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Impact of Feed Supplementation with Antimicrobial Agents on Growth Performance of Broiler Chickens, *Clostridium perfringens* and *Enterococcus* Counts, and Antibiotic Resistance Phenotypes and Distribution of Antimicrobial Resistance Determinants in *Escherichia coli* Isolates[▽]

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The effects of feed supplementation with the approved antimicrobial agents bambermycin, penicillin, salinomycin, and bacitracin or a combination of salinomycin plus bacitracin were evaluated for the incidence and distribution of antibiotic resistance in 197 commensal *Escherichia coli* isolates from broiler chickens over 35 days. All isolates showed some degree of multiple antibiotic resistance. Resistance to tetracycline (68.5%), amoxicillin (61.4%), ceftiofur (51.3%), spectinomycin (47.2%), and sulfonamides (42%) was most frequent. The levels of resistance to streptomycin, chloramphenicol, and gentamicin were 33.5, 35.5, and 25.3%, respectively. The overall resistance levels decreased from day 7 to day 35 ($P < 0.001$). Comparing treatments, the levels of resistance to ceftiofur, spectinomycin, and gentamicin (except for resistance to bacitracin treatment) were significantly higher in isolates from chickens receiving feed supplemented with salinomycin than from the other feeds ($P < 0.001$). Using a DNA microarray analysis capable of detecting commonly found antimicrobial resistance genes, we characterized 104 tetracycline-resistant *E. coli* isolates from 7- to 28-day-old chickens fed different growth promoters. Results showed a decrease in the incidence of isolates harboring *tet*(B), bla_{TEM}, *sulI*, and *aadA* and class 1 integron from days 7 to 35 ($P < 0.01$). Of the 84 tetracycline-ceftiofur-resistant *E. coli* isolates, 76 (90.5%) were positive for bla_{CMY-2}. The proportions of isolates positive for *sulI*, *aadA*, and integron class 1 were significantly higher in salinomycin-treated chickens than in the control or other treatment groups ($P < 0.05$). These data demonstrate that multiantibiotic-resistant *E. coli* isolates can be found in broiler chickens regardless of the antimicrobial growth promoters used. However, the phenotype and the distribution of resistance determinants in *E. coli* can be modulated by feed supplementation with some of the antimicrobial agents used in broiler chicken production.

Several classes of antibiotics, including glycolipids (bambermycin), polypeptides (bacitracin), ionophores (salinomycin) and β-lactams (penicillin), are used in broiler chicken production for growth promotion and prevention of infectious diseases (10, 43). Salinomycin and bacitracin are widely used in starter, grower, and finisher feeds for broilers. These antibiotics improve feed conversion and body weight gain presumably by altering the composition and activities of microflora (14, 31). This practice may modify the intestinal flora and create a selective pressure in favor of resistant bacteria (1, 43). In response to the emergence of antibiotic resistance, several European countries have restricted or banned the use of antibiotics as growth promoters (3). According to Apajalahti et al. (4), the identity of only about 10% of the chicken gastrointestinal tract bacteria is known. Little research has been conducted to systematically evaluate the potential effects antibiotics may have on the dynamics of the overall gut microflora of chicken. Much work needs to be done to study the distribution of antibiotic resistance genes among commensal bacteria in chickens fed antimicrobial growth promoter agents.

Escherichia coli is a ubiquitous organism in the chicken gastrointestinal tract and is regarded as a major pathogen of worldwide importance in commercially produced poultry (25, 47). It can cause diseases including colibacillosis and air sacculitis in poultry, resulting in significant economic losses (47). In commercial broiler chicken farms, the rations fed to chickens may legally contain up to three antimicrobial agents. However, the overall distribution of antibiotic resistance determinants among commensal bacteria isolated from healthy chickens fed with such feed is largely unknown. Publications on

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resistance to therapeutically used antibiotics do not provide a complete picture of the situation (27).

Fairchild et al. (21) showed that the oral administration of tetracycline did not induce significant changes in the chicken cecal bacterial community but that *Enterococcus* spp. and *E. coli* showed high tetracycline MICs. *Escherichia coli* isolates were found to harbor the tetracycline resistance gene *tet*(A) or *tet*(B), while *Enterococcus* isolates were positive for *tet*(M), *tet*(L), *tet*(K), and *tet*(O), with the latter gene conferring tetracycline resistance in *Campylobacter jejuni* isolates (21). The authors suggested that complex ecological and genetic factors could contribute to the prevalence and transfer of antibiotic resistance genes in the chicken production environment. Multiply drug-resistant *E. coli* strains isolated from healthy broiler chickens and humans were found to harbor similar genes encoding tetracycline resistance, suggesting the possibility that chickens may be reservoirs for tetracycline resistance genes (36).

Commensal intestinal bacteria including *E. coli* are commonly used to monitor resistance to therapeutically valuable antibiotics in food animals and in humans (16). In a previous study, we reported that multiple-antibiotic-resistant commensal *E. coli* strains carrying virulence and resistance genes can be found in samples from commercial broiler chicken farms and provide a reservoir for these genes in chicken production facilities (17). Such bacteria could later find their way into chicken products and other foods as well as manure, soil, and water. The impact of the agricultural use of antimicrobial agents on human and animal health has been the subject of several reports (3, 10, 47). For public health concerns, it is important to know the changes that occur in the intestinal flora of chickens treated with various antimicrobial growth promoters. Knowledge about the diversity and distribution of antimicrobial resistance determinants in bacteria from the chicken gut and the environment will be useful for understanding the ecology of the gut microflora as well as the epidemiology of antibiotic resistance (8). This study investigated genotypic and phenotypic changes in the intestinal *E. coli* population of broiler chickens fed with different antimicrobial agents as growth promoters.

TABLE 1. Composition of the feed used in this study

Ingredient/nutrient profile	% of inclusion in diet		
	Starter (days 0–14)	Grower (days 15–28)	Finisher (days 29–35)
Ingredient			
Wheat	34.96	35.03	40.79
Soya	23	0	0.51
Barley	10	0	0
Canola	9	22	18
Canola oil	8.6	7	7
Corn	7	25	25
Corn gluten	2.3	6	4
Limestone	1.6	1.3	1.2
Dicalcium phosphate[a]	1.6	1.5	1.4
Vitamin-mineral mixture[b]	1	1	1
Lysine	0.4	0.71	0.63
Avizyme[c]	0.05	0.05	0.05
Analyzed nutrient[d]			
Dry matter	89	88.7	88.7
Ash	6.41	5.74	5.74
Proteins	24.8	21.3	21.3
Fat	10.6	10.4	10.4
Glucose	19.3	17.3	18.6
Fructose	22.0	22.7	24.4
Acid detergent fiber	7.42	7.18	7.18
Neutral detergent fiber	13.35	13.72	13.72
Ca	1.0	1.0	1.0
Mg	0.2	0.2	0.2
K	0.89	0.55	0.55
P	0.80	0.81	0.81
Na	0.22	0.26	0.26
Fe	0.04	0.04	0.04
Zn	0.02	0.02	0.02
Mn	0.02	0.02	0.02

[a] A mixture of mono- and dicalcium phosphate containing 18% calcium and 21% phosphate.

[b] Amounts supplied per kilogram of diet: vitamin A, 9,000 IU; cholecalciferol, 1,500 IU; vitamin E, 10 IU; vitamin K, 0.5 mg; vitamin B_{12}, 0.007 mg; thiamine, 0.4 mg; riboflavin, 6 mg; folic acid, 1 mg; biotin, 0.15 mg; niacin, 135 mg; pyridoxine, 4 mg; choline chloride, 1,000 mg; DL-methionine, 1,184 mg; ethoxyquine, 125 mg; NaCl, 2 g; manganese sulfate, 60 mg; copper sulfate, 5 mg; selenium (sodium selenium), 0.1 mg; iodine, 0.35 mg; zinc sulfate, 50 mg.

