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Publisher's version / Version de l'éditeur:
https://doi.org/10.1128/AEM.01545-10
Applied and Environmental Microbiology, 76, 24, pp. 8033-8043, 2010-12-01

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2010, p. 8033–8043 Vol. 76, No. 24
0099-2240/10/$12.00 doi:10.1128/AEM.01545-10

Distribution of Antimicrobial Resistance and Virulence Genes in *Enterococcus* spp. and Characterization of Isolates from Broiler Chickens▽†‡

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Received 5 July 2010/Accepted 18 October 2010

Enterococci are now frequent causative agents of nosocomial infections. In this study, we analyzed the frequency and distribution of antibiotic resistance and virulence genotypes of *Enterococcus* isolates from broiler chickens. Fecal and cecal samples from nine commercial poultry farms were collected to quantify total enterococci. Sixty-nine presumptive enterococci were isolated and identified by API 20 Strep, and their susceptibilities to antibiotics were determined. Genotypes were assessed through the use of a novel DNA microarray carrying 70 taxonomic, 17 virulence, and 174 antibiotic resistance gene probes. Total enterococcal counts were different from farm to farm and between sample sources ($P < 0.01$). Fifty-one (74%) of the isolates were identified as *E. faecium*, whereas nine (13%), seven (10%), and two (3%) isolates were identified as *E. hirae*, *E. faecalis*, and *E. gallinarum*, respectively. Multiple-antibiotic resistance was evident in *E. faecium* and *E. faecalis* isolates. The most common multiple-antibiotic resistance phenotype was Bac Ery Tyl Lin Str Gen Tet Cip. Genes conferring resistance to aminoglycoside (*aac*, *aacA-aphD*, *aadB*, *aphA*, *sat4*), macrolide (*ermA*, *ermB*, *ermAM*, *msrC*), tetracycline (*tetL*, *tetM*, *tetO*), streptogramin (*satG_vatE8*), bacitracin (*bcrR*), and lincosamide (*linB*) antibiotics were detected in corresponding phenotypes. A range of 9 to 12 different virulence genes was found in *E. faecalis*, including *ace*, *agg*, $agrB_{Efs}$ (*agrB* gene of *E. faecalis*), *cad1*, the cAM373 and cCF10 genes, *cob*, *cpd1*, *cylAB*, $efaA_{Efs}$, and *gelE*. All seven *E. faecalis* isolates were found to carry the *gelE* gene and to hydrolize gelatin and bile salts. Results from this study showed the presence of enterococci of public and environmental health concerns in broiler chicken farms and demonstrated the utility of a microarray to quickly and reliably analyze resistance and virulence genotypes of *Enterococcus* spp.

Enterococcus spp., particularly *E. faecium* and *E. faecalis*, are important in public health; these species are responsible for approximately 12% of all nosocomial infections in the United States (11, 22). In humans, enterococci cause urinary tract infections, bacteremia, peritonitis, and endocarditis, with about 90% of all clinical infections being caused by *E. faecalis* and *E. faecium* (22). The virulence of enterococci is associated with several genes, including *ace* (collagen binding cell wall protein), *acm* (surface-exposed antigen), *agg* (aggregative pheromone-inducing adherence to extra-matrix protein), $agrB_{Efs}$ (AgrB protein of *E. faecalis*), *esp* (enterococcal surface protein), *hyl* (hyaluronidase), *cad1* (pheromone cAD1 precursor lipoprotein), the cAM373 gene (sex pheromone cAM373 precursor), the cCF10 gene (pheromone cCF10 precursor lipoprotein), *cob* (pheromone cOB1 precursor/lipoprotein, YaeC family), *cpd1* (pheromone cPD1 lipoprotein), *cylABLM* (hemolysin), $efaA_{Efs}$ (endocarditis-specific antigen), *sagA* (secreted antigen), and *gelE* (gelatinase) (22, 29). These virulence factors have been reported in enterococci isolated from food of animal origin (44).

The use of antimicrobial agents in livestock production can promote antibiotic resistance in the bacteria carried by animals (36), and antibiotic resistance in enterococci isolated from poultry has been widely documented (6, 39, 40). Although numerous mechanisms of antibiotic resistance have been described in enterococci (11, 30), little is known about the molecular ecology and genotype distribution of enterococci isolated from commercial chickens. Tetracycline-resistant enterococcus isolates harboring *tetL*, *tetM*, *tetO*, or *tetS* in association with the *ermB* gene encoding resistance to macrolide, lincosamide, and streptogramin B quinupristin-dalfopristin (MLS_B) have been isolated from the cloacae of broiler chickens (7). A previous study indicated that monensin and tylosin in feed did not affect the frequency of the macrolide (*ermB*) or tetracycline resistance (*tetM*) genes in *Enterococcus* species isolated from fecal samples or the antimicrobial susceptibility patterns of this bacterium (27). However, *Enterococcus* isolates from cattle fed monensin or tylosin had greater levels of resistance toward macrolides (27). The use of avoparcin in Europe was banned in 1997 because of the presence of vancomycin-resistant *Enterococcus* (VRE) throughout the human food supply (24, 36). Several years after the ban of avoparcin, geneti-

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† Pacific Agri-Food Research Centre contribution no. 715.

‡ Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 22 October 2010.

cally diverse strains of vancomycin-resistant *Enterococcus* possessing resistance to more than five antimicrobials have been isolated from United Kingdom broiler chickens (19).

The ability of *Enterococcus* to acquire antibiotic resistance through transfer of plasmids and transposons, chromosomal exchange, or mutation presents a significant challenge for therapeutic measures (11). Consequently, a system to simultaneously identify and genotype enterococcal species is essential for food industries (safety and security), public heath institutions (surveillance and diagnostic), and environmental agencies. Although culture-based methods in bacteriology are widely used, they are time-consuming and provide little clinical information regarding the pathogen's genotype, including antibiotic resistance genes and virulence factors. Molecular methods using DNA microarrays show great potential in research, food safety, medical, agricultural, regulatory, public health, and industrial settings (38). Other molecular typing methods, such as pulsed-field gel electrophoresis (PFGE), arbitrarily primed PCR (AP-PCR), and multilocus sequence typing (MLST), have been used to compare pathogens (32, 41). These methods are useful to understand the population dynamics and the ecology of specific organisms. However, they cannot confirm the identification of specific isolates or the carriage of important antibiotic resistance and virulence genes without additional testing. Previously, we used a DNA microarray targeting several genes to detect and describe the distribution of antibiotic and virulence determinants in *Escherichia coli* isolated from broiler chickens receiving feed supplemented with different antimicrobial agents (3, 13). The objective of the present study was to determine how broiler chicken enterococci vary in their abundance, species diversity, and characteristics according to the medicated rations used to feed them. The antibiotic resistance profiles and the biochemical phenotypes were monitored in *Enterococcus* species isolates recovered from nine commercial broiler farms using antimicrobial agents in chicken feed. A DNA microarray method was used to evaluate the distribution of various virulence and antibiotic resistance genetic determinants in studied isolates, identifying characteristics of public health concern to which humans could be exposed through contaminated food products or fecally contaminated water.

MATERIALS AND METHODS

Farm, diet, and sampling. Nine commercial broiler farms (designated a, b, c, d, e, f, g, i, and j) in the Fraser Valley of British Columbia, Canada, that varied in their use of feed from three commercial feed companies (A, B, and C) were chosen for this study. The farms used standard management practices, and the commercial feeds included different antimicrobial agents as previously described (14). Bacitracin, narasin, and nicarbazin were present in all feeds. In addition, the feed from company A contained virginiamycin, the feed from company B contained salinomycin, and that of company C contained diclazuril and virginiamycin. Briefly, 26 fecal samples were collected from each farm on days 8 to 10 and days 25 to 28. At slaughter (days 35 to 40), the cecal contents were obtained from five birds from each farm. Fecal and cecal samples were prepared for bacteriology and enterococcal isolation as previously described (13).

