Antibiotic resistance in *Escherichia coli* and *Enterococcus* spp. isolates from commercial broiler chickens receiving growth-promoting doses of bacitracin or virginiamycin

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Abstract

Antibacterial agents such as zinc bacitracin (ZB) and virginiamycin (VG) are used as growth promoting agents (GP) in broiler chicken production. The objective of this study was to evaluate the effect of the use of ZB and VG on the emergence of antibacterial resistance in a commercial broiler chicken farm. Three trials were conducted using 3 different diets: one without antibacterial agents, one containing VG, and one with ZB. *Escherichia coli* and *Enterococcus* spp. strains were isolated and tested for their susceptibility to various antibacterial agents. The occurrence of the resistance genes *vat*D, *erm*B, and *bcr*R in *Enterococcus* spp. isolates was determined by polymerase chain reaction (PCR). Comparative quantification of *vat*D and *bcr*R genes in total deoxyribonucleic acid (DNA) extracts from litter was done by SYBR Green Real-Time PCR (QPCR). *Escherichia coli* and *Enterococcus* spp. isolates from diet groups had different levels of resistance to various antibacterial agents over time. These GPs did not select for specific antibacterial agent resistance (AAR) in *Enterococcus* spp. The use of GPs seemed to lower the percentage of *E. coli* isolates resistant to some antibacterial agents. The presence of the *bcr*R gene could not explain all resistant phenotypes to ZB. Genes other than *vat*D and *erm*B might be involved in the resistance to VG in *Enterococcus* spp. Use of GPs was not associated with presence of the *bcr*R gene in DNA extracts from litter, but use of VG was associated with *vat*D presence.

Résumé

*Les agents antibactériens tels que la bacitracine de zinc (ZB) et la virginiamycine (VG) sont utilisés comme promoteurs de croissance (GP) dans la production de poulet à griller. L'objectif de la présente étude était d'évaluer l'effet de l'utilisation de ZB et VG sur l'émergence de résistance aux antimicrobiens sur une ferme commerciale de poulet à griller. Trois expériences ont été réalisées en utilisant 3 diètes différentes : une sans agent antibactérien, une dont l'aliment contenait de la VG, et une où l'aliment contenait de la ZB. Des isolats d'*Escherichia coli *et d'*Enterococcus *spp. ont été obtenus et leur sensibilité à différents agents antibactériens déterminée. La fréquence de la présence des gènes de résistance *vat*D, *erm*B et *bcr*R chez les isolats d'*Enterococcus *spp. a été déterminée par réaction d'amplification en chaîne par la polymérase (PCR). Une quantification comparative des gènes *vat*D et *bcr*R dans des extraits d'ADN total de la litière a été réalisée par PCR en temps réel utilisant le système «SYBR Green» (QPCR). Les isolats d'*E. coli *et d'*Enterococcus *spp. provenant des différents groupes avaient des degrés différents de résistance aux divers agents antibactériens dans le temps. Les GP n'ont pas sélectionné de résistance spécifique aux antimicrobiens (AAR) chez les isolats d'*Enterococcus *spp. L'utilisation de GP a semblé diminuée le pourcentage d'isolats d'*E. coli *résistants à certains agents antibactériens. La présence du gène *bcr*R ne pouvait expliquer tous les phénotypes de résistance au ZB. Des gènes autres que *vat*D et *erm*B pourraient être impliqués dans la résistance à VG observée chez *Enterococcus *spp. L'utilisation de GP n'était pas associée avec la présence du gène *bcr*R dans les extraits d'ADN de la litière, mais l'utilisation de VG était associée avec la présence de *vat*D.*

(Traduit par Docteur Serge Messier)

Introduction

Subtherapeutic doses of antibacterial agents, given as GP, are still used to improve zootechnical performances in animal husbandry (1). While regulations have been put in place in Europe to reduce the use of GP (2), the broiler chicken industry in Canada still uses compounds such as ZB and VG.