[c] Multienzyme system for wheat-based poultry feed (Halchemix Canada, Inc., Toronto, ON, Canada) containing 5,000 U/g xylanase and 1,600 U/g protease.

[d] The nutrient contents, analyzed on a dry matter basis, were determined at CRESAD.

MATERIALS AND METHODS

Broiler chickens and treatments. Studies were performed with 900 1-day-old male broiler chickens. The birds were placed in 18 pens (50 birds per pen) that were assigned at random to six treatments: a control group fed without antibiotics and five groups fed rations containing (per kg of feed) 2 mg bambermycin, 2.2 mg procaine penicillin, 60 mg salinomycin, 4.4 mg bacitracin, and a combination of 3.3 mg bacitracin plus 1.1 mg salinomycin. All of these additives are approved for use in poultry production in Canada, but salinomycin and bacitracin and their combination are among the most popular antibiotics used in British Columbia (Canada). The composition of the feed used in this study is presented in Table 1. The starter, grower, and finisher diets were formulated with wheat, barley, and corn as the principal cereals and soybean and canola meals as protein concentrates to meet the National Research Council nutrient requirements for broiler chickens (39). Analyses of dry matter, total proteins, soluble carbohydrates, fatty acids, and some of the most common minerals were performed at the Centre de Recherche en Sciences Animales de Deschambault (CRSAD, Deschambault, QC, Canada) by the usual laboratory analysis methods. Heat was provided by gas-fired brooders; water was offered through nipple drinkers and feed through tube feeders to allow for ad libitum consumption. The clean and disinfected concrete floor was covered with approximately three inches (7.6 cm) of clean softwood shavings, and the bird density was approximately 0.75 square feet (0.07 m^2) per bird, which is the industry standard. Ventilation was provided by negative pressure with fans. Performance traits (body weight, weight gain, feed consumption, and feed efficiency) were measured at days 14, 28, and 35. All experimental procedures performed in this study were approved by the Animal Care Committee of the Pacific Agri-Food Research Center and followed principles described by the Canadian Council on Animal Care (11).

Sample collection and bacteriological analysis. Ten chicks (day 0) and two birds per pen were killed by cervical dislocation at each sampling time (7, 14, 21, 28, and 35 days of age). Cecal contents and cloacal samples from the two sacrificed birds were aseptically collected and transferred to peptone buffer in test tubes and sterile "whirl-pack" plastic bags, respectively, for bacteriological culture. The samples were placed on ice and transported to the microbiology laboratory for bacteriological analysis that was carried out on the same day. Sample weights were estimated by subtracting the weight of the container without the sample from the weight with the samples.

Bacteriological analyses were performed with a total of 90 fecal and 90 cecal samples. The generic *E. coli* population was estimated using *E. coli* and coliform Petrifilms (3M, St. Paul, MN) as previously described (17, 33). After incubation at 37°C for 24 h, blue-colored, gas-producing colonies were counted as generic *E. coli*. Results were expressed as CFU per gram of material. At each sampling time, six presumptive *E. coli* samples from each treatment group (two colonies per pen) were purified on blood agar and confirmed as *E. coli* by using API20E

TABLE 2. Performance of broiler chickens fed diets containing antimicrobials[a]

Parameter	Period (days)	Value for control	Value for treatment with:					SEM	P value[b]
			BBM	PEN	SAL	BAC	SAL + BAC		
Body wt (g)	Initial	40.69	40.76	40.64	40.71	40.67	40.40	0.232	0.91
	0–14	457.31	459.89	462.87	441.32	451.67	458.77	7.224	0.38
	15–28	1,252.02	1,265.29	1,267.10	1,209.64	1,224.63	1,255.29	14.605	0.09
	29–35	1,815.09	1,811.42	1,805.12	1,783.69	1,776.75	1,825.11	27.805	0.80
Feed intake (g)	0–14	365.90	373.21	363.39	361.82	360.72	368.93	6.272	0.72
	15–28	896.23	906.58	868.70	861.47	870.33	901.47	15.969	0.27
	29–35	1,288.22	1,280.62	1,223.70	1,263.36	1,264.54	1,329.18	23.142	0.12
	0–35	3,215.03	3,237.87	3,084.20	3,118.83	3,125.57	3,239.98	47.916	0.14
Feed efficiency (g of feed/g body wt gain)	0–14	1.21	1.22	1.17	1.24	1.21	1.20	0.014	0.09
	15–28	1.78	1.77	1.69	1.76	1.76	1.77	0.018	0.06
	29–35	2.29	2.35	2.28	2.17	2.30	2.34	0.060	0.37
	0–35	1.78	1.79	1.72	1.75	1.77	1.78	0.015	0.04*
Total mortality (%)	0–35	5.48	12.64	7.12	12.77	7.65	4.47	2.682	0.26

[a] Values indicate performance parameters of broiler chickens fed diets containing bambermycin (BBM), penicillin (PEN), salinomycin (SAL), bacitracin (BAC), and a salinomycin-bacitracin combination (SAL + BAC) at concentrations specified in Materials and Methods.
[b] *P* values were obtained by analysis of variance. *, values are statistically different ($P < 0.05$).

strips (bioMérieux, St-Laurent, QC, Canada) according to the manufacturer's specifications. *Enterococcus* populations were determined by spreading 10-fold dilutions of samples on KF streptococcal agar CM0701 (Oxoid, Nepean, ON, Canada) and incubating at 37°C for 48 h (as described by Hayes et al. [29]). *Clostridium perfringens* cells were enumerated according to the description by Knarreborg et al. (31). Briefly, samples were spread on tryptose sulfite agar (Oxoid) supplemented with cycloserine (SR088E; Oxoid) and incubated under anaerobic conditions for 24 h at 37°C. At the end of the study (day 35), two litter samples were taken from each pen for bacterial analysis as described above.

Determination of antimicrobial susceptibility. Determination of the antibiotic MICs was performed with all *E. coli* isolates, using a Sensititre automated system (Trek Diagnostic Systems, Cleveland, OH), according to the Clinical Laboratory Standard Institute's (CLSI [formerly NCCLS]) guidelines with *E. coli* ATCC 25922 as the control (38). The following antimicrobials were tested on Sensititre Avian plates: amoxicillin, penicillin, ceftiofur, erythromycin, tylosin, clindamycin, spectinomycin, streptomycin, gentamicin, neomycin, oxytetracycline, tetracycline, enrofloxacin, sarafloxacin, novobiocin, sulfadimethoxime, sulfathiazole-trimethoprim-sulfadimethoxazole, and chloramphenicol. In addition, antibiotic resistance profiles were determined for all tetracycline-resistant *E. coli* isolates using a Sensititre system with National Antimicrobial Resistance Monitoring System (NARMS) plates for gram-negative bacteria. The MIC results were interpreted according to the breakpoints of the CLSI and the 2005 Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS [12]) guidelines.

DNA extraction. *E. coli* isolates were grown overnight in 3 ml of beef heart infusion broth (Becton Dickinson, Sparks, MD) at 37°C. Two hundred microliters of this culture was transferred to 1.5-ml centrifuge tubes and centrifuged at $14,000 \times g$ for 2 min. The supernatants were removed, and the bacterial pellets were resuspended in 200 μl of sterile water with vortexing. The suspension was boiled for 10 min and centrifuged as described before, and 150 μl of the supernatant containing DNA was removed for testing. *E. coli* isolates that were phenotypically resistant to tetracycline and ceftiofur were analyzed by PCR for the presence of the extended-spectrum beta-lactamase-encoding gene bla_{CMY-2} as previously described (17).

