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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

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ABSTRACT

Intestinal dysbacteriosis is increasing in broilers due to the reduced use of antibiotics in feed. This study tested the effect of daily waterline administration of a dual-strain probiotic comprising *Lactobacillus acidophilus* AG01 and *Bifidobacterium animalis* subspecies *lactis* AG02, on growth performance and intestinal health during a 3-step microbial challenge. In total, 900 Ross 308 males were assigned to 36 floor pens (25 birds/pen, 12 pens/treatment) in a completely randomized design. Birds were fed a corn, wheat and soybean-meal based diet. Diets were formulated in 3 phases (starter: 1 to 10; grower: 11 to 24; finisher: 25 to 42 d of age). Treatments comprised a non-challenged control (NC), challenged control (CC), and the CC supplemented with 1×10^8 colony forming units (**CFU**)/bird/day of the probiotic (CC+Probiotic). The challenge comprised 1×10^8 CFU/bird of Avian Pathogenic *Escherichia coli* on d 7, 4,000 oocysts/bird of *Eimeria* on d 15 and 1×10^9 CFU/bird of *C. perfringens* on d 18, 19 and 20. Growth performance was monitored over 42 d, blood samples, and digesta were collected and intestinal dysbacteriosis scoring was performed. Compared to NC birds, CC birds exhibited reduced BW (all phases), reduced feed intake (starter and grower phase), increased FCR (grower phase and overall; $P < 0.05$), reduced ileal lactic acid bacteria concentrations (d 24 and 42), and increased cecal *E. coli* (d 24; $P < 0.05$). Compared to CC birds, CC+Probiotic birds exhibited increased BW, BW gain and feed intake during grower phase ($P < 0.05$), increased ileal lactic acid bacteria at d 24 and 42 and reduced ileal *C. perfringens* at d 24, increased mucosal secretory IgA and reduced serum alpha-1-acid-glycoprotein at d 42. The overall growth performance of CC+Probiotic birds was equivalent to NC birds. These results confirm the efficacy of the dual strain probiotic for mitigating the negative effects of a multi-microbial challenge, improving gut health and growth performance in commercial broilers under dysbacteriosis challenge.

Introduction

Dysbacteriosis, an imbalance in the bacterial composition of the intestinal tract, can arise under conditions of stress (e.g. heat, overcrowding), sub-clinical infection, toxin exposure or a change in dietary composition (De Gruttola et al., 2016). During dysbacteriosis, the normal crosstalk between the host and the microbiota residing in the gut is disrupted. This leads to changes in the functional composition, metabolic activities, or distribution of bacteria along the intestinal tract, resulting in the overgrowth of potentially pathogenic bacteria, a loss of beneficial bacteria, or a loss of diversity in the gut microbiome (Fathima et al., 2022). In broilers, dysbacteriosis causes intestinal inflammation and villus atrophy (Tierlynck et al., 2011) which affects nutrient absorption and stimulates the immune system, diverting resources away from growth and reducing performance. It can also lead to more opportunistic behaviours of intestinal residents such as *Clostridium perfringens*, resulting in clinical symptoms of necrotic enteritis (**NE**), an important intestinal disease in poultry flocks. However, dysbacteriosis remains an ill-specified and non-specific condition, due to its commonly subclinical nature. Previously, antibiotic growth promoters (**AGPs**) and anticoccidials were used to prevent dysbacteriosis by inhibiting pathogenic bacteria and *Eimeria* protozoa, respectively, thereby maintaining

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homeostasis in the gut microbiome. The prohibition of AGPs in many world regions has prompted increased interest in the development of alternative approaches to supporting poultry gut health and prevent the occurrence of dysbacteriosis.

Probiotics or direct-fed microbials ('live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (FAO, 2002)) can be sourced from the host microbiome, and as such they have good adaptability and survivability to proliferate in the host. Poultry-derived probiotics include bacterial genera such as *Lactobacillus* and *Bifidobacterium*. Probiotic *Lactobacillus* strains exhibit high tolerance of acidic pH and bile salts (Shokryazdan et al., 2014; Tannock, 2004) allowing them to pass through the proventriculus and gizzard intact to colonize the intestine. They can inhibit a variety of enteric pathogens including *Escherichia coli, Aspergillus niger* and *Candida albicans* (Nallala et al., 2017). *Lactobacillus* probiotic effects are mediated by a variety of mechanisms including the production of antimicrobial compounds (Coman et al., 2014), competitive exclusion and antagonism (Jin et al., 1998; Kizerwetter-Swida and Binek, 2009) and immune cell activation (Mazziotta et al., 2023). Li et al. (2018a) observed that *L. acidophilus* supplementation in feed increased the body weight of broilers infected with *C. perfringens* and reduced intestinal NE lesion scores, mRNA expression of pro-inflammatory cytokines in the spleen and jejunum, and ileal populations of *Escherichia* compared to challenged, unsupplemented, birds. Beneficial effects from *Bifidobacterium* strains have been reported in both unchallenged and pathogen-challenged poultry via measurements of serum metabolites (Yazhini et al., 2018) growth performance and nutrient digestibility (Khabirov et al., 2022), competitive exclusion of *Salmonella enterica* serotypes and cytokine release (El-Sharkawy et al., 2020).

