Animal Nutrition 8 (2022) 82–90

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

Organic acid blends improve intestinal integrity, modulate short-chain fatty acids profiles and alter microbiota of broilers under necrotic enteritis challenge

Alip Kumar [a], Mehdi Toghyani [a, 1], Sarbast K. Kheravii [a], Lane Pineda [b], Yanming Han [b], Robert A. Swick [a], Shu-Biao Wu [a, *]

[a] School of Environmental and Rural Science, University of New England, Armidale, NSW, 2351, Australia
[b] Trouw Nutrition R & D, Stationsstraat 77, 3811, MH, Amersfoort, the Netherlands

ARTICLE INFO

Article history:
Received 20 February 2021
Received in revised form
19 April 2021
Accepted 25 April 2021
Available online 14 September 2021

Keywords:
Organic acid blend
Alternative to antibiotics
Intestinal health
Necrotic enteritis
Broiler chicken

ABSTRACT

Controlling enteric diseases of broilers is crucial. Among many additives, organic acids (OA) and their blends are gaining attention to combat diseases in the post-antibiotic era. The current study evaluated the potentials of short-chain fatty acids (SCFA) and medium-chain fatty acids (MCFA) blends and/or phenolic compounds on intestinal integrity, intestinal pH, caecal microbiota, and caecal SCFA profiles of broilers under necrotic enteritis (NE) challenge. The additives used were: (A) a blend of SCFA, MCFA, and a phenolic compound (SMP), (B) a blend of free and buffered SCFA with MCFA (SMF), and (C) a blend of free and buffered SCFA with a high concentration of MCFA (SHM). A total of 1,404 male parental chicks of Ross 308 broilers were randomly allocated to 78 floor pens on hatching day with 6 treatments replicated 13 times with 18 birds per pen. The treatments were: UCC, unchallenged control; CHC, challenged control; BAC, challenged group plus zinc bacitracin; SMP, challenged group plus additive SMP; SMF, challenged group plus additive SMF; SHM, challenged group plus additive SHM. Birds were challenged with field-strain *Eimeria* spp. on d 9 and *Clostridium perfringens* on d 14. Birds challenged with NE increased fluorescein isothiocyanate dextran (FITC-d) concentration in serum, reduced acetate and butyrate concentrations, and increased *Bacteroides* and *C. perfringens* load in the caeca ($P < 0.05$). Birds fed additives decreased FITC-d from gut to serum, reduced *Bacteroides* (d 16, $P < 0.05$) and numerically reduced *C. perfringens* load compared to CHC group. Birds fed additive SHM had higher concentrations of acetate and butyrate (d 21, $P < 0.05$) than CHC group but were not different from SMP and SMF groups. All the additives exhibited similar intestinal protection against NE compared to the BAC group indicated by FITC-d concentration in serum, acetate, propionate and butyrate concentrations in the caeca, and caecal bacterial loads except for the *C. perfringens* ($P > 0.05$). The SMP group had a higher load compared to BAC ($P < 0.05$). These findings suggest the promising effects of OA blends as alternatives to BAC to ameliorate the impact of NE challenge of broilers as indicated by improved intestinal health.

1. Introduction

Necrotic enteritis (NE) is an economically important enteric bacterial disease primarily caused by *Clostridium perfringens* along with one or more predisposing factors (M'Sadeq et al., 2015; Prescott et al., 2016). The cost impact has been estimated to be US$6 billion per annum to the poultry industry (Wade and Keyburn, 2015). *C. perfringens* is an anaerobic, Gram-positive, and rod-shaped bacterium. Whereas it presents in the normal intestinal flora, the bacteria can become pathogenic with NetB toxin-

* Corresponding author.
E-mail address: shubiao.wu@une.edu.au (S.-B. Wu).
[1] Present address: School of Life and Environmental Sciences, The University of Sydney, NSW, 2006, Australia

Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2021.04.003

producing strains causing sub-clinical or clinical forms of NE (Keyburn et al., 2008; Wu et al., 2010). Necrotic enteritis damages the intestinal mucosa and epithelial cells (Van Immerseel et al., 2004a; Palliyeguru and Rose, 2019), leading to intestinal inflammation, lesions, and a disruption of the intestinal microbial community, and short-chain fatty acid production (Gharib-Naseri et al., 2019). It impairs tight junction proteins to reduce gut barrier functions (Latorre et al., 2018) and thus, birds suffering from NE exhibit impaired intestinal permeability and poor growth performance (Awad et al., 2017). For decades, enteric diseases including NE have been controlled by using antibiotic growth promoters (AGP). However, due to the development of antibiotic resistance in bacteria, which is a threat to animal and human health (Seal et al., 2013), the use of AGP has been restricted or banned in the poultry industry leading to the re-occurrence of NE (Kaldhusdal et al., 2016). Thus, alternatives production strategies to control NE and other diseases have been explored due to the withdrawal of AGP from animal feed.

Organic acids (OA) have been used in the feed industry for decades to prevent microbial and fungal infection as a potential alternative source to AGP, particularly in the poultry industry (Adil et al., 2010; Polycarpo et al., 2017). The OA consists of short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA) and long-chain fatty acids (LCFA), and the efficacy of OA largely depend on chemical composition, form, molecular weight, pKa value, and targeted pathogen or microorganism control (Patten and Waldroup, 1988). The mode of action of OA has been suggested that the lipophilic ability of OA molecules to enter the bacterial cell membrane and detach themselves in the inner more alkaline part and the undissociated OA acids reducing the pH in the cytoplasm and therefore disrupting bacterial metabolism resulted in retarding bacterial growth and the death of bacteria (Van Immerseel et al., 2006; Papatsiros et al., 2013). Dietary addition of SCFA may be able to modulate intestinal microbiota via their bactericidal and bacteriostatic activities. It regulates the growth and proliferation of epithelial cells, which resulted in enhanced protection against diseases and improved bird performance (Corrêa-Oliveira et al., 2016; Dittoe et al., 2018). On the other hand, dietary supplementation of MCFA may inhibit the overgrowth of pathogenic bacteria via their strong antibacterial activity resulted in increased beneficial bacteria, improved digestion and absorption of nutrients, thus improved growth performance (Zentek et al., 2011; Khan and Iqbal, 2016). Different combinations of SCFA and MCFA have shown to be efficacious to ameliorate the adverse impact of enteric infection on intestinal health and reported their synergistic effects (Onrust et al., 2018; Adhikari et al., 2020; Aljumaah et al., 2020; McKnight et al., 2020; Sun et al., 2020). However, many studies have shown the positive effects of OA blends on intestinal health, but the results are not consistent, thus warrants further investigation.

It was hypothesised that the different combinations of SCFA with MCFA may promote a healthy microbial community and protect the intestinal barrier functions of birds under diseased conditions. The present study was designed to evaluate the efficacy of blended OA and/or phenolic compounds using intestinal permeability parameters and caecal microbiota analysis in combination with the caecal SCFA production profile of broilers subjected to NE challenge and to compare their potentials to ameliorate the impact of NE on intestinal health against an AGP agent.

2. Materials and methods

2.1. Ethics statement

The bird management and handling procedures were applied according to the guidelines for the use of animals for study purposes and approved by the Animal Ethics Committee, University of New England, Australia (AEC18-057).