***Enterococcus* population and isolation.** Samples (5 to 6 g) in Carry-Blair medium (Quelab, Montréal, Quebec, Canada) were vigorously vortexed for 1 min, and then 10-fold serial dilutions were prepared in sterile saline solution. Presumptive *Enterococcus* populations were enumerated by spreading 10-fold dilutions of individual samples from each farm on KF streptococcal agar CM0701 (Oxoid, Nepean, Ontario, Canada) and incubating at 37°C for 48 h (13, 23). Results were expressed as CFU per gram of fecal or cecal material. Approximately eight typical colonies per farm were randomly selected and confirmed as enterococci by Gram staining, catalase test, and API 20 Strep (bioMérieux, Saint-Laurent, Quebec, Canada). Purified *Enterococcus* colonies were frozen at −80°C in tryptic soy broth (Becton Dickinson, Mississauga, Ontario, Canada) containing 25% glycerol.

Antibiotic susceptibility. The antimicrobial MICs of all *Enterococcus* isolates were determined using the Sensititre automated system (Trek Diagnostic Systems, Cleveland, OH) according to the CLSI (Clinical and Laboratory Standards Institute, formerly the National Committee for Clinical Laboratory Standards) guidelines (9). *E. faecalis* ATCC 51299 and *E. faecalis* ATCC 29212 were used as controls. The following antimicrobials were tested on Sensititre plates: bacitracin, penicillin, chloramphenicol, erythromycin, tylosin, lincomycin, flavomycin, streptomycin, gentamicin, kanamycin, tetracycline, daptomycin, ciprofloxacin, linezolid, nitrofurantoin, quinupristin-dalfopristin, and vancomycin. The MIC results were interpreted according to the breakpoints of the CLSI and the Canadian Integrated Program for Antimicrobial Resistance Surveillance (8) guidelines.

Detection of antibiotic resistance and virulence genes. Bacterial DNA was isolated as described previously (13). A microarray carrying 70 taxonomic probes (50-mer oligonucleotide), 17 virulence probes (70-mer oligonucleotide), and 174 antibiotic resistance probes (70-mer oligonucleotide) for a total of 261 probes (see Tables S1 and S2 in the supplemental material) was constructed and used for the detection of putative target genes by using the previously described methods (5, 18). Oligonucleotide probes were printed in triplicate in each array. Taxonomic probes were designed against four genes (*ddl*, *pheS*, *atpA*, and *recA*) to identify 18 recognized *Enterococcus* species: *E. asini*, *E. avium*, *E. casseliflavus*, *E. cecorum*, *E. columbae*, *E. dispar*, *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hermanniensis*, *E. hirae*, *E. malodoratus*, *E. mundtii*, *E. pseudoavium*, *E. raffinosus*, *E. saccharolyticus*, and *E. sulfureus*. The universal eubacterial probe EUB338 was used as a positive control. The design and validation of the microarray are the subjects of a separate publication (our unpublished data). Hybridization, washing, scanning, image processing, scoring, and data analysis were done as previously described (5, 21). To confirm the results of the microarray, PCR amplifications of selected genes, including *bcrR*, *ermA*, *ermAM*, *ermB*, *msrC*, *linB*, *mefA*, *aac6-Ii*, *aadE*, *aadA9*, *aphA3*, *catA*, *tetL*, *tetM*, and the *tetO* resistance gene as well as the *ace*, *cad1*, cAM373, *cob*, and *esp* virulence genes, were performed with specific primers (12, 15, 18, 34).

BSH activity. Cultures were screened for bile salt hydrolysis (BSH) activity as previously described (17). Briefly, 10 μl of bacteria grown overnight on tryptic soy agar (Becton Dickinson) supplemented with 0.85% NaCl was plated onto MRS agar plates supplemented with 0.5% (wt/vol) sodium salt of taurodeoxycholic acid (Sigma-Aldrich, Oakville, Ontario, Canada) and 0.37 g/liter $CaCl_2$. Plates were then incubated anaerobically (atmosphere of 80% N_2, 10% CO_2, and 10% H_2) at 37°C for 48 h. The diameter of the precipitation (BSH) zone around the bacteria on the medium was measured, and isolates were categorized on a scale of zero to six for BSH activity (17), with a minimum spot diameter with no precipitation being 11 mm: group 0, 11 mm; group 1, precipitation on colony surface only; group 2, precipitation zone 2 mm beyond the colony; group 3, precipitation zone 4 mm beyond the colony; group 4, precipitation zone 7 mm beyond the colony; group 5, precipitation zone 9 mm beyond the colony; group 6, precipitation zone 12 mm beyond the colony. Groups 1 and 2 were considered to have low BSH activity, groups 3 and 4 were considered to have medium BSH activity, and groups 5 and 6 were considered to have high BSH activity.

Gelatinase and hemolysin production. The production of gelatinase was determined by streaking single colonies onto Todd-Hewitt agar (Becton Dickinson) containing 30 g of gelatin per liter and incubating overnight at 37°C (15). Plates were then placed at 4°C for 5 h before examination for turbidity zones around the colonies, indicating hydrolysis. The hemolysin production was performed by cultivating colonies onto layered fresh horse blood agar plates for 1 to 2 days at 37°C. Clearing zones around colonies indicated hemolysin production (15).

Biofilm formation. Overnight bacterial cultures in tryptic soy broth supplemented with 0.25% glucose were diluted 1:100. Then 200 μl was transferred in triplicate in polystyrene microtiter plate wells (Falcon, Franklin Lakes, NJ) and incubated for 24 h at 37°C. Wells were washed three times with phosphate-buffered saline and air dried to fix the biofilm, stained with 1% crystal violet for 30 min, and finally rinsed with distilled water. The biofilms were extracted with an 80:20 solution of ethanol-acetone for the determination of optical density at 570 nm (32).

Statistical analyses. Data were analyzed using the GLM procedure of the SAS Institute (13). *Enterococcus* counts were log transformed before analysis. The association test of Cochran-Mantel-Haenszel was used to determine the relationship between feed companies and the presence of target genes using the FREQ procedures of the SAS Institute (31). Associations between resistance

TABLE 1. Identification of enterococcal isolates by the API 20 Strep test and DNA microarray[a]

Method	Feed	No. of enterococci identified						
		E. faecium	*E. faecalis*	*E. gallinarum*	*E. durans*	*E. hirae*	*E. avium*	Total
API 20 Strep	A	12	5	4	1	0	0	22
	B	19	0	1	3	0	0	23
	C	17	2	2	2	0	1	24
	Total	48	7	7	6	0	1	69
Microarray	A	15	5	1	0	1	0	22
	B	19	0	0	0	4	0	23
	C	17	2	1	0	4	0	24
	Total	51	7	2	0	9	0	69

[a] Discrepancies were found between the two methods in the identification of *E. faecium*, *E. gallinarum*, *E. hirae*, *E. durans*, and *E. avium.*

genes and virulence genes were determined using Pearson's chi-square exact test (13, 31). The 0.05 *P* value was used to declare significance.

RESULTS

***Enterococcus* population.** Presumptive enterococci in fecal and cecal samples were enumerated by viable plate counts (data not shown). The abundance of *Enterococcus* populations did not vary with the feed companies (and therefore feed composition) that the commercial farms employed ($P > 0.05$). Populations were significantly higher ($P < 0.05$) in the fecal samples (log CFU/g = 6.8 ± 0.27 [mean ± standard deviation]) than in the cecal samples (log CFU/g = 5.5 ± 0.31).