Bacitracin is a polypeptidic antibacterial agent produced by *Bacillus licheniformis*. It inhibits the dephosphorylation of C55-isoprenylpyrophosphate, blocking the recycling of this transporter needed for the production of the peptidoglycan cell wall (3).

A resistance caused by a unidirectional pump has been observed in *Enterococcus* spp. (4). Bacitracin resistance genes *bcr* of *Enterococcus*

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spp. are thought to be regulated by *bcr*R (5). *Escherichia coli* also possesses a gene, *bac*A, that confers resistance to ZB (6).

Virginiamycin is a member of the streptogramin antibacterial agent class that is divided into 2 categories: group A — macrocyclic lactones, and group B — hexadepsipeptides. Virginiamycin is a combination of these 2 classes in a 70:30 ratio. It binds to ribosomal RNA 23S of the 50S ribosome sub-unit and inhibits protein production (7).

Resistance to VG can be the result of target modification caused by *erm* genes. The *erm*B gene is found in *Enterococcus* spp. and targets group B compounds (8). Inactivation of VG by *vat* genes (9), targeting group A compounds, and *vgb* genes (10) targeting group B compounds, can cause resistance to VG in *Enterococcus* spp. (8).

The aim of this study was to evaluate the potential of ZB and VG to promote antibacterial agent resistance (AAR) in commensal *E. coli* and *Enterococcus* spp., and to increase the AAR gene reservoir in the environment of broiler chickens. In order to do so, groups of birds were fed with or without ZB or VG. Resistance profiles of *Enterococcus* spp. and *E. coli* were evaluated. Occurrence of selected resistance genes was also assessed in *Enterococcus* spp. isolates from litter total DNA extracts by real-time PCR.

Materials and methods

Farm trials

This study was undertaken from August 2005 to February 2006 on a commercial broiler chicken farm in Farnham, Quebec. The barn was divided into 3 separate floors, each with a distinct feeding system. Each floor was dedicated to 1 specific diet group and housed 4000 day-old female Ross broiler chickens at a density of 0.08 m^2 (0.85 ft^2) per chicken. Diet types consisted of a standard commercial vegetal soy-corn diet with ZB added (Albac; Alpharma Canada, Mississauga, Ontario) 55 ppm, or with VG added (Stafac 44; Phibro Animal Health, Regina, Saskatchewan) 22 ppm; a control group, without GP, was included in the study. Before the trial initiation, treatments were randomly assigned to a specific floor; 3 consecutive trials were conducted. In the subsequent trials, each treatment was rotated to ensure that each floor would receive different treatments. The birds were fed and watered ad libitum. At the beginning of each trial, all birds were vaccinated against Marek's disease and weighed (~ 50 g); dead birds were removed daily. In each trial, birds were studied over a period of 34 d. Biosecurity measures were put in place to avoid cross contamination between treatment groups.

Upon arrival, at the start of each trial, 150 chicks were swabbed to collect cloacal samples; litter samples were also collected. On day 34, 5 samples, each consisting of cloacal swabs pooled from 3 birds, were taken randomly in each group. Five samples of approximately 100 g of litter were taken randomly from each group on days 0 and 34 of each trial.

Bacterial isolation

Enterococcus spp. and *E. coli* were isolated from samples. Briefly, for *E. coli*, samples were diluted in peptone buffered water (BD, Oakville, Ontario) at a ratio of 1:9. Peptone buffered water was incubated at 37°C overnight and 1 loop was streaked on MacConkey Agar (Fisher Scientific, Ottawa, Ontario). Typical *E. coli* colonies were isolated and purified on 5% Sheep Blood Agar (Quelab, Montreal, Quebec) and confirmed with triple iron sugar agar, citrate agar, oxidase and by catalase tests. One out of 10 isolates with typical biochemical patterns was further characterized on API 20E galleries (bioMérieux Canada, St. Laurent, Quebec) (11). For *Enterococcus* spp., samples were diluted in Enterococcosel broth (Fisher Scientific) in a 1:1 ratio. One loop of Enterococcosel broth was streaked on Enterococcosel Agar (Fisher Scientific) after an overnight incubation at 42°C. Typical *Enterococcus* spp. colonies were selected and identified as to the species by using Slanetz and Bartley Agar (Fisher Scientific), mannitol fermentation, arabinose fermentation, and methyl-α-D-glycopyranoside fermentation (11).

