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The effect of *Candida famata* and *Lactobacillus plantarum* on the number of coliforms and the antibiotic resistance and virulence of *Escherichia coli* in the gut of broilers

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ABSTRACT

This study was undertaken to determine the effect of a yeast (*Candida famata*) and a bacterium (*Lactobacillus plantarum*), administered alone or in combination in the drinking water, on the population of yeast, *Lactobacillus* sp. and coliforms, and the prevalence of antimicrobial resistance (**AMR**) and virulence genes in *Escherichia coli* (***E. coli***) isolated from digesta samples taken throughout the life of broiler chickens. Male (Ross 308) day-old chicks (220) were used. *C. famata* (isolated from a chicken) and *L. plantarum* (isolated from a pig) were administered via the drinking water. Water was provided either untreated or with *C. famata* (**CF**; 10^8/ml), *L. plantarum* (**LP**; 10^5–10^8/ml), or a combination of CF and LP (10^6–10^8/ml) in water hoppers on 2 days each week for 35 days. Administering probiotics did not affect the growth performance in broiler chickens. No significant interactions were observed between main effects, and neither CF nor LP had any effect on the population size of *Lactobacillus* sp. or coliforms. The administration of *C. famata* increased the population density of yeasts in the small intestine at these ages. The population density of coliforms, *Lactobacillus* sp. and yeast decreased with age ($P < 0.001$). There was no significant effect of probiotics on the prevalence of phenotypic AMR and virulence genes in these studies. The prevalence of *E. coli* that was resistant to ampicillin and tetracycline, as well as carrying $\geq$3 virulence-associated genes, was greatest at the end of the starter phase (around 8 days old), before declining through the grower and finisher phases. There was only limited evidence that administering either CF or LP affected either the AMR or the virulence of *E. coli* in the bird. However, tetracycline resistance in *E. coli* was associated ($P < 0.001$, $P < 0.01$, $P < 0.05$, and $P < 0.05$) with the carriage of the iron uptake systems of *E. coli* D, iron-repressible protein, increased serum survival and temperature-sensitive haemagglutinin genes respectively, suggesting that the accumulation of iron and the genetic element conferring tetracycline resistance may be intertwined.

Implications

Broiler chickens are particularly susceptible to infection by pathogenic and antimicrobial-resistant coliforms in their starter phase. Although this may be overcome naturally as the birds age (and does not appear to be assisted by the administration of either yeast or *Lactobacillus* probiotics), this does leave a window of vulnerability in early life. It is possible that increasing the supply of iron in the gut lumen may encourage the evolution of more benign (and not antimicrobial resistant) coliforms.

Introduction

Antibiotics have been used to improve animal performance and prevent disease over several decades. However, the EU banned the use of antibiotics as growth promoters in 2006 because of the increase in antimicrobial resistance (**AMR**) arising from the misuse and overuse of antimicrobial agents (Castanon, 2007). Antibiotic use in UK broiler production has decreased substantially, but the growing prevalence of AMR by bacteria is a threat to both human and animal health (Dolejska et al., 2012). Antimicrobial-resistant bacteria from animal products can be transferred, directly or indirectly, into the human food chain and thereby threaten the human consumer (Landers et al., 2012). *Escherichia coli* (***E. coli***), found in the intestinal tracts of all animals and birds, has been widely used

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as a sentinel species for the determination of AMR (Jang et al., 2017; Stromberg et al., 2017). A high prevalence of antimicrobial-resistant *E. coli* has been observed in day-old chicks in Austria (Roth et al., 2017). Many researchers have demonstrated the presence of various antibiotic-resistant genes in *E. coli* isolated from both caecal digesta and animal meats (Miles et al., 2006; Diarra et al., 2007; Smith et al., 2007; Persoons et al., 2010).

In addition to the carriage of AMR genes, some *E. coli* strains may also carry several virulence-associated genes which increase their pathogenicity and lead to disease. In poultry, the most common disease associated with *E. coli* is colibacillosis, but extraintestinal disease would include septicaemia, pericarditis, airsacculitis and perihepatitis (Delicato et al., 2003; Dziva and Stevens, 2008). The carriage of these virulence genes may increase the selection advantage of the strain; in *E. coli* known virulence genes code for the products adhesins, invasins, toxins and iron acquisition systems (siderophores) (Paixao et al., 2016).

Dietary interventions may alter the composition of the gut microbiome and the evolution of strains of *E. coli* (Olnood et al., 2015). In doing this, the proportion of AMR bacteria in the bird and poultry meats may also be altered. Probiotics such as *Lactobacillus* sp. and yeasts may help prevent the proliferation of pathogenic bacteria and potentially AMR in the gut through mechanisms such as competitive exclusion, neutralisation of toxins, bactericidal activity or enhanced immune competence (Kabir, 2009; Fijan, 2014). The aim of this experiment was therefore to determine the effect of a yeast (*Candida famata*) and a bacterium (*Lactobacillus plantarum*), administered in the drinking water, on the prevalence of phenotypic AMR status and carriage of virulence genes by *E. coli* throughout the life of broiler chickens. Preliminary results from this experiment were presented in abstract form by Lee et al. (2020).

Material and methods

Experimental design, birds and diets

A total of 220 chicks (day old, male Ross 308) were used in a 35 days feeding trial. All chicks were weighed on arrival, wing tagged, blocked by live weight, and then randomly placed in one of four brooder rings and fed a common starter diet (Table 1) until the birds were 14 days of age. No antibiotics were administered during the experimental period. *Candida famata* (isolated from a chicken) and *Lactobacillus plantarum* (isolated from a pig; University of Surrey) were administered via the drinking water. Stock cultures of each isolate in *C. famata* and *L. plantarum* were incubated overnight in Yeast Extract Peptone Dextrose (**YEPD**) broth or De Man, Rogosa and Sharpe (**MRS**) broth. Population density was then estimated by serial dilution and measurement of the optical density of the incubation medium at 600 nm. Cultures were then concentrated by centrifugation (3 354*g*, 5 min) and washed with sterile distilled water. An appropriate volume of the culture was then added to a measured volume of the drinking water in the hopper to attain the desired final concentration of probiotic. Water was therefore provided either untreated (Control) or with a preparation of *C. famata* (**CF**; 10^7–10^9 cfu/ml water offered) or *L. plantarum* (**LP**; 10^5–10^8/cfu/ml water offered), or a combination of CF and LP (**CFLP**; total 10^6–10^8 cfu/ml water offered) in water hoppers 2 days each week (days 1, 4, 8, 11, 14, 18, 22, 25, 29 and 32). Water hoppers were replenished with fresh (untreated) water when required.