2.2. Organic acid blends

The present study aimed to evaluate the potential of 3 types of commercial feed additives provided by Trouw Nutrition, a Nutreco company (Selko B.V., Amersfoort, the Netherlands), in broilers under a sub-clinical NE challenge model. The blended additives were: A) a combination of SCFA, MCFA, target release butyrates, slow-release lauric acid, and a phenolic compound (SMP; additive consisted of calcium and sodium salt of butyric acid, sorbic acid, pure distilled coconut/palm fatty acid, acacia, maltodextrin, vegetable oil from soy, starch from corn, vegetable fat from palm, silicic acid, a blend of flavouring compounds, and sepiolite); B) a combination of free and buffered SCFA with MCFA (SMF; additive consisted of formic acid, acetic acid, propionic acid, ammonium formate, lactic acid, citric acid, silicic acid, sorbic acid, and coconut/palm kernel fatty acid); C) a combination of free and buffered SCFA with a high concentration (3×) of MCFA (SHM; additive consisted of formic acid, propionic acid, acetic acid, ammonium formate, silicic acid, sorbic acid, and pure distilled coconut/palm fatty acid).

2.3. Study design, husbandry and diets

A total of 1,404 male parental chicks of Ross 308 broilers were obtained on hatching day from a commercial hatchery (Aviagen hatchery, Goulburn NSW, Australia). Upon arrival, chicks were weighed and allocated to 78-floor pens measuring 0.85 m^2 with wood shavings as bedding materials in a completely randomised design (CRD). Birds were reared in an environmentally controlled house with fresh hardwood shavings and ad libitum access to feed and clean water. The lighting, temperature, and humidity were maintained following Ross 308 guidelines (Aviagen, 2014).

Six treatments were applied in the study, and each treatment had 13 replicate pens with each pen housed 18 birds as an experimental unit. The treatments were: UCC, unchallenged control group, without additives or in-feed zinc bacitracin (BAC); CHC, challenged control group, without additives or in-feed BAC; BAC, challenged group supplemented with in-feed BAC at 0.05 g/kg in starter, grower and finisher phases; SMP, the challenged group supplemented with additive SMP at concentrations of 1.5, 1.5, 0.5 g/kg feed in starter, grower and finisher phases, respectively; SMF, the challenged group supplemented with additive SMF at concentrations of 2.5, 2.0, 1.0 g/kg feed in starter, grower and finisher phases, respectively; SHM, challenged group supplemented with additive SHM at concentrations of 2.0, 1.5, 1.0 g/kg feed in starter, grower and finisher phases, respectively. Wheat and soybean meal-based diets supplemented with xylanase and phytase were formulated to meet the nutrient requirements of birds as per Ross 308 recommendations using the manufacturer's recommended matrix values for phytase. Prior to the feed formulation, nutrient contents of feed ingredients were estimated by using near-infrared spectroscopy (NIRS) AminoProx (Evonik, Essen, Germany). Diets were cold pelleted at 65°C to 2.5 mm and crumbled for the starter phase and fed in 3 different phases following Ross 308 commercial feeding schemes for broilers; starter phase (d 0 to 10), grower phase (d 10 to 24), and finisher phase (d 24 to 35). The detail of dietary composition in each phase is shown in Table 1.

2.4. Necrotic enteritis challenge

The NE challenge model was applied following previously reported challenge procedures (Wu et al., 2014; Rodgers et al., 2015). On d 9, birds in challenged groups were inoculated with field

Table 1
Composition and nutrient contents of starter, grower and finisher diets (as-fed basis, presented in g/kg unless mentioned otherwise).

Item	Starter phase (d 0 to 10)	Grower phase (d 10 to 24)	Finisher phase (d 24 to 35)
Ingredients			
Wheat	622	651	679
Soybean meal	323	281	255
Canola oil	21	32	38
Limestone	11	11	10
Dicalcium phosphate (18P/21Ca)	8.7	7.8	6.6
Salt	1.2	1.4	1.4
Sodium bicarbonate	1.5	1.3	1.3
Vitamin premix[1]	0.9	0.9	0.9
Mineral premix[2]	1.0	1.0	1.0
Choline chloride (60%)	0.6	0.5	0.4
L-Lysine HCl	3.1	2.4	2.2
D, L-Methionine	2.8	2.1	1.9
L-Threonine	1.0	0.6	0.5
Phytase	0.1	0.1	0.1
Xylanase	0.2	0.2	0.2
Titanium di-oxide (TiO_2)	–	5.0	–
Calculated nutrients[3]			
AME, kcal/kg	3,025	3,120	3,200
Crude protein	235	217	208
Crude fat	36.0	47.3	53.6
Crude fiber	24.0	22.9	22.3
Digestible Arg	13.5	12.3	11.6
Digestible Lys	12.9	11.3	10.5
Digestible Met	5.9	5.0	4.6
Digestible Met + Cys	9.4	8.3	7.9
Digestible Trp	2.8	2.6	2.5
Digestible Ile	8.8	8.1	7.7
Digestible Thr	8.2	7.2	6.8
Digestible Val	9.5	8.8	8.4
Calcium	9.0	8.5	8.0
Phosphorus available	4.5	4.3	4.0
Phosphorus total	5.2	4.9	4.6
Sodium	1.6	1.6	1.6
Chloride	1.9	1.8	1.8
Linoleic 18:2	12.8	15.6	17.3
Choline, mg/kg	1,700	1,600	1,500
Analysed nutrients			
Dry matter	892	893	895
Gross energy, kcal/kg	3,915	4,004	4,108
Crude protein	236	219	209

AME = apparent metabolizable energy.
[1] Vitamin premix provided the following per kilogram diet: vitamin A, 12 MIU; vitamin D, 5 MIU; cyanocobalamin, 0.016 mg; vitamin E, 75 mg; vitamin K, 3 mg; folic acid, 2 mg; riboflavin, 8 mg; nicotinic acid, 55 mg; pantothenic acid, 13 mg; pyridoxine, 5 mg; biotin, 0.25 mg; thiamine, 3 mg; and antioxidant ethoxyquin, 50 mg.
[2] Mineral premix provided the following per kilogram diet: Cu (sulfate), 16 mg; Mn (sulfate), 60 mg; Mn (oxide), 60 mg; I (iodide), 0.125 mg; Se (selenite), 0.3 mg; Fe (sulfate), 40 mg; Zn (oxide and sulfate), 100 mg.
[3] Nutrient contents of wheat and soybean meal were measured using near-infrared spectroscopy (NIRS, Evonik AminoProx, Germany).

strains of *Eimeria spp*. oocysts in 1 mL dose per os consisting of *Eimeria maxima* (5000), *Eimeria acervulina* (5000), and *Eimeria brunetti* (2500) (*Eimeria* Pty Ltd., Werribee, VIC, Australia). On d 14, birds in challenged groups were inoculated with approximately 10^8 CFU/mL of *C. perfringens* in 1 mL dose per os (EHE-NE18 strain, CSIRO Livestock, Geelong, VIC, Australia). Simultaneously, birds in the unchallenged group were given 1 mL per os phosphate-buffered saline on d 9 and sterile broth on d 14.

