.2 to 8.7 log10 CFU per day for 7 d consecutive (0 to 6 d) and distributed in 4 blend dietary treatments**

|                | Control | OA.BU1 | OA.MCM2 | OA.FR3 | SEM4 | P-value |
|----------------|---------|--------|---------|--------|------|---------|
| ADG, g         |         |        |         |        |      |         |
| −10 to 0"     | 124     | 111    | 106     | 139    | 16.3 | 0.451   |
| 1 to 7         | 194     | 225    | 285     | 384    | 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"     | 717     | 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   |
| FE⁴ 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       | 0.70xy  | 0.83x  | 0.69y   | 0.73xy | 0.046| 0.086   |

1OA.BU, additional 4 kg/t organic acids plus 6 kg/t coated butyrate.
2OA.MCM, additional 4 kg/t organic acids plus 1 kg/t hydrolyzed copra meal.
3OA.FR, additional 4 kg/t organic acids plus 2 kg/t fermented rye.
4FE, feed efficiency as gram of weight gain divided by gram of feed intake.

*Means without common superscript showed a tendency for difference at time point (P ≤ 0.10).*
Reason for such limitation is unknown and could be speculated that may be related to a high load of Salmonella 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.

![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,bMeans without a common superscript are different (P ≤ 0.05).](image)

| 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 |
|---------------------------------|----------------|----------------|----------------|---------------|-----------|-------|
| SCORE ≥ 2                       | Control        | OA.BU\(^2\)   | OA.MCM\(^3\)  | OA.FR\(^4\)  | SEM       | P-value |
| −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\(^{y}\)  | 21.2\(^{x}\)  | 10.14     | 0.054  |
| 15 to 21                        | 17.7           | 33.6           | 17.7           | 0.0           | 10.22     | 0.557  |
| Post infection\(^1\)            | 28.7\(^{ab}\)  | 44.8\(^{b}\)  | 41.7\(^{a}\)  | 18.9\(^{x}\)  | 6.393     | 0.003  |
| Overall\(^3\)                   | 32.8\(^{ab}\)  | 47.3\(^{b}\)  | 47.2\(^{a}\)  | 22.2\(^{x}\)  | 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           | 17.7           | 0.0           | 5.944     | 0.267  |
| 15 to 21                        | 5.31           | 12.4           | 0.0            | 0.0           | 4.555     | 0.572  |
| Post infection\(^1\)            | 11.2           | 12.8           | 8.69           | 7.04          | 2.945     | 0.152  |
| Overall\(^3\)                   | 11.3\(^{ab}\)  | 15.5\(^{b}\)  | 13.4\(^{a}\)  | 8.27\(^{x}\)  | 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.

\(^{ab}\)Means without a common superscript showed a tendency for difference at time frame (P ≤ 0.10).

\(^{xy}\)Means without a common superscript are different at time frame (P ≤ 0.05).

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, 2018).
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 10^6 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 morphology, 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 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. *Means without a common superscript are different among time point (P ≤ 0.05). ^Means without a common superscript are different among time point (P ≤ 0.05). a–cMeans without a common superscript are different (P ≤ 0.05).
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 lower 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 BW. Furthermore, ~40% of FR is mycelium of 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 CK3) 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 derivative 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.

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Conflict of interest statement

All authors declare no conflict of interest.

Literature Cited

Ademark, P., A. Varga, J. Medve, V. Harjunpää, T. Drakenberg, F. Tjerneld, and H. Stålbrand. 1998. Softwood hemicellulose-degrading enzymes from Aspergillus niger: purification and properties of a beta-mannanase. J. Biotechnol. 63:199–210. doi:10.1016/s0168-1656(98)00086-8

Agunos, A., M. Ibuki, F. Yokomizo, and Y. Mine. 2007. Effect of dietary beta1-4 mannobiocide in the prevention of Salmonella enteritidis infection in broilers. Br. Poult. Sci. 48:331–341. doi:10.1080/00071660701370442

Akramiene, D., A. Kondrotas, J. Didziapetriene, and E. Kevelaitis. 2007. Effects of beta-glucans on the immune system. Medicina (Kaunas). 43:597–606. doi:10.3390/medicina43080076

