

ABSTRACT

Title of Dissertation: MULTIPLE ANTIBIOTIC RESISTANCES OF ENTEROCOCCI
FROM THE POULTRY PRODUCTION ENVIRONMENT AND
CHARACTERIZATION OF THE MACROLIDE-
LINCOSAMIDE-STREPTOGRAMIN RESISTANCE
PHENOTYPES OF *ENTEROCOCCUS FAECIUM*

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Enterococcus spp. were collected from poultry production and processing environments across a region of the eastern seaboard of the United States. Using a microtiter plate adaptation of traditional biochemical assays, 532 isolates were identified to the species level. *E. faecalis* was observed to be the predominant species recovered (64%), followed by *E. faecium* (24%), *E. hirae* (6%), *E. gallinarum* (5%), and less than 1% of *E. avium*, *E. casseliflavus*, *E. durans*, and indeterminate species. All isolates were tested for susceptibility to 28 antimicrobials using broth microdilution. Antimicrobial resistance was observed among the isolates with *E. faecalis* more resistant to lincosamide, macrolide, and tetracycline antimicrobials and *E. faecium* more resistant to fluoroquinolone and penicillin antimicrobials. Resistance to multiple antimicrobials was

observed among all species, with a proportionately greater diversity of resistance phenotypes among isolates of *E. faecium*. The prevalence of resistance to an important antimicrobial used in human medicine, the streptogramin quinupristin-dalfopristin, was demonstrated among 63% of *E. faecium* isolates. These observations led to the investigation of the molecular determinants among those isolates of *E. faecium* that contributed to resistance to the group of antibiotics known as the macrolide-lincosamide-streptogramin (MLS) superfamily. Ribotype analysis demonstrated that four ribotypes constituted 65% of the observed population, but displayed diverse antibiograms, suggestive of the acquisition of multiple resistance elements to other antimicrobials. This was consistent with the absence of geographic clustering of MLS phenotypes or ribotypes. Colony PCR screening for the streptogramin resistance determinants *erm*(A), *erm*(B), *mef*(A), *lnu*(B), *msr*(C), *vgb*(A), *vat*(D), and *vat*(E) was performed. Resistance to streptogramin antimicrobials was largely unaccounted for by PCR screening for specific resistance determinants. Erythromycin methyltransferase determinants *erm*(A) and *erm*(B) were observed among 7.5% and 5% of resistant isolates whereas the efflux gene *msr*(C), the streptogramin B hydrolase *vgb*(A), or the streptogramin A acetyltransferase genes *vat*(D) and *vat*(E) were not detected among resistant *E. faecium*. *Mef*(A) was detected in only 4% of macrolide-resistant isolates whereas the screening for *lnu*(B) was not successful. These results indicate that streptogramin resistance is widespread among *E. faecium* from the poultry production environment but the mechanisms of resistance within this population remain largely uncharacterized.

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MACROLIDE-LINCOSAMIDE-STREPTOGRAMIN RESISTANCE PHENOTYPES
OF *ENTEROCOCCUS FAECIUM*

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DEDICATION

I dedicate my graduate experience, represented in part by this work, to my family for their steadfast support and emotional guidance. In particular, I dedicate what this work represents to my wife Christine, for your unconditional love, understanding, and patience throughout this part of our journey and for your impatience when needed; and to my daughter Katherine, for the gifts of joy and happiness that you bring to my life every day.

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LIST OF ABBREVIATIONS

CNA	Colistin-Nalidixic Acid
Delmarva	Delaware-Maryland-Virginia
MIC(s)	Minimum Inhibitory Concentration(s)
MLS	Macrolide-Lincosamide-Streptogramin
MLSb	Macrolide-Lincosamide-Streptogramin B
NABS	Nalidixic Acid-Brain Heart Infusion-Salt
NCCLS	National Committee for Clinical Laboratory Standards
QDREF	Quinupristin-Dalfopristin-Resistant <i>Enterococcus faecium</i>
VRE	Vancomycin-Resistant <i>Enterococcus/enterococci</i>
VREF	Vancomycin-Resistant <i>Enterococcus faecium</i>

GENERAL INTRODUCTION

Microorganisms have a dynamic relationship with the biosphere after continually adapting to inconstant environmental conditions, thus generating an enormous amount of genetic diversity. The ecological niches that these forms of life occupy are limited and are under ferocious competition. Advances in the understanding of microbial ecology have only relatively recently allowed scientists to consider the bodies of many animals as rich harbors for many forms of life. Bacteria that inhabit animal niches can colonize and proliferate on or within a host and establish a similarly vigorous association. This relationship with the individual can range from beneficial to outright deadly. One particularly interesting common group of inhabitants of this environment is the genus of gram-positive cocci, *Enterococcus*.

Genus Description

Members of the genus *Enterococcus*, first described in 1899 by Thiercelin (289), are gram-positive, catalase negative, cocci that occur singly, in pairs, or short chains. Enterococci are homofermentative lactic acid bacteria that are facultatively anaerobic and can grow between 10 and 45°C, with optimum growth in most species at 35°C. All known members of the genus grow in broth containing 6.5% NaCl and hydrolyze esculin in the presence of 40% bile salts. Most described species hydrolyze pyrrolidonyl- β -naphthylamide (PYR), whereas only two species are motile. Enterococci can be α , β , or γ -hemolytic on blood agar. Twenty-six species have been proposed since the removal of *S. faecalis* and *S. faecium* from the genus *Streptococcus* to form the genus *Enterococcus* (Table 1)(256).

Ecological Distribution

Enterococci are considered important members of the intestinal microflora of mammals, reptiles, birds, fish, and insects as well as in plant environments (27,68,70,94,203). Members of *Enterococcus* spp. can also be found in the soil, water, and food (77,152,202,204).

More specifically, *E. avium*, *E. durans*, *E. faecalis*, *E. faecium* are frequently isolated from cheese products (26,110,174) whereas *E. avium*, *E. casseliflavus*, *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. hirae* have been described components of the microflora of various raw meat products (72,123,288). It is important to note that members of these species are frequently employed as starter cultures in fermented food products. The high prevalence of these species in raw meat is closely related to the fecal microflora from the food animal species (69,70,121,196,288,313). Despite the presence of enterococci in 82-100% of retail meat products (123,186), studies of cooked meat suggest that enterococci do not constitute the largest population on such products (19).

Although the enterococcal population within the human intestinal tract does not account for more than 1% of the total microflora, the population is quite diverse (262). *Enterococcus faecalis* and *E. faecium*, in particular, are by far the most prevalent species found (89,187). *E. casseliflavus*, *E. durans*, *E. gallinarum*, *E. hirae* also contribute significantly to the enterococcal fecal microflora of humans (165,197,266) whereas *E. avium* and *E. mundtii* have only been occasionally isolated (105,278).

Enterococci that have been isolated from domesticated pets include *E. avium*, *E. durans*, *E. faecalis*, *E. faecium*, and *E. hirae* (68). *E. avium*, *E. casseliflavus*, *E. durans*,

E. faecalis, *E. faecium*, *E. gallinarum*, *E. hirae*, and *E. mundtii* have been isolated from surface waters (121,238,283,297) whereas only *E. hirae* has been identified from groundwater (49). *E. casseliflavus*, *E. faecalis*, *E. faecium*, and *E. sulfureus* have also been associated with plant materials (201,222,305).