A dual strain probiotic comprising *Lactobacillus acidophilus* AG01 and *Bifidobacterium animalis* subspecies *lactis* AG02 administered daily via the waterline to broilers during a mild NE challenge [3 oral applications of 1×10^8 colony forming units (**CFU**) of *C. perfringens*] was recently found to improve bird performance (d 42 BW and overall FCR) and reduce NE lesion scores compared to a challenged, unsupplemented, control (van der Klein et al., 2023). *In vitro* studies of the dual strain (Kadekar et al., 2024) have subsequently implicated antimicrobial compounds secreted by the bacteria in these beneficial effects; cell-free supernatant (**CFS**) from *B. animalis* subsp. *lactis* AG02 reduced *C. perfringens* CFU adhesion to chicken intestinal epithelial CHIC-8E11 cells and reduced the negative effect of *C. perfringens* CFS on cell permeability, whereas both *B. animalis* subsp. *lactis* AG02 CFS and *L. acidophilus* AG01 CFS reduced *C. perfringens* CFS cytotoxicity against CHIC-8E11 cells (Kadekar et al., 2024). The present study aimed to extend our understanding of the mode of action and efficacy of the dual strain probiotic in mitigating the effects of dysbacteriosis in a commercial broiler breed. The effect of applying the probiotic every day via the waterline to broilers challenged with a 3-step subclinical microbial challenge was evaluated via measurements of growth performance, ileal, and cecal bacterial populations, dysbacteriosis scoring, immune defense indicators and microbial short chain fatty acid (**SCFA**) analysis, during 0 to 42 d of age. The 3-step microbial challenge aimed to induce a dysbacteriosis situation simulating the natural microbial dynamic occurring under field circumstances.

Materials and methods

The study was performed at Agrivet Research and Advisory Private Ltd., Kolkata, India. All experimental protocols and procedures were reviewed and approved by the Animal Ethics Committee of the Agrivet Research Center and were in compliance with the stipulations made by the Committee for the Purpose of Control and Supervision of Experiments on Animals (2020) of the Ministry of Fisheries, Animal Husbandry and Dairying, Governments of India.

Birds, housing and experimental design

In total, 900 Ross 308 male chicks were obtained on day-of-hatch from a commercial hatchery, weighed, and assigned to 36 floor pens with 25 birds per pen (each pen 2.4 × 2.4 m) and 12 pens per treatment, in a completely randomized design. Average initial weight was 43.7 g. Fresh (unused) wood shavings were used as litter. Floor pens were located in a ventilated open sided broiler house, in which the daily temperature varied with the ambient outside air temperature (ranging between 21.9 °C to 38.9 °C, averaging at 29.4 °C). The lighting regime was 23 h light and 1h dark during the first week and 20h light and 4 h dark thereafter. Birds were vaccinated against infectious bronchitis on day-of-hatch (Nobilis® IB Ma5, MSD Animal Health, Rahway, NJ), Newcastle disease at 5 and 20 d of age (Nobilis® ND Clone 30, MSD Animal Health, Rahway, NJ) and infectious bursal disease at 12 d of age (Nobilis® Gumboro 228E, MSD Animal Health, Rahway, NJ).

Treatments

The 3 treatments comprised of control (NC) which was not exposed to any bacterial dysbacteriosis, the NC group exposed to an induced dysbacteriosis (CC), and the CC supplemented with the dual strain probiotic administered via the waterline every day during 0 to 42 d of age (CC+Probiotic).

The dysbacteriosis induction comprised of a 3-step oral inoculation with 10^8 CFU/bird of Avian Pathogenic *Escherichia coli* (**APEC**) at 7 d of age, followed by administration of a mixed culture of sporulated oocysts of 3 *Eimeria* species (*E. tenella, E. maxima* and *E. acervulina*), at a dose level of 4,000 oocysts/bird at 15 d of age, and finally feeding the birds orally with a culture of *C. perfringens* (ATCC 13124) at the rate of 10^9 CFU/bird on each of 18, 19, and 20 d of age. Dysbacteriosis induction is referenced throughout this paper as challenge.

The dual strain probiotic comprised of a 50:50 blend of *Lactobacillus acidophilus* AG01 and *Bifidobacterium animalis* subspecies *lactis* AG02, produced by Danisco Animal Nutrition & Health (IFF), Oegstgeest, The Netherlands, and supplied as a powder, in sachets. Each sachet contained enough powder to supply a total dose of 1.0×10^8 CFU/bird/day once reconstituted in water, resulting in a dose of 0.5×10^8 CFU/bird/day of each strain. Sachets were stored at 4 °C until use and reconstituted in chlorine-free water according to bird age, on the morning of use. The reconstitution protocol is detailed in Table 1. The reconstituted probiotic was divided equally between supplemental drinkers in each pen and offered during a 4 h period immediately following the longest dark period (i.e. first thing in the morning) to encourage birds to drink. After the 4 h period, birds had *ad libitum* access to non-supplemented water supplied by an automated bell drinker system.

Diets

Birds in all treatments were fed the same diet. Diets were formulated

Table 1
Reconstitution protocol of the water applied dual strain probiotic. One sachet per day was reconstituted, diluted, and divided over drinkers placed in pens assigned to the probiotic treatment to deliver 1.0×10^8 CFU/bird/day.

Bird age	Water added per sachet to create stock solution, ml	Amount of stock solution added per drinker, ml	Amount of non-supplemented water added per drinker, ml	Total volume added per drinker, ml
1 to 7 d	300	10	140	150
8 to 13 d	300	10	390	400
14 to 20 d	300	10	790	800
21 to 27 d	300	10	1,190	1,200
28 to 34 d	300	10	1,390	1,400
35 to 42 d	300	10	1,590	1,600

in 3 phases: 1 to 10 d of age (starter), 11 to 24 d of age (grower) and 25 to 42 d of age (finisher) and were based on corn, soybean meal and wheat, with added full fat soya, rice bran, and rapeseed meal. Phytase was added at 750 phytase units (**FTU**)/kg during all phases. The phytase was a novel consensus bacterial 6-phytase variant manufactured and supplied by Danisco Animal Nutrition & Health (IFF), Oegstgeest, The Netherlands. The diets were reduced in available P, Ca, ME, digestible amino acids, and Na to account for the expected contribution of the phytase when added at 750 FTU/kg, according to the manufacturer's recommendations. The full ingredient composition and calculated nutrient composition of the diets, by phase, is given in Table 2. Each diet was prepared as a single batch of raw materials to which the additives were then added and mixed. Starter diets were crumbled, and grower-finisher diets were pelleted (3 mm pellet, pelleting temperature 82±2 °C, dwelling time 40 to 45 sec in the conditioner). Diets were provided to birds *ad libitum* throughout the duration of the experiment.