From the nine farms, 69 (a = 8, b = 8, c = 6, d = 7, e = 9, f = 7, g = 9, i = 8, j = 7) isolates were randomly purified and characterized, representing 22, 23, and 24 isolates for feed company A, B, and C, respectively (Table 1). The API 20 Strep identified 48 *E. faecium* isolates, seven *E. faecalis* isolates, seven *E. gallinarum* isolates, six *E. durans* isolates, and one *E. avium* isolate. Validated through the use of ATCC reference strains and by sequencing a segment of the *cpn60* gene amplified by the universal cpn60 primers, the microarray was able to confirm without ambiguity that 51 (73.9%), nine (13.0%), seven (10.1%), and two (2.9%) of the 69 *Enterococcus* species isolates were *E. faecium*, *E. hirae*, *E. faecalis*, and *E. gallinarum*, respectively. Details concerning the microarray validation are described in a separate manuscript (unpublished). There was no significant difference in the distribution of species between feed companies ($P > 0.05$), with 34 (66.7%) and 17 (33.3%) of the *E. faecium* isolates from feces and ceca, respectively. Only one *E. faecalis* isolate was from feces, and the remaining six isolates were from ceca. The nine *E. hirae* and the two *E. gallinarum* isolates were isolated from feces and ceca, respectively.

Antibiotic susceptibility. Recognizing that only a relatively small number of isolates were analyzed, no significant differences were noted between feed companies ($P > 0.05$) with respect to resistance levels to individual antimicrobials and the respective resistance spectrums (Fig. 1). The distribution of MICs and the levels of resistance to 17 different antibiotics for the 69 *Enterococcus* isolates are presented in Table 2. None of the isolates were resistant to chloramphenicol, linezolid, and vancomycin. One isolate (*E. hirae*) was resistant to gentamicin, and two isolates (one *E. faecium* isolate and one *E. faecalis* isolate) were resistant to daptomycin. Among the 17 antimicrobial agents tested, resistances to bacitracin (98.6%), lincomycin (92.8%), tetracycline (91.3%), quinupristin-dalfopristin (78.3%), tylosin (72.5%), erythromycin (68.1%), penicillin (62.3%), kanamycin (59.4%), streptomycin (55.1%), and nitrofurantoin (47.8%) were most frequent. The frequencies of resistance to ciprofloxacin and flavomycin were 30.4% and 29.0%, respectively, with 39.2 and 28.6% of *E. faecium* and *E. faecalis* isolates, respectively, being resistant to ciprofloxacin, an antibiotic of human importance. Interestingly 43 (84.3%) of the 51 *E. faecium* isolates were resistant to quinupristin-dalfopristin, another antibiotic of human importance. All nine *E. hirae* isolates were resistant to lincomycin, bacitracin, and flavomycin. Overall, *E. faecium*, *E. faecalis*, and *E. hirae* demonstrated relatively high resistance levels to bacitracin, lincomycin, tetracycline, tylosin, and penicillin, agents that are used in feed as growth promoters.

Multiple-antibiotic-resistant *Enterococcus* isolates were obtained from chickens regardless of the feed they received (Fig. 1B). The 49 antimicrobial resistance patterns found among species and sample sources are presented in Table 3. All *Enterococcus* isolates were resistant to at least two different classes of antibiotics, with 65 (94.2%) isolates being resistant to five or more antibiotics. Multiple resistance was more common among the *E. faecium* isolates, with 46 (66.7%) of them being resistant to at least seven antibiotics. Seven *E. faecium* isolates (four from ceca and three from feces) showed the resistant spectrum Bac Pen Ery Tyl Lin Str Kan Tet Cip Nit Q-D. Two (22.2%) of the nine *E. hirae* isolates were resistant to 11 antibiotics (Table 2).

Antibiotic resistance and virulence genes. The virulence and resistance genotypes of the isolates are presented in Table 4. No significant differences in the gene distributions were observed between feed companies ($P > 0.05$). Moreover, none of the *Enterococcus* isolates in this study were able to produce biofilm.

***Enterococcus faecalis*.** The seven isolates of *E. faecalis* were found to harbor bacitracin resistance *bcrR* and macrolide resistance *ermAM* genes. In addition, five of the seven isolates harbored an *ermB* gene. The combination of tetracycline resistance *tetL* and *tetM* genes was found in all four tetracycline-resistant isolates. Eight resistance genes, including the aminoglycoside resistance *sat4*, *aac6*, and *aphA3* genes, were found in one (from ceca in farm b supplied by feed company C) of the two aminoglycoside-resistant isolates.

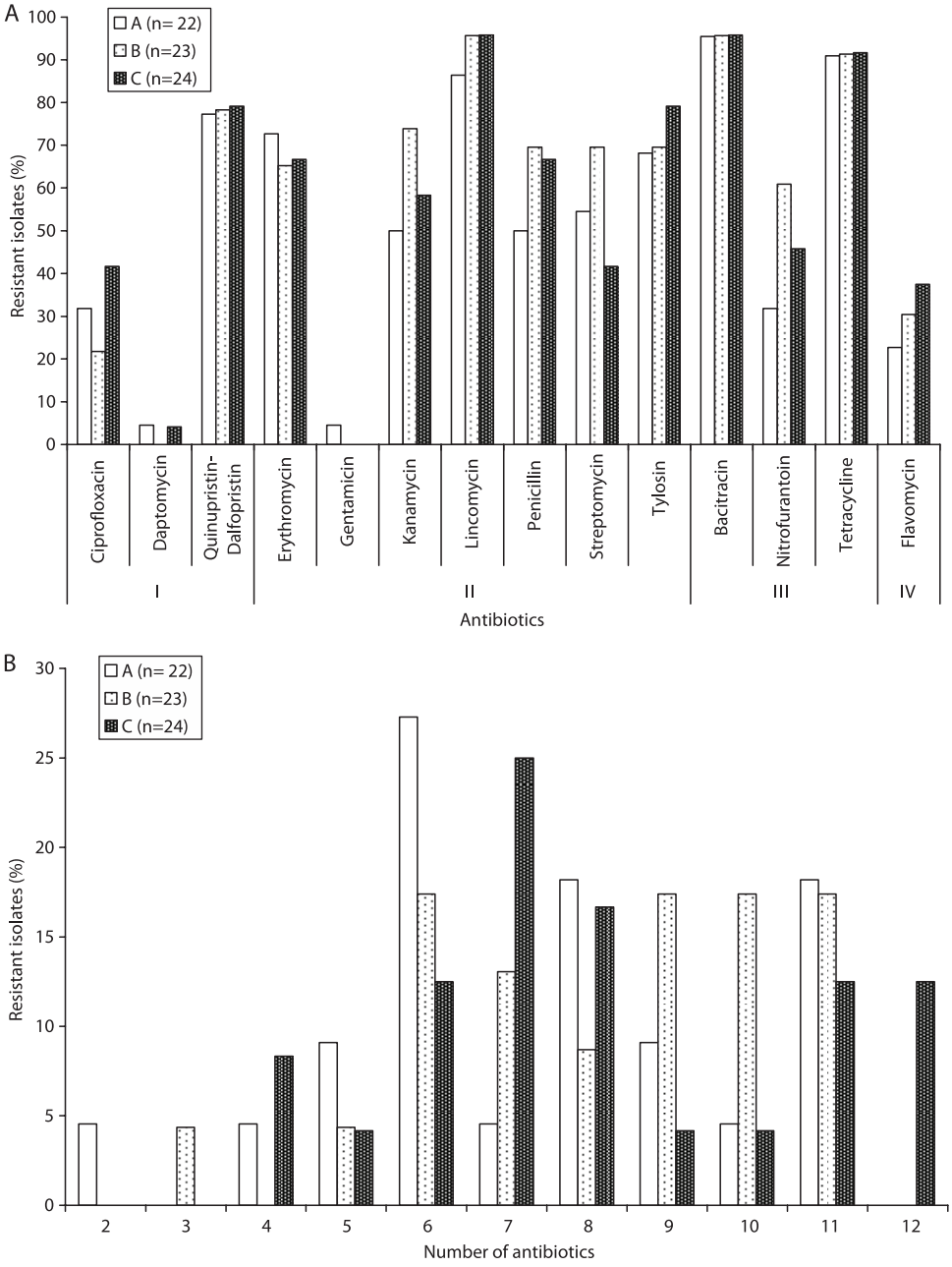

FIG. 1. Antibiotic resistance level (A) and spectrum (B) in 69 enterococcal isolates (22, 23, and 24 isolates from samples of farms receiving feed from companies A, B, and C, respectively). All isolates were susceptible to linezolid, vancomycin, chloramphenicol, and novobiocin.

