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A cross-study analysis of the effect of a dual-strain probiotic applied via the waterline on the growth performance and gut health of broilers under a mild necrotic enteritis challenge

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ABSTRACT

Probiotics offer potential as an approach for the prevention and control of poultry intestinal diseases, but external factors can influence the birds' response. Combining data from multiple trials provides greater confidence around efficacy under varying production conditions. Therefore, this study combined data from three separate trials analyzing the effect of a dual-strain probiotic comprising *Lactobacillus acidophilus* AG01 and *Bifidobacterium animalis* subspecies *lactis* AG02 on broilers during a mild necrotic enteritis (**NE**) challenge. In each, 1,440 broilers were assigned to floor-pens (40 birds/pen, 12 pens/treatment) in a completely randomized design. Treatments in each trial were a non-challenged control (**C**); challenged control (10 x dose of *Eimeria*/bird on d 14 and 1.0 x ~10^9 colony forming units (**CFU**)/bird of *C. perfringens* on d 16-20; **CC**); and CC supplemented daily via the waterline with 1 × 10^9 CFU/bird of probiotic (**CC+Probiotic**). Birds were fed corn-soybean mealbased diets by phase (starter: 0 to 14, grower: 15 to 28, finisher: 29 to 42 d of age) *ad libitum*. Growth performance was monitored over 42 d, NE lesion scoring performed on d 21 and 28 in all trials, and, in Trial 3 only, cecal microbiota composition was analyzed on d 28. From d 1 to 42, CC birds exhibited reduced BW, BW gain (**BWG**), and feed intake (**FI**) (-9.2 %, -9.5 %, -5.0 %, respectively; $P < 0.05$), increased FCR and mortality (+8.5 points and 1.3 % points, respectively; $P < 0.05$) compared to C, and increased NE induction on d 28 (67.8 vs. 9.4 %, P <0.05). Compared to CC, CC+Probiotic birds exhibited increased BW, BWG and FI (d 42: +6.9 %, +7.1 %, +4.0 %; $P < 0.05$) and reduced FCR, mortality and d 28 NE lesion scores (-0.5 points, -1.4 % points and -57.1 %, respectively; $P < 0.05$). The composition of the cecal microbiota of CC+Probiotic birds at 28 d of age exhibited higher abundance of butyrogenic bacterial genera in Trial 3, which may have contributed to the beneficial effects of the probiotic. The results demonstrate that the probiotic ameliorated the negative effects of a mild NE-challenge on growth performance and intestinal symptoms over three trials incorporating variation in season and bird breed.

Introduction

Necrotic enteritis (**NE**) is a major disease affecting commercial poultry flocks. In 2015, clinical NE was estimated to cause up to 6 billion USD/year in financial losses, equivalent to 0.0625 USD per bird (Wade and Keyburn, 2015). Subclinical NE, in which bird health and performance may be subtly impaired but objective intestinal symptoms are not always evident, may contribute an additional 370 to 740 USD/flock (Skinner et al., 2010). *Clostridium perfringens* is the causative agent of NE but is not pathogenic until one or more predisposing factors are present. The most important of these are *Eimeria* infection (Nicholds et al., 2021), changes in diet composition (for example a high fishmeal diet; Huang et al., 2018) and environmental factors such as heat stress (Tsiouris et al., 2018). These can lead to intestinal damage or microbial imbalance within the gut (dysbiosis) which makes birds more vulnerable to colonization by *C. perfringens* and its subsequent pathogenic effects. The severity of disease (and whether it manifests clinical or subclinical) depends on a variety of factors, including *C. perfringens* serotype and associated toxins produced as well as the existing health status of birds, environmental stress factors and other influencing factors.

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Antibiotic growth promoters (**AGP**) are effective in controlling *C. perfringens* abundance and pathogenicity but are no longer used in many countries. They have been largely phased out in response to consumer preference and concerns over overuse and antimicrobial resistance. The high genetic diversity of *C. perfringens* strains and rise in antimicrobial resistance among *C. perfringens* isolates has been identified as a particular concern (Bendary et al., 2022). Natural alternatives to AGPs for the control of *C. perfringens* include prebiotics, probiotics, postbiotics, plant extracts, and essential oils (Abd El-Ghany et al., 2022; Diaz Carrasco et al., 2016; Eid et al., 2020; Gomez-Osorio et al., 2021). Among these, probiotics (also known as direct-fed microbials – 'live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host'; Hill et al., 2014) have been shown to be effective in reducing *C. perfringens* ileal and cecal colonization, pathogenic symptoms (reduced occurrence of NE lesions and improved mucosal barrier integrity) and improving the composition of the gut microbiome, leading to improved growth performance (Kulkarni et al., 2022). Several mechanisms have been implicated in these beneficial effects that are not mutually exclusive. These include competitive exclusion, the production of antimicrobial compounds, enhancement of the host immune response, improvement in intestinal epithelial barrier integrity, and modulation of the intestinal microbiota that enhances immunity against intestinal inflammatory diseases such as NE (Grozina et al., 2023; Kulkarni et al., 2022; Sun and Jia, 2018).

A dual-strain probiotic comprising *Lactobacillus acidophilus* AG01 and *Bifidobacterium animalis* subspecies *lactis* AG02 has recently been shown to be effective *in vitro* in reducing NE-related pathogenic effects in broilers (Kadekar et al., 2024; van der Klein et al., 2023). It is also effective in ameliorating the negative effects of pathogen challenge (*E. coli* and *C. perfringens*) *in vivo* (van der Klein et al., 2024). The *in vitro* investigation implicated secretions present in cell-free supernatant (**CFS**) prepared from *B. animalis* subspecies *lactis* AG02 in reducing *C. perfringens* cell adhesion to host cells and ameliorating the negative effects of *C. perfringens* on host cell permeability, whereas CFS from both probiotics was shown to reduce *C. perfringens* cytotoxic effects (Kadekar et al., 2024). To establish the repeatability of beneficial effects from this dual-strain probiotic and its overall efficacy in broilers in production practice where responses will vary with time and rearing conditions, it is necessary to conduct multiple studies under varying conditions. In this study we analyzed the combined data from three separate trials. All employed the same NE challenge model and probiotic dosing regimen but were conducted at different times and varied in the commercial broiler breed used. The aim was to determine if the probiotic could ameliorate the negative effects of a mild NE challenge on growth performance and intestinal symptoms. A secondary aim was to assess the effect of the probiotic on the taxonomic composition of the cecal microflora as a potential mechanism via which its beneficial effects are mediated.

Materials and methods

All three studies were carried out at the research facilities of AH Pharma Inc. in Hebron, Maryland, USA. Trial 1 was carried out during August to October 2021, Trial 2 during November to December 2022 and Trial 3 during March to May 2023. The experimental procedures and protocols of all three trials were reviewed and approved by the Animal Ethics Committee of AH Pharma Inc. prior to the commencement of research.

Birds, Housing and Experimental design

Each trial used 1,440 male chicks obtained on day-of-hatch from a commercial hatchery. Trials 1 and 3 used Ross 308 birds whereas Trial 2 used Cobb 500 birds. All birds were vaccinated for infectious bursal disease, Marek's disease, Newcastle disease and Massachusetts type infectious bronchitis at the hatchery. In all trials, chicks were assigned to floor-pens with 40 birds per pen and 12 pens per treatment. Treatments were randomized per waterline dosing system, each system supplied 12 pens in the length of the house. Re-used litter topped with fresh wood shavings was used as bedding. Pens were located in animal houses in which the temperature was maintained initially at 30 °C, reduced to 28 °C at 7 d of age and thereafter reduced by 1 °C per day until 20 °C was reached. The lighting regime was LD 23:1 h until 7 d of age and thereafter LD 6:20 h, following EU welfare standards laid down in Council Directive 2007/43/EC (European Council, 2007).