Among the more rarely isolated species, *E. cecorum* has been isolated from domesticated pets (68) and feces from bovine and poultry sources (69,70). *E. dispar* has been isolated from poultry feces (221). Since its initial isolation from Gouda cheese (55), *E. malodoratus* has been isolated from poultry feces (221) and has been associated with spoilage of sausage (162). Although primarily associated with soil and plant material, *E. mundtii* has been isolated from fish and meat products (57,72,227). *E. mundtii* has also been identified as components of the intestinal microflora of chickens (69). *E. pseudoaerium* has been isolated from pigs and poultry (162,221). *E. raffinosus* has rarely been isolated outside of the clinical environment, but has been identified from domestic pets (68). *E. sulfureus* has been isolated only from grass and fish sources (201,222).

E. solitarius and *E. seriolicida* have been reclassified as members of the genera *Tetragenococcus* and *Lactococcus*, respectively. Recently, two new proposed species of pigmented enterococci, *E. gilvus* and *E. pallens*, were described from clinical isolates (293). These species, in addition to *E. asini*, *E. canis*, *E. haemoperoxidus*, *E. moraviensis*, *E. phoeniculicola*, *E. porcinus*, *E. ratti*, and *E. villorum*, are recent additions to the genus and their distributions in different environments are unknown.

It is worth noting that reports of the ecological distribution of *Enterococcus* spp. in different environments may be hard to compare due to differences in isolation methodology. Most surveys to date have been focused on the isolation of what are

thought to be the most abundant species of enterococci, i.e., heavily influenced by those studies conducted in clinical settings. Recent changes in the taxonomy of enterococci has revealed that an ever-increasing number of species do not conform to long-held descriptions of the genus. For example, not until the description of the PYR-negative species of *E. cecorum*, *E. columbae*, and *E. saccharolyticus* has the PYR reaction been used other than as a definitive characteristic of *Enterococcus* spp. Other factors that might influence the recovery and/or prevalence of non-dominant species include the choice of media, the temperature of incubation, composition of the incubation atmosphere, and identification methodology (39,215,322). Given the close similarity of the newly described species to long-standing members of the genus, confident identification to the species level may not always be possible using traditional biochemical testing (71).

Pathogenicity

Whereas enterococci are thought to account for no more than 1% of the adult intestinal microflora, the medical importance of enterococci outweighs their relative abundance (262). Of the 26 species that have been proposed to belong to the genus, only 11 (*E. avium*, *E. casseliflavus*, *E. durans*, *E. dispar*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae*, *E. mundtii*, *E. pseudoavium*, and *E. raffinosus*) have been described as associated with human disease (205). *E. faecalis* accounts for 80-90% of enterococcal isolates of clinical origin, with *E. faecium* the second most prevalent enterococcal species. Despite the lower frequency of isolation from clinical settings, *E. faecium* isolates are disparately resistant to treatment with antimicrobial chemotherapy (84).

Although normally commensal in nature, enterococci are responsible for approximately 10% of urinary tract infections and 16% of nosocomial urinary tract infections (96,253). They are also commonly isolated from wound infections of the abdominal area (151) as well as those from crushing injuries (64). Enterococcal bacteremia is the third leading cause of nosocomial bacteremia (114) with an estimated fatality rate of 28 to 58% (180). Enterococci are also responsible for between 5 and 20% of cases of bacterial endocarditis (188). Enterococci have been described as one of the most destructive agents that cause postoperative complications of cataract surgery (119). Up to 13% of bacteriologically confirmed cases of neonatal sepsis have been attributed to enterococci (24). Those who are elderly or have an underlying compromising situation are predisposed to enterococcal infection, especially in the hospital environment (205). This is a significant observation given the ability of enterococci to colonize surfaces of the hospital environment (333) and persist on fingertips and dry surfaces (217). As a result, enterococci seeding the clinical environment may be more easily spread if infection control measures are poorly implemented.

Virulence Factors

In addition to the hardiness of the genus, other components have been implicated as important factors in the sequence of events that lead to clinical human disease. Acid tolerance, mediated by any stimulus that causes an increase in proton pump activity, is thought to allow enterococci to survive passage through the stomach prior to colonization of the lower bowel (101,282), although it remains to be seen whether this mechanism is enhanced among isolates responsible for nosocomial infection. Aggregation substance is

thought to play a role in the translocation of enterococci from the intestinal lumen to the mesenteric lymph nodes, liver, and spleen (312) although additional mechanisms are thought to also contribute (220).

Another factor thought to be involved in adhesion is enterococcal surface protein (Esp), which has also been demonstrated to aid in the formation of a bacterial biofilm (291) and contributes to a mouse model of urinary tract infection (264). The pheromone-responsive β -hemolysin, known as cytolysin, has been shown to decrease the lethal dose of bacteria in animal models, although its mode of action in disease is unknown (191). The production of the secreted zinc metalloprotease, gelatinase, is also thought to play a role in systemic disease (76), perhaps through the modulation of the host immune response (251). An enterococcal adhesin, Ace, which mediates binding to extracellular matrix proteins, has recently been identified as a potential virulence factor that may contribute to enterococcal endocarditis (210,211,239). A similar collagen-binding adhesin, Acm, has also been described among clinical isolates (212). It is thus likely that different sets of these determinants contribute to the colonization and virulence depending upon the infection site.

Whereas typically considered to be an important member of the commensal flora that help to produce vitamins and convert toxic metabolites as well as maintain the structure and function of the intestinal epithelium, recent evidence has suggested that enterococci may help to traffic surface receptors that enhance the virulence of other pathogens (177).

Prevalence of Virulence Genes

Nearly 50% of *E. faecalis* isolates from food sources possessed the *asaI* gene, which encodes the aggregation substance (104). The prevalence of this gene among *E. faecalis* isolated from endocarditis infection has been observed at 26% of isolates and has also been detected among 18% of *E. faecalis* isolates from well-water (25). *AsaI* was observed in 64% of fecal isolates from healthy subjects whereas 32 to 40% among clinical isolates possessed the gene (131). In contrast to the observed prevalence of 63% among clinical isolates of *E. faecalis* from Germany, only 13% of *E. faecium* isolates possessed *asaI* (86). In contrast, other studies have not observed the presence of this gene among *E. faecium* from multiple environments (81,104,181).

The prevalence of the enterococcal surface protein gene *esp* ranges from 32 to 44% among *E. faecalis* and 68 to 78% of *E. faecium* of clinical origin (53,81,181,306). This is in contrast to prevalence rates of 36% of *E. faecalis* and a single *E. faecium* isolate from food sources (104). The gene is infrequently observed in isolates of *E. faecium* outside of the clinical environment (104,181).

The prevalence of cytolysin has been estimated between 11 to 56% of *E. faecalis* isolates of clinical origin, whereas it was not detected among isolates of *E. faecium* (81,86,306). Whereas this trait has been found in 17% of *E. faecalis* isolates from feces from healthy Japanese subjects, up to 60% of those of clinical origin were found to be hemolysin producers (133). It has also observed in 21% of *E. faecalis* and 8% of *E. faecium* from a variety of food sources (104).

Gelatinase, encoded by the gene *gelE*, was observed among 27 to 68% of clinical *E. faecalis* isolates (58,86,306). Up to 91% of clinical *E. faecalis* isolates may harbor

gelE (81,232), but only express the gelatinase-positive phenotype in the presence of a regulatory locus (232). Gelatinase activity has been observed in 49% of food isolates of *E. faecalis* (104). Most studies have not detected *gelE* among isolates of *E. faecium* from various environments (58,86,104,306) although in a single study *gelE* was observed in a single clinical isolate in the absence of gelatinase activity (81).