On day 14, all birds were weighed, and blocks of birds were allocated (six birds per pen, all birds originating from the same brooder ring) to one of 24 pens, with six replicate pens per probiotic treatment. All birds were then fed a common grower/finisher diet (Table 1) in the form of a mash manufactured by Target Feeds (Whitchurch, UK). Samples of each diet were analysed for starch, sugars, oil, CP and amino acids (Sciantec, York, UK). Titanium dioxide (1 mg/g) was incorporated in the diets as an indigestible marker, and feed samples (one per pen) were analysed for titanium dioxide (Short et al., 1996). Lighting was via incandescent lights with 23 h continuous light per 24 h period for the first 7 days, followed by 18 h continuous light (6 h darkness) in each 24 h period. The birds were brooded according to the breeder's recommendations using infrared lights to provide supplementary heat when necessary.

Table 1
The formulation and chemical composition (as-fed basis) of the chickens' diets.

	Starter (0–14 days)	Grower/Finisher (15–36 days)
Formulation (g/kg)		
Barley	40	40
Wheat	500	550
Soya bean meal	320	265
Rapeseed meal	42	42
Soya bean oil	50	65
L-lysine HCl	4	1
DL-methionine	3.45	2.42
L-threonine	2.05	2.02
Sodium bicarbonate	2.5	2.5
Salt	2	2.5
Limestone	11	8.56
Poultry vitamins/minerals[1]	2	2
Dicalcium phosphate	20	16
Titanium dioxide	1	1
Chemical composition (g/kg)		
ME (MJ/kg)	9.7	9.9
CP	219.0	210.0
Total Starch	244.0	265.0
Oil	47.4	49.1
Sugar as Sucrose	46.8	43.3
Cystine	3.8	3.6
Aspartate	23.1	19.6
Methionine	8.9	6.8
Threonine	11.5	10.1
Serine	11.5	10.4
Glutamate	45.9	43.1
Glycine	9.6	8.7
Alanine	9.9	8.9
Valine	11.0	10.0
Iso-Leucine	9.9	8.9
Leucine	17.0	15.2
Tyrosine	4.8	4.3
Phenylalanine	11.2	10.0
Proline	16.3	15.5
Histidine	5.8	5.3
Lysine	16.1	11.3
Arginine	15.1	13.5

Abbreviation: ME = metabolisable energy.
[1]The vitamin/mineral premix supplied per kg of starter diets: vitamin A 6 750 IU, vitamin D_3 2 500 IU, vitamin E 50 mg, vitamin B_1 1.5 mg, vitamin B_2 5 mg, vitamin B_6 1.5 mg, vitamin B_{12} 0.015 mg, nicotinic acid 30 mg, pantothenic acid 7.5 mg, folic acid 0.75 mg, biotin 0.125 mg, choline chloride 125 mg, Fe 10 mg, Mn 50 mg, Cu 5 mg, Zn 40 mg, I 0.5 mg, Se 0.125 mg, Mo 0.25 mg.
The vitamin/mineral premix supplied per kg of grower/finisher diets: vitamin A 5 000 IU, vitamin D_3 2 500 IU, vitamin E 50 mg, vitamin B_1 1.5 mg, vitamin B_2 5 mg, vitamin B_6 1.5 mg, vitamin B_{12} 0.015 mg, nicotinic acid 30 mg, pantothenic acid 7.5 mg, folic acid 0.75 mg, biotin 0.125 mg, choline chloride 125 mg, Fe 10 mg, Mn 50 mg, Cu 5 mg, Zn 40 mg, I 0.5 mg, Se 0.125 mg, Mo 0.25 mg.

Bird performance, sample collection and determination of nutrient availability

Birds were weighed individually on days 1, 14 and 35. Mean BW increase was then calculated on a pen basis (g/d) during both the starter period and the grower/finisher period. Feed intake was determined and feed conversion ratio (**FCR**) calculated on a pen

basis. All birds were sacrificed by cervical dislocation on day 36, and one bird per pen was hung in a cold room (4 °C) for 24 h. It was weighed again, and the breast muscle was then removed (excluding skin and feathers). The breast muscle was weighed, and breast meat yield was then calculated (breast muscle weight/cold carcass weight).

On day 1, a total of 16 birds were randomly selected and sacrificed by cervical dislocation. The whole intestinal tract (from proximal duodenum to ileo-caecal junction) and the yolk sac were taken. These were analysed for the determination of the population size (cfu/g) of yeasts and bacteria (coliforms and *Lactobacilli*) and for the determination of AMR and virulence genes of coliforms. An isolate of *Lactobacilli* and yeast was randomly taken from the intestinal sample for the identification of *Lactobacilli* and yeast species.

On days 8 and 14, four birds from each brooder ring were sacrificed and contents from the duodenum, ileum and caecum were taken, weighed and cultured (in the same way as above) for the determination of yeast and bacterial population density. Coliforms were not counted in the small intestine samples as earlier (unpublished) observations had detected very low populations in these segments of the gut. One isolate of *E. coli* was taken from the cultured caecal sample of each bird for the determination of antibiotic resistance (all birds) and the presence of virulence genes (with the exception of CFLP birds, as no significant interaction between CF and LP had been observed). An isolate of *Lactobacilli* and yeast was randomly taken from the intestinal sample for the identification of *Lactobacilli* and yeast species.

After the birds were allocated to their grower/finisher pens, one bird from each pen was randomly selected and sacrificed on days 22, 29 and 35. Samples of digesta from the duodenum, ileum and caecum were again taken and the yeast and bacterial population densities were determined as before. An isolate of *E. coli* was taken from each caecal sample for the determination of antibiotic resistance and (with the exception of CFLP birds, for the same reason as before) the presence of virulence genes. An isolate of *Lactobacilli* and yeast was randomly taken from the cultured intestinal samples for the identification of *Lactobacilli* and yeast species. Samples of feed and a sample of ileal digesta taken from each bird on day 35 were also analysed for titanium dioxide (Short et al., 1996) by determining the absorbance of the acid digest at 410 nm using a spectrophotometer (CECIL CE 2040, 2000 series) for the determination of DM availability.