Nine virulence genes (*ace*, $agrB_{Efs}$, *cad1*, the cAM and cCF10 genes, *cob*, *cpd1*, $efaA_{Efs}$, *gelE*) were detected in all *E. faecalis* isolates. An additional three genes, *agg*, *cylA*, and *cylB*, were detected in one isolate from farm a, supplied by feed company A. All isolates were able to hydrolyze bile salt and produce gelatinase (*gelE* genes were detected in all isolates), whereas only two isolates showed hemolytic activity (Table 4).

***Enterococcus faecium*.** The most prevalent resistance genes detected in *E. faecium* were *aac6* (49 isolates, 96.1%) for aminoglycoside resistance; *tetM* and *tetL* (48 isolates, 94.1%, and 38 isolates, 74.5%, respectively), conferring tetracycline resistance; *msrC* (47 isolates, 92.2%), implicated in resistance against 14-membered-ring macrolides and type B streptogramins; *bcrR* (46 isolates, 90.2%) for bacitracin resistance; and *vatE* (previously *satG* and identified as *satG_vatE* in this study) (43 isolates, 84.3%) for streptogramin resistance. The *ermAM* and *linB* genes involved in MLS_B resistance were detected in 33 (64.7%) and 31 (60.8%) *E. faecium* isolates, respectively. The resistance genes *ermB*, *sat4*, and *aphA3* were detected in 22 (43.1%), 21 (41.1%), and 19 (37.3%) isolates, respectively. Possession of multiple resistance genes was evident, ranging from 3 to 12 genes by isolate (Table 4). Among the 51 *E. faecium* isolates, 28 different gene combinations were detected, with *bcrR ermAM ermB msrC linB sat4 aac6*

TABLE 2. Distribution of MICs and resistances among 7 *E. faecalis*, 51 *E. faecium*, 9 *E. hirae*, and 2 *E. gallinarum* isolates from broiler chickens

Class[a]	Antibiotic	Species	MIC_{50}	MIC_{90}	Resistance (%)	Number of strains with MIC (μg/ml) of[b]: 0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>2,048
I	Ciprofloxacin	*E. faecalis*	1	4	2 (28.6)			5		2									
		E. faecium	2	4	20 (39.2)			6	25	17	3								
		E. hirae	0.5	1	0	1	4	4											
		E. gallinarum	1	1	0			2											
	Daptomycin	*E. faecalis*	1	8	1 (14.3)		1	4	1		1								
		E. faecium	2	4	1 (2.0)			11	19	20			1						
		E. hirae	2	4	0			2	5	2									
		E. gallinarum	2	2	0				2										
	Linezolid	*E. faecalis*	2	2	0			2	5										
		E. faecium	2	2	0			10	40	1									
		E. hirae	2	2	0		1	3	5										
		E. gallinarum	1	2	0			1	1										
	Quinupristin-dalfopristin	*E. faecalis*	8	8	7 (100)					1	6								
		E. faecium	8	16	43 (84.3)			4	4	7	30	5		1					
		E. hirae	2	16	4 (44.4)				5		2	2							
		E. gallinarum	2	2	0				2										
	Vancomycin	*E. faecalis*	1	2	0		2	2	3										
		E. faecium	<0.5	4	0		35	8		8									
		E. hirae	<0.5	4	0		6	2		1									
		E. gallinarum	8	8	0						2								
II	Erythromycin	*E. faecalis*	>8	>8	5 (71.4)			1	1			5							
		E. faecium	>8	>8	35 (68.6)		8	2	6		2	33							
		E. hirae	8	>8	5 (55.6)		4				2	3							
		E. gallinarum	>8	>8	2 (100)							2							
	Gentamicin	*E. faecalis*	<128	<128	0										7				
		E. faecium	<128	<128	0										50	1			
		E. hirae	<128	512	1 (11.1)										7	1	1		
		E. gallinarum	<128	<128	0										2				
	Kanamycin	*E. faecalis*	256	1,024	2 (28.6)										3	2	1	1	
		E. faecium	512	>1,024	34 (66.6)										5	12	10	1	23
		E. hirae	512	>1,024	5 (55.6)										3	1	1		4
		E. gallinarum	<128	256	0										1	1			
	Lincomycin	*E. faecalis*	>32	>32	6 (85.7)							1		6					
		E. faecium	>32	>32	47 (92.2)			1				3	5	42					
		E. hirae	>32	>32	9 (100)									9					
		E. gallinarum	>32	>32	2 (100)									2					
	Penicillin	*E. faecalis*	2	16	1 (14.3)				5		1	1							
		E. faecium	16	>16	37 (72.5)			1	1	3	9	25	12						
		E. hirae	16	>16	5 (55.6)			1	1	1	1	3	2						
		E. gallinarum	1	2	0			1	1										
	Streptomycin	*E. faecalis*	<512	>2,048	2 (28.6)												5	1	1
		E. faecium	1,024	2,048	33 (64.7)												18	21	12
		E. hirae	<512	2,048	3 (33.3)												6	2	1
		E. gallinarum	<512	<512	0												2		
	Tylosin	*E. faecalis*	>32	>32	6 (85.7)					1				6					
		E. faecium	>32	>32	37 (72.5)			1	4	8	1		1	36					
		E. hirae	32	>32	5 (55.6)				3	1			1	4					
		E. gallinarum	>32	>32	2 (100)									2					
III	Bacitracin	*E. faecalis*	>128	>128	7 (100)											7			
		E. faecium	>128	>128	50 (98.0)						1					50			
		E. hirae	>128	>128	9 (100)										1	6			
		E. gallinarum	>128	>128	2 (100)											2			
	Chloramphenicol	*E. faecalis*	8	8	0					1	6								
		E. faecium	8	8	0					18	33								
		E. hirae	4	8	0				3	4	2								
		E. gallinarum	4	8	0					1	1								
	Nitrofurantoin	*E. faecalis*	16	>64	1 (14.3)						3	2		1	1				
		E. faecium	>64	>64	27 (52.9)						1	2	2	19	27				
		E. hirae	64	>64	4 (44.4)								3	2	4				
		E. gallinarum	8	8	0						2								
	Tetracycline	*E. faecalis*	>32	>32	4 (57.1)					3				4					
		E. faecium	>32	>32	49 (96.0)					1	1	1		48					
		E. hirae	>32	>32	8 (88.8)					1			1	7					
		E. gallinarum	>32	>32	2 (100)									2					
IV	Flavomycin	*E. faecalis*	<1	8	0			4	2		1								
		E. faecium	4	>32	12 (23.5)			5	10	12	9	3	1	11					
		E. hirae	>32	>32	9 (100)									9					
		E. gallinarum	<1	>32	1 (50.0)			1						1					

[a] Class indicates antibiotic ranking based on importance in human medicine.
[b] Determined according to CLSI recommendations; dotted and solid bars indicate the breakpoints for intermediary and complete resistance, respectively.

aphA3 tetL tetM satG_vatE8 found in 11 (28.6%) isolates. The gene combinations *bcrR ermAM msrC linB aac6 tetL tetM satG_vatE8* and *bcrR ermAM ermB msrC linB aac6 tetL tetM satG_vatE8* were found in three and four isolates, respectively. The genes *msrC* and *linB* were significantly associated with *E. faecium* compared to other species ($P < 0.05$).