The *ace* gene has not been detected in any species other than *E. faecalis*, suggesting that the gene is specific only to this species (75). Another adhesin gene, *acm*, has been similarly described among clinical isolates of *E. faecium* (212). No observations of successful conjugative transfer of either the *ace* or *acm* genes have been reported.

Clinical strains of *E. faecalis* have been shown to be comparatively enriched in virulence determinants with the observation of regions of the enterococcal genome that apparently serve as a pathogenicity island (81,263). The combination of multiple virulence factors within clinical *E. faecalis* in a subsequent study did not demonstrate such a strong association or an association of virulence factors and mortality (306).

Conjugative transfer of certain virulence determinants between strains of *E. faecalis* has been well documented (52). Aggregation substance-encoding genetic determinants have been transferred *in situ* from a clinical isolate of *E. faecalis* to one used in cheese and sausage fermentation and remained a stable trait in these environments (53). The transfer of virulence determinants from clinical isolates to starter cultures of *E. faecalis* used in foods has also been observed (81).

Clinical Antimicrobial Resistance

The introduction of the newest generation of antibiotics, penicillin in 1944 (48,102) and streptomycin in 1945(255), was thought to preface the demise of infectious diseases. As early as 1940, however, enzymes responsible for antibiotic resistance had been described from laboratory studies of resistance in staphylococci (154), pneumococci (257), and *E. coli* (8,9,207). The subsequent appearance of resistant bacteria among clinical bacterial isolates was recognized as an increasingly familiar trend (80,100). Investigations of multiply resistant *Salmonella* (65) and *Shigella* (155) isolates from epidemics demonstrated that antimicrobial resistance could be spread among other enteric bacteria (307). These observations were the first to suggest that the use of antimicrobials would be accompanied by antimicrobial resistance presenting a widespread challenge to the treatment of human bacterial infections.

Multiple-drug resistant (MDR) enterococci have been on the rise for the past two decades. High-level gentamicin resistance was reported in 1979 (130) and was followed by the emergence of a serious pathogen in the nosocomial environment in the 1980s (330). With the emergence of penicillin-resistant enterococci due to the acquisition of a β -lactamase in the early 1980s, the drug of choice to treat MDR enterococci has been the glycopeptide, vancomycin. Resistance to this vanguard of antimicrobial treatment emerged first in Europe in 1986 (295), followed by emergence in the U.S. in 1989 (249).

Vancomycin-resistant enterococci (VRE) infections in the U.S. have increased twenty fold from 1989 to 1993 (43). Increases in the incidence or detection of VRE worried some that the “post-antimicrobial era” was imminent, due to fears that the elements responsible for resistance would be transferred to staphylococci (54).

Vancomycin resistance has been transferred via conjugation in the laboratory from *Enterococcus* to the more virulent *Staphylococcus aureus*, rendering them untreatable (216). This fear has recently been documented in the clinical environment, however the prevalence of vancomycin-resistant *S. aureus* is thought to be relatively low (45,46).

Studies show that the risk of death resulting from bacteremia caused by a vancomycin-resistant *Enterococcus* isolate is several fold higher than that with bacteremia due to a susceptible isolate (83). Patients that develop resistant infections can continue to carry VRE in feces for up to two years and provide a source for environmental dissemination (243). In addition, resistant bacterial infections, in general, represent a financial burden, as the estimated cost of extended in-hospital stay in the U.S. caused by six different bacteria including VRE is at least \$1.3 billion per year (219) and total cost may exceed \$4 billion per year (12).

Agricultural Antimicrobial Resistance

The origin and spread of bacterial resistance is currently an incomplete and contentious issue. In the case of VRE, resistance was first recognized among isolates of *E. faecium* recovered outside of health care settings in 1993 from sewage treatment plants in urban areas of England (23) and in small towns of Germany (159). The relationship of this troubling human pathogen to agriculture was first made when VRE were cultured from the farm in 1994 from livestock feces and from uncooked chicken purchased at retail outlets in the UK (22). The impact of agricultural use of antimicrobials, specifically the glycopeptide avoparcin, was suggested when VRE were found in fecal samples of German pigs and poultry (158). The similarity between vancomycin and

avoparcin was extended past the structural level with studies that have established that the use of avoparcin at subtherapeutic levels for growth promotion in agriculture selects for VRE (1,3).

Producers use antimicrobials at low or “subtherapeutic” doses because they promote faster animal growth on less feed. The discovery of the effect of antimicrobial agents on animal growth was first suggested in 1946 by a team of scientists working with poultry (199). By 1950, research reports had appeared in several journals claiming to document the benefits of subtherapeutic doses of tetracyclines and penicillins in other systems including swine (63) and calves (173). The current understanding of the Antibiotic Growth Effect centers on the similar growth characteristics and feed efficiency of antibiotic treated and germ-free animals (146). Antimicrobial-assisted growth is largely thought to arise from the reduction in numbers of total bacteria, which reduces normal inflammation of the tissue, thus providing a larger surface area over which nutrients can be absorbed.

Due to the small profit margins involved in poultry production and the vertical integration of the industry, producers have been reluctant to remove antimicrobials from poultry diets. As many as thirty thousand birds are raised in a poultry house and are subjected to a variety of environmental stresses, including temperature extremes, malfunctioning water equipment, or improperly mixed feed. These conditions, if persistent, can and do result in significant mortality among the flock. It is the opinion of the National Research Council that the “beneficial effects of subtherapeutic drug use are found to be greatest in poor sanitary conditions” (214). Thus, recent advances in

agricultural production, designed to maximally increase product and profit, have grown to rely heavily upon antimicrobial agents.

The consequences to human health of using antimicrobials in food have been difficult to delineate, but evidence is mounting that the use of antimicrobials for growth promotion may compromise use of either those or related antimicrobials in human therapy. Dating back to 1965, when an outbreak of drug resistant *Salmonella* in England in dairy calves that eventually spread to humans, there have been continuing concerns over the use of human therapeutics as growth promotants. This incident led to the formation of the Swann Committee to examine the use of antibacterial agents in England. The Committee recommended in 1969 the removal of antibacterial agents and antibiotics used for growth promotion from agriculture or the division of the antimicrobials into “feed” and “therapeutic” classes in order to more strictly control the use of antibacterial agents (284).

More recently, Smith et al. (272) documented the rapid rise (1 to 10 percent) in fluoroquinolone-resistant *Campylobacter jejuni* isolates from Minnesota following the 1995 approval of fluoroquinolones for use in poultry. This finding, in part, led the FDA to announce its intention to withdraw approval of the use of fluoroquinolones in poultry. In the U.S., a case of salmonellosis in a Nebraska boy caused by a ceftriaxone-resistant isolate of the multiresistant bacterium *Salmonella enterica* serotype Typhimurium DT104 was linked to an identical strain on a neighboring farm, but the evidence of transmission was circumstantial (99). Another outbreak of *S. enterica* serotype Typhimurium DT104 in humans was traced back to a swine herd infected with the same strain of bacterium, presumably the result of selection on the farm from the use of a fluoroquinolone growth

promoter (195). According to Dr. Abigail Salyers, the study is the “closest that anyone has come to a smoking gun” linking agricultural use of drugs to antimicrobial resistance that contributed to a particular human death (97).

Concern over possibly compromising the tools for efficacious antimicrobial human therapy by continuous use in agriculture led to the 1998 ban on the use of four growth promotants in the European Union. The World Health Organization subsequently recommended in 2000 the banning of antimicrobials important for human medicine from use as growth promoters in animals (328). These official actions, however, have relied upon the “Precautionary Principle” in seeking to prevent the increased prevalence of a hazard before that hazard has been scientifically established (230).