Counting of viable cells and preparation of bacterial and yeast isolates

The samples of whole intestine and yolk sac (from day-old chicks) and the samples of duodenal, ileal and caecal digesta (taken from the older birds) were weighed and serially diluted with phosphate-buffered saline (0.01 M) and 100 μl of the suspension spread on MRS agar (for the enumeration of *Lactobacillus* spp.), MacConkey agar (for the enumeration of coliforms, in caecal digesta samples only) and Saboraud dextrose agar (**SDA**, for the enumeration of yeast). Plates were incubated (37 °C, 48 h) in an anaerobic incubator (Whitly MG1000 anaerobic workstation) for the *Lactobacillus* spp. cultures and aerobically incubated overnight at 37 °C for the coliform and 30 °C for yeast cultures. Colonies were then counted (Gallenkamp Colony Counter CNW 325 030Y, UK) to determine the population sizes (cfu/g).

The preservation of *Lactobacillus* sp. isolates was done by transferring the selected isolates to a sterile Eppendorf tube containing 1 ml of 150 g/l glycerol in MRS broth. These cultures were incubated anaerobically at 37 °C for 18–24 h. After that, the *Lactobacillus* sp. suspension was stored at −80 °C. A single colony of *E. coli* and yeast was selected at random from each MacConkey and SDA plate respectively and transferred to a separate, sterile Eppendorf tube containing 500 μl of nutrient broth (*E. coli*) or YEPD broth (yeast). These cultures were aerobically incubated at 37 °C for 18–24 h. 100 μl of the resulting *E. coli* and yeast suspension was transferred to a cryobank tube (Mast group, Mastdisks, UK) and stored at −80 °C pending further analysis.

Characterisation of Lactobacilli and yeast isolates

Lactic acid bacteria (**LAB**) and yeast isolated from digesta samples were stored at −20 °C for the identification of strains. A total of 29 LAB strains were selected from the caecum for identification of the genus *Lactobacillus* and related genera by using the API 50 CHL test kit (bioMerieux UK Ltd, Basingstoke, UK). A total of 24 yeast isolates were randomly selected for identification of yeast strains by using the API system (API 20C AUX strips, bioMerieux UK Ltd, Basingstoke, UK). Identification of *Lactobacillus* and yeast strains was made using the api*web*™ identification software (https://apiweb.biomerieux.com) with database V5.1 (API, bioMerieux UK Ltd, Basingstoke, UK).

Antimicrobial resistance and extraction of genomic DNA of E. coli isolated from caecal samples

A sterile inoculation loop was inserted into the *E. coli* suspension of a cryobank tube and then streaked onto four MacConkey plates, each enriched with one of four antibiotics (ampicillin, nalidixic acid, tetracycline and chloramphenicol, each at a concentration of 20 μg/ml). Plates were then incubated (37 °C, 16 h) and growth of colonies on each plate was then determined. When growth was observed on plates containing antibiotics, it was deemed that the colony that had inoculated the plate was resistant to that antibiotic.

The genomic DNA from a total of 87 isolates of *E. coli* (taken from birds that received the control, CF or LP treatment, but not the CFLP treatment) were extracted to determine the presence of *E. coli* virulence factors. Genomic DNA was extracted using Puregene yeast/bact Kit B (Qiagen, Venlo, Netherlands) and standard protocols for fresh samples of Gram-negative bacterial cultures were followed, as described below and by Alkandari (2017). Isolated cultures of *E. coli* were streaked on nutrient agar plates and incubated at 37 °C overnight. Colonies of *E. coli* were then taken from these plates and transferred to a 1.5 ml sterile Eppendorf tube. These were then incubated at 80 °C for 5 min after adding 300 μl of cell lysis solution on the pellet. The tubes were put on ice for 20 min. 100 μl of protein precipitation solution was added and vortexed vigorously for 20 s at high speed. The mixture was centrifuged at 12 045*g* for 3 min. The supernatant was transferred to a clean 1.5 ml Eppendorf tube containing about 700 μl of 99.5% ethanol and mixed by gently inverting 50 times. The mixture was centrifuged at 12 045*g* for 1 min, and the supernatant was carefully discarded. A total of 300 μl of 70% ethanol was added to the DNA pellet and inverted several times. The mixture was centrifuged at 12 045*g* for 1 min, and again the supernatant was discarded and the pellet was allowed to air dry for 5 min. 100 μl of DNA hydration solution was added and the mixture was vortexed for 5 s, incubated at 65 °C for 1 h, followed by incubation overnight at room temperature with gentle shaking. This procedure was described by Alkandari (2017).

The purification of DNA was determined with a Nanodrop spectrophotometer (ND 2000, Nano Drop Technologies, USA). The quality of DNA was evaluated according to the method of Tonks (2018). The Nanodrop tube was cleaned by pipetting 1.5 μl of distilled water onto it and wiping with Whatman filter paper. The Nanodrop was blanked with 1.5 μl of distilled water. 1.5 μl DNA solution was added to the measuring stage and the 260:280 nm ratio was also recorded (1.82 ± 0.12). DNA stock solutions were stored

Table 2
The description of the virulence factors investigated and the primers used for their detection by PCR with *E. coli* isolated from broiler chickens.

Gene	Description	Primer sequence	Amplicon size (bp)	References
Adhesins				
*pap*C	P-fimbriae, Pyelonephritis associated pili	TGATATCACGCAGTCAGTAG CCGGCCATATTCACATA	501	(Sanger et al., 1977; Janßen et al., 2001)
tsh	Temperature-sensitive haemagglutinin	ACTATTCTCTGCAGGAAGT CTTCCGATGTTCTGAACG	824	(Dozois et al., 1992; Ewers et al., 2007)
Iron acquisition				
*iuc*D	Aerobactin synthesis, iron uptake chelate	ACAAAAAGTTCTATCGCTTC CCTGATCCAGATGATGCT	714	(Sanger et al., 1977; Janßen et al., 2001)
*irp*2	Iron-repressible protein associated with yersinabactin synthesis	AAGGATTCGCTGTTACCGGA AACTCCTGATACAGGTGG	413	(Dozois et al., 1992)
Serum survival				
iss	Increase serum survival	ATCACATAGGATTCTGCC CAGCGGAGTATAGATGCC	309	(Ewers et al., 2007)
Toxins				
*ast*A	Arginine succinyl transferase A (Enteroaggregative heat-stable toxin)	TGCCATCAACACAGTATATC TCAGGTCGCGAGTGACGG	116	(Franck et al., 1998)
*hly*A	α-haemolysin	GTCCATTGCCGATAAGTTT AAGTAATTTTTGCCGTGTTTT	1 177	(Janßen et al., 2001; Ewers et al., 2004)

at −20 °C pending the analysis of virulence genes by PCR (Alkandari, 2017).