Measurements and sampling

Birds were monitored daily for mortality and dead birds were removed and weighed. Cause of death was determined by dissection for all mortalities, including distinguishing between *Colibacillosis* and NE. Body weight was measured on a pen basis at each of d 1, 10, 24, 35 and 42 d of age, in each case at 0800 h. Data were used to calculate BW gain (**BWG**) for each period. Feed intake (**FI**) was measured on d 1, 10, 24, 35 and 42 by subtracting the quantity of feed left in each pen from the total quantity offered during that period. Feed conversion ratio corrected for mortality (**FCR**) was calculated for each period from measurements of FI and BWG.

Five birds per pen were euthanized on d 24 and 42 by cervical dislocation and eviscerated. On each of d 24 and 42, blood samples (~1 ml) were collected before euthanasia from the brachial vein of 1 bird per pen after recording BW in vacutainer tubes without any anticoagulant. The tubes were kept at room temperature to clot the blood and the serum thus harvested from the cells by centrifugation at 800 × g for 10 min. Serum samples were stored in polystyrene tubes at -20 °C. Dysbacteriosis scoring was performed using a scoring system of 1 to 10, according to the scoring criteria of de Gussem (2010). Scores were determined per bird and averaged across the 5 birds per pen. Mucosal scrapings of the ileum were taken from 1 bird/pen for secretory IgA (**sIgA**) determination on each of d 24 and 42. Scrapings were collected using a microscalpel, transferred to sterilized tubes and frozen at -80 °C until later analysis. Cecal contents were collected from 1 bird/pen, in 12 replicates of each treatment group (n=36) after 42 d, and immediately preserved using BioFreeze™ sampling kit (Alimetrics Diagnostics Ltd., Espoo, Finland). Approximately 500 mg of cecal sample was used to determine SCFA. Samples were stored at -80 °C until later analysis. Cecal and ileal digesta was obtained from 1 bird per pen for later analysis of bacterial populations. Samples of the diet (by phase) were taken for the analysis of macrominerals and proximate nutrients.

Sample analysis

Serum samples were analyzed for α-1 glycoprotein (**AAGP**) by sandwich enzyme linked immune sorbent assays (**ELISA**) in a microplate reader (Biotek 800 TS absorbance reader, Agilent Technologies Inc., Santa Clara, CA) using commercially available chicken specific ELISA kit (BT LAB Bioassay Technology Laboratory, Shanghai, China, Cat No: E0242Ch). Frozen mucosal samples were thawed, weighed, suspended in 4 volumes (wt/wt) of phosphate buffered saline, mixed, and centrifuged at 4,000 × g for 10 min at a constant temperature of 4 °C. Sandwich ELISA was used to detect mucosal sIgA in a microplate reader as mentioned above using commercially available chicken specific ELISA kit (BT LAB Bioassay Technology Laboratory, Shanghai, China, Cat No: E0110Ch). A standard curve was generated using polynomial quadratic regression equation tool (obtained from www.MyCurveFit.com) to derive the concentration of AAGP in serum and sIgA in mucosal scrapings.

The SCFA were analyzed as free acids, using pivalic acid (Sigma-Aldrich, St. Louis, MO) as an internal standard. Briefly, cecal samples were homogenized after adding 3 ml of ultrapure water and centrifuged at 10,000 × g for 10 min at 4 °C. 1 ml of supernatant was homogenized with 0.2 ml 25 % metaphosphoric acid and placed on ice for at least 30 min, followed by centrifugation at 10,000 × g for 10 min at 4 °C. Saturated oxalic acid and supernatant were mixed in 1:2 ratio (v/v) and incubate at 4 °C for 60 min followed by further centrifugation at 18,000 × g for 10 min. The supernatant was analyzed by gas chromatography (Agilent Technologies, Santa Clara, CA) using a glass column packed with 80/120 by using Carbopack B-DA/4 % Carbowax stationary phase, helium as a carrier gas under the flame ionization detector (Apajalahti et al., 2019). Lactic acid and volatile fatty acids (acetic acid, propionic

