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Antimicrobial Resistance of *Enterococcus* Species Isolated from Produce†

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The purpose of this study was to characterize the antibiotic resistance profiles of *Enterococcus* species isolated from fresh produce harvested in the southwestern United States. Among the 185 *Enterococcus* isolates obtained, 97 (52%) were *Enterococcus faecium*, 38 (21%) were *Enterococcus faecalis*, and 50 (27%) were other *Enterococcus* species. Of human clinical importance, *E. faecium* strains had a much higher prevalence of resistance to ciprofloxacin, tetracycline, and nitrofurantoin than *E. faecalis*. *E. faecalis* strains had a low prevalence of resistance to antibiotics used to treat *E. faecalis* infections of both clinical and of agricultural relevance, excluding its intrinsic resistance patterns. Thirty-four percent of the isolates had multiple-drug-resistance patterns, excluding intrinsic resistance. Data on the prevalence and types of antibiotic resistance in *Enterococcus* species isolated from fresh produce may be used to describe baseline antibiotic susceptibility profiles associated with *Enterococcus* spp. isolated from the environment. The data collected may also help elucidate the role of foods in the transmission of antibiotic-resistant strains to human populations.

Enterococcus species are ubiquitous, commensal inhabitants of the gastrointestinal tract of humans and animals. They are frequently isolated from environmental sources such as soil, surface waters, and raw plant and animal products, where their intrinsic ruggedness allows them to persist and spread in the environment. Once viewed as a genus of minimal clinical impact, enterococci, particularly *Enterococcus faecium* and *Enterococcus faecalis*, have surfaced as organisms of importance due to the emergence of multiple-drug-resistant strains that are currently responsible for approximately 12% of all nosocomial infections in the United States (10, 11). Furthermore, their ability to acquire antibiotic resistance through transfer of plasmids and transposons, chromosomal exchange, or mutation presents a significant challenge for therapeutic measures (14).

Antibiotic-resistant strains of *Enterococcus* have been isolated from raw foods (5), and some believe that water and food are possible vectors of strain transmission to human intestinal flora (28). Of recent concern is the potential development of environmental reservoirs of antibiotic resistance in farmland. Specifically, the application of untreated irrigation water or manure slurry to croplands could result in the spread of resistance to indigenous soil bacteria through horizontal transfer, which could in turn transfer resistance back to animals or humans via crops (17, 21).

While the prevalence and transmission of antibiotic resistance among bacteria associated with food animals has been well documented, research regarding resistance profiles of bacteria isolated from raw produce is lacking (1, 7, 26, 27). A few studies examining the prevalence of resistance among gram-negative microorganisms isolated from produce exist, although results are conflicting. Hamilton-Miller and Shah (6) characterized the antibiotic susceptibility of enterobacterial flora of salad vegetables, finding a high degree of resistance to ampicillin and the narrow- and expanded-spectrum cephalosporins. Alternatively, a Finnish study found that members of the *Enterobacteriaceae* family isolated from vegetables were highly susceptible to the antibiotics studied, and multidrug-resistant strains were generally not identified (18). Prazak et al. (19) studied the resistance patterns among *Listeria monocytogenes* isolates from cabbage farms, in which 98% of the isolates were resistant to at least two drugs and 85% were found to be resistant to penicillin. However, the prevalence and patterns of antibiotic resistance among *Enterococcus* strains isolated from fresh vegetables are not yet well understood.

This study was undertaken as a supplement to a larger project, the purpose of which was to determine the prevalence of selected microorganisms in fresh produce harvested from the southwestern United States. In this study, we report on the isolation, identification, and antibiotic susceptibility profiles for members of the *Enterococcus* genus isolated directly from these fresh produce samples.

Isolation, identification, and antibiotic resistance profiles of *E. faecalis* and *E. faecium*. The sampling site, located in the southwestern United States, included 13 farms and 5 packing sheds. All samples were obtained between January and May 2002. A total of 304 produce samples were collected throughout production and processing and consisted of a variety of leafy greens, herbs, and cantaloupe. Composite samples of approximately 200 g were obtained by workers wearing sterile, disposable gloves and placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, Wis.). These were then immediately shipped on ice to our location at North Carolina State University by overnight courier. All microbial analyses were initiated within 24 h of sample collection.