The only virulence gene detected in all 51 *E. faecium* isolates was *E. faecium efmA* ($efmA_{Efm}$). Hemolytic activity was found in 5 (9.8%) isolates, whereas 34 (66.7%) isolates hydrolyzed bile salts. No isolate was found to produce gelatinase (gene *gelE* was not detected in any of the *E. faecium* isolates) (Table 4).

The *bcrR* gene was found in all *E. hirae* and *E. gallinarum* isolates. The tetracycline resistance *tetO* gene was found only in *E. hirae*, with five (55.5%) isolates harboring this gene. Ten resistance genes were detected in one *E. hirae* isolate in which *tetL*, *tetM*, and *tetO* were simultaneously detected. Different aminoglycoside resistance genes (*aacA-aphD*, *aad*, *aac6*, *sat4*) were detected in *E. hirae*. In addition to *bcrR*, *ermAB*, and *tetLM*, the

TABLE 3. Distribution of antibiotic resistance patterns among *Enterococcus* spp. and sample origins (feces or ceca)

Resistance pattern	No. of *Enterococcus* species showing resistance pattern						
	E. faecium		*E. faecalis*		*E. hirae* feces	*E. gallinarum* ceca	Total
	Ceca	Feces	Ceca	Feces			
Bac Tet		1					1
Bac Lin Fla					1		1
Bac Fla Tet Nit		1					1
Bac Tyl Lin Q-D			1				1
Bac Ery Tyl Lin Q-D		1					1
Bac Ery Tyl Lin Tet						1	1
Bac Pen Lin Tet Q-D		1					1
Pen Fla Kan Cip Q-D	1						1
Bac Ery Ty Lin Tet Q-D			1	1			2
Bac Ery Tyl Lin Fla Tet					1	1	2
Bac Ery Tyl Lin Fla Tet Q-D					1		1
Bac Ery Tyl Lin Kan Q-D			1				1
Bac Ery Tyl Lin Tet Q-D		1					1
Bac Lin Str Tet Cip Q-D		1					1
Bac Pen Dap Cip Nit Q-D			1				1
Bac Pen Ery Lin Fla Tet		1					1
Bac Pen Lin Fla Tet Nit					1		1
Bac Pen Lin Str Tet Q-D		1					1
Bac Pen Lin Tet Cip Nit	1						1
Bac Pen Lin Tet Cip Q-D	1						1
Bac Pen Lin Tet Nit Q-D		1					1
Bac Ery Tyl Lin Kan Tet Q-D		3					3
Bac Ery Tyl Lin Str Tet Q-D		1					1
Bac Pen Ery Tyl Lin Tet Q-D		1					1
Bac Pen Lin Fla Kan Tet Nit		1			1		2
Bac Pen Lin Kan Tet Nit Q-D		1					1
Bac Tyl Lin Tet Cip Nit Q-D	1						1
Bac Pen Ery Tyl Lin Fla Tet					1		1
Bac Ery Tyl Lin Str Kan Tet Q-D			1				1
Bac Ery Tyl Lin Str Tet Cip Q-D			1				1
Bac Ery Tyl Lin Str Tet Q-D		1					1
Bac Lin Fla Str Gen Kan Tet Q-D					1		1
Bac Pen Ery Tyl Lin Str Kan Tet		1					1
Bac Pen Lin Fla Str Kan Tet Cip	1						1
Bac Pen Lin Fla Tet Cip Nit Q-D	1						1
Bac Pen Lin Str Tet Cip Nit Q-D		1					1
Bac Pen Tyl Lin Str Kan Tet Q-D	1						1
Bac Ery Tyl Lin Str Kan Tet Nit Q-D		1					1
Bac Pen Ery Tyl Lin Str Kan Tet Nit		2					2
Bac Pen Ery Tyl Lin Str Kan Tet Q-D	1	2					3
Bac Pen Tyl Fla Str Kan Tet Cip Q-D	1						1
Bac Ery Tyl Lin Str Kan Tet Cip Nit Q-D	1						1
Bac Pen Ery Tyl Lin Fla Str Kan Tet Q-D		1					1
Bac Pen Ery Tyl Lin Str Kan Tet Nit Q-D		4					4
Bac Pen Dap Ery Tyl Lin Str Kan Tet Nit Q-D	1						1
Bac Pen Ery Tyl Lin Fla Str Kan Tet Nit Q-D	1				2		3
Bac Pen Ery Tyl Lin Str Kan Tet Cip Nit Q-D	4	3					7
Bac Pen Ery Tyl Lin Fla Str Kan Tet Dap Nit Q-D		1					1
Bac Pen Ery Tyl Lin Fla Str Kan Tet Cip Nit Q-D	1	1					2
Total	17	34	6	1	9	2	69

vancomycin resistance *vanC* gene was detected in the two *E. gallinarum* isolates.

Among the virulence genes tested, only the pheromone cAD1 precursor lipoprotein *cad1* gene was detected in all nine *E. hirae* isolates. No virulence genes were detected in the two *E. gallinarum* isolates. Eight *E. hirae* isolates and one *E. gallinarum* isolate were found to hydrolyze bile salts. No hemolytic or gelatinase activities were observed in *E. hirae* and *E. gallinarum*.

Correlation between antibiotic resistance phenotype and genotype. Specific resistance genes were detected in corresponding antibiotic-resistant isolates (Table 5). As described above, the detected genes include those conferring resistance to aminoglycosides (*aac6*, *aacA-aphD1*, *aad9*, *aadB*, *aphA3*, *aac6 aphA3*), macrolides (*ermA*, *ermAM*, *ermB*, *msrC*), tetracycline (*tetL*, *tetM*, *tetO*), streptogramins (*sat*, *satG_vatE*), bacitracin (*bcrR*), and lincosamides (*linB*), as well as a transposon (*trans*) able to carry multiresistance gene clusters, which was found in all studied iso-

TABLE 4. Resistance and virulence genotype of enterococcal isolates from broiler fecal and cecal samples of commercial broiler farms receiving different antimicrobial-supplemented feed