2.5. Sample collection, serum FITC-d and gastrointestinal pH measurements

On d 16 and 21, 2 birds from each pen were randomly selected, weighed, colour-marked and inoculated with 1 mL per os fluorescein isothiocyanate dextran (FITC-d; average molecular weight: 4,000, Sigma–Aldrich Co., Missouri, USA) containing 4.17 mg/kg, approximately 2.5 h before euthanisation. These birds were returned to the pens where they were originally. The inoculated birds were stunned by an electric stunner (JF poultry equipment, Weltevreden Park, South Africa), and blood samples were collected in clot activator Vacutainer tubes from the jugular vein by decapitation method. For the FITC-d determination in serum, blood samples were kept at room temperature for approximately 3 h to allow clotting, centrifuged at 3,000 × *g* for 10 min to separate serum samples from whole blood, and immediately stored in a −20 °C freezer until the measurements were performed.

Fluorescence concentrations of diluted serum (1:1 in phosphate-buffered solution) were detected at the excitation wavelength of 485 nm and an emission wavelength of 528 nm on a multi-mode microplate reader SpectraMax M2e (Molecular Devices, San Jose, USA) and FITC-d concentration per mL of serum was calculated based on a standard curve constructed with known concentration FITC-d.

Caecal contents were collected from the sampled birds on d 16 for bacterial quantification and d 21 for SCFA measurements and stored in a −20°C freezer for analysis.

Gastrointestinal pH was measured following previously reported procedures (Gharib-Naseri et al., 2019). In brief, immediately after euthanasia on d 16 and 21, all gastrointestinal sections were excised from 2 sampled birds per pen. A digital pH meter,

EcoScan probe (5/6 m, Envirosensors spear tip pH probe, Eutech Instruments Pte Ltd., 139949, Singapore) was used to measure pH values by inserting into the frontal crop, gizzard, duodenum, jejunum, ileum, and caecum sections whereas assuring the probe were rinsed with purified (Ultra-pure) water between each measurement.

2.6. Microbiota analysis of caecal contents

The DNA of frozen caecal samples collected on d 16 was extracted using PowerFecal QIAcube HT Kit (Qiagen, Inc., Hilden, Germany) with some modifications. Approximately 100 mg of caecal samples and 300 mg of glass beads (0.1 mm) were placed in a 2 mL Eppendorf tube. Later, 500 μL pre-warmed PW1 was pipetted to Eppendorf tubes containing samples and placed into Tissuelyser II for 5 min at a frequency of 30 times per second to disrupt bacterial cells. The samples were incubated at 90°C and incubated for 15 min followed by centrifugation at 20,000 × *g* for 1 min. An aliquot of 400 μL supernatant was mixed with 150 μl of Buffer C3. The mixture was placed into the refrigerator at 4 °C and incubated for 5 min before centrifuging at 20,000 × *g* for 1 min. The supernatant was transferred into a loading block (S-block) containing 20 μL Proteinase K and placed at room temperature, and incubated for 10 min. Then the extraction was performed using the QIAcube HT followed by the manufacture's instruction. The quantity and quality of the resulting DNA samples were determined on a Nanodrop 8000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). DNA with standard ratios A260/A280 being >1.8 were recognised as of high purity and kept at −20 °C until required.

The caecal bacterial DNA quantification methods described previously (Wise and Siragusa, 2007; Kheravii et al., 2017) were used for this study. The stored caecal DNA was thawed and diluted in nuclease-free water (20 times), and the quantitative real-time polymerase chain reaction (PCR) of major 8 bacterial groups was performed to quantify with a real-time PCR system, Rotorgene 6000 (Corbett, Qiagen, Inc., Hilden, Germany). The SYBR-Green containing Mix (SensiMix SYBR No-Rox, Bioline, Tennessee, USA) was used for quantitative polymerase chain reaction (qPCR) and the qPCR was executed in duplicate for each sample. The reaction in an amount of 10 μL contained 2 μL of diluted caecal DNA, 300 mmol/L of forward and reverse primers, and 5 μL of 2 × SensiMix. The SYBR-Green containing Mix was used for the genomic DNA copies of *Lactobacillus* spp., *Bifidobacterium* spp., *Bacteroides* spp., *Bacillus* spp., *Ruminococcus* spp., Enterobacteriaceae, total anaerobic bacteria, and SensiFAST Probe SYBR No-ROX (Bioline, Tennessee, USA) was used for *C. perfringens* for the Taqman-based assay. The specific 16S rRNA primers applied for quantifying these bacterial groups are shown in Table 2. The number of target DNA copies was calculated, and bacteria quantity was expressed as $\log_{10}$ (genomic DNA copy number)/g digesta.

2.7. SCFA analysis of caecal contents

The caecal SCFA were analysed based on a previously reported method (Jensen et al., 1995). In brief, approximately 1 g of caecal content (stored in a −20 °C freezer) was weighed into centrifuge tubes and placed on ice, and 1 mL of internal standard, 0.01 methylbutyric acid was added. The solution was vortexed and thoroughly mixed and centrifuged at 15,000 × *g* for 20 min at 5 °C. Then 1 mL supernatant of samples was transferred to 8 mL vials with caution. After that, 0.5 mL of concentrated HCl (36%) and 2.5 mL of diethyl ether were added to the solution and vortexed, followed by centrifugation for 15 min at 1,000 × *g* (at 5°C). After centrifugation, 400 μL supernatant was transferred to 2 mL gas chromatograph (GC) vials, and then 40 μL N-tert-butyldimethylsilyl-N-methyl trifluoroacetamide was added and mixed. Samples were then vortexed gently and placed into the heating block (at 80 °C) for 20 min. The GC vials were tightened appropriately then kept at room temperature for 48 h prior to analysis on a gas Chromatograph, Varian CP3400 CX (Varian Analytical Instruments, Palo Alto, CA, USA). The concentrations of caecal SCFA were expressed as μmol/g digesta.

2.8. Data analysis

The data were analysed using one-way ANOVA as a completely randomised design where the pen was considered as a study unit ($n = 78$) with SAS 9.3 statistical package (Guide, 2010). The significant differences between means were separated by Least Significant Difference (LSD) test. The *P*-value < 0.05 was considered as statistical significance, where $P < 0.10$ was declared a tendency.

Table 2
The specific 16S rRNA primers applied for quantifying bacteria in caecal contents.

Target group of bacteria	Primer sequence (5′→3′)	Annealing temperature, °C	Reference
Lactobacillus spp.	F- CAC CGC TAC ACA TGG AG R- AGC AGT AGG GAA TCT TCC A	63	Rinttilä et al. (2004)
Bifidobacterium spp.	F- GCG TCC GCT GTG GGC R- CTT CTC CGG CAT GGT GTT G	63	Requena et al. (2002)
Bacteroides spp.	F- GAG AGG AAG GTC CCC CAC R- CGC TAC TTG GCT GGT TCA G	63	Layton et al. (2006)
Bacillus spp.	F- GCA ACG AGC GCA ACC CTT GA R- TCA TCC CCA CCT TCC TCC GGT	63	Zhang et al. (2015)
Ruminococcus spp.	F- GGC GGC YTR CTG GGC TTT R- CCA GGT GGA TWA CTT ATT GTG TTA A	63	Ramirez-Farias et al. (2008)
Enterobacteriaceae	F- CAT TGA CGT TAC CCG CAG AAG AAG C R- CTC TAC GAG ACT CAA GCT TGC	63	Bartosch et al. (2004)
Clostridium perfringens	F- ATG CAA GTC GAG CGA KG R- TAT GCG GTA TTA ATC TYC CTT T TaqMan Probe-5′-FAM-TCA TCA TTC AAC CAA AGG AGC AAT CC-TAMRA-3′	60	Rinttilä et al. (2004); Wise and Siragusa (2005)
Total bacteria	F- CGG YCC AGA CTC CTA CGG G R- TTA CCG CGG CTG CTG GCA C	63	Lee et al. (1996)

When the tendency was detected, LSD test was used to make pairwise comparisons.