The cultural methods used were recommended by the U.S.

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Food and Drug Administration, Center for Veterinary Medicine (7, 23; D. D. Wagner [Food and Drug Administration], personal communication). After 24 h at 37°C, a representative colony for each morphology, generally two or three per sample, showing esculin hydrolysis (darkened colony with black halo) was purified and screened for hemolysis by streaking on 5% sheep blood agar (Remel, Lenexa, Kans.). The colonies were then screened at the genus level by PCR-based assays and at the species level by the Vitek system (Vitek 32, GPI panel; bioMerieux, Hazelwood, Mo.). For PCR, DNA was extracted with the Ultra Clean microbial DNA isolation kit (Mo Bio Laboratories, Inc., Solana, Calif.) in accordance with manufacturer recommendations. Primers were directed to the *tuf* gene (forward primer, TACTGACAACCATTCATGATG; reverse primer, AACTTCGTCACCAACGCGAAC), yielding a 112-bp product (9). Two microliters of DNA was added to a 98-μl mixture containing 1× PCR buffer, a 200 μM concentration of each deoxynucleoside triphosphate, 2.5 U of *AmpliTaq* polymerase, 3.0 mM $MgCl_2$, and a 1 μM concentration of each primer. The PCR mixtures were subjected to predenaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. A 7-min final elongation step at 72°C concluded the PCR assay. Two positive controls, *E. faecalis* ATCC 29212 and *E. faecium* ATCC 19434, were used. Isolates producing an amplicon band of the appropriate size by agarose gel (3%) electrophoresis were considered presumptively positive for the genus *Enterococcus* and were sent on for Vitek species-level identification to the Clinical Microbiology Laboratory of the College of Veterinary Medicine at North Carolina State University.

Strains identified as *E. faecium* or *E. faecalis* were screened for antibiotic susceptibility by the microdilution broth method with Mueller-Hinton media (TREK Diagnostics, Westlake, Ohio) as outlined by the National Committee on Clinical Laboratory Standards (NCCLS) (16). A customized panel of 17 antibiotics with various concentration ranges (TREK Diagnostics), identical to that used in the National Antimicrobial Resistance Monitoring System (NARMS 2001) program, for gram-positive organisms was used in this study. The antibiotics and their concentration ranges were as follows: bacitracin, 8 to 128 IU/ml; chloramphenicol, 2 to 32 μg/ml; erythromycin, 0.5 to 8 μg/ml; bambermycin (flavomycin), salinomycin, vancomycin, quinupristin-dalfopristin, and lincomycin, 1 to 32 μg/ml; penicillin, 0.5 to 16 μg/ml; tetracycline, 4 to 32 μg/ml; tylosin tartrate, 0.25 μg/ml; ciprofloxacin, 0.12 to 4 μg/ml; linezolid, 0.5 to 8 μg/ml; nitrofurantoin, 2 to 128 μg/ml; kanamycin and gentamicin, 128 to 1,028 μg/ml; and streptomycin, 512 to 2,048 μg/ml. MICs were determined manually by assessing each antibiotic and strain combination for growth. Isolates were categorized as susceptible, intermediate, or resistant, based on the NCCLS interpretive standards, where applicable (15). The MICs, based on NCCLS breakpoints, were as follows: chloramphenicol and vancomycin, ≥32 μg/ml; erythromycin and linezolid, ≥8 μg/ml; penicillin and tetracycline, ≥16 μg/ml; quinupristin-dalfopristin and ciprofloxacin, ≥4 μg/ml; nitrofurantoin, ≥128 μg/ml; gentamicin, >500 μg/ml; and streptomycin, >1,000 μg/ml (15). Differentiations between susceptibility and resistance are based on pharmacological, clinical, and microbiological criteria. Unfortunately, both pharmacological and clinical data are lacking for most antibiotics used as growth promoters (3). Therefore, in instances where NCCLS standards were not available, a quantitative evaluation of resistance was done through calculation of MICs at which 50% and 90% of isolates were inhibited (MIC_{50} and MIC_{90}) (15, 16). Control strains included *E. faecalis* ATCC 29212 (vancomycin susceptible) and ATCC 51299 (vancomycin resistant) (7, 16). Profiles from different isolates collected from the same sample that differed by less than 2 dilutions for at least one antimicrobial MIC were considered duplicates. Consequently, only a single isolate was included for subsequent analysis.