Whereas the routes of transmission of VRE have not been clearly identified, work in the EU has demonstrated human fecal carriage in the community (113,260,304) and that the vector may be the consumption of contaminated meat (260). To date, insufficient evidence is available to conclude if this situation is mirrored in the U.S., due to the apparent complete absence of VRE in the community. This is thought to be due to the absence of selective pressure in agriculture, as avoparcin was never approved for production purposes in the U.S. This effect has also been mirrored in the observations that followed the 1986 ban of the use of any antimicrobial as a growth promotant in food animals by Sweden (298,301). A critical difference between the EU and U.S. situations is the apparent overuse of vancomycin in U.S. hospitals (88,285) which is thought to amplify the size of the VRE populations which then can spread clonally within the hospital. Thus, the impact of antimicrobial agents used in the production of food animals is thought to increase carriage in the community (e.g., in the EU) with an intense

secondary selective pressure at the hospital setting (e.g., in the U.S.) that can then lead to disease due to VRE or to seed the nosocomial environment, through which it can disseminate (185). In fact, the use of vancomycin in a hospital setting is considered a risk factor for human VRE infection (83,265).

One extremely important limiting factor in the estimation of the risk to human health posed by the agricultural usage of antimicrobial agents is the availability of usage data of antibiotics in the U.S. Traditionally, drug companies are reluctant to release information regarding antibiotic use for fear of losing “proprietary” information. Reports from trade groups such as the Animal Health Institute use the estimate of 50 million pounds of antibiotic produced in the U.S. per year as the measure for total production of antibiotics in the U.S., of which 36% are used in animals for therapeutic and non-therapeutic purposes, compared with 64% for human treatment (13). A recent report by the Union of Concerned Scientists asserts that 70% of antimicrobials are used in the non-therapeutic treatment of food animals (294), suggesting that antimicrobial usage in animals for growth promotion is much greater than previously believed. Representatives of U.S. agriculture as well as the pharmaceutical industry continue to vigorously defend the use of antimicrobials in agriculture against claims of compromising the treatment of clinical disease.

***Enterococcus* as a Model for Resistance Development**

In addition to the widespread distribution of enterococci in the environment, certain characteristics make enterococci excellent sentinels for monitoring changes in antimicrobial exposure. As early as 1899 the enterococci were described as “very hard

and tenacious of life” (178). In addition to their tolerance of heat stress, enterococci have also been shown to persist on dry surfaces for up to four months (314). As members of the normal fecal microflora of many animals, enterococci can be found at high numbers in sewage treatment plants (23) and soil (106). Isolates of common origin have been observed in humans, sewage, and poultry (23), poultry and humans (269,302,323), humans and cheese (26), and humans and meat foodstuffs (157). The preponderance of data, however, suggests that distinct populations of enterococci are found in humans and food animals.

Another important survival characteristic among enterococci is their promiscuous ability to readily acquire and transfer antimicrobial resistance elements (84). Enterococci that are not able to colonize the human colon may be able to transfer resistance determinants to those strains that may be more host or tissue-adapted (269). Porcine and avian strains can persist in the fecal flora of human volunteers between two weeks to several months (275). Additionally, identical polymorphisms of the vancomycin resistance element *vanA* among *E. faecium* of food animal and clinical environments have been demonstrated (135,326) which illustrates the potential for the transfer of resistance determinants between the two populations. Additionally, resistant *E. faecium* of food animal origin that have been observed to be transiently carried as part of the human intestinal microflora may potentially transfer resistance elements to host-adapted enterococci (275).

Therefore, these observations suggest that *Enterococcus* spp. can be useful sentinel organisms to monitor the development of resistance resulting from the usage of antimicrobial agents in animal production.

Resistance to MLS Antimicrobials

The macrolide-lincosamide-streptogramin B antibiotics constitute the MLS superfamily because of their activity against the bacterial 50S ribosomal subunit to inhibit protein synthesis. Streptogramin A components also exert an inhibitory influence on that area of the ribosome in a synergistic relationship with streptogramin B components. Cross-resistance between these important therapeutic drugs was first identified in staphylococci in 1956, only a few years after the introduction of erythromycin, and subsequently spread to France (47), the U.K. (109), and the U.S. (143). Resistance to this group of antimicrobial agents subsequently spread to other genera including *Bacillus*, *Actinobacillus*, *Lactobacillus*, *Clostridium*, *Klebsiella*, *Escherichia*, *Neisseria*, *Pediococcus*, *Staphylococcus*, and *Streptococcus* species. The MLS phenotype was first identified in *Enterococcus* in an isolate of *E. faecalis* in 1972 (61).

MLS Resistance Genes

Some of the resistance genes thus far identified in *Enterococcus faecium* that confer resistance to streptogramin combinations can also confer resistance to macrolide and lincosamide classes of antibacterial agents. The mechanisms and mode of action of MLS resistance determinants are diagrammed in Figure 1. Resistance genes identified in *E. faecium* that modify the target site include the rRNA methylases *erm(A)* and *erm(B)*. Initially identified in staphylococci, these enzymes modify a specific adenine residue, A2058, which is located in the peptidyltransferase region of the bacterial 23S rRNA. This modification confers resistance to 14-member (erythromycin) and 16-member

macrolides (tylosin), lincosamides (lincomycin), and streptogramin B, also known as the MLS_B resistance phenotype. The *erm*(B) gene alone is thought to confer macrolide resistance above the proposed testing limits (229) although its quantitative effect on lincosamide resistance has not been detailed. Resistance to a streptogramin B component does not confer resistance to a combined streptogramin and requires additional resistance elements to streptogramin A components.

The hydrolase, *vgb*(A), has been described in enterococci and has activity against streptogramin B components (137). The lincomycin and clindamycin transferase, encoded by *lnu*(B) in *E. faecium*, inactivates the antimicrobial by nucleotidylation (35). A major facilitator in *E. faecium*, which has not been thoroughly described and reduces the intracellular concentration of erythromycin, is encoded by the gene *mef*(A) (176). Similar to *mef*(A), but of questionable importance to antimicrobial resistance, is the putative ATP-binding transporter *msr*(C) (229)

Rende-Fournier et al. (236) first identified *vat*(D) from a clinical isolate of *E. faecium* from urine as an element located on a 27 kb plasmid. This gene encodes an acetyltransferase that transfers an acetyl group to streptogramin A, inactivating it. Subsequently, another acetyltransferase, *vat*(E), was described among *E. faecium* isolates (120). In a study of *E. faecium* isolated from broiler chickens and pigs, *vat*(D) was found to be localized to a large plasmid that could be conjugatively transferred, however no other genes known to confer resistance were found to be present among these isolates (117). Description of the *vat*(E) gene indicated that it is not co-localized with *vgb* even though both resistance elements were found in the same isolate (120). In Germany, the *vat*(E) gene predominates in isolates of poultry origin resistant to streptogramins (315) as

well as in isolates from human clinical samples (273). Both *vat(D)* and *vat(E)* can be transferred conjugatively, but not all isolates that possess these genes are able to transfer the determinants in vitro, suggesting that a missing component is needed for conjugation (315). Bozdogan et al. (36) have shown that with the combination of *erm(B)* and *vat(D)*, the MIC of quinupristin-dalfopristin was 8 µg/ml. Previous work involving the genes *erm(B)*, *vat(D)*, and *vgb(A)* suggests that the *erm(B)* rRNA methylase gene does not affect the combined streptogramin resistance levels in the absence of the acetyltransferase *vat(D)* (35). This conclusion is borne out by the observations of resistance to quinupristin-dalfopristin and virginiamycin being much higher in isolates that possess either *vat(D)* or *vat(E)* (MIC >64 µg/ml) than those that do not (MICs 4 to 16 µg/ml) (273). It is worthwhile to mention that some isolates that do not possess an acetyltransferase and yet are resistant to combined streptogramins represent as yet undescribed components that contribute to the MLS resistance phenotype.