Virulence genotyping

The DNA extracts taken from the *E. coli* isolates were analysed for seven avian pathogenic *E. coli* (**APEC**) virulence factors using PCR assays. The method used was described by Alkandari (2017). The virulence genes investigated (*pap*C, *iuc*D, *tsh*, *irp*2, *iss*, *ast*A, and *hly*A) are described in Table 2, with their primer sequences. PCR was performed using GE Healthcare Illustra™ PuReTaq Ready-To-Go™ PCR Beads (Thermo Fisher Scientific, UK) containing 2.5 units of recombinant PuReTaq DNA Polymerase, dATP, dCTP, dGTP, dTTP, stabilizers, bovine serum albumen and reaction buffer. PCR assays of *pap*C and *iuc*D were performed with 25 μl of PCR mixtures containing 5 μl of DNA extraction, each 1 μl (25 pmol) of forward and reverse primer pairs, and 18 μl of sterile double-distilled water. The other virulence genes (*tsh, irp*2, *iss*, *ast*A and *hly*A) were determined using 25 μl of PCR mixtures containing 5 μl of DNA, 1 μl (25 pmol) each of forward and reverse primer pairs, $MgCl_2$ (1.25 μl, 10 mM) and 16.75 μl of sterile double-distilled water.

The PCR amplification was carried out using a 96-well MJ Thermal Cycler (Bio-Rad, UK) with the following protocol: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 68 °C for 3 min, and the final extension step at 72 °C for 5 min. Analysis of the amplified products was performed by electrophoresis (75 V for 1 h) with a 1.5% agarose gel stained with ethidium bromide in 0.5 X TBE buffer. 100 bp and 1 Kbp DNA ladders were used. Gel images were captured using G-BOX Chemi-XR5, (Syngene, UK), connected to a computer. The analysis of specific band sizes for different virulence genes tested was scored as present (+) or absent (−).

Data analysis

The effect of the administration of CF, LP or the interaction between CF and LP on bird performance (feed intake, BW gain, FCR, breast meat weight and yield) and nutrient availability was determined by analysis of variance using the general linear model of Minitab (Minitab 17, Minitab Inc., PA, USA) with CF, LP and interaction between these main effects as the only terms in the model. The effect of bird age, digesta site, administration of CF, administration of LP and the interaction between all these main effects were used in the analysis of population density of yeasts, coliforms and *Lactobacillus* spp. (determined by ANOVA, general linear model, Minitab as before). Significant differences were determined at the level of $P < 0.05$.

At each sampling time, the percentage of samples (from any one treatment) that were phenotypically resistant to each antibiotic was determined. Chi square analyses were then used to determine the association between either the presence of phenotypic AMR or the presence of different virulence genes in *E. coli* and either the

Table 3
Effect of the addition of *Candida famata* and/or *Lactobacillus plantarum* in the drinking water of broiler chickens on their performance and overall DM availability.

	Control	CF	LP	CFLP	SEM	*P*-value		
						CF	LP	CF*LP
Weight gain (days 1–14, g/bird)	381	362	368	366	7.9	0.169	0.577	0.268
Weight gain (days 14–35, g/bird/d)	82	80	78	79	1.8	0.777	0.223	0.591
Feed intake (days 14–35, g/bird/d)	126	122	123	120	2.8	0.201	0.453	0.967
FCR (days 14–35)	1.54	1.52	1.57	1.52	0.02	0.070	0.370	0.322
Breast weight (g)	418	397	492	394	32.1	0.078	0.283	0.251
Breast meat yield (%)	19.4	19.5	20.5	18.4	0.82	0.238	0.993	0.196
DM availability (g/kg)	648	644	616	653	0.2	0.309	0.474	0.198

CF = birds were treated with 10^8 cfu/ml *Candida famata* (CF) in their drinking water 2 days a week throughout their life; LP = birds were treated with 10^5–10^8 cfu/ml *Lactobacillus plantarum* (LP) in their drinking water 2 days a week throughout their life; CFLP = birds were treated with a mixture of CF and LP (10^6–10^8 cfu/ml) in their drinking water 2 days a week throughout their life. CF*LP = the interaction between CF and LP. FCR = feed conversion ratio.

Table 4
Effect of treatment, digesta site and bird age on the mean population of *E. coli*, *Lactobacillus* spp., and yeast in samples of digesta taken from broiler chickens.

	Treatment[1]													*P*-value[2]							
	Control			CF			LP			CFLP			SEM								
Item	DUO	ILE	CAE	DUO	ILE	CAE	DUO	ILE	CAE	DUO	ILE	CAE		A	S	CF	LP	CF*LP	A*S	A*CF	A*LP
E. coli (log_{10} cfu/g)																					
Bird age (d)																					
8			9.14			8.91			8.26			8.53	0.32								
14			8.19			7.60			8.53			8.53	0.32								
22			8.60			8.93			8.92			9.34	0.26								
29			8.46			8.68			8.69			8.75	0.26								
35			8.07			7.89			7.95			7.95	0.26	***		0.826	0.437	0.344		0.533	0.064
Lactobacillus spp (log_{10} cfu/g)																					
Bird age (d)																					
8	7.77	9.42	9.69	7.73	9.18	10.1	8.16	8.98	8.25	7.99	9.09	9.22	0.37								
14	7.39	10.5	9.36	8.41	8.52	9.49	8.25	10.8	9.90	7.21	10.5	9.63	0.37								
22	6.97	8.58	9.04	7.32	8.54	9.33	6.97	8.88	9.48	7.24	8.72	9.69	0.30								
29	6.77	7.61	9.90	6.66	7.85	9.94	6.78	7.06	9.83	6.61	7.54	9.95	0.30								
35	6.87	8.01	9.17	6.67	7.89	9.03	6.50	7.69	8.97	6.89	7.63	8.34	0.30	***	***	0.620	0.852	0.875	***	0.245	0.045
Yeast (log_{10}cfu/g)																					
Bird age (d)																					
8	3.00	3.17		5.14	4.05		2.48	2.10		4.72	3.95		0.40								
14	3.43	4.52		3.42	470		3.45	4.95		3.89	5.10		0.40								
22	3.59	4.09		4.12	4.97		3.45	4.93		4.46	5.42		0.32								
29	3.53	3.13		5.48	4.09		3.29	2.35		4.91	3.90		0.32								
35	3.86	3.41		3.06	2.93		2.06	2.24		4.05	3.83		0.36	***	0.435	***	0.502	0.013	***	***	0.046

Abbreviations: Site of digesta collection in DUO = duodenum, ILE = ileum, CAE = caecum. Replication: $n = 4$ when birds 8 and 14 days old, $n = 6$ when birds 22, 29 and 35 days old.