Treatments

Treatments in each trial comprised a non-challenged control (**C**), a challenged control (**CC**) and the CC supplemented with the dual strain probiotic via the waterline every day during 0 to 42 d of age (**CC+Probiotic**). The NE challenge comprised a 2-step oral challenge: 1) At 14 d of age birds were given a mixed culture of sporulated oocysts of *Eimeria* (*E. tenella, E. maxima* and *E. acervulina*) administered via a 10 x dose (equal to 0.1 ml per bird) of B52 COCCIVAC (Merck Animal Health) to cause tissue damage and predispose birds to NE; 2) At each of 16, 17, 18, 19, and 20 d of age, birds received 1.0 x ~10^9 CFU of *C. perfringens* (NetB+) in 1.0 ml of thioglycolate broth, administered by oral gavage. Birds in the CC treatment were inoculated at 14 d of age with 0.1 ml of sterile phosphate buffered saline containing no *Eimeria* oocysts and at 18, 19 and 20 d of age with 1.0 ml of sterile thioglycolate broth containing no *C. perfringens*, as a control.

The dual strain probiotic comprised a 50:50 blend of *L. acidophilus* AG01 and *B. animalis* subspecies *lactis* AG02 and was obtained in sachets from Danisco Animal Nutrition & Health (IFF), Oegstgeest, The Netherlands. Each sachet contained enough probiotic to supply a total dose of 1.0 × 10^8 CFU/bird once reconstituted in water. Sachets were stored at 4 °C until use and reconstituted in 1,000 ml chlorine-free water on the morning of use to produce a stock solution. This stock solution was further diluted with chlorine free water according to bird age as detailed in Table 1, so that older birds would receive the same dose of probiotic as younger birds but in a larger volume of water proportionate to their greater drinking capacity. The diluted probiotic solutions were administered to birds in pens via an automated waterline dosing system. The application occurred every morning and the dosing container was checked at the end of each day to confirm it was fully emptied.

Diets

In all trials, diets were formulated in three phases (1 to 14 d of age, starter; 15 to 28 d of age, grower; 29 to 42 d of age, finisher). The ingredient and calculated nutrient composition of the diets is presented in Table 2. Birds in Trial 1 were fed a corn-soybean meal-based diet,

Table 1
Reconstitution and dilution protocol for the dual-strain probiotic prior to its administration to birds via an automated waterline dosing system.

Bird age, d	Water added per sachet to create stock solution, ml	Volume of stock solution added to the dosing system, ml	Volume of chlorine-free water added to the dosing system, ml	Total volume of the dosing system, ml
0 to 6	1,000	15	0	15
7 to 13	1,000	15	35	50
14 to 20	1,000	15	85	100
21 to 27	1,000	15	135	150
28 to 34	1,000	15	185	200
35 to 42	1,000	15	235	250

Table 2
Ingredient and calculated nutrient composition of the trial diets, by phase.

	Trial 1			Trial 2			Trial 3		
	Starter (1 to 10 d of age)	Grower (11 to 21 d of age)	Finisher (22 to 42 d of age)	Starter (1 to 10 d of age)	Grower (11 to 21 d of age)	Finisher(22 to 42 d of age)	Starter (1 to 10 d of age)	Grower (11 to 21 d of age)	Finisher (22 to 42 d of age)
Ingredients, g/kg as fed, unless otherwise stated									
Corn	568	633	660	432	466	481	432	466	481
Soybean meal (48 %)	368	314	290	341	303	279	341	303	279
Wheat	-	-	-	150	150	150	150	150	150
Soybean hull	-	7.9	-	-	-	-	-	-	-
Soybean oil	0.5	0.5	6.8	34	41	50	34	41	50
Limestone	30.8	13.0	12.2	12.8	12.1	11.2	12.8	12.1	11.2
Dicalcium phosphate	15.3	14.3	13.6	13.5	12.3	11.8	13.5	12.3	11.8
Salt	6.4	6.0	5.9	3.98	2.71	3.17	3.98	2.71	3.17
L-Lysine HCL	4.5	4.0	3.8	2.11	2.91	3.25	2.11	2.91	3.25
DL-methionine	1.0	2.4	2.8	5.94	5.54	5.38	5.94	5.54	5.38
Vitamin-mineral premix[1]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Phytase FTU/kg[2]	750	750	750	750	750	750	750	750	750
Calculated nutrients, %, unless otherwise stated									
Crude protein	22	20	19	21.4	19.9	18.9	21.4	19.9	18.9
Metabolizable energy, kcal/g	3,086	3,142	3,197	3,029	3,095	3,161	3,029	3,095	3,161
Calcium	1.56	0.85	0.80	0.70	0.65	0.60	0.70	0.65	0.60
Total phosphorus	0.79	0.74	0.71	0.75	0.71	0.68	0.75	0.71	0.68
Available phosphorus	0.45	0.42	0.40	0.45	0.42	0.40	0.45	0.42	0.40
Total lysine	1.37	1.32	1.29	1.28	1.24	1.20	1.28	1.24	1.20
Digestible lysine	1.20	1.17	1.14	1.16	1.14	0.11	1.16	1.14	0.11
Methionine + cysteine	1.25	1.16	1.12	1.25	1.16	1.17	1.25	1.16	1.17
Digestible methionine + cysteine	0.88	0.82	0.79	0.83	0.77	0.75	0.83	0.77	0.75

[1] Supplied per kilogram of diet: 35,280 MIU vitamin A, 13,230 MIU vitamin D3, 276 mg 25-hydroxyvitamin D3, 221 MIU vitamin E, 5.8 g vitamin K3, 8.8 g thiamin, 31 g riboflavin, 176 g niacin, 49 g pantothenic acid, 14 g pyridoxine, 0.8 g biotin, 3.5 g folic acid, 53 mg vitamin B12, 0.105 g manganese, 0.105 g zinc, 0.138 iron, 13.95 mg copper, 0.3 mg selenium, 1.5 mg iodine.
[2] A novel consensus bacterial 6-phytase variant produced in *Trichoderma reesei* (Danisco Animal Nutrition & Health, IFF).
FTU, phytase units.

whereas those in Trial 2 and 3 received a corn-wheat soybean meal-based diet. Diets were formulated to meet the broilers' nutrient requirement. The phytase used was a novel consensus bacterial 6-phytase variant produced in *Trichoderma reesei* (Danisco Nutrition & Health (IFF), Oegstgeest, The Netherlands). All final diets were pelleted and provided *ad libitum*.

Measurements and Sampling

In all trials, birds were weighed at d 0, 14, 21, 38, 35 and 42. Weights were measured manually on a per pen basis in the same order every weigh day. Pens were observed daily for any dead birds and dead birds were removed and weighed. Body weight gain (**BWG**) was calculated per phase. Feed disappearance was measured on each of d 0, 14, 21, 28, 35 and 42 and used to calculate feed intake (**FI**). Feed conversion ratio (**FCR**) was calculated from measurements of BWG and FI. All measures were corrected for mortality.

At 21 and 28 d of age, 5 birds per pen were euthanized by cervical dislocation, eviscerated, and NE lesion scoring performed on the entire length of the intestine. Birds were assigned an NE lesion score of between 0 (least severe) and 4 (most severe) based on the following scoring criteria: 0) Normal: no NE lesions, small intestine has normal elasticity, 1) Mild: small intestinal wall is thin and flaccid, excess mucus covering mucus membrane, 2) Moderate: noticeable reddening and swelling of the intestinal wall, minor ulceration and necrosis of the intestine membrane, excess mucus, 3) Severe: extensive areas of necrosis and ulceration of the small intestinal membrane, significant hemorrhage, layer of fibrin and necrotic debris on the mucus membrane, 4) Dead or moribund: bird that would likely die within 24 h and has an NE lesion score of 2 or more, or birds that died due to NE prior to NE lesion scoring.