***E. coli* DNA labeling.** Bacterial DNA was labeled using Bioprime DNA labeling system (Invitrogen Life Technologies, Burlington, ON, Canada). Fifteen microliters of the supernatant containing DNA was added to a final volume of 32.5 μl containing 10 μl of a random primer solution, 0.5 μl of high-concentration DNA polymerase (Klenow fragment, 40 U/μl), 5 μl of a deoxyribonucleoside triphosphate (dNTP) mixture (1.2 mM dATP, 1.2 mM dGTP, 1.2 mM dTTP, and 0.6 mM dCTP in 10 mM Tris [pH 8.0] and 1 mM EDTA), and 2 μl of 1 mM Cy5-dCTP. Labeling reactions were performed in the dark at 37°C for 3.5 h and stopped by the addition of 5 μl Na_2EDTA 0.5 M (pH 8.0). The labeled samples were then purified with a PureLink PCR purification kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The amount of incorporated fluorescent Cy5 dye was then quantified by scanning the DNA sample with a NanoDrop ND-1000 spectrophotometer from 200 to 700 nm. Data were analyzed using a Web-based percent incorporation calculator (http://www.pangloss.com/seidel/Protocols/percent_inc.html).

DNA microarrays. The antimicrobial resistance determinants in selected tetracycline-resistant isolates were detected using specific probes. The microarray used in this study is based on earlier published work (9) and carries oligonucleotides of 70 bases in length targeting 38 antimicrobial resistance or antimicrobial resistance-related genes. The microarray also carries five positive controls for *E. coli* derived from the sequences of genes encoding tryptophanase (*tnaA*), beta-glucuronidase (*uidA*), lactose permease (*lacY*), beta-galactosidase (*lacZ*), and glutamate decarboxylase (*gad*). Negative controls added to this microarray consist of oligonucleotides derived from the gene sequences for the green fluorescent protein of *Aequorea victoria*, the lactose permease of *Citrobacter freundii*, and the chlorophyll synthase from *Arabidopsis thaliana*.

Hybridization of labeled DNA. Prehybridization and hybridization were performed as previously described by Hamelin et al. (28), with the following modifications: the habitation was performed using a SlideBooster hybridization workstation (model SB800; Advalytix, Germany), and scanning was performed at a resolution of 5 μm at 95% laser power, using a ScanArray Lite fluorescent microarray analysis system (Perkin-Elmer, Mississauga, ON, Canada). Acquisition of fluorescent spots and quantification of fluorescent spot intensities were performed as described by Hamelin et al. (28).

Statistical analysis. Data were analyzed according to a randomized complete block design using the GLM procedure of SAS software (33, 45), with the individual pens as experimental units (three pens per treatment group). The association test of Cochran-Mantel-Haenszel and logistic analysis (proportional odds model) were used to determine the relationship among feed supplementation, phenotype, and genotype by using the FREQ procedure of SAS Institute (45). Associations between resistance genes and class 1 integrons were determined using Pearson's chi-square exact test (35). The *P* value of 0.05 was used to declare significance.

RESULTS

Broiler performance. The effects of diet supplementation with bambermycin, penicillin, salinomycin, bacitracin, and salinomycin plus bacitracin on body weight, feed intake, feed efficiency, and mortality are presented in Table 2. No significant differences were noted between the treatment groups for body weight and feed intake ($P > 0.05$). Although bambermycin and penicillin increased the body weight from days 15 to 28, these increases were not statistically significant ($P = 0.09$). From days 0 to 14 and from days 15 to 28, penicillin improved

TABLE 3. *C. perfringens*, *Enterococcus*, and *E. coli* counts obtained from cecal, cloacal, and litter samples[a]

Bacterium	Day	Sample	Mean CFU/g of sample in treatment						SEM	*P* value[b]
			Control	BBM	PEN	SAL	BAC	SAL + BAC		
C. perfringens	7	CE	2.15	2.22	2.93	0.62	1.86	1.76	0.530	0.39
		CL	1.30	0.57	1.23	1.16	1.20	1.36		
	14	CE	2.27	0.82	1.86	2.57	1.55	1.71	0.462	0.286
		CL	1.22	0.92	0.00	0.83	0.88	0.54		
	21	CE	2.45	2.54	2.54	2.54	2.00	2.54	0.306	0.127
		CL	1.43	2.34	1.15	2.29	2.55	2.67		
	28	CE	3.23	2.53	2.76	3.28	3.11	3.04	0.512	0.701
		CL	2.54	2.30	1.56	1.38	2.44	2.28		
	35	CE	3.95	3.55	3.66	3.29	3.76	3.28	0.341	0.831
		CL	2.65	2.54	2.54	2.78	2.95	2.67		
Enterococcus spp.	7	CE	9.41	9.55	8.06	7.63	7.51	8.40	0.782	0.7047
		CL	5.73	5.73	5.73	5.73	5.73	5.73	0.958	
	14	CE	6.95	4.90	4.92	6.26	5.93	6.55	0.683	0.217
		CL	4.76	5.78	4.45	6.62	5.39	6.04	0.837	
	21	CE	6.50	6.52	5.66	5.68	5.81	5.23	0.512	0.173
		CL	5.87	5.40	5.49	5.20	5.50	4.20	0.627	
	28	CE	6.09	5.28	5.73	4.73	5.16	5.22	0.472	0.072
		CL	4.47	5.68	3.17	3.41	4.59	4.20	0.578	
	35	CE	5.23	5.76	5.60	5.46	5.74	5.03	0.400	0.115
		CL	6.61	7.06	4.87	5.99	6.93	6.48	0.490	
		Litter	7.86	7.45	7.60	7.28	7.80	7.47	0.28	0.671
E. coli	7	CE	10.50	9.90	9.62	10.09	9.86	10.39	0.357	0.533
		CL	7.22	7.32	6.98	0.26	7.28	7.60	0.437	
	14	CE	8.41	8.00	8.80	8.77	7.98	8.70	0.425	0.106
		CL	6.71	5.73	6.16	6.64	5.73	7.17	0.520	
	21	CE	7.68	7.55	9.69	8.52	7.52	7.88	0.372	0.041*
		CL	7.01	6.58	6.63	6.63	6.63	6.09	0.456	
		CE	7.89	8.71	9.27	7.92	9.74	8.06	0.643	0.52
		CL	5.93	5.24	5.81	5.71	5.95	5.73	0.788	
	35	CE	9.57	8.67	9.43	8.29	9.17	9.10	0.542	0.493
		CL	6.75	6.31	6.80	6.53	7.38	6.47	0.664	
		Litter	8.47	8.87	8.72	8.43	8.57	8.44	0.14	0.197

[a] Values indicate $\log_{10}$ bacterial counts of *C. perfringens*, *Enterococcus*, and *E. coli* cells obtained from cecal (CE), cloacal (CL), and litter (analyzed on day 35 only) samples of broiler chickens fed diets containing bambermycin (BBM), penicillin (PEN), salinomycin (SAL), bacitracin (BAC), and a salinomycin-bacitracin combination (SAL + BAC) at concentrations specified in Materials and Methods.

[b] *P* values were obtained by analysis of variance. *, values are statistically different ($P < 0.05$).

feed efficiency (with reduced feed intake required to achieve a kilogram of gain). This improvement in feed efficiency with penicillin was significant on a cumulative response from days 0 to 35 ($P < 0.05$). No significant differences were observed for the cumulative mortality rate ($P > 0.05$), with 12% and 5% mortality recorded for the salinomycin-treated and the untreated control group, respectively, but less than 5% in the group receiving salinomycin-plus-bacitracin treatment.