[1] Treatment: CF = birds were treated with 10^8 cfu/ml *Candida famata* (CF) in their drinking water 2 days a week throughout their life; LP = birds were treated with 10^5–10^8 cfu/ml *Lactobacillus plantarum* (LP) in their drinking water 2 days a week throughout their life; CFLP = birds were treated with a mixture of CF and LP (10^6-10^8 cfu/ml) in their drinking water 2 days a week throughout their life.

[2] Abbreviations: A = bird age, S = site of digesta sampling, CF = effect of CF treatment, LP = effect of LP treatment; * = denotes the interaction between main effects. There was no significant interaction ($P > 0.05$) between site and either CF or LP. Three way and four way interactions were all not statistically significant ($P > 0.05$). *** denotes $P < 0.001$.

birds' age or its treatment (Control, CF, LP, CFLP). Associations between the carriage of virulence genes and the phenotypic expression of AMR were also determined by Chi square analysis.

Results

Bird performance and characterisation of E. coli, Lactobacillus and yeasts in the gut

The administration of probiotics did not affect bird performance or DM availability (Table 3). Even as day-old chicks, there was a large population in the gut of both coliforms (log_{10} 5.65 ± 0.35 cf u/g) and lactic acid bacteria (log_{10} 6.87 ± 0.13 cfu/g), which was larger than the populations observed in the yolk sac (log_{10} cfu/g 4.27 ± 0.13 and 4.49 ± 0.08 for coliforms and lactic acid bacteria, respectively). No yeast was detected in the yolk sac but some chicks (6 out of 16) had a small population of yeasts in the gut (log_{10} 3.18 ± 0.42 cfu/g).

The administration of *C. famata* and *L. plantarum*, either alone or in combination, had no effect on the population of coliforms or *Lactobacilli*. Administering *L. plantarum* did not affect the population of yeasts either, but the administration of *C. famata* did increase the population density of yeasts (from 3.4 to 4.2 log_{10}cfu/g, $P < 0.001$). As birds aged, there was a general decline in the population density of coliforms ($P < 0.001$, Table 4). There was also a significant ($P < 0.001$) interaction between the age of the bird and the site of the gut where digesta were collected with respect to the population density of *Lactobacilli*. The population density increased from the duodenum, through the ileum and was greatest in the caecum, and this difference between the different sections of the intestine was more apparent as the birds got older (Table 4).

There were some interesting interactions between bird age, site (duodenum, ileum and caecum) and the administration of CF or LP on the population size of yeasts. On days 14 and 22, the population density of yeasts was greater in the ileum and caecum than in the duodenum. However, by day 29, the yeast population was greater in the duodenum than in the ileum and caecum. By day 35, the population density of yeasts was lower in all three sites compared with the density observed in those same sites when the birds were younger ($P < 0.001$, Table 4). Administering *C. famata* increased the population of yeasts when the birds were younger (days 8, 22 and 29) but by day 35, the population density of yeasts was lower and was not affected by the administration of *C. famata* ($P < 0.001$, Table 4). There was also an interaction between the administration of *C. famata* and digesta site on the yeast population ($P < 0.05$). Administering *C. famata* increased the population of yeast in the duodenum and ileum but not in the caecum (Table 4). In addition, there was an interaction between the administration of CF and LP ($P < 0.01$, Table 4). Administering *C. famata* increased the population of yeast in the gut, and yeast counts increased even further, when both *C. famata* and *L. plantarum* were administered. Administering *L. plantarum* alone, however, had no effect on the yeast population.

The identification of the *Lactobacilli* and yeast isolates is presented in Tables 5 and 6. A variety of *Lactobacillus* species were identified, with only two isolates (one of which was from a control bird) being identified as *L. plantarum*. The other positive *L. plantarum* identification was from a bird treated with LP. With respect to yeasts, a number of different *Candida* species were identified including *C. rugosa*, *C. zeylanoides*, *C. tropicalis* and *C. glabrata*. *Geotrichum candidum*, *Saccharomyces cerevisaea* and *Rhodotorula mucilaginosa* were also identified.

Antimicrobial resistance and virulence genotyping of E. coli from the caecum

Fig. 1 shows the proportion of caecal *E. coli* samples that were resistant to antimicrobials throughout the experimental period. Resistance to nalidixic acid and chloramphenicol was low throughout the study. Tetracycline resistance was high (79%) at 8 days and then declined to 21% at 35 days ($P < 0.001$) with no effect of treatment. All of the samples taken from the gut of day-old birds were resistant to ampicillin and this persisted to day 8. However, it then decreased to 38% at 35 days ($P < 0.001$). There was no effect of treatment on the resistance of *E. coli* to antibiotics.

The percentage of *E. coli* isolates (n = 84) carrying different virulence-associated genes is also presented in Fig. 1. The *pap*C gene was not detected in any of the samples. There was a significant association ($P < 0.01$) between bird age and the carriage of virulence genes by *E. coli*. Isolates of *E. coli* taken from the youngest birds (days 1 and 8 of age) had a much higher incidence of *iuc*D, *irp*2, *iss* and haemolysin *hly*A than in isolates taken from older birds. There was no significant association between treatment and the carriage of different virulence genes. There was a significant association between phenotypic resistance of *E. coli* to tetracycline and the carriage of the genes *iuc*D ($\chi^2 = 12.27$, $P < 0.001$), *irp2* ($\chi^2 = 9.573$, $P < 0.01$), *iss* ($\chi^2 = 5.25$, $P < 0.05$) and *tsh* ($\chi^2 = 6.34$, $P < 0.05$) (Fig. 2).

Table 5
Identification (by API system, bioMerieux UK Ltd, Basingstoke, UK) of the *Lactobacillus* isolates taken from samples of caecal digesta of the broiler chickens.