Antibacterial agent resistance

All *Enterococcus* spp. (n = 211) and *E. coli* (n = 214) isolated from litter and from birds were tested for phenotypical AAR (Table I). Because of logistical reasons, birds of replicate 1 could not be sampled and only litter was sampled. Resistance to selected antibacterial agents (Oxoid, Nepean, Ontario) was determined by disk diffusion according to Clinical and Laboratory Standards Institute's (CLSI) guidelines (12). The following antimicrobial agents were used for all isolates: amikacin (AK), amoxicillin/clavulanic acid (AMC), ampicillin (AMP), apramycin (APR), bacitracin (B), cefoxitin (FOX), ceftiofur (XNL), ceftriaxon (CRO), cephalothin (KF), chloramphenicol (C), ciprofloxacin (CIP), clindamycin (DA), enrofloxacin (ENR), erythromycin (E), gentamycin (CN), kanamycin (K), nalidixic acid (NA), neomycin (N), quinupristin/dalfopristin (QD), streptomycin (S), sulfamethoxazole (RL), tetracycline (TE), trimethoprim-sulfamethoxazole (STX), vancomycin (VA) and virginiamycin (VG). *Escherichia coli* ATCC #25922 and *Enterococcus faecalis* ATCC #29212 were used as control strains.

Bacitracin was used in the disk diffusion assay to evaluate ZB phenotypical resistance. Virginiamycin was not available in disk format for the agar disk diffusion assay, so resistance to VG was assessed using a minimal inhibitory concentration (MIC) agar dilution method. Virginiamycin was extracted using acetone from the premix Stafac 44, containing 44 g of virginiamycin per kg, to a concentration of 20 mg/mL (13,14). After extraction, acetone was filtered with a 0.22 μm filter and added aseptically into 50°C non-solidified Mueller-Hinton Agar (Oxoid) to obtain serial 2-fold dilutions of VG in agar plates ranging from 2 to 256 μg/mL.

In a previous study, the breakpoint for resistance to VG was 8 μg/mL against *E. faecium* (15). In this study, VG was extracted from the premix and tested against *Enterococcus* spp. including both *E. faecalis* and *E. faecium*. For this reason, a VG breakpoint was fixed according to the (Gaussian) distribution of MICs of the isolates. The MIC for VG was determined to be 32 μg/mL, corresponding to one standard deviation over the means of the MICs obtained.

DNA extractions

Isolates of *Enterococcus* spp. (n = 90) were grown overnight on 5% (v/v) Sheep Blood Agar (Quelab). One loop of bacteria was resuspended in 200 μL of lysis buffer [Tris-HCl 20 mM, EDTA 2 mM, Triton X-100 1.2% (v/v), pH 8.0] containing 10% (v/v) of Chelex-100

Table I. Numbers of bacteria isolated from 150 broiler chicken cloacae and 275 litter samples[a]

		Bacterial number in treatment											
		Control				Virginiamycin				Zinc bacitracin			
		E. coli		Enterococci		*E. coli*		Enterococci		*E. coli*		Enterococci	
Collection	Sample	T0[b]	T34[c]	T0	T34	T0	T34	T0	T34	T0	T34	T0	T34
1	Litter	5	13	10	12	7	14	7	13	3	14	10	11
	Cloacae	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2	Litter	0	8	6	9	1	11	4	10	2	11	7	10
	Cloacae	11	3	10	2	14	0	13	3	14	0	10	4
3	Litter	1	11	6	9	2	10	5	9	3	10	7	9
	Cloacae	15	0	7	0	15	0	9	0	13	0	7	0
Total		32	32	39	32	39	35	38	35	35	35	41	34

nd — no data.
[a] A total of 211 *Enterococcus* spp. (65 and 146 from cloacae and litter samples, respectively) and 214 *E. coli* (85 and 129 from cloacae and litter samples, respectively) were isolated.
[b] Time 0.
[c] Time 34 d.