Species	Feed	Farm	Isolate	Hybridization signal[a]																																					Phenotype[b]
				Antibiotic resistance genes																						Virulence genes																
				bcrR	*ermA*	*ermAM*	*ermB*	*msrC*	*linB*	*sat4*	*aac6*	*aacA-aphD1*	*aad9*	*aadB*	*aphA3*	*tetL*	*tetM*	*tetO*	*satG_vatE8*	*vanC*	F_IS1182	F_IS150	F_trans	F_trans1	*ace*	*agg*	$agrB_{Efs}$	*cad1*	cAM373	cCF10	*cob*	*cpd1*	*cylA*	*cylB*	$efaA_{Efs}$	$efmA_{Efm}$	*gelE*		
E. faecalis	A	a	A 1 CEC	+	−	+	+	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	+	−	+	−	+	+	+	+	+	+	−	−	+	−	+	GEL-B12	
	A	a	A 2 CEC	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	+	+	+	+	+	+	+	+	+	−	+	GEL-B14	
	A	c	C 64	+	−	+	+	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	+	−	+	−	+	+	+	+	+	+	−	−	+	−	+	GEL-B14	
	A	c	C 1 CEC	+	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	+	−	+	−	+	+	+	+	+	+	−	−	+	−	+	GEL-B12	
	A	c	C 2 CEC	+	−	+	+	−	−	+	+	−	−	−	+	+	+	−	−	−	−	−	+	−	+	−	+	+	+	+	+	+	−	−	+	−	+	H-GEL-B14	
	C	b	B 4 CEC	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+	−	+	+	+	+	+	+	−	−	+	−	+	H-GEL-B13	
	C	f	F-5 CEC	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+	−	+	+	+	+	+	+	−	−	+	−	+	GEL-B12	
E. faecium	A	a	A 3 CEC	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B13	
	A	a	A 5	+	−	+	+	+	+	−	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	
	A	a	A 15	+	−	+	+	+	+	−	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	
	A	a	A 136	+	−	+	+	+	+	+	+	−	−	+	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B13	
	A	a	A 156	+	−	+	−	+	−	−	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B20	
	A	c	C 55	+	−	+	+	+	−	+	−	−	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	H-B14	
	A	c	C 191	+	−	+	−	+	+	−	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B19	
	A	c	C 203	+	−	−	−	+	−	−	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B20	
	A	i	I 421	+	−	−	−	+	−	−	+	−	−	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B21	
	A	i	I 431	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B12	
	A	i	I 473	+	−	+	−	+	+	+	+	−	−	−	+	+	+	−	+	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−		
	A	i	I 490	+	−	−	−	+	−	−	+	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B21	
	A	i	I 1 CEC	+	−	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B12	
	A	i	I 4 CEC	+	−	−	−	−	+	+	+	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−		
	A	i	I 5 CEC	+	−	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B15	
	B	d	D 82	+	−	−	−	+	+	+	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B13	
	B	d	D 209	+	−	−	−	+	−	−	−	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B22	
	B	d	D 218	−	−	−	−	+	−	−	+	−	−	−	−	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B16	
	B	d	D 223	+	−	−	−	+	−	−	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B18	
	B	d	D 231	+	−	−	−	+	+	−	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B19	
	B	d	D 2 CEC	+	−	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B12	
	B	e	E 243	+	−	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	H	
	B	e	E 249	+	−	+	−	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	H-B17	
	B	e	E 256	+	−	+	+	+	+	−	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−		
	B	e	E 1 CEC	+	−	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−		
	B	e	E 2 CEC	+	−	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B15	
	B	e	E 3 CEC	+	−	+	−	+	+	−	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B17	
	B	e	E 4 CEC	+	−	+	−	+	+	−	+	−	−	−	−	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−		
	B	j	J 451	+	−	+	+	+	+	−	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B22	
	B	j	J 1 CEC	+	−	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B12	
	B	j	J 3 CEC	+	−	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B14	
	B	j	J 499	+	−	+	+	+	−	+	+	−	−	−	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B16	
	B	j	J 505	+	−	+	+	+	−	+	+	−	−	−	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−		
	B	j	J 511	+	−	−	−	+	−	−	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B18	
	C	b	B 36	+	−	−	−	+	−	−	+	−	−	−	−	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−		
	C	b	B 166	+	−	+	+	+	+	−	+	−	−	−	−	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−		
	C	b	B 174	+	−	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B15	
	C	b	B 182	+	−	+	+	+	−	−	+	−	−	−	−	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−		
	C	b	B 2 CEC	−	−	−	−	+	−	−	+	−	−	−	−	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	H-B22	
	C	b	B 3 CEC	+	−	−	−	+	−	−	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	H	
	C	f	F 296	−	−	+	+	+	+	−	+	−	−	−	−	+	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B16	
	C	f	F Lit #2	+	−	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−		
	C	f	F 2 CEC	+	−	−	−	+	+	−	+	−	−	−	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B13	
	C	f	F 3 CEC	+	−	−	−	+		−	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B14	
	C	g	G 314	+	−	+	−	+	+	−	+	+	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B13	
	C	g	G 367	+	−	+	−	−	−	−	+	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B22	
	C	g	G 376	−	−	+	−	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B17	
	C	g	G 384	+	−	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−		
	C	g	G 390	+	−	+	+	+	+	+	+	−	−	−	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	B20	
	C	g	G 1 CEC	+	+	−	−	−	−	−	+	−	−	−	+	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−		
	C	g	G 4 CEC	+	−	+	−	+	−	+	+	−	−	−	+	+	+	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−		
E. hirae	A	a	A 4	+	−	−	−	−	+		+	+	−	−	−	+	+	+	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	B13	
	A	d	D 92	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	B12	
	B	e	E 109	+	−	+	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	B12	
	B	e	E 123	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	B12	
	B	j	J 458	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	B13	
	B	b	B 48	+	−	+	−	−	−	−	−	−	+	−	−	−	+	−	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−		
	C	f	F 268	+	−	−	−	−	+	−	+	−	−	−	−	+	+	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	B13	
	C	f	F 280	+	−	+	+	−	−	+	+	+	−	−	+	+	+	+	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	B11	
	C	g	G 323	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	B12	
E. gallinarum	A	i	I 2 CEC	+	−	+	+	−	−	−	−	−	−	−	−	+	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−		
	C	g	G 3 CEC	+	−	+	+	−	−	−	−	+	−	−	−	+	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	B13	

[a] Isolates are shown with their positive (+) or negative (−) hybridization signals for each gene.
[b] H, hemolytic activity on blood agar; GEL, gelatinase production; B, bile salt hydrolysis activity (number indicates the size [mm] of the zone around the colonies).

TABLE 5. Distribution of antibiotic resistance genes among the 69 enterococcal isolates showing correlations between resistant phenotype and genotype

Antibiotic resistance gene group and gene(s)	No. (%) of enterococcal isolates[a]				
	E. faecalis ($n = 7$)	*E. faecium* ($n = 51$)	*E. hirae* ($n = 9$)	*E. gallinarum* ($n = 2$)	Total ($n = 69$)
Polypeptide (Bac^r)	7 (100)	50 (98.0)	9 (100.0)	2 (100)	68 (98.6)
bcrR	7 (100)	46 (90.2)	9 (100)	2 (100)	64 (92.8)
Macrolide (Ery^r)	6 (85.7)	35 (68.6)	5 (55.6)	2 (100)	48 (69.6)
ermA	0 (0.0)	1 (2.0)	0 (0.0)	0 (0.0)	1 (1.4)
ermAM	7 (100)	34 (66.7)	3 (33.3)	2 (100)	46 (66.6)[c]
ermB	5 (71.4)	22 (43.1)	1 (1.1)	2 (100)	30 (43.5)[c]
msrC	0 (0.0)	47 (92.2)	0 (0.0)	0 (0.0)	47 (68.1)[c]
ermAM ermB	0 (0.0)	10 (19.6)	0 (0.0)	0 (0.0)	10 (14.5)[c]
ermAM ermB msrC	0 (0.0)	18 (35.3)	0 (0.0)	0 (0.0)	18 (26.1)[c]
Lincosamide (Lin^r)	6 (85.7)	48 (94.1)	9 (100)	2 (100)	64 (92.8)
linB	0 (0.0)	31 (60.8)	2 (22.2)	0 (0.0)	33 (47.8)[c]
Tetracycline (Tet^r)	4 (57.1)	49 (96.1)	8 (88.9)	2 (100)	63 (91.3)
tetL	4 (57.1)	38 (74.5)	4 (44.4)	2 (100)	49 (70.0)
tetM	4 (57.1)	48 (94.1)	5 (55.6)	2 (100)	59 (85.5)[c]
tetO	0 (0.0)	0 (0.0)	5 (55.5)	0 (0.0)	5 (7.2)[c]
tetL tetM	4 (57.1)	37 (72.5)	4 (44.4)	2 (100)	47 (68.1)
tetL teM tetO	0 (0.0)	0 (0.0)	1 (1.1)	0 (0.0)	1 (1.4)
Aminoglycoside (Kan^r)[b]	2 (28.6)	35 (68.2)	5 (55.6)	0 (0.0)	42 (60.9)
aac6	1 (14.3)	49 (96.1)	3 (33.3)	0 (0.0)	53 (76.8)[c]
aacA-aphD1	0 (0.0)	1 (2.0)	2 (22.2)	1 (50.0)	4 (5.8)
aad9	0 (0.0)	1 (2.0)	2 (22.2)	0 (0.0)	3 (4.3)
aadB	0 (0.0)	1 (2.0)	0 (0.0)	0 (0.0)	3 (4.3)
aphA3	1 (14.3)	19 (37.3)	1 (1.1)	0 (0.0)	21 (30.4)[c]
aac6 aphA3	1 (14.3)	19 (37.3)	1 (1.1)	0 (0.0)	21 (30.4)[c]
Streptogramin (Q-D^r)	7 (100)	43 (84.3)	4 (44.4)	0 (0.0)	54 (78.3)
satG_vatE	0 (0.0)	43 (84.3)	4 (44.4)	0 (0.0)	47 (68.1)
Glycopeptide (Van^r)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
vanC	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	2 (2.3)
Transposon (plasmid pUW786) multiresistance gene cluster	7 (100)	51 (100)	9 (100)	2 (100)	69 (100)