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

	Starter (1 to 10 d of age)	Grower (11 to 24 d of age)	Finisher (25 to 42 d of age)
Ingredients, % as fed, unless otherwise stated			
Corn	40.12	41.07	40.83
Soybean meal (50 % CP)	22.88	20.49	16.38
Wheat	20.00	20.00	20.00
Full Fat Soya	7.00	8.00	10.00
Rice bran	3.00	3.00	5.00
Rapeseed meal	2.50	2.50	2.50
Limestone (fine)	1.41	1.45	1.35
Dicalcium phosphate	0.90	0.71	0.41
Maize gluten meal	0.56	0.00	0.00
Soybean oil	0.47	1.65	2.59
DL-methionine	0.29	0.27	0.22
L-Lys HCl	0.28	0.25	0.20
Salt	0.25	0.20	0.15
Vitamin-mineral premix[1]	0.15	0.15	0.15
Sodium bicarbonate	0.09	0.13	0.13
L-Thr	0.09	0.09	0.04
Choline Chloride	0.02	0.03	0.04
Phytase (750 FTU/kg)[2]	0.01	0.01	0.01
Calculated nutrients, % unless otherwise stated			
AME, kcal/kg	2,950	3,050	3,150
Dry matter	88.91	88.96	88.97
Ether extract	4.16	5.61	7.45
Crude protein	22.21	21.31	20.68
Crude fibre	3.01	2.99	3.20
Ash	6.02	5.75	5.36
Digestible Lys	1.22	1.15	1.04
Digestible Met	0.61	0.57	0.51
Digestible Cys	0.33	0.32	0.32
Digestible Met+Cys	0.94	0.89	0.83
Digestible Thr	0.81	0.77	0.69
Digestible Trp	0.22	0.21	0.21
Digestible Leu	1.60	1.47	1.33
Digestible Iso	0.82	0.78	0.74
Digestible Val	0.92	0.87	0.83
Digestible Arg	1.27	1.21	1.15
Calcium	0.90	0.86	0.76
Total phosphorus	0.79	0.74	0.70
Available phosphorus	0.45	0.42	0.38
Sodium	0.19	0.18	0.16
Chlorine	0.24	0.21	0.17
Potassium	0.91	0.88	0.85
Choline	0.15	0.15	0.15

[1] Supplied per kilogram of diet: 13.5 MIU Vitamin A, 4.5 MIU Vitamin D3, 60 g Vitamin E, 3.5 g Vitamin K3, 3.5 g Thiamin, 8 g Riboflavin, 60 g Niacin, 14.5 g Pantothenic acid, 3.5 g Pyridoxine, 0.145 g Biotin, 2.25 g Folic acid, 0.02 g Vitamin B12, 60 g manganese, 60 g zinc, 30 g iron, 10 g copper, 0.6 g selenium, 4 g iodine, 1 g chromium.

[2] A novel consensus bacterial 6-phytase variant produced in *Trichoderma reesei* and supplied by Danisco Animal Nutrition & Health (IFF), Oegstgeest, The Netherlands.

acid, isobutyric acid, butyric acid, 2-methylbutyric acid, isovaleric acid and valeric acid) were derivatized to the respective phenyl esters by using phenyl chloroformate reagent. Resulting esters were analyzed by Agilent GC-FID (Agilent, Santa Clara, CA). Matrix-matched internal calibration standards were used for detection of acetic acid and butyric acid.

Bacterial counts in cecal and ileal digesta samples were determined by culture dependent techniques. The tubes were stored at 4 °C and, within 48 h, were cultured in specific media for *Escherichia coli* (Luria Bertani Agar, Miller or Miller Luria Bertani Agar, M1151, Hi Media Laboratories, Mumbai, India), and *Lactobacillus* spp. (*Lactobacillus* MRS Agar, M641, Hi Media Laboratories, Mumbai, India). All the cultures were incubated at 37 °C for 36 h to develop visible colonies. *Lactobacillus* culture was performed in the presence of 5 % carbon dioxide. The number of visible colonies was enumerated manually under a colony counter, and the values were expressed in $\log_{10}$ CFU per g of ileal and caecal digesta (Muthusamy et al., 2011).

All diets were analysed for dry matter (AOAC 934.01), organic matter (AOAC 942.05), moisture (AOAC 930.15-2012), crude protein (EN ISO 9001:2008), ether extract (DIN EN ISO 9001), total ash (AOAC 942.05-2012), crude fibre (AOAC 920.102), calcium (AOAC 927.02-1990) and phosphorus [AOAC 965.17-1966 (1996)].

Statistical analysis

A pen was the experimental unit in all data analyses. Differences between treatment groups were determined using a linear model function, lm() in R (version 4.1.2). Treatment was included as a fixed effect. Residuals were checked for normality and Levene's test was used to assess the homogeneity of variances. Tukey's HSD test was used to separate pairs of means where the linear model showed a significant effect of treatment. A P value of > 0.05 was considered statistically significant. P-value between 0.1 and 0.05 was considered a tendency.

Results

Diet analysis

Analyzed concentrations of all proximate nutrients and Ca in the diets were close to (within 15 % of) formulated values (Table 3). Analyzed concentrations of total P were close to formulated levels in starter and grower diets but moderately below the formulated (expected) level (-18.6 %) in the finisher diet.

Growth performance

The effects of treatment on growth performance are shown in Table 4. Initial BW at d 1 was higher ($P < 0.05$) in CC compared to NC birds but the effect size was small (0.7 %). During 1 to 10 d of age, FI, BWG and d 10 BW were all lower in CC vs. NC birds (BW by 14 g/bird or 4.9 %; $P < 0.05$), whereas FCR was unaffected. These response measures were also lower in CC+Probiotic vs. NC birds (BW by 12 g/bird or 4.2 %; $P < 0.05$), similar as the responses observed in CC birds.

During 11 to 24 d of age, FI, BWG and d 24 BW were again lower in CC vs. NC birds, to a greater extent than during starter phase (BW reduced by 141 g/bird or 10.8 %; $P < 0.05$). The addition of the probiotic in CC+Probiotic increased BW at 24 d of age, BWG and FI compared to birds in treatment CC (BW was 49 g/bird or 4.2 % higher in CC+Probiotic vs the CC at 24 d of age; $P < 0.05$). The responses did not reach the levels achieved by NC birds ($P < 0.05$; Table 4). The FCR during 11 to 24 d of age was also higher in CC+Probiotic birds vs. NC birds, similar as the CC birds.