Table II. Primers developed during this work and used for growth promoter resistance gene screening

Gene	Gene bank accession number	Primer sequence (5′–3′)	Hybridization temperature	Amplicon length (bp)
*erm*B	AY827545.1	F-CATTTAACGACGAAACTGGC R-GGAACATCTGTGGTATGGCG	55°C	400
*vat*D	L12033.1	F-CAAATCATAGAATGGATGGC R-TTTCGTTAGCAGGATTTCC	53°C	251
*bcr*R	AY496968.1	F-GTTACCCTAACATGGAGTCG R-AAACATAACCGCCAACAGAG	55°C	215

(Bio-Rad, Mississauga, Ontario) and then heated for 8 min at 95°C. Samples were centrifuged at 5000 × *g* for 2 min and supernatants were used directly for conventional PCR (16).

Total DNA extraction from litter (*n* = 36) was achieved by centrifugating 2 mL of litter, thoroughly mixed 1:9 in peptone buffered water, at 8000 × *g* for 10 min. The pellet containing bacteria was washed with 1 mL of washing buffer (Tris-HCl pH 8.0, NaCl 150 mM). After another centrifugation step, the pellet was resuspended in 200 μL of lysis buffer supplemented with 2% (v/v) SDS and added to 30 mg of 0.1 mm glass beads (Biospecs Products, Bartlesville, Oklahoma, USA) in a 1.5 mL microfuge tube. Microfuge tubes were then vortexed for 5 min using a Turbomixer (Fisher Scientific) to allow cell breakdown (17). Chelex-100 was added to obtain to a final concentration of 10% (v/v) after lysis. Tubes were then heated for 8 min at 95°C, cooled down for 2 min, and then centrifuged at 10 000 × *g* for 2 min. The DNA in the supernatant was cleaned up and concentrated using the Qiagen DNAeasy Tissue kit (Qiagen, Mississauga, Ontario). The DNA extraction protocol was initiated with a proteinase K digestion step and an incubation at 70°C for 15 min, following the manufacturer's instructions. Prior to extraction, a loopful of a *Enterococcus faecium* isolate (#crsv-ent01), previously characterized in our laboratory, containing *erm*B, *vat*D and *bcr*R genes was added to 1 litter sample as a positive control.

Polymerase chain reaction

Enterococcus spp. (*n* = 90) isolates, randomly selected from litter and bird sampling, were screened for growth promoter AAR genes by conventional PCR. The GP-resistant and GP-susceptible phenotypes from day 0 and day 34 were used to determine the carriage of the selected genes in both *E. faecium* and *E. faecalis*. The PCR for VG resistant *vat*D and *erm*B genes and for the ZB resistant *bcr*R gene was performed using the Invitrogen *Taq* polymerase kit (Invitrogen Canada, Burlington, Ontario) using 1 unit of *Taq* polymerase, 1.5 mM of magnesium chloride ($MgCl_2$), 1 mM of deoxynucleotide triphosphates (dNTPs), 0.5 μM of each primer, and 0.1 μg/μL of bovine serum albumin (BSA). New PCR primers (Table II) were designed using GenBank sequences with the software PerlPrimer (18). Amplification conditions in an Eppendorf Mastercycler gradient system (Brinkmann Instruments Canada, Mississauga, Ontario) were as follows: initial denaturation 1 step of 5 min at 95°C, followed by 40 cycles of 5 s at 95°C, 15 s at annealing temperature (Table II), 5 s at 72°C, and a final extension step of 2 min at 72°C. Amplicons were visualized after electrophoresis on 2% (v/v) agarose gels using SybrSafe (Invitrogen). Positive controls for *vat*D and *erm*B were provided by Dr. Michel Bergeron, Centre Hospitalier Universitaire de Québec (CHUQ, QC). Positive controls for *bcr*R were provided by Dr. Gregory M. Cook, New-Zealand University of Otago (9).