Soltani et al. demonstrated that the *erm(B)* gene was detected in 100% of European isolates that possessed *vat(D)* and in 79% of isolates that possessed *vat(E)*, but the location of these resistance elements were not reported (273). The combination of *erm(B)*, *vgb(A)*, and *vat(D)* has been observed among individual clinical isolates of *E. faecium* (35).

Prevalence of MLS Resistance Genes

Estimates of *erm(A)* prevalence among macrolide-resistant isolates from Spain reveal that it is infrequent (229). There was an observed *erm(A)* prevalence in 1% of poultry and porcine isolates from Denmark, Finland, and Norway (6) and 5% of EU

clinical isolates (258). The prevalence is apparently not different among streptogramin-resistant isolates from Danish poultry (138). *Erm(B)* is estimated to represent up to 88% of macrolide-resistant isolates of poultry and porcine origin in the EU (5,6). The high prevalence of *erm(B)* was similarly observed among clinical isolates resistant to macrolides from European countries (229,258). Analogous to the situation with *erm(A)*, the prevalence of *erm(B)* is similar among streptogramin-resistant isolates from Danish poultry and pigs (138), and as well as those of clinical origin (35,273). Less resistance was observed in a U.S. study of retail poultry products in which *erm(B)* was observed in 41% of streptogramin-resistant isolates (267).

The prevalence of the efflux pump *mef(A)* has been estimated to be as high as 22% of U.S. clinical isolates of *Enterococcus* spp. (176), which is in contrast to the observed non-detection among enterococci from Belgian pork production environments (183). A *mef*-like gene has been observed in a single clinical isolate from Spain (229). The *msr(C)* gene was initially described among clinical isolates of *E. faecium* as a likely intrinsic feature of the species (229). Subsequent studies on the *msr(C)* confirmed the previous observation of conservation among *E. faecium* of clinical origin (270). The gene contributed to a rise in erythromycin MIC by six-fold, but was not solely responsible for macrolide resistance. Although the gene has been ascribed activity against lincosamide antimicrobials, the MIC was not markedly affected (270). The incorporation of *E. faecium* from multiple sources in Germany revealed that the *msr(C)* gene could not be detected in 42% of isolates (315) with a subsequent study indicating its absence in 31% of isolates from poultry (316). The nucleotidyltransferase *lnu(B)* [previously known as *lin(B)*] was described in a clinical isolate of *E. faecium* from

France. This genetic element was demonstrated to be on a 240-kb plasmid and parenthetically described as present among all 14 isolates of *E. faecium* of clinical origin that inactivated lincosamides (34). Upon curing of the plasmid, this isolate also lost its resistance to erythromycin, suggesting that the element that conferred macrolide resistance is either co-localized with *lnu(B)* or is located on another resident plasmid. Since the publication of this observation, *lnu(B)* has been described in only a single, lincosamide-resistant isolate of group B Streptococcus of clinical origin from Canada (66). The initial description of the streptogramin B hydrolase *vgb(A)* among enterococci was in a clinical isolate of streptogramin-resistant *E. faecium* from The Netherlands (137). Since its description, it has been identified in only one other instance, a clinical isolate from France (36).

Known resistance elements that confer streptogramin A resistance to *E. faecium* include the acetyltransferases encoded by the genes *vat(D)* and *vat(E)*. The prevalence of *vat(D)* among isolates of poultry origin from Europe is estimated between 10 to 13% (6,138). The gene was not detected in a recent survey of resistant isolates from U.S. retail poultry (267). Among resistant isolates from pigs in European production operations, *vat(D)* has been found among 2 to 7% of isolates (5,6,138). Comparative studies suggest that in addition to its more frequent isolation from pigs, *vat(D)* also is found more frequently among isolates of human origin (137,273,317). A survey of clinical isolates from Spain, however, suggests that the predominant resistance mechanism may vary by region (241). *Vat(E)* has been observed to be more frequently encountered among isolates of poultry origin and accounts for 72 to 100% of streptogramin-resistant poultry isolates from Europe (6,120,138,317). In contrast, *vat(E)*

is estimated to be present among only 4 to 7% of resistant-isolates from pigs (5,138).

Among resistant isolates from U.S. retail poultry, *vat*(E) was observed in 37% of resistant isolates (267).

The *erm*(B) and *vat*(E) genes have been proven to be closely linked in a collection of *E. faecium* isolates from Germany (136). This linkage is reported in 74% of all streptogramin resistant *E. faecium* from this region, notably from poultry sources. Given that reports have long suggested transfer of resistance elements is both geographically and phylogenetically widespread (198,244) and that the *erm*(B) element has been found to have been duplicated in the clostridial genome (95), it is not unexpected that any of the targeted genes will be found in a similar arrangement in *E. faecium*.

Scope of Dissertation

The hypothesis examined in this dissertation is that the use of antimicrobial agents in the agricultural environment selects for resistant isolates of bacteria, especially the enterococci. The increased prevalence of these resistant organisms in animal production or processing facilities may have the potential to compromise the therapeutic efficacy of related antimicrobials used in human treatment. Enterococcal resistance to the clinically important macrolide, lincosamide, and streptogramin classes of antimicrobials is of acute interest due to the decline in antimicrobial treatment options. Elements conferring resistance to these antimicrobials from U.S. poultry production isolates of *Enterococcus faecium* may not mirror human-derived isolates, but may have variable potential for the dissemination and subsequent establishment of resistance. This hypothesis will be tested by pursuing three specific aims:

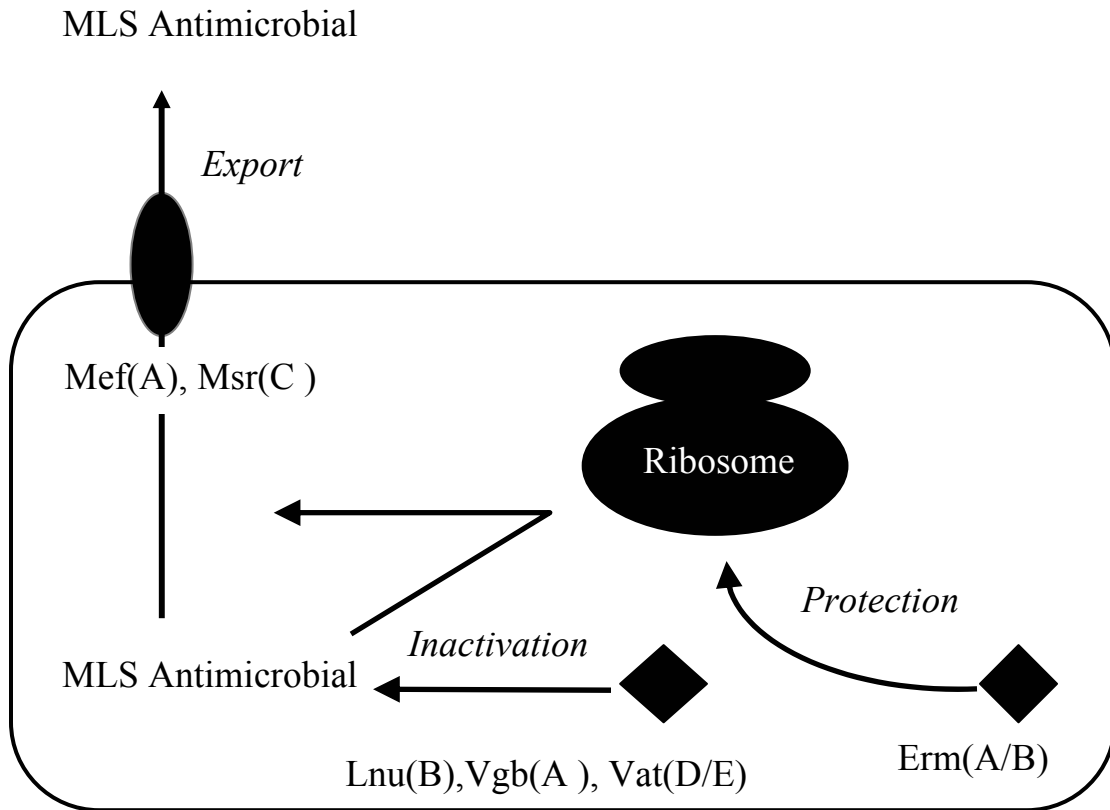
- I. Establish the populations of *Enterococcus* spp. from the poultry production environments of the Delmarva Peninsula.
- II. Establish a baseline of resistance among the isolated *Enterococcus* spp. to a number of antimicrobial agents used in animal production as well as the treatment of human disease.
- III. Characterize molecular determinants that confer the Macrolide-Lincosamide-Streptogramin (MLS) resistance phenotypes among *Enterococcus faecium* from the poultry production environment.

Table 1. Current and proposed *Enterococcus* spp.

<i>E. asini</i>	<i>E. hirae</i>
<i>E. avium</i>	<i>E. malodoratus</i>
<i>E. canis</i>	<i>E. moraviensis</i>
<i>E. casseliflavus</i>	<i>E. mundtii</i>
<i>E. cecorum</i>	<i>E. pallens</i>
<i>E. columbae</i>	<i>E. phoeniculicola</i>
<i>E. dispar</i>	<i>E. porcinus</i>
<i>E. durans</i>	<i>E. pseudoavium</i>
<i>E. faecalis</i>	<i>E. raffinosus</i>
<i>E. faecium</i>	<i>E. ratti</i>
<i>E. flavescens</i>	<i>E. saccharolyticus</i>
<i>E. gallinarum</i>	<i>E. seriolicida</i> ^a
<i>E. gilvus</i>	<i>E. solitarius</i> ^a
<i>E. haemoperoxidus</i>	<i>E. sulfureus</i>
	<i>E. villorum</i>

^a Species have been shown to belong to other genera

Figure 1. Macrolide-lincosamide-streptogramin resistance mechanisms in *E. faecium*



Mechanism of action of resistance proteins is indicated in italics; arrows denote the target of the resistance mechanisms.

CHAPTER 1

Isolation, Identification, and Antimicrobial Susceptibility Profiling of *Enterococcus* spp. from the Poultry Production Environment of the Delmarva Peninsula

ABSTRACT

The potential impact on the bacterial population of food animals in the production environment as a result of antimicrobial drug use for growth enhancement continues to be a cause for concern. Enterococci from 82 farms within a poultry production region on the eastern seaboard were isolated to establish a baseline of susceptibility profiles to a number of antimicrobials used in production as well as clinical environments. Of the 541 isolates recovered, *E. faecalis* (53%) and *E. faecium* (31%) were the predominant species isolated, whereas multiresistant antimicrobial phenotypes were observed among all species with a proportionately greater diversity of resistance phenotypes observed among isolates of *E. faecium*. Isolates of *E. faecalis* were more resistant to lincosamide, macrolide, and tetracycline antimicrobials whereas isolates of *E. faecium* were more resistant to fluoroquinolones and penicillins. Notably, 63% of the *E. faecium* isolates were resistant to the streptogramin quinupristin-dalfopristin, whereas high-level gentamicin resistance was observed only among the *E. faecalis* population, of which 7% of the isolates were resistant. The primary observations are that enterococci can be frequently isolated from the poultry production environment and can be multi-resistant to antimicrobials used in human medicine. The high frequency with which resistant enterococci are isolated from this environment suggests that these organisms might be

useful as sentinels to monitor the development of resistance resulting from the usage of antimicrobial agents in animal production.

INTRODUCTION

Our anthropocentric view of human pathogens has historically caused us to think of bacterial resistance to antimicrobials as a problem arising purely out of clinically related events. In fact, it is being increasingly recognized that antimicrobial resistance develops at a high frequency in food animal production. The conundrum is whether the prevalence in this environment contributes to the problem being observed in the clinical sphere. Enterococci can persist for long periods of time on surfaces in the clinical environment and can readily be transferred among the patient population, many of whom may be prone to colonization (218) with significant morbidity and mortality among predisposed patients (205). *Enterococcus* spp., particularly *E. faecalis* and *E. faecium*, have presented serious challenges to the control of antimicrobial resistance as they are the second leading cause of nosocomial infections in intensive care units in the United States (44) and are becoming increasingly resistant to treatment with antimicrobials. Additionally, infections caused by other *Enterococcus* species (*E. durans*, *E. avium*, *E. raffinosus*, *E. gallinarum*, and *E. casseliflavus*) occasionally occur and warrant attention (205). Over twenty-four percent of nosocomial infections are complicated by the intrinsic resistance of this organism to many antibiotics as well as acquired resistance to vancomycin (132).

The unprecedented cooperation of various clinical institutions has led to the development and maintenance of highly developed surveillance programs such as the National Nosocomial Infections Surveillance (NNIS) system of the Centers for Disease Control, while program monitoring of other environments is still in development, e.g., the National Antimicrobial Resistance Monitoring System (NARMS). In spite of the efforts

in the clinical arena to monitor the incidence of antimicrobial resistance, the frequency of resistance continues to climb. Description of the antimicrobial susceptibility profiles of pathogens isolated from non-clinical environments is comparatively limited because of the focused interest of investigators only on resistance to particular antimicrobial agents. The ability to evaluate the threat posed by antimicrobial resistance to the treatment of human disease is also complicated by differences seen geographically. For example, in the case of *Enterococcus* spp., the high prevalence of vancomycin-resistant enterococci (VRE) observed in non-hospitalized human (community) populations of EU members has not been mirrored in the United States. In contrast, the U.S. has a comparatively higher prevalence of VRE in the hospital environment (206,310).

Past surveillance has also demonstrated a high prevalence of VRE in the food animal production environments of the EU as opposed to the U.S. where no VRE have been reported from studies of U.S. farms (185,310). These observations strongly implicate the agricultural use of the glycopeptide avoparcin in EU operations in the development of resistance. Explanations for the higher rate of occurrence of VRE among hospitalized U.S. patients have been ascribed to extensive use of vancomycin in the hospital environment.

Increased concern over the selection for resistance through the use of analogues of human antimicrobials for growth promotion has led the EU to ban the use of all antimicrobials as feed additives. Although existing evidence has not yet suggested that enterococci of environmental or foodborne origin should be regarded as bacterial pathogens, they could serve as potential reservoirs of virulence and antimicrobial resistance genes to host-adapted strains (103). The 1999 FDA approval of quinupristin-

dalfopristin (Q-D or Synercid[®]) for treatment of vancomycin-resistant *E. faecium* infections in humans has been met with similar concern due to the use of the analogue virginiamycin in U.S. agriculture for over 25 years. The demonstration of resistance in the food production environment (118,124,313), food products (273), and the community (2) has raised concerns about the temporal efficacy of this drug in the clinical environment.