The average final (d 42) BW of NC birds was 2,640 g. During 25 to 35 and 36 to 42 d of age, BW at the end of each phase was lower in CC vs. NC birds. BW at 42 d of age was higher in CC+Probiotic compared to the CC, similar as the NC whereas at 35 d of age BW of CC+Probiotic remained below that of the NC, equivalent to CC. Other performance response measures did not differ among treatments during or at the end of these phases.

Table 3
Analyzed nutrient concentrations (as-fed basis) in the diet.

	Starter (0 to 10 d of age)	Grower (11 to 24 d of age)	Finisher (25 to 42 d of age)
Dry matter	89.44	89.12	89.24
Organic matter	95.08	96.51	95.41
Crude protein	21.69	20.66	19.88
Ether extract	4.08	6.17	7.89
Moisture	10.56	10.88	10.76
Ash	4.92	3.49	4.59
Crude fiber	3.47	3.31	3.15
Phosphorus	0.70	0.63	0.59
Calcium	0.90	0.79	0.73

Table 4
Effect of treatment on growth performance, by phase and cumulatively.

	NC	CC	CC+Probiotic	SEM	*P*-value
Initial BW	43.6^{b}	43.9^{a}	43.7^{ab}	0.082	0.025
Starter, 0 to 10 d of age					
BW, 10 d of age, g/bird	284^{a}	270^{b}	272^{b}	1.81	<0.001
BWG, g/bird	240^{a}	226^{b}	228^{b}	1.81	<0.001
FI, g/bird	262^{a}	252^{b}	251^{b}	1.96	<0.001
FCR, g:g	1.092	1.112	1.101	0.008	0.223
Mortality, %	0.000	0.000	0.000	NA	NA
Grower, 11 to 24 d of age					
BW, 24 d of age, g/bird	1,305^{a}	1,164^{c}	1,213^{b}	10.56	<0.001
BWG, g/bird	1,021^{a}	894^{c}	941^{b}	10.81	<0.001
FI, g/bird	1,300^{a}	1,189^{c}	1,249^{b}	14.24	<0.001
FCR, g:g	1.275^{b}	1.349^{a}	1.335^{a}	0.010	<0.001
Mortality, %	0.334	2.667	2.000	0.767	0.102
Finisher 1, 25 to 35 d of age					
BW, 35 d of age, g/bird	2,019^{a}	1,868^{b}	1,926^{b}	25.11	0.001
BWG, g/bird	715	705	713	27.03	0.962
FI, g/bird	1,519	1,460	1,458	29.63	0.263
FCR, g:g	2.244	2.209	2.217	0.064	0.918
Mortality, %	2.917	3.875	3.025	1.202	0.827
Finisher 2, 36 to 42 d of age					
BW, 42 d of age, g/bird	2,640^{a}	2,479^{b}	2,547^{ab}	33.15	0.006
BWG, g/bird	621	611	621	25.35	0.948
FI, g/bird	1,198	1,240	1,214	33.84	0.671
FCR, g:g	1.975	2.101	1.984	0.055	0.211
Mortality, %	2.158	2.625	1.742	0.919	0.795
0 to 24 d of age					
BWG, g/bird	1,261^{a}	1,120^{c}	1,169^{b}	10.57	<0.001
FI, g/bird	1562^{a}	1440^{c}	1500^{b}	14.82	<0.001
FCR, g:g	1.240^{b}	1.300^{a}	1.289^{a}	0.008	<0.001
Mortality, %	0.333	2.667	2.000	0.768	0.102
0 to 35 d of age					
BWG, g/bird	1,976^{a}	1,825^{b}	1,882^{b}	25.13	0.001
FI, g/bird	3,082^{a}	2,900^{b}	2,958^{b}	32.62	0.001
FCR, g:g	1.590	1.633	1.617	0.016	0.180
Mortality, %	2.667	5.667	4.333	1.214	0.231
0 to 42 d of age					
BWG, g/bird	2,597^{a}	2,435^{b}	2,503^{ab}	33.14	0.006
FI, g/bird	4,280	4,140	4,172	56.93	0.206
FCR, g:g	1.679^{b}	1.742^{a}	1.707^{ab}	0.016	0.024
Mortality, %	4.33	7.67	5.667	1.251	0.182

^{a,b}Means within a row bearing different superscript letters are significantly different at $P < 0.05$.
*Standard error of the mean.
NC, non-challenged control; CC, challenged control; CC+probiotic, challenged control + dual strain probiotic; SEM, standard error of the mean.

For the overall period from 0 to 24 d of age, BWG was lower d in CC vs. NC birds (by 141 g/bird or 11.2 %; $P < 0.05$) and higher in CC+Probiotic birds vs the CC (by 40 g/bird or 4.4 %; $P < 0.05$). FCR was higher in CC vs. NC birds ($P < 0.05$) but not in CC+Probiotic birds vs. NC bird. For the overall period from 0 to 35 d of age, BWG was also lower d in CC vs. NC birds (by 151 g/bird or 7.6 %; $P < 0.05$), but CC+Probiotic birds did not differ from CC. FCR did not significantly differ between treatments. For the overall period from 0 to 42 d of age, BWG was lower in CC vs. NC birds (by 162 g/bird or 6.2 %; $P < 0.05$) and higher in CC+Probiotic birds, to a level similar as the control (NC) but not significantly above the CC treatment. Overall (d 0 to 42) FCR was increased in CC vs. NC birds ($P < 0.05$) but not in CC+Probiotic birds vs. NC birds. Mortality was unaffected by treatment during any individual phase or overall.