Table III. Significant ($P < 0.05$) changes (day 0 vs day 34) in the percent of isolates of *Escherichia coli* resistant to antibacterial agents for each type of diet

	Antibacterial agents with significant increase of resistant isolates percentage			Antibacterial agents with significant decrease of resistant isolates percentage		
Diets	Antibacterial agent[a]	d = 0 (%)	d = 34 (%)	Antibacterial agent	d = 0 (%)	d = 34 (%)
No additive	KF	5.5 (± 11)	43.0 (± 21.4)	CN	15.7 (± 18)	2.0 (± 3)
	STX	0.0 (± 0)	17.7 (± 20.0)			
ZB	—	—	—	AMC	33.3 (± 46)	14.0 (± 14)
	—	—	—	AMP	82.5 (± 21)	27.3 (± 5)
	—	—	—	KF	31.3 (± 28)	15.7 (± 18)
VG	K	0.0 (± 0)	16.7 (± 29)	AMP	32.5 (± 36)	9.7 (± 10)
	N	0.0 (± 0)	9.7 (± 17)	CN	12.5 (± 25)	1.0 (± 1)
	NA	0.0 (± 0)	21.7 (± 22)			

Average (replicate 1, 2, 3) percentage of isolates resistant to the antibacterial agent (± standard deviation).
[a] AMC — amoxicillin/clavulanic acid, AMP — ampicillin, KF — cephalothin, CN — gentamycin, K — kanamycin, NA — nalidixic acid, N — neomycin, STX — trimethoprim-sulfamethoxazole.

SYBR green real-time polymerase chain reaction

The *bcr*R and *vat*D genes, associated with GP resistance, were cloned following the manufacturer's recommendations using the TopoTA cloning kit with electrocompetent Top10 *E. coli* cells (Invitrogen). Plasmids from selected clones were then extracted with the NucleoSpin Plasmid kit (BioLynx, Brockville, Ontario). Plasmids were linearized with Bam HI enzyme (Roche, Laval, Quebec) and used for optimization, standardization and generation of standard curves ranging from 10^6 to 10^1 copies. The quantitative polymerase chain reaction (QPCR) assays were performed using the same primers as conventional PCR (Table II). Stratagene Brilliant SYBR Green QPCR Core Reagent Kit (VWR, Mont-Royal, Quebec) was used as recommended by the manufacturer with a primer concentration of 750 nM. The QPCR was performed using Stratagene Mx4000 system (Stratagene, La Jolla, Ontario). Cycling conditions consisted of an initial denaturation step of 10 min at 95°C followed by 45 cycles of 5 s at 95°C, 15 s at annealing temperature (Table II), and 20 s at 72°C with 2 fluorescence acquisitions at 72°C. The temperature ramping rate was set to 2°C/s. Melting curve analysis was performed at the end of amplification using 1°C/min for a 30-min segment. Obtained threshold cycle (Ct) values were expressed as grams of extracted litter. Low Ct/g values correspond to high copy numbers of the target gene. Four litter samples retrieved at T = 34 days for each diet for each replicate were compared in duplicate on the same QPCR reaction plate.

Statistical analyses

The Cochran-Mantel-Haenszel test was used to determine the relationship between the feed used and the percentage of strains resistant to each antibiotic. The relationship between time and the percentage of resistance to each antibiotic was done using the FREQ procedures of SAS (SAS 2000; SAS Institute, Cary, North Carolina, USA). A linear mixed model was used for the QPCR experiments to assess significant differences between diet groups. The statistical significance was set at a value of $P < 0.05$.