Bacteriological analyses. Cloacal, cecal, and litter samples were collected as described in Materials and Methods and analyzed in order to determine the concentrations of generic *E. coli*, *C. perfringens*, and *Enterococcus* (Table 3). The concentrations of each bacterial group were higher in the cecal samples than in the cloacal samples ($P > 0.05$). At day 35, counts of *C. perfringens* cells in cecal and cloacal samples were significantly higher than those obtained at day 7 ($P < 0.05$), although counts of this bacterium remained lower than those of *E. coli* or *Enterococcus*. The numbers of these two bacteria (*E. coli* and *Enterococcus*) were highest on day 7 and slowly declined thereafter. At day 35, analysis of litter samples showed higher *E. coli* and *Enterococcus* concentrations compared to the cloacal samples. There was no significant effect of any treatment on bacterial counts in any sample (cloacal, cecal, or litter; $P > 0.05$). One hundred ninety-seven presumptive *E. coli* isolates were obtained (32 from the salinomycin-plus-bacitracin treatment group and 33 from each of the five other treatment groups). Antibiotic susceptibility was determined for these 197 isolates.

Antibiotic susceptibility. Antibiotic-resistant *E. coli* isolates were obtained from chickens regardless of the diets they received (Fig. 1). All 197 *E. coli* isolates tested were multiresistant to penicillin, erythromycin, tylosin, clindamycin, and novobiocin and displayed different resistance levels to the other antibiotics. Low levels of resistance to enrofloxacin, sarafloxacin, and trimethoprim-sulfamethoxazole were noted. Among the 19 antimicrobial agents on the Sensititre Avian plate, resistance levels to tetracycline (68.5%), amoxicillin (61.4%), ceftiofur (51.3%), spectinomycin (47.2%), sulfathiazole (42.1%), and sulfadimethoxime (41.6%) were the most frequent. The levels of resistance to streptomycin, chloramphenicol, and gentamicin were 33.5%, 35.5%, and 25.3%, respectively. Overall resistance levels were highest on day 7 and decreased thereafter ($P < 0.001$; Fig. 1). Interestingly, resistance to ceftiofur (69.0%) was significantly higher in *E. coli*

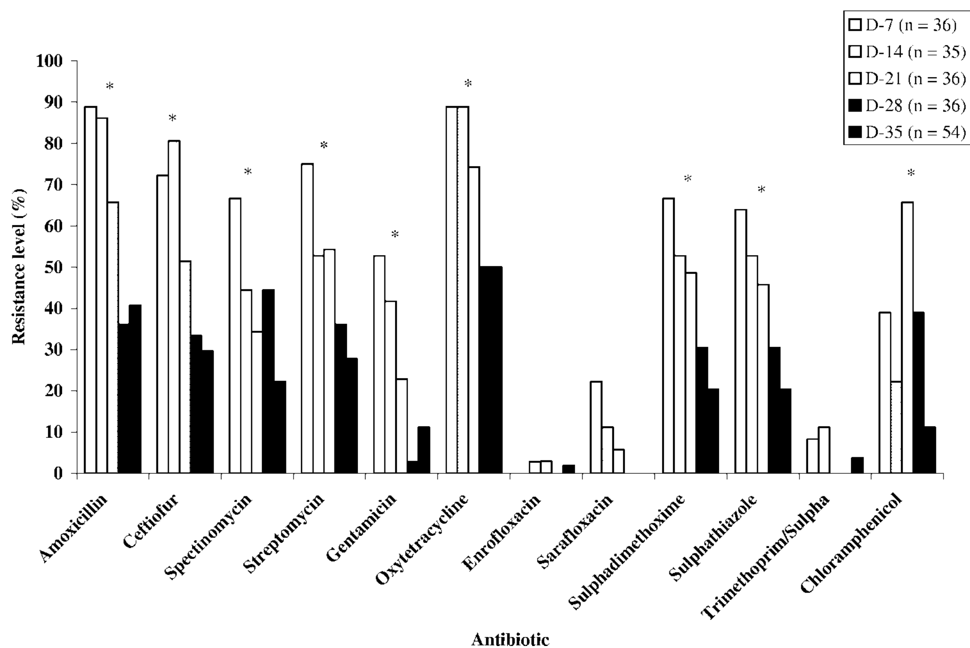

FIG. 1. Effect of age on resistance profiles of 197 *E. coli* isolates from broiler chickens. The percentage of resistance to most antibiotics decreased significantly ($P < 0.001$) from day 7 to day 35). Asterisks indicate the antibiotics against which the resistance percentages between treatments were statistically different ($P < 0.001$).

isolates from chickens receiving feed supplemented with salinomycin than from the other groups ($P < 0.001$). Resistance levels to spectinomycin and to gentamicin obtained from salinomycin- and bacitracin-fed chickens were similar and higher than those from the other groups ($P < 0.05$; Fig. 2).

Distribution of antibiotic resistance phenotypes and their determinants. Of the 197 *E. coli* isolates tested, 135 (68.5%) were resistant to tetracycline. One hundred four (77%) of these 135 tetracycline-resistant *E. coli* isolates were obtained between days 7 and 28 and were further characterized for resistance to antibiotics of importance in human and food animal medicine and for the presence of antibiotic resistance genes (Tables 4 and 5). All were susceptible to ceftriaxone, kanamycin, and amikacin and displayed low resistance levels to

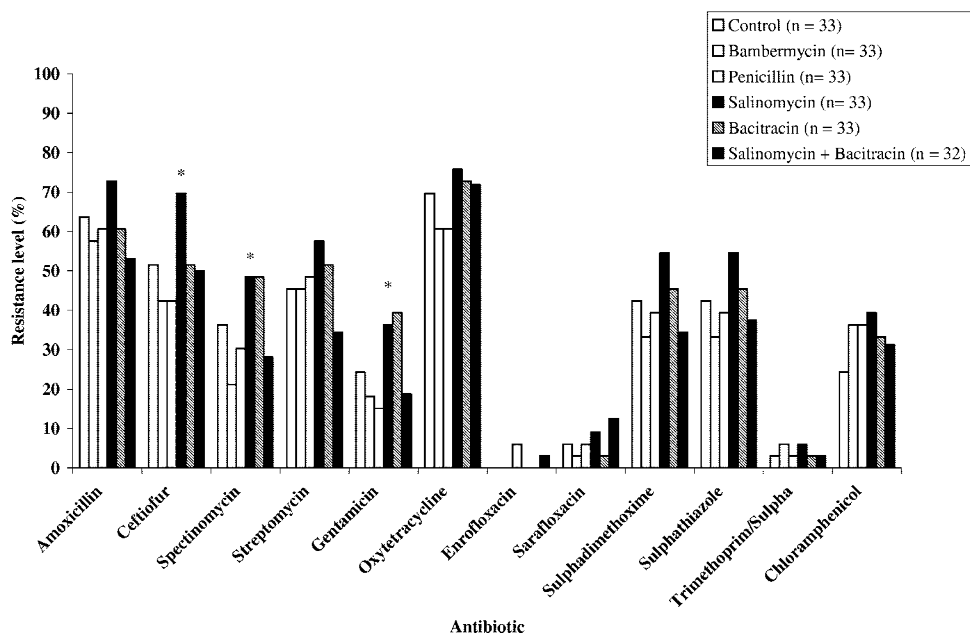

FIG. 2. Effect of growth promoter diet agents on the frequency of antibiotic resistance levels in 197 *E. coli* isolates from broiler chickens. The percentage of resistance to most antibiotics decreased significantly ($P < 0.001$) from day 7 to day 35. Asterisks indicate the antibiotics against which the resistance percentages between treatments were statistically different ($P < 0.001$).