Bird age, d	Treat	Identification	% C[1]	Bird age, d	Treat	Identification	% C
1	N/A	*Lactococcus lactis* ssp. *lactis* 1	95.8	22	LP	*Lactobacillus paracasei* ssp. *paracasei* 1	99.9
1	N/A	*Lactobacillus paracasei* ssp. *paracasei* 1	92.8	22	LP	*Lactococcus lactis* ssp. *lactis* 2	61.1
1	N/A	*Lactococcus lactis* ssp. *lactis* 1	95.8	29	CON	*Lactococcus lactis* ssp. *lactis* 1	48.6
1	N/A	*Lactobacillus paracasei* ssp. *paracasei* 1	88.1	29	CON	*Lactococcus lactis* ssp. *lactis* 1	64.1
1	N/A	*Lactobacillus paracasei* ssp. *paracasei* 1	99.6	29	CF	*Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum* 1	69.2
8	CON	*Lactococcus lactis* ssp. *lactis* 1	64.3	29	CF	*Lactococcus lactis* ssp. *lactis* 1	90.0
8	CF	*Lactococcus lactis* ssp. *lactis* 1	64.3	29	LP	*Lactococcus lactis* ssp. *lactis* 1	93.2
8	LP	*Lactococcus lactis* ssp. *lactis* 1	64.3	29	LP	*Lactococcus lactis* ssp. *lactis* 1	64.3
14	CON	*Lactococcus lactis* ssp. *lactis* 1	64.3	35	CON	No result	–
14	CF	*Lactococcus lactis* ssp. *lactis* 2	69.8	35	CON	*Lactococcus lactis* ssp. *lactis* 1	72.7
14	LP	*Lactobacillus plantarum* 1	97.3	35	CF	*Lactobacillus paracasei* ssp. *paracasei* 3	79.9
22	CON	No result	-	35	CF	*Lactococcus lactis* ssp. *lactis* 1	51.1
22	CON	*Lactobacillus plantarum* 1	76.9	35	LP	*Lactococcus lactis* ssp. *lactis* 1	92.2
22	CF	*Lactobacillus paracasei* ssp. *paracasei* 1	99.9	35	LP	*Lactobacillus brevis* 1	93.0
22	CF	*Lactococcus lactis* ssp. *lactis* 1	64.3				

CON = control; N/A = not applicable; CF = birds were treated with 10^8 cfu/ml *Candida famata* (CF) in their drinking water 2 days a week throughout their life; LP = birds were treated with 10^5–10^8 cfu/ml *Lactobacillus plantarum* (LP) in their drinking water 2 days a week throughout their life; Treat = treatment.
[1]%C: % confidence in identification.

Table 6
Identification (by API system, bioMerieux UK Ltd, Basingstoke, UK) of the yeast isolates taken from samples of deudonal, ileal and caecal digesta of the broiler chickens.

Bird age (d)	Treatment	Gut section	Identification
14	CON	Duodenum	*Candida rugosa*
14	CON	Ileum	*Candida rugosa*
14	CON	Ileum	*Geotrichum candidum*
14	CF	Duodenum	*Candida zeylanoides*
14	CF	Duodenum	*Candida tropicalis*
14	CF	Ileum	*Candida rugosa*
14	LP	Ileum	*Candida rugosa*
14	LP	Caecum	*Candida rugosa*
14	CFLP	Ileum	*Candida rugosa*
14	CFLP	Caecum	*Candida rugosa*
22	CF	Ileum	*Candida rugosa*
22	CF	Ileum	*Candida rugosa*
22	LP	Ileum	*Candida rugosa*
22	CFLP	Ileum	*Candida rugosa*
29	CF	Ileum	*Candida glabrata*
29	CF	Caecum	*Candida glabrata*
29	LP	Caecum	*Rhodotorula glutinis*
29	CFLP	Duodenum	*Candida rugosa*
35	CON	Duodenum	*Saccharomyces cerevisaea*
35	CF	Ileum	*Candida glabrata*
35	LP	Caecum	*Rhodotorula mucilaginosa 2*
35	CFLP	Duodenum	*Candida spherica*
35	CFLP	Ileum	*Saccharomyces cerevisaea 1*
35	CFLP	Caecum	*Candida pelliculosa*

CON = control; CF = birds were treated with 108 cfu/ml *Candida famata* (CF) in their drinking water 2 days a week throughout their life; LP = birds were treated with 105–108 cfu/ml *Lactobacillus plantarum* (LP) in their drinking water 2 days a week throughout their life; CFLP = birds were treated with a mixture of CF and LP (106–108 cfu/ml) in their drinking water 2 days a week throughout their life.

Discussion

If probiotics are to have any effect at all on the health and performance of the host animal, then they must colonise and proliferate within the gut (Angelakis et al., 2013; Peng et al., 2016). The absence of any effect of *L. plantarum* administration on the population density of *Lactobacilli* in any segment of the gut, and the further observation that only one of the samples of *Lactobacilli* isolated from the chickens showed any evidence of them being related to the administered source would suggest that colonisation by *L. plantarum* had been unsuccessful. The only caveat to that analysis would be to note that the samples that were taken were of digesta (squeezed from the lumen of the intestinal section). Bacteria colonise the gut by adhering to the mucosal wall, and it is possible that the population adhering to the gut wall was different from the luminal population. However, Herias et al. (1999) observed no such distinction which would suggest that the colonisation of the gut by administered *L. plantarum* was indeed limited. This may explain the lack of effect of LP on bird performance and the populations of either *E. coli* or LAB. In contrast, Peng et al. (2016) administered a similar dose of *L. plantarum* to broilers but did so every day and in the feed (rather than water). They reported improvements in FCR and a reduction in *E. coli* when broilers were aged 42 days. However, no effect on coliform populations was observed when the birds were at a more vulnerable age (day 21 of age).

There was an increased yeast population in response to the administration of *C. famata*, and it was notable that this occurred mostly in the small intestine. *C. famata* administration may therefore have stimulated the proliferation of yeasts, but it was other yeast populations such as *C. rugosa*, *C. zeylanoides*, *C. tropicalis*, *Geotrichum candidum*, *C. glabrata*, *Saccharomyces cerevisaea* and *Rhodotorula mucilaginosa* that also appeared to be stimulated. However, as it was the small intestine in which the yeasts proliferated it is perhaps unsurprising that there was no associated effect on the caecal population of *E. coli.* Future research might investigate what effect, if any, the administration of *C. famata* had on the duodenal coliform population.