3. Results

3.1. Performance

The performance results in detail have been reported in a previous paper (Kumar et al., 2021). Briefly, the birds fed additive SHM had higher body weight gain (BWG) compared to the CHC and BAC groups ($P < 0.05$; d 10 to 24), and all the 3 additive groups had lower feed conversion ratio (FCR) compared to the CHC group ($P < 0.05$; d 0 to 35). The BWG and feed intake (FI) of the UCC group were significantly higher than all the challenged groups ($P < 0.001$ for both; d 0 to 35). The livability of the birds was not different among the treatment groups ($P > 0.05$; d 0 to 35).

3.2. Serum FITC-d concentration

The effects of NE challenge and blended additives on serum FITC-d in broilers are shown in Table 3. One-way ANOVA analysis indicated that FITC-d concentration in the serum showed significant differences in both d 16 and 21 ($P < 0.001$ and 0.001, respectively). The UCC group had lower serum FITC-d on d 16 and d 21 compared to the CHC group. On d 16, birds fed blended additives (SMP, SMF and SHM) had a lower concentration of serum FITC-d compared to the CHC group. On d 16 and 21, birds fed additives had similar serum FITC-d compared to the BAC group but higher than the UCC group. On d 21, birds fed additive SMF had lower serum FITC-d compared to the CHC group but not different from additive SMP and SHM groups.

3.3. Gastrointestinal pH

The effects of NE challenge and blended additives on gastrointestinal pH in broilers on d 16 and 21 are presented in Table 4. One-way ANOVA analysis indicated that ileal pH on d 16 and caecal pH on d 21 showed significant differences ($P = 0.001$ and 0.017, respectively). The UCC group had higher ileal pH on d 16 and lower caecal pH on d 21 compared to the CHC group. Birds in additive groups had similar pH measurements in gizzard, duodenum, jejunum, ileum and caeca on d 16, and crop, gizzard, jejunum, ileum, and caeca on d 21 compared to CHC and BAC groups. However, there was a tendency in crop pH on d 16 ($P = 0.084$) where additive SMP (5.11) had the highest pH whereas the CHC group had the lowest pH (4.72). In addition, there was a tendency for lower gizzard pH on d 21 ($P = 0.069$) in birds fed additive SHM (5.73) group compared to UCC (5.92), CHC (5.97), BAC (5.92) and SMF (5.94) groups.

3.4. Caecal bacterial quantification

The effects of NE challenge and blended additives on caecal microbiota in broilers on d 16 are shown in Table 5. One-way ANOVA analysis indicated that the quantification of *Bacteroides* spp. ($P = 0.013$), *Ruminocccus* spp. ($P = 0.025$), Enterobacteriaceae

Table 3
Effect of additives and NE challenge on serum FITC-d (μg/mL) on d 16 and 21.[1]

Item	UCC	NE challenged[2]					SEM	*P*-value
		CHC	BAC	SMP	SMF	SHM		
Day 16	0.199^{c}	0.547^{a}	0.487^{b}	0.451^{b}	0.455^{b}	0.460^{b}	0.021	<0.001
Day 21	0.127^{c}	0.220^{a}	0.207^{ab}	0.205^{ab}	0.187^{b}	0.198^{ab}	0.009	<0.001

NE = necrotic enteritis; FITC-d = fluorescein isothiocyanate dextran.
$^{a-c}$ Values in a row with no common superscripts differ significantly ($P < 0.05$). Mean values are based on 2 birds per replicate and 13 replicates per treatment.
[1] UCC, unchallenged control; CHC, challenged control; BAC, zinc bacitracin; SMP, a blend of short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA) and phenolic compound; SMF, a blend of buffered SCFA with MCFA; SHM, a blend of buffered SCFA with a high concentration of MCFA.
[2] NE challenged birds were gavaged with *Eimeria* spp. at d 9 and *C. perfringens* at d 14.

Table 4
Effect of additives and NE challenge on pH on d 16 and 21.[1]

Item	UCC	NE challenged[2]					SEM	*P*-value
		CHC	BAC	SMP	SMF	SHM		
Day 16								
Crop	4.97	4.72	4.86	5.11	4.94	4.89	0.09	0.084
Gizzard	2.69	2.83	2.81	2.75	2.76	2.76	0.11	0.945
Duodenum	5.84	5.72	5.73	5.78	5.66	5.75	0.05	0.231
Jejunum	5.79	5.60	5.65	5.72	5.67	5.63	0.06	0.264
Ileum	5.56^{a}	5.31^{bc}	5.29^{bc}	5.47^{ab}	5.13^{c}	5.31^{bc}	0.07	0.001
Caeca	5.51	5.57	5.52	5.47	5.50	5.49	0.08	0.969
Day 21								
Crop	4.75	4.85	4.95	4.87	4.92	4.86	0.09	0.717
Gizzard	2.57	2.82	2.76	2.86	2.65	2.88	0.11	0.310
Duodenum	5.92	5.97	5.92	5.85	5.94	5.73	0.06	0.068
Jejunum	6.04	6.05	6.05	6.12	6.05	5.97	0.04	0.224
Ileum	5.71	5.77	5.69	5.69	5.81	5.76	0.07	0.736
Caeca	5.85^{b}	6.08^{a}	6.14^{a}	6.21^{a}	6.04^{ab}	6.17^{a}	0.07	0.017

NE = necrotic enteritis.
$^{a-c}$ Values in a row with no common superscripts differ significantly ($P < 0.05$). Mean values are based on 2 birds per replicate and 13 replicates per treatment.
[1] UCC, unchallenged control; CHC, challenged control; BAC, zinc bacitracin; SMP, a blend of short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA) and phenolic compound; SMF, a blend of buffered SCFA with MCFA; SHM, a blend of buffered SCFA with a high concentration of MCFA.
[2] NE challenged birds were gavaged with *Eimeria* spp. at d 9 and *C. perfringens* at d 14.

Table 5
Effect of additives and NE challenge on caecal bacterial genomic DNA copies (expressed as $\log_{10}$ copies/g digesta) on d 16.[1]

Item	UCC	NE challenged[2]					SEM	*P*-value
		CHC	BAC	SMP	SMF	SHM		
Lactobacillus spp.	9.31	9.36	9.50	9.82	9.57	9.38	0.12	0.058
Bifidobacteria spp.	8.68	8.66	8.70	8.75	8.52	8.67	0.11	0.741
Bacteroides spp.	5.03^{b}	5.65^{a}	5.05^{b}	5.18^{b}	5.11^{b}	5.08^{b}	0.13	0.013
Bacillus spp.	7.85	7.04	6.94	7.34	7.16	7.02	0.24	0.114
Ruminococcus spp.	9.64^{a}	9.31^{b}	9.23^{b}	9.26^{b}	9.26^{b}	9.24^{b}	0.09	0.025
Enterobacteriaceae	7.36^{b}	8.72^{a}	9.69^{a}	9.52^{a}	9.15^{a}	8.97^{a}	0.35	<0.001
Clostridium pefringens	0.84^{c}	9.31^{a}	5.96^{b}	8.96^{a}	8.48^{ab}	8.04^{ab}	0.61	<0.001
Total bacteria	11.40	11.16	11.21	11.36	11.23	11.12	0.08	0.120

NE = necrotic enteritis.
$^{a-c}$ Values in a row with no common superscripts differ significantly ($P < 0.05$). Mean values are based on 2 birds per replicate and 7 replicates per treatment.
[1] UCC, unchallenged control; CHC, challenged control; BAC, zinc bacitracin; SMP, a blend of short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA) and phenolic compound; SMF, a blend of buffered SCFA with MCFA; SHM, a blend of buffered SCFA with a high concentration of MCFA.
[2] NE challenged birds were gavaged with *Eimeria* spp. at d 9 and *C. perfringens* at d 14.