TABLE 4. Distribution of antibiotic resistance phenotypes among tetracycline-resistant *E. coli* isolates from chickens fed antimicrobials[a]

Class	No. (%) of tetracycline-resistant isolates relative to the total no. of isolates (*n*) tested per treatment						
	Control (*n* = 19)	BBM (*n* = 14)	PEN (*n* = 16)	SAL (*n* = 20)	BAC (*n* = 19)	SAL + BAC (*n* = 16)	Total (*n* = 104)[b]
β-Lactams							
Amoxicillin	17 (89.5)	14 (100)	16 (100)	20 (100)	16 (84.2)	12 (75)	95 (91.3)
Ampicillin	17 (89.5)	14 (100)	15 (93.7)	19 (95)	15 (78.5)	12 (75)	92 (88.5)
Amoxicillin-clavulanic acid	17 (89.5)	12 (85.7)	15 (93.7)	19 (95)	15 (78.5)	12 (75)	90 (86.5)
Cefoxitin	17 (89.5)	12 (85.7)	15 (93.7)	19 (95)	14 (73.7)	12 (75)	89 (85.6)
Ceftiofur	14 (73.7)	12 (85.7)	14 (87.5)	19 (95)	13 (68.4)	12 (75)	84 (80.8)*
Aminoglycosides							
Streptomycin	13 (68.4)	11 (78.6)	12 (75)	15 (75.0)	15 (78.9)	7 (43.7)	73 (70.2)
Spectinomycin	11 (57.9)	6 (42.8)	6 (37.5)	15 (75.0)	12 (63.1)	6 (37.5)	56 (53.8)*
Gentamicin	7 (36.8)	6 (42.8)	4 (25.0)	11 (55.0)	11 (57.9)	5 (31.2)	44 (42.3)*
Sulfonamides							
Sulfadimethoxime	12 (63.1)	10 (71.4)	10 (62.5)	17 (85.0)	13 (68.4)	7 (43.7)	69 (66.3)
Sulfathiazole	12 (63.1)	10 (71.4)	10 (62.5)	17 (85.0)	13 (68.4)	5 (31.2)	67 (64.4)
Sulfizoxazole	12 (63.1)	9 (64.3)	10 (62.5)	16 (80.0)	13 (68.4)	5 (31.2)	65 (62.5)
Trimethoprim-sulfamethoxazole	2 (10.5)	1 (7.1)	0 (0.0)	2 (10.0)	1 (5.3)	0 (0.0)	6 (5.8)
Phenicols							
Chloramphenicol	10 (52.6)	9 (64.3)	9 (56.2)	13 (65.0)	12 (63.2)	5 (31.2)	58 (55.8)
Quinolones							
Nalidixic acid	2 (10.5)	2 (14.3)	4 (25.0)	3 (15)	2 (10.5)	2 (12.5)	15 (14.4)
Sarafloxacin	2 (10.5)	1 (7.1)	3 (18.7)	3 (15)	2 (10.5)	2 (12.5)	13 (12.5)
Ciprofloxacin	0 (0.0)	1 (7.1)	3 (18.5)	0 (0.0)	0 (0.0)	0 (0.0)	4 (3.8)
Enrofloxacin	0 (0.0)	0 (0.0)	2 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.9)

[a] Distribution of antibiotic resistance phenotypes among tetracycline-resistant *E. coli* isolates from chickens fed a control diet and diets containing bambermycin (BBM), penicillin (PEN), salinomycin (SAL), bacitracin (BAC), and a salinomycin-bacitracin combination (SAL + BAC) at the concentrations specified in Materials and Methods. All the isolates were resistant to penicillin, erythromycin, tylosin, clindamycin, oxytetracycline, and novobiocin, and all were susceptible to ceftriaxone, kanamycin, and amikacin.

[b] *, values are statistically different ($P < 0.05$).

quinolones. Higher levels of resistance to β-lactams, aminoglycosines, sulfonamides, and chloramphenicol were noted. In general, a correlation between resistance phenotype and genotype was observed. Antibiotic resistant phenotypes and corresponding resistance genes were found in similar proportions in *E. coli* isolates from untreated controls and from chickens treated with the test antimicrobial agents.

Tetracycline. Of the 104 tetracycline-resistant isolates, at least one of three tetracycline resistance genes, *tet*(A), *tet*(B), or *tet*(C), was found in each isolate. The *tet*(A) and *tet*(B) genes were found in 76 (73.1%) and 59 (56.7%) isolates, respectively. The combinations of *tet*(A) plus *tet*(B) and *tet*(A) plus *tet*(C) were found in 31 (29.8) and 6 (5.8) isolates, respectively. Eight isolates were positive for all three tetracycline resistance genes *tet*(A) plus *tet*(B) plus *tet*(C). None of the tetracycline-resistant isolates was found to harbor the *tet*(D), *tet*(E), or *tet*(Y) gene. No significant differences were observed for the number of isolates in which the genes were detected ($P > 0.05$) between the treatment and the control groups.

β-Lactams. Ninety-five of the 104 tetracycline-resistant *E. coli* isolates were also resistant to amoxicillin. Of the six resistance genes screened in these 95 isolates, only bla_{TEM} and bla_{SHV} were detected in 42.1% and 8.4% of isolates, respectively. No significant differences were observed for the distribution of these genes ($P > 0.05$) between treatment and control groups. Both genes were found in two isolates from the bambermycin treatment group. Among the 104 selected tetracycline-resistant *E. coli* isolates, 84 (80.8%) were resistant to ceftiofur. Among these 84 ceftiofur-resistant *E. coli* isolates, PCR detection showed that 76 (90.5%) were positive for bla_{CMY2} (Fig. 3).

Aminoglycosides. Seventy-three of the tetracycline-resistant isolates were phenotypically resistant to streptomycin. Of the six aminoglycoside resistance genes, only *ant(3″)-Ia* (*aadA*) was detected in 43 (58.9%) of these 73 isolates. The proportion of isolates carrying this gene was significantly higher from the salinomycin (80%) and bacitracin (66.7.%) treatment groups than from the other groups ($P = 0.05$). In the isolates from the control group, 61.5% of streptomycin-resistant isolates were also found to harbor the *ant(3″)-Ia* (*aadA*) gene. The *ant(2″)-Ia* (*aadB*) gene encoding the kanamycin, neomycin, and gentamicin resistance phenotypes was not detected, even though 42% of the 104 tetracycline-resistant isolates were also resistant to gentamicin (Table 4).

Sulfonamides. Among the 69 tetracycline-resistant isolates that were also resistant to sulfadimethoxime, *sulI* and *sulII* were found in 40 (58.0%) and 63 (91.3%) isolates, respectively. The proportions of isolates carrying the *sulI* gene were significantly higher in isolates from the bacitracin (76.9%) and salinomycin (70.6%) treatment groups than from the other groups ($P < 0.05$). The combination of *sulI* plus *sulII* was found in 35 (50.7%) of the 69 sulfadimethoxime-resistant isolates, with a significantly higher proportion found in isolates from the salinomycin (58.8%) and bacitracin (76.9%) treat-

TABLE 5. Distribution of antibiotic resistance genes among 104 selected tetracycline-resistant *E. coli* isolates[a]