The impact of *C. famata* on bird performance was also limited, with only a tendency ($P = 0.07$) for FCR to be improved. The optimal dose and route of administration therefore need further consideration to enable these species to colonise the gut and effect any changes in the microbiome. Other studies (with admittedly different probiotics) have demonstrated that administering probiotics in water improved performance in broiler chickens. Eckert et al. (2010) showed that intermittent *Lactobacillus* based probiotic treatment via drinking water significantly improved FCR and

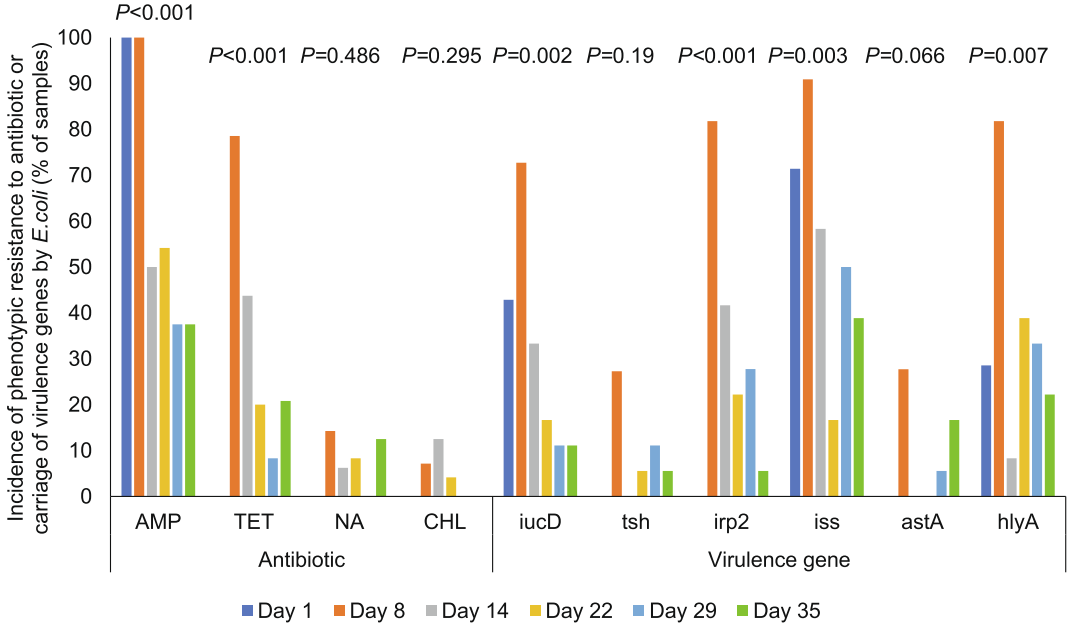

Fig. 1. The percentage of *E. coli* isolates taken from broiler chickens of different ages (days of life) that were phenotypically resistant to different antimicrobials or carried different virulence genes. AMP = ampicillin; TET = tetracycline; NA = nalidixic acid; CHL = chloramphenicol; For identification and description of genes, see Table 2.

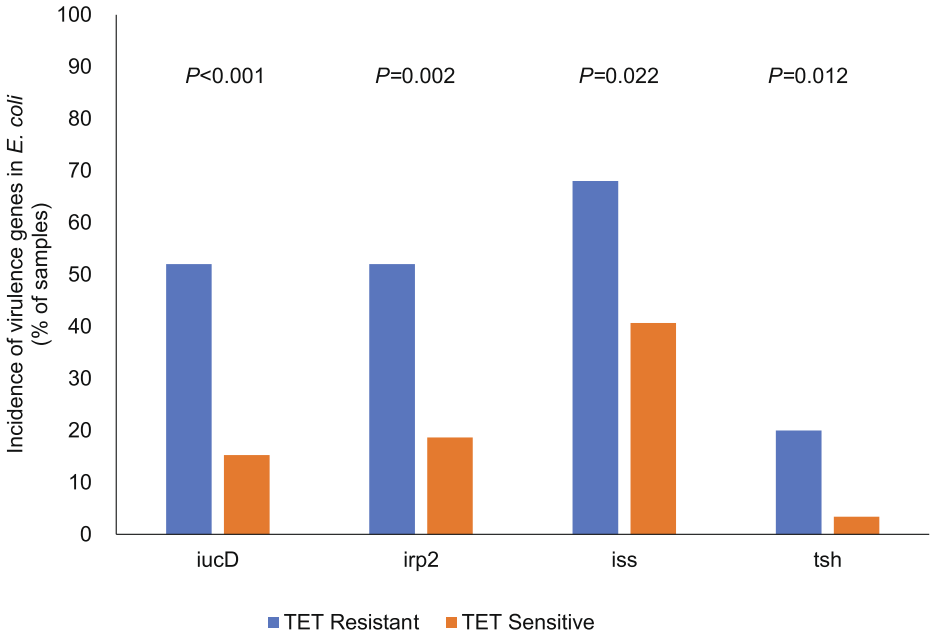

Fig. 2. The association between the carriage of the genes coding for iron uptake chelate, iron-repressible protein, increased serum survival and temperature-sensitive haemagglutinin and the phenotypic sensitivity or resistance by isolates of *E. coli* to tetracycline (20 μg/ml) in broiler chickens. TET = tetracycline; For identification and description of genes, see Table 2.

increased BW compared with administration in feed or continuously in drinking water. A possible explanation for the beneficial effect of intermittent administration may be a consequence of the immunostimulatory properties of probiotics. Administering probiotics continuously would result in a sustained high state of immune stimulation and the energy for activation of the immune system might partition nutrients away from bird growth. Karimi Torshizi et al. (2010) also observed that the administration of nine different probiotics (*Aspergillus oryzae*, *Lactobacillus acidophilus*, *L. rhamnosus*, *L. plantarum*, *L. bulgaricus*, *Bifidobacterium bifidum*, *Enterococcus faecium*, *Streptococcus thermophilus* and *Candida pintolopesii*) in water resulted in improved performance (in terms of BW gain, feed intake and FCR) compared with no probiotics or administering them in the feed. Administering probiotics in the water intermittently may therefore be the optimum means of delivery, but in this study, the dose (certainly of the *L. plantarum*) was insufficient to produce an observable effect on bird performance.

There was a high degree of resistance of *E. coli* to tetracycline at 8 days and to ampicillin at both 1 and 8 days even though the birds were not exposed to antibiotics at any time in their life. Ampicillin and tetracycline resistance then decreased as the birds aged, and these results reflect those of Roth et al. (2017) who also observed *E. coli* in faecal contents of day-old chicks was highly resistant to ampicillin, ciprofloxacin, streptomycin, sulfamethoxazole and tetracycline but that the prevalence of resistant *E. coli* by day 17 and 38 decreased (although only to ciprofloxacin, streptomycin and sulfamethoxazole). A high prevalence of AMR in day-old chicks was also observed by da Costa et al. (2011), who reported resistance to ampicillin, cefotaxime, tetracycline, streptomycin, gentamicin and enrofloxacin by *E. coli* isolates from cloacal samples from day-old chicks. *E. coli* (both commensal and pathogenic, potentially carrying antibiotic resistance genes) may transfer from breeding birds to chicks via the eggshell or egg yolk during oviposition. However, in the absence of any exposure to antibiotic, it is unclear why the prevalence of resistance then increased still further in the first week of life (although it did then decrease after that). Clearly there must be some other selective advantage for *E. coli* carrying antibiotic resistance genes.