TABLE 1. Isolation of *E. faecium* and *E. faecalis* isolates from produce samples

Commodity (n = 304)	No. (%) of samples		No. (%) of *Enterococcus* isolates			
	Total n	With *Enterococcus* spp.	Total	*E. faecium* (n = 97)	*E. faecalis* (n = 38)	Other species
Celery	20	1 (5)	1	1 (100)	0	0
Cilantro	25	4 (16)	5	2 (40)	0	3
Mustard greens	70	39 (56)	56	29 (52)	7 (13)	20
Spinach	12	2 (17)	2	0	0	2
Collards	12	4 (33)	4	2 (50)	0	2
Parsley	48	24 (50)	31	19 (61)	2 (6)	10
Dill	12	9 (75)	11	9 (82)	0	2
Cabbage	15	12 (80)	18	11 (61)	4 (22)	3
Cantaloupe	90	48 (53)	57	24 (42)	25 (44)	8

Prevalence and antibiotic resistance patterns of *E. faecalis* and *E. faecium* isolates from produce. The distribution of produce samples and *Enterococcus* species is shown in Table 1. Mustard greens, parsley, and cantaloupe represented nearly 70% (208 of 304) of the total produce items collected. At least one *Enterococcus* strain was isolated from over half of these samples. Among the 185 *Enterococcus* isolates obtained from all of the samples, a total of 97 (52%) were *E. faecium*, 38 (21%) were *E. faecalis*, and 50 (27%) were other *Enterococcus* species. Ninety-one percent of the *E. faecium* isolates and 32% of the *E. faecalis* isolates were resistant to at least one of the antibiotics tested, excluding intrinsic resistance. A summary of resistance profiles is provided in Table 2.

Inherent resistance. The treatment of *Enterococcus* infections is limited by the intrinsic resistance among enterococci. In general, enterococci show intrinsic resistance to cephalosporins, lincosamides, and many synthetic β-lactams, such as the penicillinase-resistant penicillins (5, 20). *Enterococcus* species are also resistant to low levels of aminoglycosides, due to the decreased uptake of this antibiotic class (5). In this study, a majority of the *E. faecium* and *E. faecalis* isolates showed inherent resistance patterns which were consistent with previous studies with farm animals and pets (2, 4). For instance, both *E. faecium* and *E. faecalis* had intrinsic resistance to bacitracin, i.e., 90% of the isolates were inhibited at concentrations greater than 128 IU/ml. Also, for *E. faecium* the MIC_{90} of flavomycin was greater than 32 μg/ml. Consistent with reported data (8, 22), a majority (97%) of *E. faecalis* isolates were resistant to quinupristin-dalfopristin when species identification was based on PCR alone but less so (87%) if identification was based on Vitek. Results also showed that isolates were resistant to low levels of lincomycin.

Resistance relevant to animal agriculture. It has been suggested that the overuse of antibiotics in livestock production

TABLE 2. Antibiotic resistance profiles among *E. faecium* and *E. faecalis* isolates from produce