[a] Numbers and percentages of isolates carrying the target gene out of all enterococcal isolates.
[b] Fifteen isolates (10 *E. faecium*, two *E. faecalis*, one *E. hirae* and the two *E. gallinarum* isolates) presented intermediary resistance to kanamycin.
[c] Values in this row are statistically different ($P \leq 0.05$).

lates. Significant differences ($P \leq 0.05$) in gene distribution were observed between the different species. The *msrC* and *tetO* genes were found only in *E. faecium* and *E. hirae*, respectively (Table 5).

DISCUSSION

Enterococci are Gram-positive bacteria ubiquitously found in the gastrointestinal tracts of animals, birds, and humans, as well as in soil and water (16, 22). In the human intestine, the density of enterococci ranges from 5 to 8 log CFU/g of intestinal content (42). The mean (±standard deviation) *Enterococcus* numbers found in our present study were 6.82 ± 0.27 and 5.53 ± 0.31 log CFU/g of fecal and cecal samples, respectively. Because of their relative abundance and their resistance to environmental adversity, enterococci have been proposed as indicators of bacteria for antimicrobial resistance, as well as indicators for the hygienic quality of food and water (37, 40). However, factors affecting numbers of enterococci in the intestinal tract and feces are not well established. The present study showed a significant difference between cecal and fecal *Enterococcus* densities regardless of feed type used by farmers, as previously reported (13), therefore suggesting that a significant number of fecal enterococci could be present in the litter.

Commensal bacteria, including *Enterococcus* spp. in commercial livestock and poultry, could contaminate the food chain during processing or find their way into the environment (13, 14). A survey of retail raw meat revealed that *E. faecium* was the predominant *Enterococcus* species recovered from ground turkey (60%), ground beef (65%), and chicken breast (79%), while *E. faecalis* was the predominant species (54%) recovered from pork chops (23). Although in our study 74.0% and 10.0% of all 69 *Enterococcus* isolates were *E. faecium* and *E. faecalis*, respectively, the DNA microarray taxonomic data combined with the variability observed with the virulence factor and antibiotic resistance gene distribution clearly revealed that the 69 isolates in this study were not clonal in nature. Six

of the seven *E. faecalis* isolates and 17 of the 51 *E. faecium* isolates were from ceca, while all *E. hirae* isolates were from feces. Despite a relatively small number of isolates analyzed in this study, *E. faecalis* and *E. faecium*, the two most prevalent enterococci involved in human infection (22), were found in broiler cecal and fecal material. It would be interesting to use our microarray method to analyze the distribution of *Enterococcus* species in larger studies that could help identify ecological niches (ceca or feces) of different species. Overall, fecally contaminated chicken litter should be handled appropriately to minimize environmental and public health risk.

Antibiotic-resistant *Enterococcus* isolates from animal production have been reported (26, 39, 40). The resistance levels observed in our enterococcal isolates were higher than those reported in a Slovakian study of 82 isolates (4). A low percentage of gentamicin resistance and a relative high frequency of resistance to kanamycin and streptomycin were found in the present study. Daptomycin resistance was found in two isolates (one *E. faecium* isolate and one *E. faecalis* isolate). Overall, the resistance patterns in the fecal and cecal isolates were similar. To our knowledge, this study provides, for the first time, detailed antibiotic resistance genotypes of a variety of enterococci isolated from the feces and ceca of commercial broiler chickens in British Columbia.

Bacitracin is one of the antimicrobial agents used as a growth promoter in all of the farms in this study. The *bcrR* gene was the only bacitracin resistance gene in our DNA microarray. This gene encodes a transcriptional activator of the bacitracin resistance genes *bcrA* and *bcrB*, which are actively involved in pumping bacitracin from the bacterial cell (33). The *bcrR* gene was found in 46 (90.2%) *E. faecium* isolates and all (100%) *E. hirae*, *E. faecalis*, and *E. gallinarum* isolates. This *bcrR* gene, which also was previously reported in broiler chicken and clinical enterococcal isolates (34, 43), was simultaneously detected with several other resistant genes, including tetracycline resistance genes *tetLM* and *ermB*, encoding resistance to the MLS_B antibiotics. Such broad distribution of the *bcrR* gene among the different species determined in this study suggests a correlation between bacitracin use and the appearance of this gene. Further studies are required to confirm this observation.

In *Enterococcus*, the methylation of the 23S RNA by methylase enzymes encoded by the *erm* genes is involved in the resistance of these bacteria to MLS_B antibiotics (30). In the present study, *ermAM* and *ermB* were detected in 33 (64.7%) and 22 (43.1%) *E. faecium* isolates, respectively. All seven *E. faecalis* isolates harbored *ermAM*, and five *E. faecalis* isolates harbored *ermB*. These two genes were also detected in *E. hirae* and *E. gallinarum*. The *msrC* gene, conferring resistance to macrolides, was found in 47 (92.2%) *E. faecium* isolates, confirming that this gene is intrinsic to this species. The lincomycin resistance gene *linB* (also known as *lnuB*) was also found only in 31 (60.78%) *E. faecium* and two (22.2%) *E. hirae* isolates. It is noteworthy that none of the sampled farms in this study used macrolides or lincomycin for therapy or as a growth promoter.

A high prevalence of tetracycline resistance in *Enterococcus* isolates from broilers has been reported (7). The high level of tetracycline resistance observed in the present study was in agreement with a high detection frequency of *tetLM* genes. Furthermore, the *tetO* gene was detected in five *E. hirae* isolates. The *tetL* gene product is a large protein with 14 transmembrane domains and confers resistance to tetracycline by active efflux (30). The *tetM* gene, the most common and frequently detected tetracycline resistance determinant found in enterococci, and the *tetO* gene, also reported in *Campylobacter*, *Listeria*, and streptococci, both confer resistance due to ribosome protection by large cytoplasmic proteins similar to elongation factors (7, 30). The possible physical link between *tet* and *erm* genes was not evaluated in the present study. However, *ermB* was detected in 38 (55.3%) *E. faecium* isolates and in 2 of 3 *E. faecalis* isolates harboring *tetM*. Cauwerts et al. (7) reported that resistance to tetracycline is often present in poultry enterococci carrying the *ermB* gene. Our data confirm that *E. faecium* and *E. faecalis* isolates from poultry feces and ceca may constitute reservoirs of MLS_B and tetracycline resistance that could be spread by a transposon, such as *trans*, known to carry multiresistance gene clusters. This *trans* transposon was detected in all of our isolates.