In Trial 3 only, on d 28, 2 bird per pen from 12 pens per treatment was selected at random, euthanized by cervical dislocation, eviscerated and the intestine tied off with string at the proventricular-duodenal junction and terminal end of the rectum. The excess was cut off and the occluded gut sections placed in an open Whirl-Pak bag containing 20 ml of sterile saline to keep the intestinal tract moist. The sealed bags were transported on ice to the laboratory and then frozen at -20 °C until later analysis. Cecal content swabs were taken from the thawed gut sections and analyzed as described below.

DNA extraction, 16S rRNA Gene amplification from Cecal Swab samples

All reagents and materials were purchased from Thermo Fisher Scientific, Waltham, MA, unless otherwise stated. Microbial genomic DNA was extracted from cecal swab samples (Trial 3 only) using the Qiagen MagAttract PowerSoil DNA Kit. Extractions were performed using the automated Thermo KingFisher Flex instrument. Primers 515F (5′-GTGCCAGCMGCCGCGGTRA-3′) and 806R (5′- GGACTACHVGGGTWTCTAAK-3′) were used to amplify the V4 region of the 16S rRNA gene. Purified metagenomic DNA was processed as follows prior to microbial community sequencing: 2 µl of metagenomic DNA was added to a PCR reaction along with 25 µl of ABI Universal TaqMan Reaction mix without UNG, 0.1 µl (100 µM) of each PCR primer, and 24.8 µl of Molecular Biology Grade water, to give a total volume of 52 µl. The PCR thermocycling parameters were as follows; 10 min at 95°C followed by 35 cycles of 15 s at 95°C, 30 s at 55°C and 2 min at 72°C. Amplified reactions were purified using Ampure XP Magnetic Beads (Beckman Coulter (Brea, CA) A63881) in an Agilent Bravo Automated Robotic Workstation. A 2 µl sample of each amplicon was then indexed in a second PCR reaction using the following thermocycling conditions: 10 min at 95°C followed by 15 cycles of 30 s at 95°C, 30 s at 55°C and 2 min at 72°C, followed by 5 min at 72°C, using Illumina DNA/RNA UD Index Sets A-D, Tagmentation (Illumina (San Diego, CA) 20091654,

20091656, 20091658 and 20091660). Indexed amplicons were then pooled and purified as before. Pooled, indexed amplicons were quantitated using the Agilent's 4200 TapeStation System (Santa Clara, CA), in accordance with the manufacturer's instructions. Purified, quantitated, indexed, pools were loaded on to an Illumina MiSeq sequencing system at a final concentration of 6 pM along with 4.5 % Illumina PhiX (Illumina (San Diego, CA) FC-110-3001). Sequencing was run for 2 × 250 paired-end cycles.

Amplicon Sequence Analysis

Amplicon data generated by the Illumina MiSeq sequencing were processed by an in-house pipeline: Paired-end reads were merged by FLASH in accordance with the procedures described by Magoč and Salzberg (2011), with parameters "-O -M 800". The forward and reverse primers were removed from the merged reads and reads with an overall quality score of < 20 were discarded using the Ribosomal Database Project (RDP) Initial Process Tool, as described by Fish et al. (2013), with parameters "-F 2 -R 1 -m 220 -x 270". The length of the trimmed reads was ~253 bp. Reads passing the above quality processing steps were clustered at 99 % using the CD-HIT program, as described by Li et al. (2006), to obtain operational taxonomic units (**OTU**). To reduce the error rate, reads from OTUs of size one or two were removed from the subsequent analysis. The representative sequence from each OTU was assigned to the genetically closest species using the RDP Alignment Tools, as described by Fish et al. (2013), against the latest RDP Classifier training set No. 19 established by Wang and Cole (2024), a vetted 16S reference database containing mostly 16S genes from type strains and public genomes. The classification assignment of the reads at genus and higher ranks was performed by RDP Classifier as described by Wang et al. (2007), using the same training set.

Differentially Abundant Genera Analysis

Microbial abundance matrices (expressed as the percentage composition or count of the given species, genus, or higher taxonomic rank within each cecal swab sample) were determined based on the classification assignments performed above. The differentially abundant taxa (genera) between pairs of treatment groups (C vs. CC, CC vs. CC+Probiotic, and C vs. CC+Probiotic) were detected by DESeq2 which uses a generalized linear model with gene-specific dispersion estimation and normalization of the data, as described by Love and Huber (2014). The DESeq2 computed the log2 foldchanges (Log2FoldChange) between the estimated coefficients for each treatment pair comparison for each taxa level (in this case genus). A positive value of Log2FoldChange indicated the corresponding taxon is more abundant in the second group than the first group. The software also calculated *P* values and adjusted *P* values to correct for false discovery due to multiple comparison testing, using the Benjamini-Hochberg correction. An adjusted *P* value of < 0.05 was considered statistically significant. $0.1 > P > 0.05$ was considered a tendency.

Statistical Analysis

Pen was the experimental unit for all growth performance and intestinal pathology data analyses. For average NE lesion scores, score values were averaged per pen. Performance were analyzed by one-way ANOVA followed by Tukey's HSD test to separate pairs of means where the ANOVA result was statistically significant. Trial was included as a random effect in the ANOVA and treatment as a fixed effect. For the NE lesion scores, NE induction, and lesion scores of NE + birds, the non-parametric Friedman test and post hoc Nemenyi test were used (friendman.test() and frdAllPairsNemenyiTest() function in R). A *P* value of < 0.05 was considered statistically significant. $0.1 > P > 0.05$ was considered a tendency.

Results and discussion

Growth Performance

The effect of treatment on growth performance is presented in Table 3. BW at d 42 of the Control treatment (C) was 527 g and 246 g below the performance objectives for Cobb 500 and Ross 308 birds, respectively (Aviagen Inc., 2022; Cobb-Vantress Inc., 2022). This slight suppression of growth performance is unlikely to have been due to nutritional deficiency because the control diet was formulated to supply adequate nutrients. The use of re-used litter as well as environmental conditions could have been responsible. Previous studies have shown that the microbial composition of re-used litter can have a reciprocal effect on the ileal and (less so) the cecal microbiome of broilers (Cressman et al., 2010; Wang et al., 2016) and the microorganism composition of the gastrointestinal tract is known to be closely linked to broiler performance responses (Dittoe et al., 2022). In addition, environmental temperature was controlled but humidity was not. Humidity fluctuations can influence the severity of coccidial infections (Mesa-Pineda et al., 2021) and therefore suppress performance.

The Necrotic Enteritis Challenge

The NE challenge had the expected effect on growth performance.

Table 3
Effect of treatment on growth performance across all three trials (data pooled), by phase and cumulatively.