($P < 0.001$) and *C. perfringens* ($P < 0.001$) in caecal content were significantly affected by treatments. The UCC group had lower *Bacteroides* spp., Enterobacteriaceae, *C. perfringens*, and higher *Ruminocccus* spp. loads compared to the CHC group. Birds in the BAC group had lower *C. perfringens* loads than the CHC group. Birds fed blended additives (SMP, SMF and SHM) had lower *Bacteroides* spp. compared to the CHC group. *C. perfringens* loads were not different between bird-fed additives and CHC groups but had a numeric reduction in all the additive groups. *Lactobacillus* spp., *Bifidobacteria* spp., *Bacteroides* spp., *Bacillus* spp., *Ruminococcus* spp., Enterobacteriaceae, and total bacteria loads in caecal content were not different between birds fed additives and BAC groups. In addition, *C. perfringens* loads in birds fed additives SMF and SHM were not different compared to the BAC group, whereas additive SMP had significantly higher loads than the BAC group but not different from SMF and SHM groups. There was a tendency for *Lactobacillus* spp. ($P = 0.058$) to be present at higher loads in birds fed additive SMP (9.82) group compared to the UCC (9.31), CHC (9.36) and SHM (9.38) groups.

3.5. Caecal SCFA profiles

The effects of NE challenge and blended additives on caecal SCFA in broilers on d 21 are shown in Table 6. One-way ANOVA analysis indicated that acetate ($P = 0.017$), isobutyrate ($P = 0.019$) and butyrate ($P = 0.002$) concentrations in caecal content showed significant differences. The UCC group had higher acetate and butyrate, and lower isobutyrate concentrations in caecal content compared to the CHC group. Caecal concentrations of birds fed all additives were not different in SCFA compared to the BAC group except for the isobutyrate, wherein birds supplemented with SHM, isobutyrate concentration was higher compared to the birds supplemented with BAC. Birds fed additive SHM had higher concentrations of acetate and butyrate compared to the CHC group but not different from the UCC group. In addition, caecal SCFA concentrations were not different among the additive groups except for the butyrate, where additive SHM had a higher concentration than the additive SMP group. There was a tendency in lactate concentration ($P = 0.076$) in caecal content where the additive SMP group (3.50) had the highest concentration, whereas the UCC group (2.40) had the lowest concentration. However, there was a tendency in propionate ($P = 0.098$) and total SCFA ($P = 0.066$) concentrations in caecal content to being higher in the UCC and additive SHM groups compared to the CHC group.

4. Discussion

Organic acids (OA) and their combinations with proven beneficial impacts on intestinal health have been used as potential alternatives to AGP to control the enteric infection of broilers. However, in commercial settings, the mechanism of OA to ameliorate the impact of the enteric disease on intestinal health is still not very clear. This study evaluated the effects of different combinations of SCFA with MCFA and/or phenolic compounds on intestinal permeability, caecal microflora and SCFA, and the potential of these blended additives to modulate the intestinal

Table 6
Effect of additives and NE challenge on caecal SCFA profiles on d 21 (μmol/g)[1].

Item	UCC	NE challenged[2]					SEM	*P*-value
		CHC	BAC	SMP	SMF	SHM		
Formate	6.12	4.17	3.89	3.67	4.17	6.52	1.22	0.417
Acetate	169^{a}	149^{c}	159^{abc}	155^{bc}	154^{bc}	163^{ab}	4	0.017
Propionate	4.72	3.66	4.27	4.45	3.82	4.59	0.31	0.098
Isobutyrate	1.17^{c}	1.57^{ab}	1.37^{bc}	1.56^{ab}	1.67^{ab}	1.78^{a}	0.13	0.019
Butyrate	51.1^{a}	35.4^{c}	42.8^{bc}	37.8^{c}	39.3^{bc}	46.9^{ab}	2.90	0.002
Isovalerate	1.84	2.61	2.59	2.88	2.01	2.02	0.74	0.897
Valerate	1.27	1.15	1.13	1.24	1.21	1.33	0.10	0.680
Lactate	2.40	2.73	3.05	3.50	3.42	3.07	0.29	0.076
Succinate	33.5	37.9	36.2	37.3	38.5	37.9	5	0.990
Total SCFA	272	238	254	247	248	267	9	0.066

NE = necrotic enteritis; SCFA = short-chain fatty acids.
$^{a-c}$ Values in a row with no common superscripts differ significantly ($P < 0.05$). Mean values are based on 2 birds per replicate and 13 replicates per treatment.
[1] UCC, unchallenged control; CHC, challenged control; BAC, zinc bacitracin; SMP, a blend of short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA) and phenolic compound; SMF, a blend of buffered SCFA with MCFA; SHM, a blend of buffered SCFA with a high concentration of MCFA.
[2] NE challenged birds were gavaged with *Eimeria* spp. at d 9 and *C. perfringens* at d 14.

environment were explored under a sub-clinical NE challenge. Results showed that birds fed blended additives had reduced intestinal permeability, reduced pathogenic *Bacteroides* spp. and numerically lower *C. perfringens* loads and had increased concentrations of caecal acetate and butyrate compared to the CHC group. Birds in additive groups also maintained similar gut status to the BAC group. Moreover, all 3 additives improved the status of intestinal health under the NE challenge condition. These findings support the hypothesis that dietary supplementation with blended SCFA in combination with MCFA and/or phenolic compound improves intestinal permeability and helps to promote intestinal health by altering the intestinal bacterial load and increasing caecal SCFA concentrations of broilers subjected to NE challenge.

In the current study, the birds were challenged with *Eimeria* spp. and *C. perfringens*, and a successful sub-clinical NE challenge was introduced as the typical signs of NE such as impaired FCR, reduced BWG, FI and digestibility, and mild intestinal lesions with low mortality were observed in previous work on this experiment (Kumar et al., 2021). It has been well established that birds infected with NE show reduced BWG, FI and impaired FCR (Timbermont et al., 2011; M'Sadeq et al., 2015; Gharib-Naseri et al., 2019). The effects of NE on the intestinal health of broilers, especially microbiota and bacterial metabolites, have been reported previously (Stanley et al., 2014; Wu et al., 2016; Kheravii et al., 2018). Furthermore, it has been demonstrated that NE infection of birds increases the permeability of the gut, as evidenced by the increased concentration of FITC-d in the bloodstream (Latorre et al., 2018). The results obtained in the present study showed that birds in the CHC group had increased intestinal permeability, reduced caecal SCFA concentrations and altered bacterial populations including increased *C. perfringens*. This further confirms the introduction of a successful NE challenge in addition to the performance and lesion results that have been reported previously (Kumar et al., 2021).