Antibiotic	*E. faecium* (n = 97)				*E. faecalis* (n = 38)			
	No. (%) resistant	No. (%) intermediate	MIC_{50}	MIC_{90}	No. (%) resistant	No. (%) intermediate	MIC_{50}	MIC_{90}
Bacitracin	NA[a]	NA	>128	>128	NA	NA	128	>128
Chloramphenicol	5 (5)	5 (5)			1 (3)	2 (5)		
Erythromycin	10 (10)	73 (75)			1 (3)	26 (68)		
Flavomycin	NA	NA	>32	>32	NA	NA	1	4
Penicillin	7 (7)	NA			0	NA		
Salinomycin	NA	NA	1	2	NA	NA	1	2
Quinupristin-dalfopristin	16 (16)	48 (49)			37 (97)	1 (3)		
Tetracycline	28 (29)	3 (3)			0 (0)	0		
Vancomycin	0	5 (5)			0	0		
Lincomycin	NA	NA	16	32	NA	NA	32	32
Tylosin tartrate	NA	NA	2	8	NA	NA	1	4
Ciprofloxacin	27 (28)	22 (23)			2 (5)	8 (21)		
Linezolid	0	9 (9)			0	2 (5)		
Nitrofurantoin	23 (24)	55 (57)			0 (0)	2 (5)		
Kanamycin	NA	NA	256	1,024	NA	NA	128	128
Gentamicin	0	NA			0	NA		
Streptomycin	3 (3)	NA			0	NA		

[a] NA, not applicable.

may provide an environmental reservoir of antibiotic resistance (21). Among the panel of 17 antibiotics screened in this study, 7 are used in animal feed for growth promotion. These include bacitracin, flavomycin, penicillin, salinomycin, tetracycline, lincomycin, and tylosin (12). Both *E. faecium* and *E. faecalis* demonstrated a high degree of susceptibility to salinomycin, lincomycin, and tylosin. As mentioned above, *E. faecium* is intrinsically resistant to flavomycin; however, *E. faecalis* isolates were susceptible to flavomycin (MIC_{90} = 4 μg/ml). Less than 10% of the *E. faecium* isolates were resistant to penicillin, and all *E. faecalis* isolates were susceptible to penicillin. Twenty-nine percent of the *E. faecium* isolates and no *E. faecalis* isolates were resistant to tetracycline. Erythromycin is also used in livestock production, specifically for therapeutic purposes in chickens and turkeys (12). In this case, 10% of the *E. faecium* isolates were resistant to erythromycin, while only 3% of the *E. faecalis* isolates were resistant. We can generally conclude that there was not a high degree of resistance to the antibiotics commonly used in animal agriculture among the *Enterococcus* isolates collected in this study, especially for *E. faecalis*. However, *E. faecium* demonstrated a higher degree of resistance to tetracycline (29%) than did *E. faecalis.*

Resistance relevant to human medicine. All of the antibiotics used in the NARMS 2001 panel are of importance for human therapeutic use except for tylosin tartrate, salinomycin, and flavomycin (29). Penicillin, vancomycin, aminoglycosides, chloramphenicol, ciprofloxacin, and quinupristin-dalfopristin all have been used in the treatment of enterococcal infections either in combination therapy, for optimal killing, or monotherapeutically (4). Synergistic treatment includes the use of an aminoglycoside with the addition of a cell wall-active agent, such as vancomycin or penicillin (4). In the present study, there was an extremely low level of resistance to the aminoglycosides (*E. faecium*, 3%; *E. faecalis*, 0%), vancomycin (*E. faecium*, 0%; *E. faecalis*, 0%), and penicillin (*E. faecium*, 7%; *E. faecalis*, 0%). Chloramphenicol, also used synergistically in documented cases, was shown to inhibit a majority of all isolates; 5% of *E. faecium* strains and only 3% of *E. faecalis* strains were resistant to chloramphenicol (4). According to Chow and Shlaes, enterococcal infections of less severity have been treated with a single antibiotic (4). Among the drugs in the NARMS 2001 panel, an example of such an antibiotic is ciprofloxacin, to which 27 (28%) of the *E. faecium* strains and 2 (5%) of the *E. faecalis* strains were found to be resistant. Quinupristin-dalfopristin can also be used for the treatment of *E. faecium* infections in humans, and in our study, 16 (16%) isolates were found to be resistant and 48 (49%) were intermediately resistant to these drugs. There is evidence that during the therapeutic use of quinupristin-dalfopristin for *E. faecium* bacteremia, superinfection of *E. faecalis* can occur, posing concerns regarding such a high proportion of *E. faecium* resistance to these drugs (4, 13). Finally, and consistent with the literature (25), we found that all *E. faecalis* strains were susceptible to nitrofurantoin, a drug frequently used for the treatment of *E. faecalis* urinary tract infections. When the data are taken together, there was a relatively low prevalence of resistance to most of the drugs used in clinical treatment of enterococcal infections in humans, especially for *E. faecalis*.