Results

Antibacterial agent resistance profiles

The level of resistance (day 0 vs day 34) was compared for each diet group for each microorganism. Significant ($P < 0.05$) variations in the percentage of isolates resistant to antibacterial agents were found for *E. coli* in all conditions (Table III). As expected, all isolates were resistant to VG and ZB. For *Enterococcus* spp. ($P < 0.05$), variations in the percentage of resistant isolates over time were observed in all diet groups (Table IV). The percentage of resistance to 4 antibacterial agents (bacitracin, erythromycin, quinupristin/dalfopristin, and apramycin) increased over time regardless of the type of diet used. Resistance to VG and ZB in *Enterococcus* spp. isolates was also variable.

The level of resistance between groups was evaluated at the end of each trial. Some significant ($P < 0.05$) differences were found for *E. coli*. Surprisingly, the percentage of resistant isolates to cephalothin was higher in the control group than in the ZB and VG groups. The proportion of isolates resistant to nalidixic acid was higher in the control group than in the ZB group, and a higher percentage of resistant isolates to nalidixic acid was found in the VG group compared with the ZB group. The percentage of isolates resistant to sulfamethoxazole was higher in birds fed with the control diet than in those that were fed the ZB diet. There was no significant effect of diet type on the frequency of *Enterococcus* spp. resistant to any of the antibiotics ($P < 0.05$).

Occurence of antibacterial agent resistance genes

Screening of *Enterococcus* spp. isolates by PCR revealed the presence of *bcr*R, *vat*D, and *erm*B in 50.5%, 14.0%, and 67.0% of the isolates. Phenotypical resistance to ZB was observed in 52.0% of isolates, resistance to VG in 64.0% of isolates, and resistance to QD in 70.0% of isolates.

Table IV. Significant ($P < 0.05$) changes (day 0 vs day 34) in the percent of isolates of *Enterococcus* spp. resistant to antibacterial agents for each type of diet

	Antibacterial agents with significant increase of resistant isolates percentage			Antibacterial agents with significant decrease of resistant isolates percentage		
Diets	Antibacterial agent[a]	d = 0 (%)	d = 34 (%)	Antibacterial agent	d = 0 (%)	d = 34 (%)
No additive	AK	69.0 (± 31)	94.0 (± 10)	ENR	18.0 (± 36)	0.0 (± 0)
	APR	67.8 (± 30)	91.3 (± 9)			
	B	35.0 (± 18)	67.0 (± 24)			
	E	18.0 (± 15)	72.7 (± 25)			
	K	88.0 (± 18)	97.3 (± 5)			
	N	58.8 (± 44)	91.3 (± 9)			
	QD	28.5 (± 24)	90.7 (± 2)			
	TE	72.8 (± 22)	97.3 (± 5)			
	VG	58.0 (± 39)	79.3 (± 19)			
ZB	APR	80.3 (± 29)	97.0 (± 5)	CN	48.8 (± 37)	29.0 (± 43)
	B	43.0 (± 31)	98.0 (± 4)			
	E	54.3 (± 33)	81.0 (± 33)			
	KF	20.0 (± 14)	58.0 (± 31)			
	QD	47.0 (± 24)	91.0 (± 16)			
	VG	62.3 (± 18)	86.7 (± 23)			
	AK	85.5 (± 17)	97.5 (± 5)			
VG	APR	77.3 (± 19)	91.3 (± 5)	ENR	12.5 (± 25)	0.0 (± 0)
	B	37.5 (± 43)	77.7 (± 22)			
	E	49.8 (± 17)	86.0 (± 8)			
	N	72.3 (± 20)	95.0 (± 9)			
	QD	63.5 (± 25)	93.7 (± 26)			

Average (replicate 1, 2, 3) percentage of isolates resistant to the antibacterial agent (± Standard Deviation).
[a] AK — amikacin, APR — apramycin, B — bacitracin, KF — cephalothin, ENR — enrofloxacin, E — erythromycin, CN — gentamycin, K — kanamycin, N — neomycin, QD — quinupristin/dalfopristin, TE — tetracycline, VG — virginiamycin.