Ileal and cecal bacterial populations

The effect of treatment on ileal and cecal bacterial concentrations (Log_{10} CFU/g of digesta) is shown in Table 5. At 24 d of age, ileal lactic acid bacteria concentrations were reduced in CC vs. NC birds (by 0.44 Log_{10} CFU/g, or 6.4 %; $P < 0.05$) whereas cecal concentrations of *E. coli* were increased (by 0.76 Log_{10} CFU/g or 12.1 %; $P < 0.05$). The addition of the probiotic in CC+Probiotic increased ileal lactic acid bacteria concentrations above the level observed in CC birds ($P < 0.05$), equivalent to NC, and reduced ileal *C. perfringens* concentrations ($P < 0.05$) to a level equivalent to NC. At 42 d of age, ileal lactic acid bacteria concentrations were reduced in both CC and CC+Probiotic treatments vs. NC ($P < 0.05$).

Cecal short chain fatty acids composition

The content of individual and total SCFA in the cecal digesta, by treatment, at 24 d of age, is presented in Table 6. For some individual SCFA there was wide variation in the numerical value of the means across treatments (e.g. 2.55 to 9.70 for lactic acid; SEM 3.47) but there were no statistically significant differences among treatments.

Immune defense measures and dysbacteriosis scores

The effect of treatment on dysbacteriosis scores, intestinal mucosal sIgA and serum AAGP concentrations is presented in Table 7. There was no effect of treatment on dysbacteriosis scores. Mucosal secretory IgA levels tended to be higher in CC+Probiotic birds than CC or NC birds ($P = 0.052$) at 24 d of age and were higher ($P < 0.05$) in CC+Probiotic birds than CC or NC birds at 42 d of age (by 16.8 µg/ml or 20.0 % vs. NC). Concentrations of AAGP were increased in CC vs. NC birds at both 24 and 42 d of age (+34.2 % and +26.9 %, respectively, $P < 0.05$). At 24 d of age, the addition of the probiotic in CC+probiotic resulted in AAGP levels that were not significantly different from those achieved by the NC but which also did not differ from those achieved by the CC, whereas at 42 d of age AAGP levels in CC+Probiotic birds were reduced compared to those achieved by CC birds (-146 µg/ml or 33.0 %), equivalent to NC.

Table 6
Effect of treatment on cecal short chain fatty acids (SCFA) concentrations at 24 d of age.

Compound, mmol/kg	NC	CC	CC+Probiotic	SEM	P-value
Volatile fatty acids (total)	140	124	126	10.44	0.536
Valeric acid	2.12	1.79	1.70	0.255	0.474
SCFA (total)	144	125	135	11.72	0.538
Propionic acid	24.1	27.7	28.3	3.02	0.581
Lactic acid	4.27	2.55	9.70	3.47	0.347
Isovaleric acid	0.85	1.23	1.13	0.223	0.477
Isobutyric acid	1.22	1.39	1.30	0.215	0.870
Branch chain fatty acids (total)	2.83	3.61	3.33	0.575	0.648
Butyric acid	16.8	13.5	12.2	1.95	0.241
Acetic acid	93.9	77.4	80.9	6.71	0.220
2-methylbutyric acid	0.76	0.99	0.90	0.153	0.596

NC, non-challenged control; CC, challenged control; CC+probiotic, challenged control + dual strain probiotic; SEM, standard error of the mean

Table 7
Effect of treatment on dysbacteriosis scoring, secretory IgA (sIgA) and serum alpha-1-acid-glycoprotein (AAGP) concentrations.

	NC	CC	CC+Probiotic	SEM	P-value
Dysbacteriosis scores					
24 d of age	0.53	1.00	0.47	0.194	0.140
42 d of age	1.30	1.33	1.00	0.280	0.659
sIgA, µg/ml					
24 d of age	73.7	73.2	82.8	3.01	0.052
42 d of age	84.2[b]	87.4[b]	101[a]	2.42	<0.001
AAGP, µg/ml					
24 d of age	325[b]	436[a]	360[ab]	23.15	0.006
42 d of age	349[b]	443[a]	297[b]	15.87	<0.001

NC, non-challenged control; CC, challenged control; CC+Probiotic, challenged control + dual strain probiotic; SEM, standard error of the mean

[a] Means within a row bearing different superscript letters are significantly different at $P < 0.05$.

[b] Means within a row bearing different superscript letters are significantly different at $P < 0.05$.

Discussion

The need for effective alternatives to AGPs in poultry production is well recognized and a major current topic of research (Abd El-Hack et al., 2021; Attia et al., 2023; Rafiq et al., 2021). Demonstrating the efficacy of potential probiotic strains in conditions simulating the field environment where multiple pathogens may be encountered even when biosecurity and hygiene measures are in place, is a key part of that research. In the present study, birds were challenged consecutively with

Table 5
Effect of treatment on ileal and cecal digesta bacterial populations (Log_{10} CFU/g).

		24 d of age					42 d of age				
Gut region	Species	NC	CC	CC+Probiotic	SEM	*P*-value	NC	CC	CC+Probiotic	SEM	*P*-value
Ileum	Lactic acid bacteria	6.92[ab]	6.48[b]	6.99[a]	0.140	0.032	6.35[a]	5.38[b]	5.48[b]	0.156	0.000
Ileum	*Clostridium perfringens*	4.75[ab]	5.20[a]	4.43[b]	0.161	0.006	4.64[ab]	4.98[a]	4.45[b]	0.145	0.044
Ileum	*Escherichia coli*	5.55	5.94	5.84	0.290	0.618	4.66	4.88	4.48	0.232	0.474
Cecum	Lactic acid bacteria	6.84	6.58	6.84	0.191	0.543	6.67	6.44	6.95	0.165	0.106
Cecum	*Clostridium perfringens*	4.70	5.16	4.86	0.168	0.159	4.68	4.97	4.53	0.164	0.168
Cecum	*Escherichia coli*	6.29[b]	7.05[a]	6.68[ab]	0.169	0.012	6.17	6.28	5.98	0.225	0.637

NC, non-challenged control; CC, challenged control; CC+probiotic, challenged control + dual strain probiotic; CFU, colony forming units; SEM, standard error of the mean

[a] Means within a row bearing different superscript letters are significantly different at $P < 0.05$.