In other research, AMR of *E. coli* isolated from caecal and faecal samples has been observed to continue to increase throughout the bird's life. The prevalence of coliform resistance to penicillins was high and reported to be increasing in intensively farmed broilers (Majewski et al., 2020). Bezerra et al. (2016) observed that all *E. coli* isolated from cloacal samples were resistant to trimethoprim-sulfamethoxazole and 95.4% to tetracycline, 91.4% to ciprofloxacin and 87.3% to ampicillin with birds of preslaughter age (35–8 days). *E. coli* isolates from excreta samples of birds had a high frequency of resistance to tetracycline, kanamycin and nalidixic acid throughout the birds' life in a study reported by Miles et al. (2006). The high prevalence (frequency or density) of resistant *E. coli* arose from genomic evolution as a result of successful competition with susceptible strains (Kolář et al., 2001; Smith et al., 2007), although if no antibiotics are administered, the basis for this successful competition is unclear. Another possible explanation for this is that resistance genes are associated with mobile genetic elements, such as plasmids. These may confer resistance to antibiotic sensitive bacteria by conjugation, transformation and transduction, but also confer other selective advantages. In this study, AMR declined after 8 days, suggesting that while chicks may have been infected with highly resistant *E. coli* (often APEC types), over the lifespan of the birds, these types were replaced by more robust non-antibiotic-resistant commensal types.

Roth et al. (2017) added an organic acid additive to the birds' feed and observed that this was associated with a reduced prevalence of ampicillin- and tetracycline-resistant *E. coli*. This finding is consistent with this study, as the administration of *C. famata* (which in addition to ethanol was also, *in vitro*, observed to produce ethyl acetate, data not shown) tended to reduce the ampicillin resistance of *E. coli*. Organic acids produced by probiotics or added directly to the diet may inhibit the growth of coliforms, particularly those carrying antibiotic-resistant genes (Edens, 2003; Alagawany et al., 2018). Further research is clearly needed to investigate the appropriate selection of probiotics to reduce the prevalence of resistant *E. coli* in broiler chickens.

The seven virulence genes used in this study are generally analysed to differentiate between commensal and pathogenic *E. coli* in

poultry. The *iss* gene was the most prevalent of the genes investigated, but *iuc*D, *irp*2, *iss* and *hly*A were also very prevalent in birds that were 8 days old before declining as the birds got older. In recent years, there has been considerable interest in the detection of virulence genes in commensal and pathogenic *E. coli* isolates in poultry (Zhao et al., 2009; Karami et al., 2017). Janßen et al. (2001) reported that a high frequency of the genes *fim*C, *iuc*D and *tsh* was observed in APEC isolates.

According to Paixao et al. (2016), APEC isolates had a higher prevalence of the iron uptake-related genes and the serum survival genes compared with commensal strains of *E. coli*. This is because iron is an essential nutrient for *E. coli* and is of more limited availability in extraintestinal tissues compared with digesta. There is therefore a selective advantage for APEC to carry genes such as *iuc*D and *irp*2. What was interesting in this study was the association between *iuc*D, *irp*2, *iss* and *tsh* with tetracycline resistance. As observed before, it is unclear what selective advantage there is to the carriage of genes coding for tetracycline (or any other antibiotic) resistance when no antibiotics have been administered. However, it is possible that these virulence genes are carried on the same plasmid (or other mobile genetic element) as the genes coding for tetracycline resistance and the advantage (to APEC) is in the greater ability to scavenge and accumulate iron, with the resistance to tetracycline being a side benefit.

Associations between virulence and AMR have been observed before; Johnson et al. (2012) observed that APEC had a higher proportion of plasmid mediated multidrug resistance than their avian commensal counterparts. They also observed that certain virulence genes, including *iss*, were positively associated with multidrug resistance. Szmolka et al. (2012) also reported that *tet*A (coding for tetracycline resistance) was highly correlated with the virulence genes *iro*N and *iss* in both commensal and avian pathogenic *E. coli*. These findings suggest that there is a positive relationship between mechanisms for iron accumulation by *E. coli* and the resistance by *E. coli* to a range of antimicrobials including tetracycline. This may provide a means of controlling the maintenance and spread of *E. coli* resistant to antimicrobials. The avoidance of antibiotics may not be sufficient to control this spread, since the prevalence of *E. coli* resistant to antibiotics is so widespread even when no antibiotics are administered. Altering the iron availability (in the gut) for *E. coli*, conceivably by altering the dietary iron content or removing any chelating agents, may serve as a means of altering the selective advantage that APEC currently has, particularly in the starter period of the bird's life. This might then encourage the proliferation of commensal *E. coli* (carrying fewer genes encoding either antibiotic resistance or virulence).

This study has confirmed that the strains of *E. coli* which are prevalent in the young broiler chicken (up to 8 days of age) are resistant to ampicillin and tetracycline and they also carry a number of virulence-associated genes. This poses a considerable threat to the bird when it is at a vulnerable stage of development. What was positive, however, was the evolution of the *E. coli* population as the birds aged. By the finishing stage, the dominant strains of *E. coli* were commensal and sensitive to antibiotics. The carriage of *iuc*D, *irp*2, *iss* and *tsh* genes was strongly associated with tetracycline-resistant *E. coli*. Interventions to encourage the early proliferation of commensal *E. coli* (without the resistance to antimicrobials) would make the young growing chick less susceptible to antibiotic-resistant disease. One such intervention may be through the manipulation of iron availability in the chicken gut.

Ethics approval

Animals in this study were handled according to the recommendations of the Code of Recommendations for the Welfare of Livestock: Meat chickens and breeding chickens (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/69372/pb7275meat-chickens-020717.pdf) and the Ross Broiler Management Handbook (http://en.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-Broiler-Handbook-2014i-EN.pdf). No further ethical approval was required as no pain, suffering, distress or lasting harm was imposed.

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available upon request to the authors.

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Declaration of interest

None.

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