Ribosomal target modification, transport alteration, and enzymatic modification are the three known acquired mechanisms of aminoglycoside resistance in enterococci (30). In our isolates, the *aac6*, *aad9*, and *aphA3* genes were detected, encoding aminoglycoside modifications and conferring high-level resistance to aminoglycoside and to synergism between cell wall-active agents and aminoglycoside (1, 25). The *aac6′-Ii* gene intrinsic to *E. faecium* was found in 49 of the 51 (96.0%) *E. faecium* isolates. The remaining two *E. faecium* isolates, in which the *aac6′-Ii* gene was not detected, suggests the possible loss of this gene or possible mutations (variant of the gene, deletion, insertion) in these isolates. The nondetection of this gene in 4.0% of the isolates is not due to the accuracy of the detection method as described above, since another gene (*msrC*) intrinsic to *E. faecium* has been detected in 92.2% of the isolates of this species. Kanamycin resistance, common in enterococci, is associated with the *aphA3* gene encoding an enzyme that phosphorylates kanamycin, neomycin, and amikacin (30). The use of bacitracin or tetracycline as a growth promotion or therapeutic agent may therefore coselect for resistance to MLS_B antibiotics and high-level resistance to aminoglycoside, which may be important as an alternative therapy for enterococcal infections in humans (7).

Quinupristin-dalfopristin (streptogramin antibiotic) is bacteriostatic against *E. faecium* but ineffective against *E. faecalis* due to intrinsic resistance. In the United States, this compound is approved for some serious VRE infections (10, 28). Two of the poultry feeds in our study contained virginiamycin, another streptogramin antibiotic, in finisher diet. The contribution of virginiamycin use in poultry to quinupristin-dalfopristin resistance in humans remains unclear. However, 43 (84.3%) *E. faecium* isolates were resistant to quinupristin-dalfopristin and were all found to harbor *satG_vatE*, a gene conferring resistance to streptogramin A by inactivation. Quinupristin-dalfopristin resistance was not related to virginiamycin use on the farms. Interestingly, the *satG_vatE* gene was found in four of the nine *E. hirae* isolates and in 19 of 22 (86.4%), 18 of 31 (58.1%), and 12 of 19 (63.2%) *E. faecium* isolates harboring the *ermB*, *linB*, and *aphA3* genes, respectively. Likewise, all *satG_vatE*-positive *E. faecium* isolates also harbored *tetM*, suggesting their possible association. Various quinupristin-dalfopristin-resistant *Enterococcus* spp. (*E. hirae*, *E. casseliflavus*,

and *E. faecium*) have been isolated from environmental and animal (including chicken) samples, and several of these isolates carried the *ermB* (55.5%), *msrC* (3%), *vatE* (2%), and *vatD* (0.3%) genes (26). Virginiamycin resistance seems to develop slowly, but continuous virginiamycin exposure seems to be required to maintain a stable streptogramin-resistant population of *E. faecium* in the chickens (35).

Despite the presence of several vancomycin resistance gene probes (*vanABCDEGHRXYZ*) on our microarray, the *vanC* gene was detected only in the two *E. gallinarum* isolates which had a vancomycin MIC of 8 μg/ml. This species is usually known to demonstrate low-level resistance to vancomycin (30). The absence of other vancomycin resistance genes among *E. faecalis*, *E. faecium*, and *E. hirae* isolates is not surprising. The use of the glycopeptide antibiotic avoparcin in animal production has been considered an important factor in the emergence and spread of VRE (2). This antibiotic has not been used in poultry production in Canada. However, due to the multiresistance genes detected in our isolates and possible lateral gene transfer (11), active surveillance is recommended. Despite some resistance phenotype to penicillin (beta-lactam), ciprofloxacin (quinolone), daptomycin (cyclic lipopeptide), and nitrofurantoin, none of the beta-lactamase (*bla*) or quinolone resistance (*qnr*) gene probes on the microarray were positive. Studies on the mechanisms of daptomycin resistance and the development of resistance to this antibiotic in *Enterococcus* are rare. Nitrofurantoin belongs to a group of antibiotics characterized by the presence of nitro groups on a nitro-aromatic or nitro-heterocyclic backbone. The specific mode of action and mechanism of resistance need to be established. Probes to detect resistance to daptomycin or nitrofurantoin were not on our microarray.

The virulence of enterococci appears to be multifactorial and associated with several genes, and resistance alone cannot be a predictor of disease frequency and outcome (22, 29). The presence of the *esp* gene was reported to be highly associated with biofilm formation in enterococci (22). This gene was not detected in our isolates, explaining, in part, that none of them were found to produce biofilm. All of our *E. faecalis* isolates and a large proportion of *E. faecium* and *E. hirae* isolates were found to hydrolyze bile salt, suggesting an adaptive response to the gut environment (17). The $efmA_{Efm}$ and *cad1* genes were found in all *E. faecium* and *E. hirae* isolates, respectively, suggesting a lower virulence potential of these isolates. In contrast, a higher number of virulence genes (*ace*, $agrB_{Efs}$, *cad1*, the cAM and cCF10 genes, *cob*, *cpd1*, $efaA_{Efs}$, and *gelE*) and gelatinase activity were found only in *E. faecalis*. The degradation of host extracellular matrix proteins by gelatinase is important in *E. faecalis* pathogenesis, indicating the potential virulence of our *E. faecalis* isolates (22). An additional three genes (*agg*, *cylA*, and *cylB*) were found in an *E. faecalis* isolate that was not hemolytic, suggesting the existence of gene expression silencing (20). Hemolytic activity was observed in two and four *E. faecalis* and *E. faecium* isolates, respectively. None of these isolates were positive for *cylA*, *cylB*, and *cylC*, suggesting the existence of other possible hemolytic determinants.

Rapid and efficient gene detection is one of the strategies against potential resistant pathogens. The DNA microarray was able to provide useful information on genotypes (the virulence potential and antibiotic resistance gene contents) of *Enterococcus* species that could lead to the development of effective approaches in the control and spread of antibiotic resistance. The detection methodology described in this study allowed a clear differentiation between chicken enterococci having a high virulence potential that could later find their way into natural environments. Although other molecular methods can provide epidemiological or virulence potential information, our novel enterococcal DNA microarray provides taxonomic, virulence, and antibiotic resistance information simultaneously with a high level of resolution and could represent a valuable tool to assess safety of environmental, food, and water enterococci. The *E. faecium* isolates were found to have a higher prevalence of resistance phenotypes and genotypes, while *E. faecalis* isolates had a high prevalence of virulence genes ($P \leq 0.05$) that are expressed. Additional tests need to be performed to evaluate the potential of these *E. faecalis* isolates to cause disease. The high prevalence of resistance to antibiotics of human and animal importance in our isolates as well as their corresponding genes and mobile genetic elements, such as a transposon, is of concern, knowing that *Enterococcus* is capable of disseminating these genes to other bacteria (11, 45). The absence of VRE suggests that the broiler farms in our study do not contribute to the dissemination of vancomycin resistance. Overall, the present study underscores the importance of risk management measures taken at the time of slaughter to minimize the risk of food contamination and the fact that poultry fecal material should be handled appropriately to minimize environmental contamination.

ACKNOWLEDGMENTS

This study was funded by Agriculture and Agri-Food Canada.

We acknowledge the technical assistance of J. Takizawa and thank the British Columbia Chicken Marketing Board and participating farmers from Fraser Valley for their support.

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