Item	C	CC	CC+Probiotic	SEM	ANOVA *P* value
Initial BW	66.5	66.7	66.7	3.096	0.62
Starter, 0 to 14 d of age					
BW, 14 d of age, g/bird	533	535	536	4.34	0.41
BWG, g/bird	467	468	470	1.61	0.51
FI, g/bird	552	552	556	5.57	0.67
FCR, g:g	1.183	1.179	1.183	0.009	0.85
Mortality, %	0.764	0.972	0.833	0.124	0.78
Grower 1, 15 to 21 d of age					
BW, 21 d of age, g/bird	1,010[a]	981[b]	999[a]	4.53	<0.001
BWG, g/bird	477[a]	447[b]	462[a]	3.11	<0.001
FI, g/bird	708	701	699	5.22	0.75
FCR, g:g	1.486[b]	1.570[a]	1.513[b]	0.009	<0.001
Mortality, %	0.425	0.491	0.283	0.095	0.67
Grower 2, 22 to 28 d of age					
BW, 28 d of age, g/bird	1,617[a]	1,499[c]	1,580[b]	7.07	<0.001
BWG, g/bird	606[a]	518[b]	581[a]	5.29	<0.001
FI, g/bird	1,047[a]	964[b]	1,033[a]	10.07	<0.001
FCR, g:g	1.729[b]	1.873[a]	1.778[b]	0.018	<0.001
Mortality, %	0.381[a]	1.094[b]	0.311[a]	0.116	0.008
Finisher, 29 to 42 d of age					
BW, 42 d of age, g/bird	2,975[a]	2,701[c]	2,888[b]	12.97	<0.001
BWG, g/bird	1,359[a]	1,202[c]	1,308[b]	9.78	<0.001
FI, g/bird	2,875[a]	2,715[b]	2,833[ab]	24.11	0.003
FCR, g:g	2.123[b]	2.262[a]	2.175[b]	0.019	<0.001
Mortality, %	0.161	0.620	0.192	0.094	0.082
0 to 42 d of age					
BWG, g/bird	2,909[a]	2,634[c]	2,821[b]	13.64	<0.001
FI, g/bird	5,135[a]	4,878[b]	5,075[a]	25.87	<0.001
FCR, g:g	1.768[b]	1.853[a]	1.802[b]	0.010	<0.001
Mortality, %	1.667[a]	2.917[b]	1.528[a]	0.216	0.013

a,bMeans within a row bearing different superscript letters are significantly different at $P < 0.05$.
C, non-challenged control; CC, challenged control; CC+probiotic, challenged control plus dual strain probiotic.

Weight gain and feed efficiency were reduced in C vs. CC birds from Grower 1 phase (15 to 21 d of age) onwards, this being directly after the *Eimeria* challenge was administered (on d 14). During Grower 1, d 21 BW and BWG were reduced by 31 g/bird or 3.1 % and by 30 g/bird or 6.3 %, respectively, and FCR was increased by 8.4 points or 5.6 %, in CC vs. C birds ($P < 0.05$; Table 3). During Grower 2 and Finisher phases (22 to 28 and 29 to 42 d of age, respectively), BW and BWG were also reduced and FCR was increased in CC vs. C birds but with greater effect sizes than in Grower 1. For example, BWG was reduced by 14.5 % in Grower 2 and 11.6 % in Finisher compared with 6.3 % in Grower 1. Feed intake was unaffected by challenge during Starter and Grower 1 phases but was reduced ($P < 0.05$) during Grower 2 and Finisher phase (-83 g/bird or 7.9 % and -160 g/bird or 5.6 %, respectively, in CC vs. C birds). The greater negative effect of the challenge during Grower 2 and Finisher phases likely reflects the cumulative effect of infection with *C. perfringens* (which was administered towards the end of Grower 1 phase), on top of the preceding *Eimeria* challenge. In particular, the decreased appetite of birds during Grower 2 and Finisher phases is a key indicator of NE infection (Cooper et al., 2013) that will have contributed to the greater reductions in BWG that were observed during these phases compared with Grower 1. Overall, the growth performance response of birds in treatment CC is consistent with the findings of other NE-challenge studies in which reduced weight gain, FI and feed efficiency have been reported following a similar 2-step oral challenge with *Eimeria* followed by *C. perfringens* (Akerele et al., 2022; Rodrigues et al., 2018; Xue et al., 2018). As such, the results indicate that the challenge was effective in inducing mild NE. This was further confirmed by the mortality results (Table 3). Mortality was increased significantly but moderately from Grower 2 phase onwards [2.91 % vs. 1.67 % in CC vs. C birds for the overall period (0 to 42 of age); $P < 0.05$], indicating a low level of infection that significantly but not markedly elevated mortality, consistent with a mild NE challenge.

The Probiotic Effect

The probiotic had a positive effect on growth performance from Grower 1 phase onwards. During Grower 1, BW and BWG were increased and FCR was reduced in CC+Probiotic vs. CC birds (+18Akerele et al., 2022; g/bird or 1.8 %, +15 g/bird or 3.3 % and -5.7 points or 3.6 %, respectively; $P < 0.05$; Table 3). In each case, the improvements brought the responses to levels that were not different from those achieved by the control. The same measures were improved during Grower 2 and Finisher phase but with larger effect sizes. For example, BWG during Grower 2 and Finisher phase were increased by 63 g/bird or 12.1 % and by 106 g or 8.8 %, respectively, vs. 25Akerele et al., 2022; g or 3.3 % in Grower 1 phase, in CC+Probiotic vs. CC birds ($P < 0.05$). Again, these measures were improved up to the level of the C, except for BWG during Finisher phase which remained below the level achieved by C birds (-51Akerele et al., 2022; g/bird; $P < 0.05$). Feed intake and mortality were unaffected by probiotic supplementation during Starter and Grower 1 but were increased or reduced, respectively, during Grower 2 phase (+69Akerele et al., 2022; g or 7.2 % and -0.8 %, respectively, in CC+Probiotic vs. CC birds; $P < 0.05$). For the overall period (0 to 42 d of age), all measures except BWG did not differ in CC+Probiotic compared with C birds (BWG remained 88 g/bird lower in CC+Probiotic vs. C birds). The improvements vs. CC in CC+Probiotic birds for the overall period were 7.1 %, 4.0 %, 2.7 % and 1.4 % points for BW, BWG, FI, FCR and mortality, respectively ($P < 0.05$; Table 3).

Results from the wider literature on probiotics in broiler chickens are variable and somewhat inconsistent. A recent meta-analysis of 54 studies published between 2012 and 2022 (Yosi and Metzler-Zebeli, 2023) indicated an overall beneficial effect in pathogen-challenged birds on gut integrity and morphology, but not on growth performance (average daily feed intake, average daily gain and FCR). The present results are not in line with the previous findings. We have demonstrated a measurable beneficial effect of the dual-strain probiotic on growth performance of broilers under a mild NE challenge, across three separate trials that incorporated variation in bird breed, season, and diet. The standard error values associated with the CC+Probiotic treatment means for the overall period were relatively low for the majority of growth performance response measures (0.5 to 1.4 % of the mean, data not shown) indicating a consistency of effect across the different trial settings. The exception to this was mortality, which is likely due to the relatively large impact of one morality case on the percentage mortality within a pen size of only 40 birds. Notwithstanding this, the results support a general repeatability of beneficial response to the probiotic that was sufficient, in this mild NE-challenge setting, to bring growth performance up to the same level as that achieved by unchallenged birds, over 0 to 42 d. Further work is now needed to extend the variation in production conditions under which the probiotic is tested, in order to further evaluate the impact of different production settings on bird responses.

Necrotic Enteritis Lesion Scores

The effect of treatment on NE induction and lesion scores is shown in Table 4. At both 21 and 28 d of age, CC birds exhibited a higher percentage of NE induction (defined as the percentage of birds having an NE lesion score >1) than control birds; 14.4 % vs. 5.0 % at 21 d of age ($P = 0.05$) and 67.78 % vs. 9.4 % at 28 d of age ($P < 0.05$). Similarly, compared to control birds, the average lesion score of CC birds was increased almost 3-fold at 21 d of age ($P = 0.05$) and markedly increased 11-fold at 28 d of age ($P < 0.05$). This demonstrates a time effect of the challenge in inducing gut pathology characteristic of NE disease that was markedly greater at d 28, compared to d 21 (immediately after the *C. perfringens* part of the challenge). As such, these results are consistent with the growth performance results. They support the notion that the effects on gut pathology contributed to impaired nutrient absorption and utilization for growth. A marked increase in NE lesions following *C. perfringens* infection in broilers pre-exposed to *Eimeria* is characteristic of the etiology of NE in which the initial *Eimeria* infection damages host mucosal cells, increases cell permeability, disrupts nutrient digestion and absorption, and causes dysbiosis (Madlala et al., 2021), all of which enable *C. perfringens* to colonize, proliferate, release toxins, and exert multiple pathogenic effects.