Antibacterial	Gene	No. (%) of tetracycline-resistant *E. coli* isolates detected relative to no. tested (*n*) carrying the indicated gene per treatment[b]						
		C (*n* = 19)	BBM (*n* = 14)	PEN (*n* = 16)	SAL (*n* = 20)	BAC (*n* = 19)	SAL + BAC (*n* = 16)	Total (*n* = 104)[d]
β-Lactam (amoxicillin)		17 (89.5)[c]	14 (100.0)	16 (100.0)	20 (100.0)	16 (84.2)	12 (75)	95 (91.3)
	bla_{TEM}		8 (57.14)	4 (25.00)	8 (40.0)	8 (50.0)	6 (50.5)	40 (42.1)
	bla_{SHV}	1 (5.9)	5 (35.7)	0 (0.00)	0 (0.0)	0 (0.00)	2 (16.7)	8 (8.4)
	bla_{TEM} + bla_{SHV}	0 (0.0)	2 (14.3)	0 (0.0)	0 (0.0)	0 (0.00)	0 (0.0)	2 (2.1)
Aminoglycoside (streptomycin)		13 (68.4)	11 (78.6)	12 (75)	15 (75.0)	15 (78.9)	7 (43.7)	73 (70.2)
	ant(3″)-Ia (*aadA*)	8 (61.5)	5 (45.4)	4 (30.7)	12 (80.0)	10 (66.7)	4 (57.1)	43 (58.9)*
Tetracycline[c]								
	tet(A)	13 (68.4)	12 (85.7)	9 (56.2)	17 (85.0)	15 (78.9)	10 (62.5)	76 (73.1)
	tet(B)	11 (57.9)	8 (57.1)	9 (56.2)	12 (60.0)	11 (57.9)	8 (50.0)	59 (56.7)
	tet(A) + *tet*(B)	5 (26.3)	6 (42.8)	2 (12.5)	9 (45.0)	7 (36.8)	2 (12.5)	31 (29.8)
	tet(A) + *tet*(C)	9 (5.3)	0 (0.0)	0 (0.0)	2 (10.0)	1 (5.3)	2 (12.5)	6 (5.8)
	tet(A) + *tet*(B) + *tet*(C)	2 (10.5)	1 (7.1)	0 (0.0)	3 (15.0)	2 (10.5)	0 (0.0)	8 (7.7)
Sulfonamide (sulfadimethoxime)		12 (63.1)	10 (71.4)	10 (62.5)	17 (85.0)	13 (68.4)	7 (43.7)	69 (66.3)
	sulI	7 (58.3)	5 (50.0)	2 (20.0)	12 (70.6)	10 (76.9)	4 (57.1)	40 (58.0)*
	sulII	10 (83.3)	9 (90.0)	10 (100.0)	16 (94.1)	13 (100.0)	5 (71.4)	63 (91.3)
	sulI + *sulI*	5 (41.7)	5 (50.0)	2 (20.0)	10 (58.8)	10 (76.9)	1 (14.3)	35 (50.7)*
	dhfrI + *sulII*	1 (8.3)	0 (0.0)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.9)
Phenicol (chloramphenicol)		10 (52.6)	9 (64.3)	9 (56.2)	13 (65.0)	12 (63.2)	5 (31.2)	58 (55.8)
	floR	8 (80.0)	9 (100.0)	9 (100.0)	13 (100.0)	12 (100.0)	5 (100.0)	56 (96.6)
Class 1 integron in the 104 *tetR* genes	*int1(1)* + *int1(2)* + *int1(3)*	7 (36.84)	5 (35.71)	3 (18.75)	12 (60.00)	10 (52.63)	4 (25.00)	41 (39.42)*

[a] Table shows the distribution of antibiotic resistance genes among 104 selected tetracycline-resistant *E. coli* isolates from the guts of chickens fed with antibiotic growth promoters.
[b] C, control; BBM, bambermycin; PEN, penicillin; SAL, salinomycin; BAC, bacitracin; SAL + BAC, salinomycin with bacitracin.
[c] None of the isolates were positive to *tet*(C) alone or to the *tet*(B)-*tet*(C) combination.
[d] *, values are statistically different ($P < 0.05$).

ment groups. Two isolates (one in the control and one in the penicillin treatment groups) were positive for the combination of *dhfrI* plus *sulII*.

Phenicols. Fifty-eight of the 104 tetracycline-resistant isolates were also resistant to chloramphenicol (Tables 4 and 5). Of these 58 isolates, only the *floR* gene was found in 56 (96.6%) isolates. No significant differences were observed between the groups ($P > 0.05$) for the presence of this gene. The other phenicol resistance genes, *catI*, *catII*, and *catIII*, were not found in our isolates.

Class 1 integron. The 104 tetracycline-resistant isolates were screened for the presence of genes related to the class 1 integron in order to investigate the distribution of this resistance-disseminating element. A class 1 integron (*qacED1-sulI* and integrase gene) was found in 41 (39.4%) of the 104 *E. coli* isolates. These 41 strains were isolated at different ages (days 7, 14, 21, and 28) from all the experimental groups, and their phenotypes and genotypes are presented in Table 6. Some pens had two or three different isolates over the course of the study. Compared to the isolates of the other groups, significantly higher proportions of class 1 integron were found in isolates from the salinomycin (60.0%) and bacitracin (52.6%) treatment groups ($P < 0.05$). All 41 isolates were multiresistant to several of the antibiotics tested (Table 6). However, all integron-bearing isolates were susceptible to ceftriaxone, kanamycin, amikacin, and ciprofloxacin. The aminoglycoside [*ant(3″)-Ia* (*aadA*)], tetracycline [*tet*(A), *tet*(B), or *tet*(C)], and sulfonamide (*sulI* or *sulII*) resistance genes were found in 100% of the integron-positive isolates. The β-lactamase gene (*tem*) and the phenicol resistance gene *floR* were found in 78 and 93% of the isolates, respectively (Table 6).

Association between genetic resistance determinants. The significance of the association between resistance determinants was analyzed. The β-lactamase gene bla_{SHV} and the sulfonamide (trimethoprim) resistance gene *dhfrI* were not associated with any of the resistance genes or class 1 integron. Associations between the β-lactamase (*tem*), tetracycline (*tet*), sulfonamide (*sulI* or *sulII*), aminoglycoside [*ant(3″)-Ia* (*aadA*)], and phenicol resistance (*floR*) genes and class 1 integron were found.

DISCUSSION

The poultry industry has developed in recent years due to the continuous integration of various disciplines for production such as poultry health, nutrition, breeding, husbandry, and knowledge of poultry products (3). However, poultry production in Canada and in the United States is facing constraints. The consequences of poultry production for environmental, food safety, and animal welfare issues are now part of consum-

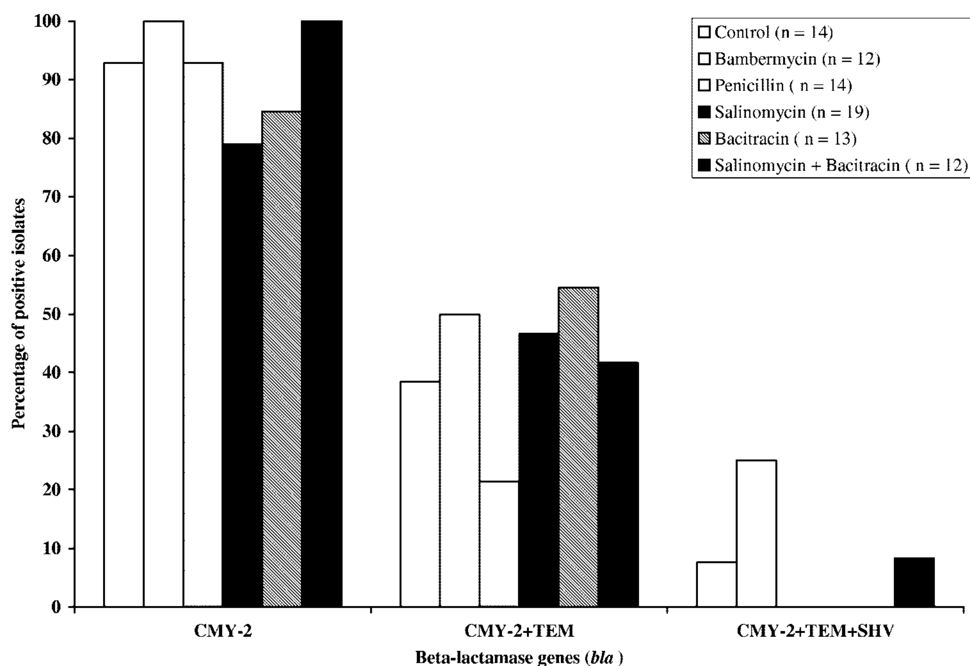

FIG. 3. Prevalence of the *bla* (CMY-2, TEM, and SHV) genes in 84 tetracycline-ceftiofur-resistant *E. coli* isolates (14, 12, 14, 19, 13, and 12 for treatment control, bambermycin, penicillin, salinomycin, bacitracin, and salinomycin plus bacitracin, respectively). Detection of the bla_{CMY-2} gene was performed by PCR as previously described (17).