Multiple-drug resistance. Fifty-nine (61%) of the *E. faecium* isolates and 4 (11%) of the *E. faecalis* isolates showed multidrug resistance, i.e., resistance to two or more drugs, although no specific patterns of multidrug resistance were readily apparent. In general, the *E. faecium* isolates had a greater degree of multidrug resistance than did the *E. faecalis* isolates. Twenty-five percent of the *E. faecium* strains had simultaneous resistance to three or more drugs. As previously mentioned, significant resistance was found among the *E. faecium* isolates to ciprofloxacin, tetracycline, and nitrofurantoin. Interestingly, at least one of these clinically important antibiotics was represented in over 75% of the multidrug-resistant *E. faecium* strains, suggesting the possibility of gene linkage, although this was not confirmed in our study. For all *E. faecium* and *E. faecalis* isolates, 24 (18%) multidrug resistant strains were resistant to ciprofloxacin, 22 (16%) strains were resistant to tet-

racycline, and 21 (16%) strains were resistant to nitrofurantoin. Six (6%) *E. faecium* strains were found to be simultaneously resistant to all three antibiotics.

Conclusions. Multiple-drug-resistant strains of *E. faecalis* and *E. faecium* have been increasingly associated with nosocomial infections. Of particular interest has been the potential for foods as a vehicle for transmission of these strains to humans or, alternatively, as a reservoir for horizontal transfer between strains. This might be considered credible since once ingested, enterococci can survive gastric passage, multiply, and colonize the gastrointestinal tract for a significant amount of time (24). Indeed, there is strong epidemiological evidence to link the use of antibiotics in human medicine and animal agriculture with the presence of resistant strains in animal products. In many cases where high rates of resistance have been shown to occur in food and humans, there is also a link to drug use in animals, conferring cross-resistance from avoparcin to vancomycin (27, 28). In general, the prevalence of antibiotic-resistant enterococci in farm animals and their meat is high (>60%) (5). Moreover, Hayes et al. (7), applying the panel of antibiotics used in our study, reported that resistant *Enterococcus* spp. commonly contaminate retail meat and their resistance patterns reflect the use of antimicrobial agents in the production of such products. The patterns of resistance to antibiotics are similar between the work of Hayes et al. (7) and this study; however, the prevalence (or degree) of antibiotic resistance in produce is lower than that found in retail meats.

In our study, resistance patterns differed among species of the genus *Enterococcus*. Overall, *E. faecium* was found to have a higher prevalence of resistance among the panel antibiotics, particularly tetracycline, ciprofloxacin, and nitrofurantoin, while *E. faecalis* isolates had a relatively lower prevalence of resistance to antibiotics of both clinical and agricultural relevance, excluding their inherent resistance to quinupristin-dalfopristin. A high percentage of the *E. faecium* isolates were found to be resistant to multiple drugs, a factor that contributes to the challenge of selecting therapeutic measures. While *Enterococcus* resistance to glycopeptides is among current clinical concerns, the absence of vancomycin-resistant enterococci in the present study suggests that raw produce does not contribute to the dissemination of vancomycin resistance.

The present study provides evidence that can be used in subsequent risk assessment exercises to elucidate the role of raw produce in the dissemination of antibiotic resistance to human populations. The findings indicate that while fresh produce items do harbor strains of enterococci that are resistant to many commonly used antibiotics, the resistance patterns are not significantly different from those reported for *Enterococcus* strains isolated from animal products such as poultry and pork. However, animal products are usually cooked prior to consumption, which should theoretically inactivate most of the native microflora, including enterococci, in those products. Fresh produce, in many instances, is consumed without a terminal heating step. Clearly, the role of food in the transmission of these strains is a question for which there is no definitive answer. However, data such as those presented here offer evidence that should be helpful in the identification of future study topics and initiatives aimed at reducing the public health burden of antibiotic-resistant pathogens.

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