Andrés-Barranco, S., J. P. Vico, M. J. Grilló, and R. C. Maina-Jaime. 2015. Reduction of subclinical Salmonella infection in fattening pigs after dietary supplementation with a β-galactomannan oligosaccharide. J. Appl. Microbiol. 118:284–294. doi:10.1111/jam.12713

Ariandi, Y., and A. Meryandini. 2015. Enzymatic hydrolysis of copra meal by mannanase from Streptomyces sp. BF3.1 for the production of mannooligosaccharides. HAYATI J. Biosci. 22:79–86. doi:10.4308/hjb.22.2.79

Baptista, F. M., J. Dahl, and L. R. Nielsen. 2010. Factors influencing Salmonella carcass prevalence in Danish pig abattoirs. Prev. Vet. Med. 95:231–238. doi:10.1016/j.prevetmed.2010.04.007

Becker, P. M., S. Galletti, P. J. Roubos-van den Hil, and P. G. vanWikelaar. 2007. Validation of growth as measurand for bacterial adhesion to food and feed ingredients. J. Appl. Microbiol. 103:2686–2696. doi:10.1111/j.1365-2672.2007.03524.x

Biagi, G., A. Piva, M. Moschini, E. Vezzali, and F. X. Roth. 2007. Performance, intestinal microflora, and wall morphology of weaning pigs fed sodium butyrate. J. Anim. Sci. 85:1184–1191. doi:10.2527/jas.2006-378

Boyen, F., F. Haesebrouck, A. Vanparys, J. Wolff, M. Mahu, F. Van Immerseel, I. Rychlik, J. Dewulf, R. Ducatelle, and F. Pasmans. 2008. Coated fatty acids alter virulence properties of Salmonella Typhimurium and decrease intestinal colonization of pigs. Vet. Microbiol. 132:319–327. doi:10.1016/j.vetmic.2008.05.008

Carvajal, A. E. S., E. A. Koehnlein, A. A. Soares, G. J. Eler, A. T. Nakashima, A. Bracht, and R. M. Peralta. 2012. Bioactives of fruiting bodies and submerged culture mycelia of Agaricus brasiliensis (A. blazei) and their antioxidant properties. LWT-Food Sci. Technol. 46:493–499. doi:10.1016/j.lwt.2011.07.023

Casanova-Higes, A., S. Andrés-Barranco, and R. Maina-Jaime. 2017. Influence of on-farm pig salmonella status on salt nemaella shedding at slaughter. Zoonoses Public Health. 64:328–336. doi:10.1111/zph.12301

Chapman, M. A., M. F. Grahn, M. Hutton, and N. S. Williams. 1995. Butyrate metabolism in the terminal ileal mucosa of patients with ulcerative colitis. Br. J. Surg. 82:36–38. doi:10.1002/bjs.1800820115

Choi, E. Y., S. S. Lee, J. Y. Hyeon, S. H. Choe, B. R. Keum, J. M. Lim, D. C. Park, I. S. Choi, and K. K. Cho. 2016. Effects of β-glucan on the release of nitric oxide by macrophages stimulated with lipopolysaccharide. Asian-Australas. J. Anim. Sci. 29:1664–1674. doi:10.5713/ajas.16.0418

CVB. 2006. Tabellenboek Vervoeding 2006 voedernormen Varkens en voederwaarden voedermiddelen voor Varkens, ed. The Netherlands: Federatie Nederlandse Diervoede kerken. De Ridder, L., D. Maes, J. Dewulf, F. Pasmans, F. Boyen, F. Haesebrouck, E. Méric, P. Butaye, and Y. Van der Stede. 2013. Evaluation of three intervention strategies to reduce the transmission of Salmonella Typhimurium in pigs. Vet. J. 197:613–618. doi:10.1016/j.tvjl.2013.03.026

Fang, C. L., H. Sun, J. Wu, H. H. Niu, and J. Feng. 2014. Effects of sodium butyrate on growth performance, haematological and immunological characterisitics of weaning piglets. J. Anim. Physiol. Anim. Nutr. (Berl.). 98:680–685. doi:10.1111/jpn.12122