The results obtained in the present study indicate that the blended SCFA in combination with MCFA has improved intestinal health under a NE challenge condition. The study observed reduced pathogenic *Bacteroides* spp. and numerically reduced *C. perfringens* in birds fed additives SMP, SMF, and SHM compared to the challenged birds without additives or BAC suggesting the bactericidal and bacteriostatic effects of the blended additives on the gut against pathogens. Similarly, other studies have shown that diets supplemented with MCFA exhibited antibacterial effects against both Gram-positive and Gram-negative bacteria (Zentek et al., 2011; Onrust et al., 2018). Several studies reported the antibacterial effects of OA against *Clostridia* (Timbermont et al., 2010; McKnight et al., 2020; Stefanello et al., 2020) and *Salmonella* (Van Immerseel et al., 2004b; Aljumaah et al., 2020). The current study showed improved intestinal integrity in additive groups compared to the CHC group that is in accordance with the boosted immunity responses as observed earlier (Kumar et al., 2021) and the reduced load of pathogenic bacteria overserved in this study. It has been shown that diets supplemented with OA are protecting the epithelial cells from disruption via reduced production of toxic substances in the intestine, thus reduced mucosal permeability in the intestine (Adil et al., 2010; Stefanello et al., 2020). Birds fed blended additives had similar caecal SCFA concentrations to the antibiotic group. However, birds fed additive SHM had higher concentrations of acetate, butyrate and tended to have higher concentrations of propionate, lactate, and total SCFA compared to the CHC group. A similar observation was reported by Aljumaah et al. (2020), where birds fed a diet supplemented with OA blend had higher intestinal isobutyric, butyric, and acetic acid concentrations compared to the control diet under the *Salmonella* challenged condition. Altogether, the data of the current study suggest positive effects of blended additives on intestinal health contributed to the improved FCR, digestibility, immune response, and footpad health as reported earlier (Kumar et al., 2021).

The healthy intestinal mucosa is essential for optimum digestion and absorption of nutrients and acts as a barrier against pathogenic bacterial infection (Balda and Matter, 2008; Sánchez de Medina et al., 2014). Studies have reported that enteric inflammation damages intestinal mucosa and tight junctions, thus increasing intestinal permeability (Vicuña et al., 2015; Barekatain et al., 2019). In the current study, NE challenged birds had higher serum FITC-d concentration compared to the UCC group on d 16, indicating compromised gut integrity by NE. The reduced serum FITC-d concentrations in birds fed blended additives demonstrate the beneficial effects of a blend of SCFA with MCFA and/or phenolic compound on intestinal integrity of the birds challenged with *E. spp.* and *C. perfringens*. Similarly, a recent study reported that the birds fed OA in combination with essential oils had lower serum FITC-d with upregulated tight junction proteins of the birds with NE (Stefanello et al., 2020).

It is widely accepted that the microbiota composition of birds can be dramatically impacted by enteric infection (Wu et al., 2014; Clavijo and Florez, 2018; Gharib-Naseri et al., 2019). In turn, intestinal microbiota can largely affect the productive performance and health of the birds. This study has shown that birds infected with NE led to increased *Bacteroides* spp., Enterobacteriaceae and *C. perfringens* loads, and reduced *Ruminococcus* spp. load. Similar changes have been reported previously where birds infected with NE had higher *C. perfringens* and *Bacteroides* spp., and lower *Ruminococcus* spp. in the caeca (Gharib-Naseri et al., 2021). Further, it has been shown that increased proliferation of *Clostridia* spp. and *Bacteroides* spp. can enhance enteric diseases (Bondarenko et al., 2003; Loy, 2005). *Bacteroides* spp. is commensal intestinal flora in birds that can emerge to be pathogenic to the birds (Wexler, 2007) and can be increased in the impaired intestine when the birds are infected with pathogens (Phong et al., 2010). It is likely that the higher load of *Bacteroides* spp. is due to the *Eimeria* colonisation and/or increased *C. perfringens* in the birds under challenge in the current study. These bacteria have shown proteolytic and excessive immunostimulatory activities that affect the immune response and disrupt the intestinal health of the hosts (Wells et al., 1996; Kleessen et al., 2002; Wexler, 2007). Therefore, a reduced load of *Bacteroides* spp. by blended additives application revealed the beneficial effects of blended SCFA in combination with MCFA and/or phenolic compound against pathogenic bacteria under the condition in the current study.

It is well known that birds cannot completely metabolise fibrous carbohydrates in the feed (Bedford, 2002; Choct, 2002). The intestinal microbiota plays an important role to ferment undigested carbohydrates throughout the intestinal tract, primarily in the caeca due to the high density and diversity of the bacterial populations. Such fermentation produces SCFA including acetate, propionate, and butyrate (Dunkley et al., 2007). Reports have shown that SCFA i.e. acetate, propionate and butyrate, can promote intestinal morphology, tight junction, and immunity status of birds (Wang et al., 2012; Corrêa-Oliveira et al., 2016). They can also be used as an energy source, and lead to an increased absorption surface in the intestine via the improved proliferation of epithelial cells (Dibner and Richards, 2005; Hijova and Chmelarova, 2007). Thus, the concentration of SCFA in the caeca might be used as an intestinal health indicator which signals enriched beneficial bacteria in the caeca and acts as a contributor of energy for improved bird performance (Rehman et al., 2007). In the current study, the increased concentrations of acetate and butyrate in the SHM fed birds and the tendency of increased propionate, lactate and total SCFA in all additive groups revealed the beneficial effects of blended SCFA in combination with MCFA on intestinal homeostasis.

Such effects have also been reported previously that the OA blend supplementation in diets increased acetate and butyrate concentration in the caeca (Aljumaah et al., 2020). It can be speculated that the positive impacts of the fatty acid treatments might be realised, at least partially, by the increased concentrations of SCFA in the caeca. In fact, previous studies stated that OA in diets improved BWG, FCR and health of broilers (Adil et al., 2010), and butyric acid supplemented to the diets ameliorate the impacts of NE (Timbermont et al., 2010) and *Salmonella* infection (Van Immerseel et al., 2005), and enhanced intestinal barrier function (Wang et al., 2012) in broilers.

5. Conclusions

Dietary supplementation of different blends of OA and/or phenolic compounds can alleviate the detrimental impact of NE challenge on intestinal health through improved intestinal barrier function, intestinal microbiota, and production of volatile SCFA. Altogether, these findings illustrate the potential of OA blends to ameliorate the negative impact of NE when in-feed AGP is restricted to be used in poultry production.

Author contributions

Alip Kumar: Animal trial, Feed formulation, Laboratory analysis, Statistical analysis, Study design and Writing; **Mehdi Toghyani:** Study design, Feed formulation, Animal trial, Data evaluation, Manuscript review; **Sarbast K. Kheravii:** Data evaluation, Manuscript review; **Lane Pineda:** Methodology, Data evaluation, Manuscript review; **Yanming Han:** Methodology, Data evaluation, Manuscript review; **Robert A. Swick:** Study design, Data evaluation, Manuscript review; **Shu-Biao Wu:** Coordinator, Study Design, Data collection, Statistical analyses, Critical manuscript review.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgments

The study was funded by Trouw Nutrition, a Nutreco company, The Netherlands. The authors thank Shuyu Song and Jonathon Clay for their technical assistance. The authors thank Ms. Petrina Young for providing *Eimeria* spp. oocysts and Prof. Robert Moore for providing *Clostridium perfringens* EHE-18.