ers' opinions and demands (2, 18). In the present study, the effects of feed supplementation with bambermycin, penicillin, salinomycin, bacitracin, and a combination of salinomycin plus bacitracin on the growth performance and gut microflora of broiler chickens were investigated over a 35-day period. In accordance with findings by Feighner et al. (22), penicillin was found to increase growth performance. No effects were induced by bambermycin, salinomycin, and/or bacitracin. The lack of significant effects of these compounds on growth performance is likely due to the low repetition numbers (three repetitions/treatment group) and the high hygienic and biosecurity practices used before and throughout the experimental protocol. In a similar experiment conducted with virginiamycin, Dumonceaux et al. (19) also found no significant growth promotion effects for broiler chickens.

The growth of normal intestinal bacteria varies with the gut environment, and there is an increasing interest in the commensal components of the gut microfloras associated with food-producing animals (19, 20, 21, 32, 44). *Escherichia coli* and *Enterococcus* and *Clostridium* spp. are normal inhabitants of the gastrointestinal tract of the chicken. In the present study, feed supplementation with antimicrobial agents had no effect on the concentration of these commensal bacterial species in the cecum and cloaca of the broiler chicken. *Enterococcus* and *E. coli* viable counts were higher at day 7 than at day 35, in contrast to *C. perfringens* counts, which increased from days 7 to 35. Our data confirmed that bacterial numbers in the chicken gut change as a function of age (18). However, *E. coli* counts recovered from the ceca in the present study were higher than those reported in the study by Gabriel et al. (23). The pathogenicity of the bacteria found in our study needs to be investigated to establish their potential health risks for chickens or humans.

The development of antibiotic resistance in *E. coli* isolates from poultry is a well-known phenomenon (5, 47, 48). All of the *E. coli* isolates in the present study were susceptible to ceftriaxone, kanamycin, and amikacin; however, all were multiresistant to several antibiotics. A high rate of resistance to β-lactam (amoxicillin and ceftiofur), tetracycline, streptomycin sulfonamides, and chloramphenicol was noted in the present work. In contrast, Smith et al. (44) detected a low prevalence of resistance to amoxicillin in poultry. The chickens in our study did not receive any anticoccidial or antibacterial agents, other than those used in the experimental design. Care was taken to avoid contamination, and clean pens and fresh wood shavings were used. Our results agree with data reported by Smith et al. (44), who showed a high prevalence of resistance to antimicrobials that are not commonly used in broiler chicken production.

We also found that antibiotic resistance levels decreased with increasing bird age. In chickens, the diets and the environments can affect the microbial status of the gastrointestinal tract (4). Litter and other management practices also can change microbial composition of the chicken gut directly by providing a continuous source of bacteria or indirectly by influencing the defense mechanisms of the birds (4). The reason for the decreased antibiotic resistance level in this study is unclear and may be due to the composition of diets fed at different growth phases, from the starter to the finisher (Table 1), or to other unknown parameters resulting in microbial flora turnover. Our data suggest that day-old chicks are colonized with some resistant strains that are replaced by the normal

TABLE 6. Antibiotic resistance phenotypes and genotypes of 41 class 1 integron-positive *E. coli* isolates from broiler chickens[a]

Strain	Treatment[b]	Pen	Age (days)	Phenotype (resistance pattern)[c]	Genotype			
					bla	*tet*	*sul*	*floR*
2440-CE-2	Control	2	7	Amo-AmoCla-Amp-Cefx-Spec-Stre-Gent-Sulx-Sulz		A, C	*sulI*	−
2447-CE-10	Control	10	14	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2448-CE-17	Control	17	14	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B, C	*sulI*, *sulII*	+
2451-CL-17	Control	17	14	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B, C	*sulI*, *sulII*	+
2453-CE-10	Control	10	21	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2454-CE-17	Control	17	21	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2458-CE2	Control	2	28	Amo-AmoCla-Amp-Cefx-Spec-Stre-Gent-Sulx-Sulz		A	*sulI*	−
2465-CE-5	BBM	5	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2466-CE-18	BBM	18	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*, *shv*	A, B	*sulI*, *sulII*	+
2467-CL-1	BBM	1	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*, *shv*	A, B	*sulI*, *sulII*	+
2468-CL-5	BBM	5	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2478-CE-18	BBM	18	21	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B, C	*sulI*, *sulII*	+
2488-CE-6	PEN	6	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2489-CE-9	PEN	9	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2492-CL-9	PEN	9	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A	*sulII*	+
2511-CE-3	SAL	3	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sara-Nali-Sulx-Sulz-Trm/Sul		A	*sulI*, *sulII*	−
2513-CE-14	SAL	14	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2514-CL-3	SAL	3	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Sara-Nali-Sulx-Sulz-Trm/Sul		A	*sulI*, *sulII*	−
2515-CL-4	SAL	4	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2516-CL-14	SAL	14	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2519-CE-14	SAL	14	14	Amo-AmoCla-Amp-Cefx-Cefti-Clho		A, B	*sulI*, *sulII*	+
2520-CL-3	SAL	3	14	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sara-Nali-Sulx-Sulz		A	*sulI*, *sulII*	−
2521-CL-4	SAL	4	14	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B, C	*sulI*, *sulII*	+
2522-CL-14	SAL	14	14	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B, C	*sulI*, *sulII*	+
2523-CE-3	SAL	3	21	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B, C	*sulI*, *sulII*	+
2525-CE-14	SAL	14	21	Amo-AmoCla-Amp-Cefx-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2534-CL-14	SAL	14	28	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2535-CE-7	BAC	7	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sul-Clho		A	*sulI*, *sulII*	+
2536-CE-11	BAC	11	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2537-CE-16	BAC	16	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B, C	*sulI*, *sulII*	+
2538-CL-7	BAC	7	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B, C	*sulI*, *sulII*	+
2541-CE-7	BAC	7	14	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2542-CE-11	BAC	11	14	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2543-CE-16	BAC	16	14	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2544-CL-7	BAC	7	14	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sara-Nali-Sulx-Sulz-Trm/Sul		A, C	*sulI*, *sulII*	−
2545-CL-11	BAC	11	14	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2550-CL-7	BAC	7	21	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho		A	*sulI*, *sulII*	+
2560-CE-12	SAL + BAC	12	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sara-Nali-Clho	*tem*	A, C	*sulI*, *sulII*	+
2563-CL-12	SAL + BAC	12	7	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sara-Nali-Sulx-Sulz-Clho	*tem*	A	*sulI*, *sulII*	+
2566-CE-12	SAL + BAC	12	14	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+
2569-CL-12	SAL + BAC	12	14	Amo-AmoCla-Amp-Cefx-Cefti-Spec-Stre-Gent-Sulx-Sulz-Clho	*tem*	A, B	*sulI*, *sulII*	+

[a] Table shows antibiotic resistance phenotypes and genotypes of the 41 class 1 integron-positive *E. coli* isolates from broiler chickens. All the isolates were resistant to penicillin, erythromycin, tylosin, clindamycin, oxytetracycline, novobiocin, and sulfizoxazol. All were susceptible to ceftriaxone, kanamycin, amikacin, and ciprofloxacin.