Whereas the extent to which the selection and distribution of resistant human pathogens is related to the use of antimicrobials in agricultural is still hotly debated, few studies have actually detailed the multiresistant nature of enterococci from the food animal production environment to drugs used in production. The purpose of this study was to characterize the species and related broad antimicrobial susceptibility profiles of a large number of *Enterococcus* spp. isolated from poultry production operations located on the eastern seaboard of the U.S.

MATERIALS AND METHODS

Sample collection. Samples were either poultry litter or swabs of poultry transport containers. Surface poultry litter was collected from 55 roaster and broiler chicken houses located on the eastern seaboard of the U.S., as described previously (16). Over a period of eleven weeks in the summer of 1998, two swabs from each of 103 poultry transport containers representing 27 farms were periodically collected at a regional processing facility. Fecal material from six surface sites of each of the poultry transport containers at the facility was swabbed with sterile gauze using a five-inch diameter metal template, as described previously (235). Swabs were immersed in 50 ml of Cary Blair medium in sterile specimen cups and litter samples were stored in sealed Whirl-Pak[®] bags (Nasco, Fort Atkinson, WI) and transported to the laboratory. Two sets of pooled swabs were processed independently as shown in Figure 2. The locations of the sampled commercial poultry production houses for which geographic data was available on the Delmarva Peninsula are diagrammed in Figure 3. Data on the identity and quantity of antimicrobial use among the production environments were not available.

Isolation and identification of *Enterococcus* spp. Surface poultry litter was added at a 1:4 dilution to 40 ml of nalidixic acid-brain heart infusion-salt (NABS) enrichment broth for incubation at 35°C with agitation in a Series 25 rotary shaker incubator (New Brunswick Scientific, Edison, NJ). Similarly, swabs of the poultry containers were removed from the Cary-Blair media, pooled in sets of three and placed in 40 ml of NABS broth and incubated as described above. Enterococci were cultured by

transferring 100 µl of broth to using colistin-nalidixic acid (CNA) and bile esculin azide (BEA) agars (Difco).

Isolates were presumptively characterized as enterococci based on Gram stain, catalase reaction, tolerance to 6.5% NaCl and growth at 45°C, the production of pyrolidonylarylamidase (PYR), and hydrolysis of esculin in the presence of bile. Confirmation to the genus level was accomplished using the *Enterococcus* AccuProbe[®] Culture Identification kit (Gen-Probe, Inc., San Diego, CA) according to the manufacturer's specifications.

Identification to species or group was performed using a miniaturized identification system based on the biochemical tests recommended by Facklam and Collins (93), which included the utilization of R-mannitol, R-sorbitol, L-sorbose, R-raffinose, and L-(+)-arabinose (Sigma-Aldrich, St. Louis, MO) performed in Costar[®] 96 well cell culture plates (Corning, Inc., Corning, NY), as well as assays for the presence of methyl- α -D-glucopyranosidase and arginine dihydrolase. Twenty microliters of a visually prepared McFarland 1 suspension of fresh bacterial growth was added to 200 µl of each test well of a 96-well microtiter plate. The plate was covered and incubated at 35 °C for 18±1 h. Supplementary testing included ribose, sucrose, and inulin utilization as well as assays for motility when additional discrimination was required between two or more species. These results were then compared to those of conventional tube tests run in parallel. Control strains used in identification included ATCC strains *E. faecalis* 51299, *E. avium* 35665, *E. pseudoavium* 49372, *E. raffinosus* 49427, *E. malodoratus* 43197, *E. faecium* 35667, *E. mundtii* 43186, *E. casseliflavus* 25788, *E. gallinarum* 49573, *E. durans* 49479, *E. hirae* 10541, *E. dispar* 51266, *E. sulfureus* 49903, *E. saccharolyticus*

43076, *E. columbae* 51263, and *E. cecorum* 43198. The VITEK (bioMérieux) microbial identification system was used to supplement identification. Isolates and control strains were frozen at -80°C in Trypticase Soy broth supplemented with 20% glycerol.

Susceptibility testing. The minimum inhibitory concentrations (MIC) for 28 antimicrobials were determined for each of the isolates using the Sensititre™ antimicrobial susceptibility testing system (Trek Diagnostic Systems, Inc., Westlake, OH) as seen in Figure 4. Particular antimicrobials and/or analogs used in human and food animal production environments are listed in Table 2 with test concentrations and interpretative criteria for resistance breakpoints for each drug as available (213). Additionally, isolates were tested for resistance to vancomycin and for high-level resistance to gentamicin, kanamycin, and streptomycin. Approximately 5×10^5 CFU/ml of each isolate, suspended in Mueller-Hinton broth, were inoculated into microtiter plates containing the test antimicrobials and incubated at 37°C for 18 ± 1 h. in ambient air. *E. faecalis* strains ATCC 29212 and ATCC 51299 were used as quality controls. The plates were removed and read manually for growth to score the MIC determinations using available NCCLS interpretive criteria (213). Ambiguous or inconclusive observations were repeated.

Antibiogram exclusion analysis. Antibiograms of the isolates from all samples were compared in relation to the farm and species designation. Enterococcal antibiograms from different isolates from the same farm that differed by less than two

dilutions for one or more antimicrobial MICs were considered duplicate isolates and only the data from a single representative isolate were used for subsequent analyses.

RESULTS

Performance of the microtiter identification method. To evaluate the consistency of the microtiter adaptation to traditional biochemical assays, five repetitions of the ten biochemical tests were conducted using the ATCC control strains of *Enterococcus* spp. listed in Table 3. The results of the microtiter adaptation essentially were identical to those of the traditional tube-based biochemical tests. No variability was observed within the species with the exception of the raffinose reactions of *E. faecium* and *E. dispar*, in which aberrant reactions were observed in 1/5 tests of the biochemical panel. Observations were most discriminatory when the plates were observed at 18 h compared with observations at 16 and 24 h.

Isolation and identification of enterococcal species. Of the over 1000 isolates screened from over 100 litter and 70 crate swab samples, 541 were subsequently identified as enterococci. A large proportion of those isolates that were initially isolated and later discarded appeared to be gram-positive pleomorphic rods that were catalase positive. Additionally, there were numerous occasions in which a rapidly swarming and mucoid microorganism overgrew the plate and precluded the isolation of other resident bacteria.

All enterococcal isolates were identified to species and antimicrobial susceptibility profiles were established. This collection was reduced to 331 unique isolates after the removal of isolates of the same species from the same farm with non-distinct susceptibility patterns (Table 4). There were no apparent differences in the species prevalence or their associated susceptibility profiles of enterococci isolated from

litter and from poultry transport containers. *E. faecalis* was the predominant species (53.2%) identified followed by *E. faecium* (31.4%), *E. gallinarum* (6.0%), *E. hirae* (3.9%), *E. durans* (1.5%), *E. casseliflavus* (1.2%), and *E. avium* (0.3%). Eight isolates that were not clearly identifiable to species using biochemical means were placed into groups established by Facklam and Collins (93) based on the fermentation of mannitol and the activity of arginine dihydrolase.