The probiotic reduced the percentage NE induction, average lesion score and average lesion score of NE-positive birds at 28 d of age (by 30.6 % points ($P < 0.05$), 57.1 % ($P < 0.05$) and 22.9 % ($P = 0.05$), respectively CC+Probiotic birds vs. CC birds). At 21 d of age, the percentage of NE induction and average lesion score were not significantly reduced in CC+Probiotic birds vs. CC birds but were also not

Table 4
Effect of treatment on necrotic enteritis (NE) induction and lesion scores across all three trials (data pooled).

Item	C	CC	CC+ Probiotic	SEM	Friedman test *P* value
21 d of age					
% NE induction[1]	5.00	14.44	7.78	1.189	0.05
Lesion score[2]	0.05	0.14	0.08	0.012	0.05
28 d of age					
% NE induction	9.44[a]	67.78[b]	37.22[ab]	2.864	0.049
Lesion score	0.09[a]	0.98[b]	0.42[ab]	0.042	0.049
Lesion score of NE+ birds[3]	1.00	1.44	1.11	0.033	0.05

[1] Defined as the percentage of birds having an NE lesion score >1.
[2] Defined as the average NE lesion score of all birds.
[3] Defined as the average NE lesion score of NE positive birds (those with an NE lesion score >1).
[ab]Means within a row bearing different superscript letters are significantly different at $P < 0.05$.

C, non-challenged control; CC, challenged control; CC+probiotic, challenged control plus dual strain probiotic.

significantly different from those of control birds. This indicates that by 28 d of age, the probiotic had ameliorated the negative effect of the challenge in inducing NE lesions, whereas at 21 d of age a clear beneficial effect was not fully evident. Clearly, 21 d of age was only 1 day after the end of the *C. perfringens* challenge so may have been too soon to observe an effect. However, it is interesting that a significant beneficial effect on growth performance (BWG and FCR) was already evident by then. This could suggest that there are other mechanisms via which the probiotic exerted its beneficial effect on growth performance beyond its action in reducing the prevalence and severity of NE lesions. Knowledge of the mode(s) of beneficial action of this probiotic is still developing. The *in vitro* studies of the same two probiotic strains (*L. acidophilus* AG01 and *B. animalis* AG02) carried out by Kadekar et al. (2024) have implicated antimicrobial compounds secreted by these bacteria in reducing key pathogenic traits of *C. perfringens* against poultry intestinal epithelial cells (Kadekar et al., 2024). However, there may be other routes of effect. Other probiotic bacteria, including strains of *Lactobacillus*, have been shown to beneficially modulate the intestinal microbiome composition, the immune system and increase the production of short chain fatty acids by the microbiota (Gao et al., 2022; Jha et al., 2020; Obianwuna et al., 2023), all of which could influence growth performance via separate mechanisms to direct effects on the intestinal pathology.

Effect on the cecal microbiome

The effect of the pathogen challenge and probiotic on the taxonomic composition of the cecal microbiota in Trial 3 is presented in Table 5. Changes in the abundance of individual bacterial genera (expressed as log2-fold changes) between pairs of treatments are shown only for those cases where the change was statistically significant (cases where no significant effect was found are not shown). The calculation of the log2-fold change by DESeq2 involves the identification and removal of outliers from individual treatment groups; the mean abundance values are normalized before the fold-change is calculated. This is why in certain case comparisons, the mean percentage abundance values between a given pair of treatment means do not agree with the direction of the fold-change (e.g. *Achromobacter*, C vs. CC+Probiotic).

Compared to unchallenged birds (C), the NE challenge (CC) altered the balance of the cecal microbiota. Birds in the CC treatment exhibited reduced ($P < 0.05$) abundance of three bacterial genera (*Achromobacter, Bordetella* and to a lesser extent *Anaerotignum*) and increased ($P < 0.05$) abundance of eight genera (*Butyricicoccus, Campylobacter Coprobacter, Enteroscipio, Gallalistipes, Rikenella, Subdoligranulum*, and especially

Table 5
List of differentially abundant bacterial genera in the cecal content identified in at least one of the three treatment comparisons, at 28 d of age in Trial 3.

Family[1]	Genus[2]	Mean abundance %			Pair-wise comparisons					
		C	CC	CC +Probiotic	C vs. CC		CC vs. CC+Probiotic		C vs. CC+Probiotic	
					Log2-FoldChange[2]	*P* value[3]	Log2-FoldChange[2]	*P* value[3]	Log2-FoldChange[2]	*P* value[3]
Bifidobacteriaceae[a]	*Bifidobacterium*	0.03	0.02	0.99			-4.725	0.018	5.365	0.038
Eggerthellaceae[a]	*Adlercreutzia*	0.10	0.09	0.13			-1.390	<0.001	1.284	0.011
	Enteroscipio	0.01	0.06	0.01	2.487	<0.001	2.010	0.003		
	Gordonibacter	0.17	0.14	0.25			-1.125	0.001		
	Rubneribacter	0.38	0.33	0.53			-1.188	<0.001	0.919	0.012
Bacteroidaceae[b]	*Bacteroides*	0.25	0.23	2.80			-4.087	<0.001	3.343	<0.001
	Mediterranea	0.11	0.05	0.01					-3.049	0.001
Porphyromonadaceae[b]	*Parabacteroides*	0.54	0.70	1.09			-1.162	0.024	1.423	0.011
Barnesiellaceae[b]	*Coprobacter*	0.76	1.49	0.57	0.902	0.034	1.072	0.007		
Rikenellaceae[b]	*Gallalistipes*	0.12	0.20	0.13	0.659	0.010				
	Rikenella	0.13	0.36	0.28	1.474	0.014				
Lactobacillaceae[c]	*Ligilactobacillus*	5.40	3.00	8.46			-1.599	0.013		
Enterococcaceae[c]	*Enterococcus*	0.10	0.10	0.40					2.322	0.002
Bacillota incertae sedis[c]	*Negativibacillus*	0.14	0.18	0.08			0.806	0.017		
Sutterellaceae[d]	*Parasutterella*	0.39	0.56	0.18			1.249	0.044		
Alcaligenaceae[d]	*Achromobacter*	5.01	0.01	6.70	-8.620	<0.001	-3.288	<0.001	-4.718	<0.001
	Bordetella	0.14	<0.01	0.19	-9.036	0.025				
Campylobacteracea[e]	*Campylobacter*	0.01	0.05	0.04	2.198	0.014				
Helicobacteraceae[e]	*Helicobacter*	0.63	0.36	0.14			1.352	0.015	-2.172	<0.001
Peptococcaceae[c]	*Peptococcus*	<0.01	0.34	<0.01	8.933	<0.001	10.253	<0.001		
Lachnospiraceae[c]	*Anaerotignum*	0.02	0.14	0.29	-0.542	0.032	-1.112	0.002		
	Anthropogastromicrobium	0.26	0.24	0.12					-1.095	0.041
	Catenibacillus	0.04	0.04	0.07					1.287	0.011
	Merdimonas	0.20	0.17	0.35			-1.806	<0.001	1.579	<0.001
Butyricicoccaceae[c]	*Butyricicoccus*	0.42	0.83	1.12	0.939	0.014	-1.351	0.013	2.194	<0.001
Oscillospiraceae[c]	*Acutalibacter*	0.11	0.12	0.05			1.063	<0.001	-0.893	0.032
	Dysosmobacter	0.33	0.29	0.17			0.573	0.027	-0.770	0.043
	Fournierella	0.40	0.41	0.59			-1.695	<0.001	1.618	<0.001
	Pseudoflavonifractor	1.01	1.10	1.39			-1.124	0.011	0.93	0.038
	Subdoligranulum	0.06	0.13	0.08	0.974	0.014				
Enterobacteriaceae[d]	*Escherichia Shigella*	0.03	0.01	2.09			-5.000	<0.001	2.938	0.038