[b] BBM, bambermycin; PEN, penicillin; SAL, salinomycin; BAC, bacitracin; SAL + BAC, salinomycin with bacitracin.

[c] Amo, amoxicillin; AmoCla, amoxicillin-clavulanic acid; Amp, ampicillin; Cefx, cefoxitin; Cefti, ceftiofur; Spec, spectinomycin; Stre, streptomycin; Gent, gentamicin; Sara, saraloxacin; Nali, nalidixic acid; Sulx, sulfadimethoxime; Sulz, sulfathiazole;Trm/Sul, trimethoprim-sulfamethoxazole; Clho; chloramphenicol.

susceptible bacteria as the birds age. The origin of resistance to the other antibiotics is unknown and could be derived from the broiler production environment, including the litter (37), feed, or caretakers (15).

It has been reported that selection and maintenance of tetracycline-streptomycin-sulfonamide-resistant *E. coli* may be due to environmental components independent of antibiotic selection (30). Interestingly, we found higher incidences of ceftiofur, spectinomycin, and gentamicin resistance in *E. coli* isolates from chickens receiving feed supplemented with salinomycin than with other feeds. Higher percentages of gentamicin-resistant isolates were also observed for bacitracin-fed chickens. Coliforms from birds fed salinomycin were found to have more multiresistance patterns with significant numbers resistant to streptomycin, ampicillin, carbenicillin, and cephalothin (24). Our results confirm that multiresistant commensal *E. coli* strains may be present in conventional broiler chicken production independently of specific antibiotic selection pressure.

Characterization of 104 selected tetracycline-resistant *E. coli* isolates showed resistance to several antibiotics of human importance. We used a DNA microarray hybridization method to evaluate the presence and distribution of antibiotic resistance determinants among these tetracycline-resistant isolates (9, 28). In *E. coli* isolates, tetracycline resistance is frequently regulated by several efflux genes on large plasmids that frequently carry other antibiotic and heavy metal resistance genes (13). At least one of three tetracycline resistance genes, *tet*(A), *tet*(B), or *tet*(C), was found in all the 104 tetracycline-resistant isolates. The *tet*(D), *tet*(E), and *tet*(Y) genes were not found in any of the isolates, while the *tet*(A) and *tet*(B) genes were detected in 76 and 59 isolates, respectively. Few isolates were positive for *tet*(C), which was seen only in combination with *tet*(A) or *tet*(A) plus *tet*(B). Fairchild et al. (21) reported the

presence of *tet*(A) and *tet*(B) but not *tet*(C) or *tet*(D) in intestinal *E. coli* isolates after oral administration of tetracycline to chickens.

β-Lactams are among the most clinically important antibiotics in both human and veterinary medicine, and yet resistance to this class of antibiotics is increasing at an alarming rate (34). Previously, we reported the presence of the extended-spectrum-β-lactamase bla_{CMY-2} gene in a large percentage of avian *E. coli* isolates that were resistant to ceftiofur (17). In this study, the bla_{CMY-2} gene was detected in 90.5% of ceftiofur-resistant isolates, indicating that this gene is widespread in commensal *E. coli* isolates from chickens. However, bla_{TEM}, and bla_{SHV} were found in 41 (43%) and 9 (10%) of 95 tetracycline-amoxicillin-resistant isolates, respectively. These genes were similarly distributed among the treatment groups. These results indicate that other resistant genes may be implicated in the resistance to this class of antibiotics in our isolates.

Of the 73 aminoglycoside-resistant isolates, 43 were positive for the aminoglycoside nucleotidyltransferase gene *ant*(*3*″)-*Ia* (*aadA*). The remaining aminoglycoside-resistant isolates, in which none of the other six genes was detected, suggests the presence of different aminoglycoside resistance determinants. Interestingly, we found that there was a higher incidence of *ant*(*3*″)-*Ia* (*aadA*)-positive *E. coli* (80%) in the chickens receiving salinomycin than in the other antimicrobial treatment groups. This result suggests that salinomycin may play a role in the selection and maintenance of streptomycin/spectinomycin resistance in broiler chickens.

Chloramphenicol has not been used in chicken production in Canada since 1980 (26). Nevertheless, 56 of the 58 tetracycline-resistant isolates that were also resistant to chloramphenicol bore the *floR* gene, with a similar distribution among the treatment groups. The chloramphenicol resistance genes *floR*, *cat*, and *cml* were also reported in enterotoxigenic *E. coli* and nonenterotoxigenic *E. coli* isolated from swine in Ontario, Canada (46). In avian species, *flo* was detected in phenicol-resistant *E. coli* isolates in the United States, where chloramphenicol is likewise not used (47). We did not find the phenicol resistance genes *catI*, *catII*, and *catIII* in any of our isolates.

Historically, sulfonamides played an important role in the development of broiler chicken production systems by allowing birds to be raised in higher densities. However, the development of resistance to this class of antibiotic has reduced its role in poultry production (40). In the present work, the *sulI* and *sulII* genes were the sulfonamide resistance genes most frequently found, alone or in combination. More than 70% of the sulfonamide-resistant *E. coli* isolates from the salinomycin (70.6%) and bacitracin (76.9%) treatment groups were positive for *sulI*. A high incidence (76.9%) of the *sulI*-plus-*sulII* combination was found in the bacitracin treatment group.

The ability of bacteria to acquire and disseminate exogenous genes is a major factor in the development of multiple antibiotic resistance. Integrons are gene expression elements that contribute to the spread of antimicrobial resistance by gene transfer in a variety of enteric bacteria (6, 35, 42). The presence of integrons in enteric bacteria from poultry has been previously reported (17, 37, 41). The 41 integron-positive isolates found in our study were all multiresistant. They all harbored the genes *ant*(*3*″)-*Ia* (*aadA*), *sulI*, and/or *sulII* and *tet*(A), *tet*(B), or *tet*(C). The β-lactam (bla_{TEM})- and phenicol (*floR*)-resistant genes were found in 32 (78%) and 35 (85%) isolates, respectively. We did not determine if these genes are physically linked; however, the statistical analysis clearly showed significant associations not only between them but also with the class 1 integron. Associations have been observed between the *tet*(A), *sulI*, and *aadA* genes in porcine *E. coli* isolates (7). The coexistence of antimicrobial resistance genes in association with integrons may increase the selection and dissemination of multidrug-resistant bacteria (34). We found that isolates from the salinomycin and bacitracin treatment groups showed the highest incidence of the class 1 integron. Phenotypic and genotypic analyses suggested that these two growth promoters may play a role in the development and/or maintenance of antibiotic resistance in broiler chicken production.

Our data confirm that the gastrointestinal tract of broiler chickens can be colonized by multidrug-resistant *E. coli* bacteria and that the use of growth promoter agents like salinomycin or bacitracin may exercise pressure for selection for such bacteria. The presence of the class 1 integron in *E. coli* indicates a potential for lateral antibiotic resistance gene transfer between this bacterium and other chicken gut bacteria. These bacteria have the potential to spread in the environment through the litter (37) and subsequently to farm workers and processing plants. Our results also suggest that in the absence of specific antibiotic selection pressure, some specific resistance genes can be maintained due to the association with the genes encoding resistance to other antimicrobials that are currently approved for use in broiler chicken production.

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