Real-time polymerase chain reaction

Standard curve parameters obtained with the *bcr*R clone were $y = -3.558 \times \log(x) + 42.55$ with an efficacy of 91% and a regression coefficient of 0.999. Comparative quantification data for *bcr*R in the litter samples harvested at the end of the experiment gave the following Ct/g of extracted litter: diet without antibacterial agents = 134.62, ZB diet = 132.78, and VG diet = 131.03. As expected, only 1 amplification product was visible on the denaturation curve. When the Ct/g values for each diet group were compared, no significant difference was found for the presence of the *bcr*R gene.

Standard curve parameters for the *vat*D clone were $y = -4.531 \times \log(x) + 49.85$ with a low efficacy of 66%, but a regression coefficient of 0.988. Comparative quantification data obtained from the same amplification plate for *vat*D in the litters harvested at the end of the experiment were 198.53, 200.69, and 185.16 Ct/g of extracted litter, respectively for diet without antibacterial agents, ZB diet, and VG diet. Once again, only 1 amplification product was visible on the denaturation curve. Statistical analysis revealed that litter extracts from the VG diet group contained significantly ($P < 0.05$) more *vat*D gene copies (lower Ct/g values) than the other 2 diet groups.

Discussion

One objective of the present study was to determine if the use of GP would increase the reservoir of AAR in selected enteric bacteria of broiler chickens. In a similar experiment, McDermott et al (19) observed an increase in the resistance to QD in *E. faecium* isolated from VG fed broiler chickens; this effect was not observed in the present study. It is difficult, however, in a commercial-scale experiment, such as in the current study, to control all aspects related to a possible bacterial cross-contamination among groups of birds. Even if rigorous biosecurity measures were taken, cross-contamination by flies or dust carried on clothing, skin, or through ventilation systems may have affected the results.

The percentages of resistance at day = 0 are not evenly distributed in all test conditions (Tables III and IV). This might reflect lack of uniformity in the distribution of bacterial strains within the environment. Relatively few isolates were found in fresh clean litter at day 0. The AAR still increased significantly over time and there were also significant changes among diet groups.

In this field study, a decrease in gentamycin resistance was observed for ZB and VG diet groups. Decrease of resistance to

gentamycin at broiler chicken farms was also reported in a recent study (20). This decrease could have resulted from the use of this antimicrobial at the hatchery level. Gentamycin was not used in the present study, possibly decreasing the advantage conferred by gentamycin resistance aquired by bacteria at the hatchery, and thus decreasing the presence of resistant isolates to gentamycin (21). This explanation could also be applied to other antibacterial agents with decreased percentage of resistance (Tables III and IV). Unfortunately, information on the use of antibacterial agents at the hatchery was not available. On the other hand, variations of antibacterial agents resistance, over time, may also be associated with animal age and the changes in the gut flora during the growing period (21).

Escherichia coli is considered intrinsically resistant to ZB and VG; however, *E. coli* was monitored in this study, as it is a common indicator microorganism used in most AMR studies (DANMAP). Also, we wanted to explore the possibility that resistance to antibacterial agents in *E. coli* may be affected by growth promoters, either directly by co-selection mechanisms or indirectly by alteration of enteric bacterial populations.

Playdell et al (22) observed that resistance to ampicillin increased in the abscence of GP in organically grown broilers. In the study herein, resistance of *E. coli* isolates to some antibacterial agents varied significantly in the various feed conditions, possibly because of inhibition of AAR gene transfer, as reported for some GPs (23). This observation needs to be thoroughly investigated in future studies.

Variation of the antimicrobial resistance profiles may also result from factors other than the presence or absence of GPs. There may be some resistance to metals such as zinc, which may co-select for AAR (24). In this study, zinc levels (data not shown) found in ZB feed were not identical to those in VG and control diets. Differences observed between ZB condition and the other feed conditions may thus be due to an effect of zinc rather than the sole presence of bacitracin. Selection of phenotypical resistance to antibacterial agents may also be due to co-selection of linked gene by factors other than GP presence (25). For example, the resistance gene *erm*B in *E. faecium* has been found linked to other important resistance genes (26).