[b] Means within a row bearing different superscript letters are significantly different at $P < 0.05$.

3 common poultry pathogens: APEC, Eimeria and *C. perfringens*. These were administered at levels designed to generate a subclinical challenge resulting in dysbacteriosis without significant mortality, so that the effects of the probiotic could be assessed both at bird and tissue level. Hence, the dose of APEC given on d 7 (10^8 CFU/bird) was an order of magnitude below that (10^9 CFU/bird) which causes significant (>5 %) 7-d mortality among chicks (Helmy et al., 2023). Even at this level, the challenge was sufficient to significantly reduce appetite (FI) and growth (BW and BWG) within 3 days of administration (as measured during 0 to 7 d of age). This highlights the known fast-acting nature of APEC infections in commercial broilers in the field (Swelum et al., 2021). By the end of grower phase, when CC birds had received all 3 pathogen challenges, birds were consuming 8.5 % less feed and had attained 10.8 % lower BW than unchallenged birds, indicating a marked negative effect of the challenge on growth. These results are consistent with other studies that have shown marked negative effects of oral challenge with APEC, Eimeria or *C. perfringens* on weight gain 7 to 14 days post-infection, with or without clinical signs of disease (Akerele et al., 2022; Leung et al., 2019; Xu et al., 2024). The effects of the challenge on BW were cumulative, equating to a 6.1 % reduction over the entire growth cycle (0 to 42 d of age). They were accompanied by modulations in the ileal and cecal microbiome (increased cecal *E. coli* and reduced ileal lactic acid bacteria concentrations) and by increased serum levels of the acute phase protein AAGP which is produced in the liver and is a biomarker of inflammation and subclinical disease (Chamanza et al., 2019; Fournier et al., 2000). However, they were not accompanied by any increase in dysbacteriosis scores. This highlights the very real effect that a subclinical pathogen infection can have on production outcomes that would not necessarily be picked up by routine dysbacteriosis scoring designed to monitor infection status in the field.

The dual strain probiotic improved the growth performance (FI, BWG, and BW) of challenged birds during grower phase and, by the end of the study, had improved d 42 BW and overall FCR up to levels that were not different from those achieved by the non-challenged control treatment. It should be noted, however, that the final (d 42) BW of non-challenged birds was below the current performance objective for Ross 308 male broilers (by 582 g, Aviagen Inc., 2022). The relative high temperature (averaging at 29.4 °C) and humidity (averaging at 78.3 %) during the growing conditions, respectively 6.8 °C and 8.3 % higher compared to breeder recommendations, could have caused heat stress. Heat stress is known to reduce broiler growth performance (Andretta et al., 2021). Under microbially unchallenged conditions, the current combination of probiotic strains is known to reduce mortality caused by heat stress (van der Klein et al., 2024). Notwithstanding this, the performance results demonstrate the *in vivo* efficacy of the probiotic in compensating for the adverse effects of the 3-step subclinical pathogen challenge on performance over an entire growth cycle, in the tested setting.

Comparison of the performance results with studies that have used other probiotic strains is not informative because bird responses to probiotics, especially in conditions of challenge, are highly strain and disease specific (Yosi and Metzler-Zebeli, 2023). However, the observation of improved BW in challenged birds supplemented with the dual-strain probiotic compared to challenged unsupplemented birds (during all phases; +2.7 % at 42 d of age) is broadly consistent with the findings reported in two other publications that employed the same dual-strain probiotic, albeit with a lower effect size. Van der Klein et al. (2023) reported a 9.4 % improvement in final (d 42) BW in broilers supplemented daily (via the waterline) with the probiotic following oral challenge with *Eimeria* (10 x overdose of COCCIVAC®-B52, Merck Animal Health, Rahway, NJ) followed by netB+ *C. perfringens* (1×10^8 CFU/bird) to induce a mild NE infection, and van der Klein and Gibbs (2024) reported an 7.8 % improvement in BW at d 42 (on average across 3 studies) in broilers supplemented daily with the probiotic following *Eimeria* and *C. perfringens* challenge with the same dosing protocol. These two studies also reported improved mortality corrected FCR up to d 42 in the probiotic-supplemented birds (+4.7 % and +3.0 % in van der Klein et al. (2023) and van der Klein and Gibbs (2024), respectively). No significant difference in overall FCR between CC and CC+Probiotic birds was evident in the present study although the numerical values of the treatment means suggest that the response in CC+Probiotic birds was intermediate between that of NC and CC birds. Intestinal NE lesion scores were reduced in probiotic-supplemented challenged birds at 28 d of age (vs. unsupplemented challenged birds) in both of the previous studies (van der Klein et al., 2023; van der Klein and Gibbs, 2024). This was not replicated in the present study, although again the numerical values of the treatment means suggest that the response in CC+Probiotic birds was intermediate between that of CC and NC birds. The absence of any statistically significant effect on dysbacteriosis scores in the present study may indicate that the dysbacteriosis challenge induced was milder, which could be due to the different commercial broiler breed used in the present compared with the earlier studies (Ross 308 vs. Cobb 500) or the difference in the dosage and virulence of the *Eimeria* challenge compared to van der Klein et al. (2023).