[1] Associated Class is denoted by lower case superscript letters:
[a] *Actinomecetota*;
[b] *Bacteroidota*;
[c] *Bacillota*;
[d] *Pseudomonadota*
[e] *Campylobacterota*
[2] Taxonomical assignment was based on RDP Classifier training set No. 19
[3] A positive value of Log2FoldChange indicates the corresponding taxon is more abundant in the second group than the first group.
[4]Displayed *P* values are after adjustment for false discovery rate (FDR) using the Benjamini-Hochberg procedure.

Peptococcus (+8.9 Log2-FoldChange; $P < 0.001$). The functional significance of these changes is unknown. However, it may be speculated that the increase in *Campylobacter*, a pathogen itself, may have been facilitated by the negative effects of *Eimeria* and *C. perfringens* on the gut mucosa (measured by the increase in NE lesion scores in CC birds) which could have enabled the pathogen to colonize the gut lining and proliferate. Such an effect could potentially have contributed to the negative effects of the challenge on bird performance by disrupting nutrient digestion and absorption; impaired nutrient absorption is an established pathogenic trait of *Campylobacter jejuni* colonization (Awad et al., 2022). The increased abundance of *Enteroscipio* could also have contributed to the negative effect of the challenge on bird performance; *Eggerthellaceae* (of which *Enteroscipio* is a member) in the cecum and ileum have previously been negatively correlated with broiler weight at both 14 and 21 d of age (Johnson et al., 2018). Meanwhile, *Butyricicoccus* and *Subdoligranulum* are both SCFA producers, mainly producing butyrate (Eeckhaut et al., 2016; Holmstrom et al., 2004) that has well described beneficial effects on gut health and barrier integrity in broilers (Matis et al., 2022). An increase in the abundance of these bacteria in CC birds may therefore be associated with a beneficial effect, although the relative Log2-FoldChanges were not large for these genera (0.939 and 0.974, respectively; Table 5). *Coprobacter* is also a SCFA (propionic acid) producer (Shkoporov et al., 2013), and to our knowledge has not been previously identified in chicken microbiota, whereas *Achromobacter* is potentially associated with pathogenic phenotype in humans (Crone et al., 2022) and in animals (broilers and carps, Ke et al., 2024).

Several bacterial genera were also modulated in the cecum of CC+Probiotic birds. Birds in treatment CC exhibited a lower abundance ($P < 0.05$) of 15 bacterial genera and a higher abundance ($P < 0.05$) of eight genera compared with CC+Probiotic birds (Table 5). The genera that were more abundant in CC+Probiotic birds included multiple major SCFA producers: *Anaeromassilibacillus, Fournierella, Pseudoflavonifractor* (from the *Oscillospiraceae* family previously known as *Ruminococcaceae)* and *Anaerotignum, Merdimonas*, and *Butyricoccus,* all genera belonging to the *Bacillota* phylum-previously called *Firmicutes*). *Pseudoflavonifractor, Anaerotignum* and *Merdimonas* belong to different families within the *Clostridium* cluster of the phylum *Bacillota* and are major butyrate producers (Singh et al., 2023). *Anaeromassilibacillus* is also a butyrate producer that has been positively correlated with the expression of immune relevant genes in lean-line broilers and may help to maintain a healthy gut environment with less abdominal fat deposition (Jing et al., 2021). As well as having beneficial effects on gut-associated metabolic processes, gut immune responses and barrier integrity, butyrate also acts as a nutrient source for other colonocytes in the gut and itself beneficially modulates the gut microbiota by secreting antimicrobial and anti-inflammatory molecules (Singh et al., 2023). Further studies are needed to investigate the relationship between the dual-strain probiotic and the observed increase in butyrogenic bacteria in the cecum of NE-challenged birds and how this influences nutrient digestion and utilization for growth and immune responses to pathogen challenge. *Fournierella* is a major producer of acetic acid but also produces butyric acid, isobutyric acid, and propionic acid. It has been identified as being associated with broiler immune system development and having immunomodulatory properties (Liu et al., 2023) as well as being associated with increased BW (Farkas et al., 2022). The *Helicobacter* abundance was reduced in CC+Probiotic vs. CC birds, a genera containing species that are pathogenic to both broilers and humans (Javed et al., 2017; Kusters et al., 2006). Other genera were modulated in a way that cannot be explained as beneficial. These include *Escherichia/Shigella* and *Bacteroides,* both of which can proliferate in birds infected with *Eimeria* (Martynova-Van Kley et al., 2012), and both of which were increased in CC+Probiotic vs. CC (or vs. control) birds ($P < 0.05$). Similarly, *Peptococcus* and *Parasutterella*, which are bacterial genera that are transferred from hen to chick during the first week of life, were reduced in CC+Probiotic vs. CC birds ($P < 0.05$). The functional significance of these changes is unknown and requires further study.

In conclusion, daily administration a dual-strain waterline probiotic containing *L. acidophilus* AG01 and *B. animalis* subspecies *lactis* AG02 ameliorated the negative effects of a mild NE challenge on growth performance and gastrointestinal NE pathology in broilers during 0 to 42 d of age. As such, they provide greater confidence in the efficacy of the probiotic to reduce the negative effects of a mild NE challenge under production conditions. The microbiome data additionally demonstrated that the probiotic altered the taxonomic composition of the cecal microflora which may have contributed to its beneficial effect on growth performance. In particular, the mode-of-action may have come through the dual-strain probiotic increasing the abundance of SCFA-producing bacterial genera. Further studies are needed to confirm these effects and determine their contribution to the overall beneficial effects of this probiotic in broiler chickens.

Disclosures

Sasha van der Klein, Marion Bernardeau, Qiong Wang and Kirsty Gibbs are employees of Danisco Animal Nutrition & Health (IFF), Oegstgeest, The Netherlands, a global supplier of feed additive solutions.

Declaration of competing interest

All authors declare that they have no conflicts of interest.

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References

Abd El-Ghany, W.A., Abdel-Latif, M.A., Hosny, F., Alatfeehy, N.M., Noreldin, A.E., Quesnell, R.R., Chapman, R., Sakai, L., Elbestawy, A.R., 2022. Comparative efficacy of postbiotic, probiotic, and antibiotic against necrotic enteritis in broiler chickens. Poult. Sci. 101, 101988. https://doi.org/10.1016/j.psj.2022.101988.

Akerele, G., Al Hakeem, W.G., Lourenco, J., Selvaraj, R.K., 2022. The effect of necrotic enteritis challenge on production performance, cecal microbiome, and cecal tonsil transcriptome in broilers. Pathogens. 11. https://doi.org/10.3390/pathogens11080839.

Aviagen Inc., 2022. Ross 308 broiler performance objectives. Accessed on the 6th April 2024 from: https://aviagen.com/assets/Tech_Center/Ross_Broiler/RossxRoss308-BroilerPerformanceObjectives2022-EN.pdf.