References

Adhikari P, Kiess A, Adhikari R, Jha R. An approach to alternative strategies to control avian coccidiosis and necrotic enteritis. J Appl Poultry Res 2020;29:515–34.
Adil S, Banday T, Bhat GA, Mir MS, Rehman M. Effect of dietary supplementation of organic acids on performance, intestinal histomorphology, and serum biochemistry of broiler chicken. Vet Med Int 2010:479485. https://doi.org/10.4061/2010/479485.
Aljumaah MR, Alkhulaifi MM, Abudabos AM, Alabdullatifb A, El-Mubarak AH, Al Suliman AR, et al. Organic acid blend supplementation increases butyrate and acetate production in *Salmonella* Enterica serovar *typhimurium* challenged broilers. PLoS One 2020;15:e0232831.
Aviagen. Ross broiler management handbook. Newbridge, Scotland, UK: Aviagen Ltd.; 2014.
Awad WA, Hess C, Hess M. Enteric pathogens and their toxin-induced disruption of the intestinal barrier through alteration of tight junctions in chickens. Toxins 2017;9:60.
Balda MS, Matter K. Tight junctions at a glance. J Cell Sci 2008;121:3677–82.
Barekatain R, Nattrass G, Tilbrook AJ, Chousalkar K, Gilani S. Reduced protein diet and amino acid concentration alter intestinal barrier function and performance of broiler chickens with or without synthetic glucocorticoid. Poultry Sci 2019;98:3662–75.
Bartosch S, Fite A, Macfarlane GT, McMurdo ME. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol 2004;70:3575–81.
Bedford M. The role of carbohydrases in feedstuff digestion. In: McNab JM, Boorman KN, editors. Poultry feedstuffs – supply, composition and nutritive value. England: CABI Publ.; 2002. p. 319–36.
Bondarenko V, Chervinets V, Vorob'ev A. Role of persisting opportunistic bacteria in the pathogenesis of the gastric and duodenum ulcer. Zh Mikrobiol Epidemiol Immunobiol 2003:11–7.
Choct M. Non-starch polysaccharides. In: McNab JM, Boorman KN, editors. Poultry feedstuffs – supply, composition and nutritive value. England: CABI Publ.; 2002. p. 221–35.
Clavijo V, Florez MJV. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: a review. Poultry Sci 2018;97:1006–21.
Corrêa-Oliveira R, Fachi JL, Vieira A, Sato FT, Vinolo MAR. Regulation of immune cell function by short-chain fatty acids. Clin Transl Immunology 2016;5:e73.
Dibner J, Richards J. Antibiotic growth promoters in agriculture: history and mode of action. Poultry Sci 2005;84:634–43.
Dittoe DK, Ricke SC, Kiess AS. Organic acids and potential for modifying the avian gastrointestinal tract and reducing pathogens and disease. Front Vet Sci 2018;5:216.
Dunkley K, Dunkley C, Njongmeta N, Callaway T, Hume M, Kubena L, et al. Comparison of in vitro fermentation and molecular microbial profiles of high-fiber feed substrates incubated with chicken cecal inocula. Poultry Sci 2007;86:801–10.
Gharib-Naseri K, Kheravii S, Keerqin C, Morgan N, Swick R, Choct M, et al. Two different *Clostridium perfringens* strains produce different levels of necrotic enteritis in broiler chickens. Poultry Sci 2019;98:6422–32.
Gharib-Naseri K, Dorigam JC, Doranalli K, Morgan N, Swick RA, Choct M, et al. *Bacillus amyloliquefaciens* CECT 5940 improves performance and gut function in broilers fed different levels of protein and/or under necrotic enteritis challenge. Anim Nutr 2021;7:185–97.
Guide SUs. Statistic. Cary, NC, USA: Sas institute. Inc; 2010., version 9.3.
Hijova E, Chmelarova A. Short chain fatty acids and colonic health. Bratisl Lek Listy 2007;108:354.
Jensen M, Cox R, Jensen BB. Microbial production of skatole in the hind gut of pigs given different diets and its relation to skatole deposition in backfat. Anim Sci 1995;61:293–304.
Kaldhusdal M, Benestad SL, Løvland A. Epidemiologic aspects of necrotic enteritis in broiler chickens–disease occurrence and production performance. Avian Pathol 2016;45:271–4.
Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, Parker D, et al. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathog 2008;4:e26.
Khan SH, Iqbal J. Recent advances in the role of organic acids in poultry nutrition. J Appl Anim Res 2016;44:359–69.
Kheravii SK, Swick RA, Choct M, Wu S-B. Coarse particle inclusion and lignocellulose-rich fiber addition in feed benefit performance and health of broiler chickens. Poultry Sci 2017;96:3272–81.
Kheravii SK, Swick RA, Choct M, Wu S-B. Effect of oat hulls as a free choice feeding on broiler performance, short-chain fatty acids and microflora under a mild necrotic enteritis challenge. Anim Nutr 2018;4:65–72.
Kleessen B, Kroesen A, Buhr H, Blaut M. Mucosal and invading bacteria in patients with inflammatory bowel disease compared with controls. Scand J Gastroenterol 2002;37:1034–41.
Kumar A, Toghyani M, Kheravii SK, Pineda L, Han Y, Swick RA, et al. Potential of blended organic acids to improve performance and health of broilers infected with necrotic enteritis. Anim Nutr 2021;7(2):440–9. https://doi.org/10.1016/j.aninu.2020.11.006.
Latorre JD, Adhikari B, Park SH, Teague KD, Graham LE, Mahaffey BD, et al. Evaluation of the epithelial barrier function and ileal microbiome in an established necrotic enteritis challenge model in broiler chickens. Front Vet Sci 2018;5:199.
Layton A, McKay L, Williams D, Garrett V, Gentry R, Sayler G. Development of *Bacteroides* 16S rRNA gene taqman-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl Environ Microbiol 2006;72:4214–24.
Lee D-H, Zo Y-G, Kim S-J. Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. Appl Environ Microbiol 1996;62:3112–20.
Loy C. Antibiotic-associated diarrhoea: an overlooked aetiology? Br J Biomed Sci 2005;62:166–9.
McKnight L, Page G, Han Y. Effect of replacing in-feed antibiotics with synergistic organic acids, with or without trace minerals and/or water acidification, on growth performance and health of broiler chickens under a *Clostridium perfringens* type A challenge. Avian Dis 2020;64(3):374–8.
M'Sadeq SA, Wu S-B, Swick RA, Choct M. Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide. Anim Nutr 2015;1:1–11.