Recent *in vitro* studies have highlighted several potential modes of action of the dual-strain probiotic in reducing the pathogenesis of NE in broilers (Kadekar et al., 2024). The authors tested the effect of CFS from *L. acidophilus* AG01 separately from *B. animalis* subsp. *lactis* AG02 on the cytotoxicity, cell adhesion, and permeability effects of *C. perfringens* strains against chicken intestinal epithelial (CHIC-8E11) cells. They reported that *B. animalis* CFS reduced the negative effects of *C. perfringens* CFS on cell permeability whereas both strains reduced its cytotoxic effects and numerically reduced pathogen cell adhesion. It is plausible that such effects could have contributed to the observed amelioration of the negative effects of pathogen challenge on bird growth performance by the probiotic in the present study. The increase in cecal *E. coli* and ileal *C. perfringens* concentrations and reduction in ileal lactic acid bacteria concentrations observed in CC vs. NC birds, that was not evident in CC+Probiotic birds, does suggest that the probiotic strains (or compounds secreted by them) reduced pathogen adhesion and retention in the cecum. However, whether this was via a cytotoxic effect, competitive exclusion, or a different mechanism is unknown. Li et al. (2018a) showed that probiotic *L. acidophilus* supplemented to *C. perfringens*-challenged broilers reduced the negative effects of NE challenge on performance (ADG, FCR, and mortality) whilst concurrently reducing ileal *E. coli* populations and increasing intestinal lactic acid bacteria populations. This implicates an improved gut microbiome in the beneficial effects of probiotic *Lactobacillus* on broilers, which is consistent with the present study findings.

In the wider literature, there are other mechanisms via which probiotics can reduce the effects of NE challenge in broilers, in addition to those already mentioned above. These include regulation of the immune system (including increasing the secretion of intestinal immunoglobulins and enzymes, reduction in pro-inflammatory cytokines, and increase in anti-inflammatory cytokine production) and enhancing the immune system functioning by modulating the Toll-like receptor (TLR)/NF-$_K$ signaling pathway (Obianwuna et al., 2023). In the present study, intestinal mucosal sIgA production was increased by the probiotic in CC+Probiotic birds relative to NC and CC birds at 42 d of age. Secretory IgA is an antibody produced by plasma cells situated in the lamina propria just below the epithelium. Its secretion is upregulated immediately following the first signs of an infection and it plays a crucial role in maintaining epithelial barrier integrity and mucosal homeostasis which in turn influence the gut microbiota and the induction of systemic immunity (Mantis et al., 2011). In particular, sIgA prevents the attachment of bacteria to the gut epithelium thereby preventing a key step in disease pathogenesis (Mcpherson et al., 2008). Certain probiotics including strains of *Lactobacillus* have previously been shown to increase secretion of ileal and cecal IgA in broilers which serves to enhance the immune response and host resistance to intestinal damage (Gao et al., 2022; Gyawali et al., 2022). The increased secretion of muscoal sIgA in birds supplemented with the probiotic is therefore suggestive of an effective

immune response having been raised following the pathogen challenge. Given its key role in the first line of host-defence against pathogens in the gut, the reduced serum AAGP concentrations at d 42 in CC+Probiotic birds vs. CC birds (equivalent to NC birds) are also consistent with the notion of the probiotic having beneficially enhanced the immune response leading to reduced inflammation. Clearly, sIgA and AAGP are just two of many chemical mediators involved in the immune response to pathogens. Other key mediators such as cytokines were not measured in the current study, but beneficial effects of *L. acidophilus* on the expression of cytokines and other secreted compounds involved in the immune system following pathogen challenge have been reported by other studies, including reduced expression of IL-8, IL-1B and iNOS following challenge with *E. coli* (Wu et al., 2021).

Short chain fatty acids are important metabolites produced by cecal microbes, some of which (e.g. butyric acid) serve as key energy sources for absorptive epithelial cells (enterocytes) and have beneficial anti-inflammatory effects on immune endothelial cells (Li et al., 2018b). *L. acidophilus* AG01 produces lactate as a primary end-product of fermentation and *B. animalis* produces both acetate and lactate (De Vuyst and Leroy, 2011). As both of these SCFA can be converted into butyrate by other residing gut microbes (De Vuyst and Leroy, 2011; Rivière et al., 2016) these probiotic strains have the capacity, in theory, to enhance the availability of beneficial butyrate in the gut. Although no significant effect was evident in the present results, the numerical values of the means obtained for cecal lactic acid content may suggest that the probiotics increased its production in the cecum. Further research is needed to confirm an effect, perhaps conducted at a later timepoint (42 d of age) which would enable the detection of cumulative effects. *In vitro*, probiotic *Lactobacillus* strains have been shown to increase the production of lactate, propionate and butyrate in a batch culture system initiated with cecal digesta samples, over 24 h (Meimandipour et al., 2009; Onrust et al., 2015).

In conclusion, daily waterline administration of a dual-strain probiotic comprising *L. acidophilus* AG01 and *B. animalis* subsp. lactis AG02 to broilers challenged sequentially and subclinically with APEC, *Eimeria* and *C. perfringens*, improved growth performance, reduced the intestinal host systemic inflammatory marker AAGP, increased the ileal mucosal immune defense marker sIgA, and beneficially altered the intestinal microbial composition. These results require verification under field conditions but suggest potential for the dual-strain probiotic to be used to support gut health, prevent dysbacteriosis and maintain production outcomes in commercial broiler flocks without AGPs.

Disclosures

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Declaration of competing interest

All authors declare that they have no conflicts of interest.

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Supplementary materials

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