The use of enrichment media for *Enterococcus* spp. isolation could also have selected for AAR isolates; this could explain the lack of significant differences between groups (27).

The apparent spread of resistance to bacitracin, erythromycin, quinupristin/dalfopristin, and apramycin may result from the colonization of the farm environment by a specific genotype of *Enterococcus* spp. (21). Clonal analyses of *Enterococcus* spp. isolates required to verify this hypothesis would be beneficial to explore the possibility that some isolates from birds and litter are identical.

Enterococcus faecalis is considered to be resistant to VG since it possesses the chromosomal resistance gene *Lsa* (28). This bacterial species is thus generally not used in VG resistance studies. In this experiment, the MIC of VG against *E. faecalis* varied from 32 μg/mL to > 256 μg/mL, and resistance genes *vat*D and *erm*B were found in a variable proportion of these isolates (data not shown). It is possible that naturally resistant bacteria may acquire additional resistance genes present in the environment; therefore, they should be considered more frequently in AAR studies. Monitoring of AAR could be assessed by global surveillance of resistance genes (29).

Prevalence of antibacterial agent resistance genes

The prevalence of AAR genes in the poultry environment was variable. To our knowledge, ZB resistance gene *bcr* has only been found in *Enterococcus* spp. and its prevalence is currently unknown. The presence of the streptogramin resistance gene *vat*D is variable, ranging from 0% to 100% in *Enterococcus* spp.; the presence of *erm*B also varies from 0% to 100% (19,30–32). The *bcr*R gene was not found in all phenotypically ZB resistant *Enterococcus* spp. isolates, suggesting that other genetic determinants are associated with this resistance.

Resistance to streptogramin cannot be explained soley by the presence of the *vat*D gene, since this gene was not found in all VG or quinupristin/dalfopristin resistant isolates. The streptogramin resistance gene *erm*B was also found in some susceptible *Enterococcus* spp. isolates. The presence of only 1 of these genes is not likely to confer full resistance to streptogramin since they target only 1 of the GP components (33). Only 12.6% of the strains harbored both *vat*D and *erm*B genes (data not shown). These findings suggest that other genes responsible for VG resistance may have been present.

Real-time polymerase chain reaction

Since it is likely that the changes in AAR observed by the use of indicator microorganisms would not completely reflect the impact of GP use on the AAR in the various enteric bacterial species, a QPCR was developed for a comparative quantification, in total DNA extracts, of the resistance genes that may be present in other bacterial species. This approach has already been used in the study of other AAR genes (34,35).

The ZB resistance gene *bcr*R has not yet been reported in bacteria other than *Enterococcus* spp. Lack of difference in comparative quantification between strains from birds exposed to the different diet conditions for *bcr*R suggests that this gene is present in other bacteria that might not have been selected by GP. Other bacterial genera with various resistance levels to ZB should be evaluated for the presence of the *bcr*R gene in order to compare the phenotypic results of ZB resistance with the results obtained by QPCR.

Diets containing VG did not select for VG resistant *Enterococcus* spp., but did contribute to the selection of the streptogramin resistance gene *vat*D in total litter DNA extracts. This result reinforces the hypothesis that resistance to VG in this experiment could not only be the result of *vat*D gene presence, but also to the presence of many other genes that are not necessarily selected for by growth promoting doses of VG.

Conclusion

Under the field conditions of this study, the use of GPs seemed to lower the percentage of *E. coli* isolates resistant to some antibacterial agents, but did not significantly influence the overall resistance phenotype of *E. coli* and *Enterococcus* spp. from broiler chickens. However, the use of VG may select for specific resistance genes in total litter DNA extracts. Future studies undertaken over a longer period and within other premises should be conducted to validate these results. This study also suggests that QPCR can be a useful

tool for monitoring the level of AAR and in future experiments for characterizing the risk posed by the presence of specific AAR genes in the broiler chicken farm context.

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