Awad, W.A., Ruhnau, D., Gavrau, A., Dublecz, K., Hess, M., 2022. Comparing effects of natural betaine and betaine hydrochloride on gut physiology in broiler chickens. Poult. Sci. 101, 102173. https://doi.org/10.1016/j.psj.2022.102173.

Bendary, M.M., Abd El-Hamid, M.I., El-Tarabili, R.M., Hefny, A.A., Algendy, R.M., Elzohairy, N.A., Ghoneim, M.M., Al-Sanea, M.M., Nahari, M.H., Moustafa, W.H., 2022. *Clostridium perfringens* associated with foodborne infections of animal origins: insights into prevalence, antimicrobial resistance, toxin genes profiles, and toxinotypes. Biology. 11. https://doi.org/10.3390/biology11040551.

Cobb-Vantress Inc, 2022. Cobb500 broiler performance & nutrition supplement. Accessed on the 6th April 2024 from: https://cobbgenetics.com/assets/Cobb-Files/2022-Cobb500-Broiler-Performance-Nutrition-Supplement.pdf.

Cooper, K.K., Songer, J.G., Uzal, F.A., 2013. Diagnosing clostridial enteric disease in poultry. J. Vet. Diagn. Invest. 25, 314–327. https://doi.org/10.1177/1040638713483468.

Cressman, M.D., Yu, Z., Nelson, M.C., Moeller, S.J., Lilburn, M.S., Zerby, H.N., 2010. Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. Appl. Environ. Microbiol. 76, 6572–6582. https://doi.org/10.1128/AEM.00180-10.

Crone, C.G., Rezahosseini, O., Schultz, H.H.L., Qvist, T., Johansen, H.K., Nielsen, S.D., Perch, M., 2022. Achromobacter spp. in a cohort of non-selected pre- and post-lung transplant recipients. Pathogens. 11. https://doi.org/10.3390/pathogens11020181.

Diaz Carrasco, J.M., Redondo, L.M., Redondo, E.A., Dominguez, J.E., Chacana, A.P., Fernandez Miyakawa, M.E., 2016. Use of plant extracts as an effective manner to control *Clostridium perfringens* induced necrotic enteritis in poultry. Biomed. Res. Int. 2016, 3278359. https://doi.org/10.1155/2016/3278359.

Dittoe, D.K., Olson, E.G., Ricke, S.C., 2022. Impact of the gastrointestinal microbiome and fermentation metabolites on broiler performance. Poult. Sci. 101, 101786. https://doi.org/10.1016/j.psj.2022.101786.

Eeckhaut, V., Wang, J., Van Parys, A., Haesebrouck, F., Joossens, M., Falony, G., Raes, J., Ducatelle, R., Van Immerseel, F., 2016. The probiotic *Butyricicoccus pullicaecorum* reduces feed conversion and protects from potentially harmful intestinal