Onrust L, Van Driessche K, Ducatelle R, Schwarzer K, Haesebrouck F, Van Immerseel F. Valeric acid glyceride esters in feed promote broiler performance and reduce the incidence of necrotic enteritis. Poultry Sci 2018;97:2303–11.
Palliyeguru MWCD, Rose SP. Sub-clinical necrotic enteritis: its aetiology and predisposing factors in commercial broiler production. World Poult Sci J 2019;70:803–16.
Papatsiros V, Katsoulos P-D, Koutoulis K, Karatzia M, Dedousi A, Christodoulopoulos G. Alternatives to antibiotics for farm animals. CAB Rev Ag Vet Sci Nutr Res 2013;8:1–15.
Patten J, Waldroup P. Use of organic acids in broiler diets. Poultry Sci 1988;67:1178–82.
Phong S, Shanmugavelu S, Thayalini K, Noraini S, Wong H. Detection of *Lactobacillus, Bacteroides* and *Clostridium perfringens* in the gastrointestinal contents of chicken fed different diets by real-time PCR. J Trop Agric Food Sci 2010;38:81–7.
Polycarpo GV, Andretta I, Kipper M, Cruz-Polycarpo VC, Dadalt JC, Rodrigues PHM, et al. Meta-analytic study of organic acids as an alternative performance-enhancing feed additive to antibiotics for broiler chickens. Poultry Sci 2017;96:3645–53.
Prescott JF, Smyth JA, Shojadoost B, Vince A. Experimental reproduction of necrotic enteritis in chickens: a review. Avian Pathol 2016;45:317–22.
Ramirez-Farias C, Slezak K, Fuller Z, Duncan A, Holtrop G, Louis P. Effect of inulin on the human gut microbiota: stimulation of *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii*. Br J Nutr 2008;101:541–50.
Rehman HU, Vahjen W, Awad WA, Zentek J. Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. Arch Anim Nutr 2007;61:319–35.
Requena T, Burton J, Matsuki T, Munro K, Simon MA, Tanaka R, et al. Identification, detection, and enumeration of human *Bifidobacterium species* by PCR targeting the transaldolase gene. Appl Environ Microbiol 2002;68:2420–7.
Rinttilä T, Kassinen A, Malinen E, Krogius L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 2004;97:1166–77.
Rodgers NJ, Swick RA, Geier MS, Moore RJ, Choct M, Wu S-B. A multifactorial analysis of the extent to which *Eimeria* and fishmeal predispose broiler chickens to necrotic enteritis. Avian Dis 2015;59:38–45.
Sánchez de Medina F, Romero-Calvo I, Mascaraque C, Martínez-Augustin O. Intestinal inflammation and mucosal barrier function. Inflamm Bowel Dis 2014;20:2394–404.
Seal BS, Lillehoj HS, Donovan DM, Gay CG. Alternatives to antibiotics: a symposium on the challenges and solutions for animal production. Anim Health Res Rev 2013;14:78–87.
Stanley D, Wu S-B, Rodgers N, Swick RA, Moore RJ. Differential responses of cecal microbiota to fishmeal, *Eimeria* and *Clostridium perfringens* in a necrotic enteritis challenge model in chickens. PLoS One 2014;9:e104739.
Stefanello C, Rosa DP, Dalmoro YK, Segatto AL, Vieira MS, Moraes ML, et al. Protected blend of organic acids and essential oils improves growth performance, nutrient digestibility, and intestinal health of broiler chickens undergoing an intestinal challenge. Front Vet Sci 2020;6:491.
Timbermont L, Lanckriet A, Dewulf J, Nollet N, Schwarzer K, Haesebrouck F, et al. Control of *Clostridium perfringens*-induced necrotic enteritis in broilers by target-released butyric acid, fatty acids and essential oils. Avian Pathol 2010;39:117–21.
Sun Y, Han Y, Chen J, Ni A, Jiang Y, Li Y, et al. Effect of replacing in-feed antibiotics with synergistic organic acids on growth performance, health, carcass, and immune and oxidative status of broiler chickens under *Clostridium perfringens* type A challenge. Avian Dis 2020;64:393–400.
Timbermont L, Haesebrouck F, Ducatelle R, Van Immerseel F. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Pathol 2011;40:341–7.
Van Immerseel F, De Buck J, Boyen F, Bohez L, Pasmans F, Volf J, et al. Medium-chain fatty acids decrease colonization and invasion through hila suppression shortly after infection of chickens with *Salmonella* Enterica serovar *enteritidis*. Appl Environ Microbiol 2004a;70:3582–7.
Van Immerseel F, Fievez V, De Buck J, Pasmans F, Martel A, Haesebrouck F, et al. Microencapsulated short-chain fatty acids in feed modify colonization and invasion early after infection with *Salmonella enteritidis* in young chickens. Poultry Sci 2004b;83:69–74.
Van Immerseel F, Boyen F, Gantois I, Timbermont L, Bohez L, Pasmans F, et al. Supplementation of coated butyric acid in the feed reduces colonization and shedding of *Salmonella* in poultry. Poultry Sci 2005;84:1851–6.
Van Immerseel F, Russell J, Flythe M, Gantois I, Timbermont L, Pasmans F, et al. The use of organic acids to combat *Salmonella* in poultry: a mechanistic explanation of the efficacy. Avian Pathol 2006;35:182–8.
Vicuña E, Kuttappan V, Tellez G, Hernandez-Velasco X, Seeber-Galarza R, Latorre J, et al. Dose titration of FITC-d for optimal measurement of enteric inflammation in broiler chicks. Poultry Sci 2015;94:1353–9.
Wade B, Keyburn A. The true cost of necrotic enteritis. Poultry World 2015;31:16–7.
Wang H-B, Wang P-Y, Wang X, Wan Y-L, Liu Y-C. Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein *Claudin-1* transcription. Dig Dis Sci 2012;57:3126–35.
Wells CL, Van de Westerlo E, Jechorek RP, Feltis B, Wilkins T, Erlandsen S. *Bacteroides fragilis* enterotoxin modulates epithelial permeability and bacterial internalization by HT-29 enterocytes. Gastroenterology 1996;110:1429–37.
Wexler HM. *Bacteroides*: the good, the bad, and the nitty-gritty. Clin Microbiol Rev 2007;20:593–621.
Wise MG, Siragusa GR. Quantitative detection of *Clostridium perfringens* in the broiler fowl gastrointestinal tract by real-time PCR. Appl Environ Microbiol 2005;71:3911–6.
Wise MG, Siragusa GR. Quantitative analysis of the intestinal bacterial community in one- to three-week-old commercially reared broiler chickens fed conventional or antibiotic-free vegetable-based diets. J Appl Microbiol 2007;102:1138–49.
Wu S-B, Rodgers N, Choct M. Optimized necrotic enteritis model producing clinical and subclinical infection of *Clostridium perfringens* in broiler chickens. Avian Dis 2010;54:1058–65.
Wu S-B, Stanley D, Rodgers N, Swick RA, Moore RJ. Two necrotic enteritis predisposing factors, dietary fishmeal and *Eimeria* infection, induce large changes in the caecal microbiota of broiler chickens. Vet Microbiol 2014;169:188–97.
Wu S-B, Rodgers NJ, Cui G, Sun Y, Choct M. Dynamics of intestinal metabolites and morphology in response to necrotic enteritis challenge in broiler chickens. Avian Pathol 2016;45:346–56.
Zentek J, Buchheit-Renko S, Ferrara F, Vahjen W, Van Kessel A, Pieper R. Nutritional and physiological role of medium-chain triglycerides and medium-chain fatty acids in piglets. Anim Health Res Rev 2011;12:83.
Zhang Y, Chen D, Yu B, He J, Yu J, Mao X, et al. Spray-dried chicken plasma improves intestinal digestive function and regulates intestinal selected microflora in weaning piglets. J Anim Sci 2015;93:2967–76.