microorganisms and necrotic enteritis in broilers. Front. Microbiol. 7, 1416. https://doi.org/10.3389/fmicb.2016.01416.
Eid, S., Atfehy, N., Amer, F., Tolba, H., Hamed, R., 2020. Prevention of necrotic enteritis in broiler chickens by prebiotics and probiotics vs control by antibiotics, in vivo study. Alex. J. Vet. Sci. 64. https://doi.org/10.5455/ajvs.76994.
European Council, 2007. Council Directive 2007/43/EC laying down minimum rules for the protection of chickens kept for meat production. OJ L 182/19.
Farkas, V., Csitari, G., Menyhart, L., Such, N., Pal, L., Husveth, F., Rawash, M.A., Mezolaki, A., Dublecz, K., 2022. Microbiota composition of mucosa and interactions between the microbes of the different gut segments could be a factor to modulate the growth rate of broiler chickens. Animals 12, 1296. https://doi.org/10.3390/ani12101296, 10.
Fish, J.A., Chai, B., Wang, Q., Sun, Y., Brown, C.T., Tiedje, J.M., Cole, J.R., 2013. FunGene: the functional gene pipeline and repository. Front. Microbiol. 4, 291. https://doi.org/10.3389/fmicb.2013.00291.
Gomez-Osorio, L.M., Yepes-Medina, V., Ballou, A., Parini, M., Angel, R., 2021. Short and medium chain fatty acids and their derivatives as a natural strategy in the control of necrotic enteritis and microbial homeostasis in broiler chickens. Front. Vet. Sci. 8, 773372. https://doi.org/10.3389/fvets.2021.773372.
Grozina, A.A., Ilina, L.A., Laptev, G.Y., Yildirim, E.A., Ponomareva, E.S., Filippova, V.A., Tyurina, D.G., Fisinin, V.I., Kochish II, Griffin, D.K., Surai, P.F., Romanov, M.N., 2023. Probiotics as an alternative to antibiotics in modulating the intestinal microbiota and performance of broiler chickens. J. Appl. Microbiol. 134. https://doi.org/10.1093/jambio/lxad213.
Gao, J., Wang, R., Liu, J., Wang, W., Chen, Y., Cai, W., 2022. Effects of novel microecologics combined with traditional Chinese medicine and probiotics on growth performance and health of broilers. Poult. Sci. 101, 101412. https://doi.org/10.1016/j.psj.2021.101412.
Hill, C., Guarner, F., Reid, G., Gibson, G.R, Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C., Sanders, M.E., 2014. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. Rev. Gastroenterol. Hepatol. 11, 506–514. https://doi.org/10.1038/nrgastro.2014.66.
Holmstrom, K., Collins, M.D., Moller, T., Falsen, E., Lawson, P.A., 2004. *Subdoligranulum variabile* gen. nov., sp. nov. from human feces. Anaerobe 10, 197–203. https://doi.org/10.1016/j.anaerobe.2004.01.004.
Huang, T., Gao, B., Chen, W.L., Xiang, R., Yuan, M.G., Xu, Z.H., Peng, X.Y., 2018. Temporal effects of high fishmeal diet on gut microbiota and immune response in *Clostridium perfringens*-challenged chickens. Front. Microbiol. 9, 2754. https://doi.org/10.3389/fmicb.2018.02754.
Javed, S., Gul, F., Javed, K., Bokhari, H., 2017. *Helicobacter pullorum*: An emerging zoonotic pathogen. Front. Microbiol. 8, 604. https://doi.org/10.3389/fmicb.2017.00604.
Jha, R., Das, R., Oak, S., Mishra, P., 2020. Probiotics (direct-fed microbials) in poultry nutrition and their effects on nutrient utilization, growth and laying performance, and gut health: A systematic review. Animals 10 (10), 1863. https://doi.org/10.3390/ani10101863.
Jing, Y., Yuan, Y., Monson, M., Wang, P., Mu, F., Zhang, Q., Na, W., Zhang, K., Wang, Y., Leng, L., Li, Y., Luan, P., Wang, N., Guo, R., Lamont, S.J., Li, H., Yuan, H., 2021. Multi-omics association reveals the effects of intestinal microbiome-host interactions on fat deposition in broilers. Front. Microbiol. 12, 815538. https://doi.org/10.3389/fmicb.2021.815538.
Johnson, T.J., Youmans, B.P., Noll, S., Cardona, C., Evans, N.P., Karnezos, T.P., Ngunjiri, J.M., Abundo, M.C., Lee, C.W., 2018. A consistent and predictable commercial broiler chicken bacterial microbiota in antibiotic-free production displays strong correlations with performance. Appl. Environ. Microbiol. 84. https://doi.org/10.1128/AEM.00362-18.
Kadekar, D., Udrea, A.C., Bak, S.Y., Christensen, N., Gibbs, K., Shen, C., Bernardeau, M., 2024. Cell-free culture supernatant of *Lactobacillus acidophilus* AG01 and *Bifidobacterium animalis* subsp. *lactis* AG02 reduces the pathogenicity of NetB-positive *Clostridium perfringens* in a chicken intestinal epithelial cell line. Microorganisms. 12. https://doi.org/10.3390/microorganisms12040839.
Ke, K., Sun, Y., He, T., Liu, W., Wen, Y., Liu, S., Wang, Q., Gao, X., 2024. Effects of feather hydrolysates generated by probiotic *Bacillus licheniformis* WHU on gut microbiota of broiler and common carp. J Microbiol. Feb 29. https://doi.org/10.1007/s12275-024-00118-z.
van der Klein, S.A.S., Yitbarek, A., Gibbs, K., 2023. A comparison between two application strategies of a dual strain waterline probiotic during a mild necrotic enteritis challenge. In: Poultry Science Association 112th Annual meeting abstracts, pp. 84–85 abstract no.
van der Klein, S.A.S., Arora, S.S., Haldar, S., Dhara, A.K., Gibbs, K., 2024. A dual strain probiotic administered via the waterline beneficially modulates the ileal and cecal microbiome, sIgA and acute phase protein levels, and growth performance of broilers during a dysbacteriosis challenge. Poult. Sci. 103, 104462. https://doi.org/10.1016/j.psj.2024.104462.
Kulkarni, R.R., Gaghan, C., Gorrell, K., Sharif, S., Taha-Abdelaziz, K., 2022. Probiotics as alternatives to antibiotics for the prevention and control of necrotic enteritis in chickens. Pathogens. 11. https://doi.org/10.3390/pathogens11060692.
Kusters, J.G., van Vliet, A.H., Kuipers, E.J., 2006. Pathogenesis of *Helicobacter pylori* infection. Clin. Microbiol. Rev. 19, 449–490. https://doi.org/10.1128/CMR.00054-05.
Li, W., Godzik, A., 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics. 22, 1658–1659. https://doi.org/10.1093/bioinformatics/btl158.
Liu, Y., Feng, Y., Yang, X., Lv, Z., Li, P., Zhang, M., Wei, F., Jin, X., Hu, Y., Guo, Y., Liu, D., 2023. Mining chicken ileal microbiota for immunomodulatory microorganisms. ISMe J. 17, 758–774. https://doi.org/10.1038/s41396-023-01387-z.
Love, M.I., Huber, W., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome biol. 15, 1–21.
Madlala, T., Okpeku, M., Adeleke, M.A., 2021. Understanding the interactions between Eimeria infection and gut microbiota, towards the control of chicken coccidiosis: a review. Parasite 28, 48. https://doi.org/10.1051/parasite/2021047.
Magoč, T., Salzberg, S., 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27 (21), 2957–2963.
Martynova-Van Kley, M.A., Oviedo-Rondon, E.O., Dowd, S.E., Hume, M., Nalian, A., 2012. Effect of Eimeria infection on cecal microbiome of broilers fed essential oils. Int. J. Poult. Sci. 11, 747–755.
Matis, G., Mackei, M., Boomsma, B., Febel, H., Nadolna, K., Szymanski, L., Edwards, J.E., Neogrady, Z., Kozlowski, K., 2022. Dietary protected butyrate supplementation of broilers modulates intestinal tight junction proteins and stimulates endogenous production of short chain fatty acids in the caecum. Animals 12, 1940. https://doi.org/10.3390/ani12151940, 15.
Mesa-Pineda, C., Navarro-Ruiz, J.L., López-Osorio, S., Chaparro-Butiérrez, J.J., Gómez-Osorio, L.M., 2021. Chicken coccidiosis: From the parasite lifecycle to control of the disease. Front. Vet. Sci. 8, 787653. https://doi.org/10.3389/fvets.2021.787653.
Nicholds, J.F., McQuain, C., Hofacre, C.L., Mathis, G.F., Fuller, A.L., Telg, B.E., Montoya, A.F., Williams, S.M., Berghaus, R.D., Jones, M.K., 2021. The effect of different species of Eimeria with *Clostridium perfringens* on performance parameters and induction of clinical necrotic enteritis in broiler chickens. Avian Dis. 65, 132–137. https://doi.org/10.1637/aviandiseases-D-20-00106.
Obianwuna, U.E., Agbai Kalu, N., Wang, J., Zhang, H., Qi, G., Qiu, K., Wu, S., 2023. Recent trends on mitigative effect of probiotics on oxidative-stress-induced gut dysfunction in broilers under necrotic enteritis challenge: A review. Antioxidants 12, 911. https://doi.org/10.3390/antiox12040911, 4.
Rodrigues, I., Svihus, B., Bedford, M.R., Gous, R., Choct, M., 2018. Intermittent lighting improves resilience of broilers during the peak phase of sub-clinical necrotic enteritis infection. Poult. Sci. 97, 438–446. https://doi.org/10.3382/ps/pex315.
Shkoporov, A.N., Khokhlova, E.V., Chaplin, A.V., Kafarskaia, L.I., Nikolin, A.A., Polyakov, V.Y., Shcherbakova, V.A., Chernaia, Z.A., Efimov, B.A., 2013. *Coprobacter fastidiosus* gen. nov., sp. nov., a novel member of the family *Porphyromonadaceae* isolated from infant faeces. Int. J. Syst. Evol. Microbiol. 63, 4181–4188. https://doi.org/10.1099/ijs.0.052126-0.
Singh, V., Lee, G., Son, H., Koh, H., Kim, E.S., Unno, T., Shin, J.H., 2023. Butyrate producers, "The Sentinel of Gut": Their intestinal significance with and beyond butyrate, and prospective use as microbial therapeutics. Front. Microbiol. 13, 1103836. https://doi.org/10.3389/fmicb.2022.1103836.
Skinner, J.T., Bauer, S., Young, V., Pauling, G., Wilson, J., 2010. An economic analysis of the impact of subclinical (mild) necrotic enteritis in broiler chickens. Avian Dis. 54, 1237–1240. https://doi.org/10.1637/9399-052110-Reg.1.
Sun, X., Jia, Z., 2018. Microbiome modulates intestinal homeostasis against inflammatory diseases. Vet. Immunol. Immunopathol. 205, 97–105. https://doi.org/10.1016/j.vetimm.2018.10.014.
Tsiouris, V., Georgopoulou, I., Batzios, C., Pappaioannou, N., Ducatelle, R., Fortomaris, P., 2018. Heat stress as a predisposing factor for necrotic enteritis in broiler chicks. Avian Pathol. 47, 616–624. https://doi.org/10.1080/03079457.2018.1524574.
Wade, B., Keyburn, A., 2015. The true cost of necrotic enteritis. Poultry World 31.
Wang, L., Lilburn, M., Yu, Z., 2016. Intestinal microbiota of broiler chickens as affected by litter management regimens. Front. Microbiol. 7, 593. https://doi.org/10.3389/fmicb.2016.00593.
Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–5267. https://doi.org/10.1128/AEM.00062-07.
Wang, Q., Cole, J.R., 2024. Updated RDP taxonomy and RDP Classifier for more accurate taxonomic classification. Microbiol. Resour. Announc. 13, e0106323. https://doi.org/10.1128/mra.01063-23.
Xue, G.D., Barekatain, R., Wu, S.B., Choct, M., Swick, R.A., 2018. Dietary L-glutamine supplementation improves growth performance, gut morphology, and serum biochemical indices of broiler chickens during necrotic enteritis challenge. Poult. Sci. 97, 1334–1341. https://doi.org/10.3382/ps/pex444.
Yosi, F., Metzler-Zebeli, B.U., 2023. Dietary probiotics modulate gut barrier and immune-related gene expression and histomorphology in broiler chickens under non- and pathogen-challenged conditions: A meta-analysis. Animals 13, 1970. https://doi.org/10.3390/ani13121970, 12.