Multiresistance phenotypes of *Enterococcus* spp. Reduced susceptibility to antimicrobials used in the poultry production environment was prevalent among the isolates (Table 5). Reduced susceptibility to lincosamide antimicrobials was most often encountered, occurring in 98.5% across all species, followed by streptogramin (78.3%), tetracycline (68.0%), macrolide (54.3%), and penicillin (26.7%). No isolate was resistant to all five classes examined, but 52.7% were co-resistant to four antimicrobials. Multiresistance profiles to the selected antimicrobials were quite diverse with the lincosamide-macrolide-streptogramin-tetracycline (36%), lincosamide-streptogramin-tetracycline (19%), and lincosamide-penicillin-streptogramin-tetracycline (11%) phenotypes, most commonly observed on a percentage basis owing largely to the purported intrinsic resistance of the large number of *E. faecalis* isolates to streptogramin antimicrobials in the data set. The lincosamide-penicillin -streptogramin-tetracycline (23%), lincosamide-streptogramin-tetracycline (14%), and lincosamide-macrolide-penicillin -streptogramin-tetracycline (11%) resistance phenotypes were otherwise the most common multiresistance patterns observed among all isolates. Isolates of *E. faecium* demonstrated the largest diversity of multiresistance phenotypes (18) followed

by *E. hirae* (8) and *E. gallinarum* (6) compared to the five observed among the larger population of *E. faecalis* isolates. There were no observed isolates of vancomycin-resistant *E. faecium* or *E. faecalis*.

Resistance to high-level aminoglycosides was prevalent across all species except for a single isolate of *E. avium* (Table 6). The observed frequency was highest among isolates of *E. faecium* (68%), followed by Group III *Enterococcus* spp. (67%), *E. faecalis* (53.7%), *E. casseliflavus* (50%), *E. durans* and *E. gallinarum* (40%), *E. hirae* (30%), and Group II *Enterococcus* spp (20%). The patterns of resistance to high-level aminoglycosides revealed that resistance to streptomycin was most prevalent across all of the isolates except for *E. hirae*, followed by kanamycin and co-resistance to streptomycin and kanamycin. Resistance to high-levels of gentamicin was only observed among isolates of *E. faecalis* and only occurred in conjunction with high-level kanamycin resistance.

Susceptibility profiles of *E. faecalis* and *E. faecium* isolates. The susceptibility profiles to antimicrobial agents used in the food animal production environment and their human analogues were examined using the two largest populations recovered from sampling: *E. faecalis* and *E. faecium*. There was no overlap of MICs for bambarmycin (flavomycin), between *E. faecalis* and *E. faecium*, which had modes of 2 and >32 µg/ml, respectively (Figure 5).

Among the cephalosporin antimicrobials cefazolin and cephalothin, there were no observed differences between *E. faecalis* and *E. faecium* with 94 – 100% resistance among both populations (Figures 6 and 7). Similarly, no differences in susceptibility

profiles were observed between the two populations to chloramphenicol with both populations susceptible to chloramphenicol although more variance was observed among *E. faecium* isolates (Figure 8).

Fifty-two percent of *E. faecium* isolates were resistant to ciprofloxacin at ≥ 4 $\mu\text{g/ml}$, whereas only 1.7% of the *E. faecalis* isolates were resistant (Figure 9). As seen in Figures 10-12, the greater resistance of *E. faecium* compared to *E. faecalis* was consistent among the other tested fluoroquinolones (lomefloxacin, norfloxacin, and ofloxacin). The susceptibility profiles of both populations were remarkably consistent to lomefloxacin and ofloxacin. Norfloxacin, in contrast, appeared to be the most active fluoroquinolone with only 2.9% of *E. faecium* resistant.

Whereas *E. faecalis* had a broader distribution of vancomycin MIC values (≤ 0.5 -4 $\mu\text{g/ml}$) than *E. faecium* (≤ 0.5 -1 $\mu\text{g/ml}$), no isolate of either species was resistant (Figure 13). In contrast, MIC values to the ionophore salinomycin were more evenly distributed among *E. faecalis* isolates than those of *E. faecium* (Figure 14). In addition, over 48% of *E. faecium* isolates were one dilution away from the resistance breakpoint.

Among the lincosamide class of antimicrobials, *E. faecalis* isolates were resistant to clindamycin (Figure 15) and by analogy to lincomycin which does not have interpretive criteria (Figure 16) whereas the profile of the population of *E. faecium* appeared highly resistant (84%) to the tested lincosamide antimicrobials. Greater dispersion of MIC values was observed among isolates of *E. faecium*.

Among the tested macrolide antimicrobials, resistance was higher among *E. faecalis* isolates (67-69%) compared to *E. faecium* isolates (13.5-34%). The distributions of MIC values among both populations to clarithromycin (Figure 17) and erythromycin

(Figure 18) were comparatively more uniformly distributed than the distribution observed to tylosin (Figure 19). Two modes were observed among all macrolide distributions of *E. faecalis* isolates whereas three modes were observed among *E. faecium* susceptibility profiles to erythromycin and two modes for clarithromycin and tylosin.

Only a single isolate of *E. faecium* was observed to be resistant to ampicillin, although 50% of the entire population of *E. faecium* had an ampicillin MIC of 8 µg/ml, one dilution less than the NCCLS breakpoint (Figure 20). Both species were observed to be almost uniformly resistant to oxacillin (Figure 21). Differences were apparent among susceptibility profiles of penicillin with 71% of *E. faecium* isolates resistant to penicillin compared with none of the isolates of *E. faecalis* (Figure 22). Interestingly, the distribution of penicillin MIC values among *E. faecalis* was tightly clustered (1-4 µg/ml) whereas *E. faecium* MICs were broadly distributed (≤ 0.03 ->32 µg/ml).

Over 93% of *E. faecalis* and 100% of *E. faecium* isolates were observed to be resistant to the peptide bacitracin (Figure 23). Interestingly, 90% of *E. faecalis* and 92% of *E. faecium* isolates had MICs in excess of the upper limit of the tested range (>256 IU/ml). A larger proportion of *E. faecalis* isolates (10.2%) were observed to be more resistant than *E. faecium* isolates (2.9%) to rifampin (Figure 24). Additionally, the population of *E. faecalis* isolates had more evenly distributed MIC values across the tested range than *E. faecium*.

Only seven isolates of *E. faecalis* were observed to have a MIC to quinupristin-dalfopristin less than 4 µg/ml, whereas the resistance rate among *E. faecium* was 63% (Figure 25). The distributions of virginiamycin MIC values of both populations were more dispersed than those of quinupristin-dalfopristin (Figure 26). In particular, two

modes were observed among MIC distributions of *E. faecium* to the streptogramin antimicrobials: 2 and 16 µg/ml (quinupristin-dalfopristin) and 1 and 16 to 32 µg/ml (virginiamycin).

Both species were observed to have high resistance rates to tetracycline (79-91%) as well as a distinct separation of resistant and sensitive populations (Figure 27). The susceptibility profiles of both species to ampicillin/sulbactam were unremarkable in that no resistance and very little variance were observed (Figure 28). Whereas both species presented a similar susceptibility profile to trimethoprim/sulamethoxazole, only a single isolate of *E. faecalis* was found to be resistant (Figure 29).

DISCUSSION

The identification of enterococci isolated from the commercial poultry production environment did not reveal any unusual species, although eight isolates require more discriminant analyses prior to definitive identification. Whereas multiple isolates were occasionally recovered from the same sample, the elimination of isolates with indistinguishable antibiograms from the same farm provided a collection that was conservative in its estimation of diversity, but did not substantively affect the relative proportions of species isolated.

The finding of *E. faecalis* predominance in this study was similar to that previously reported for poultry production environments in other parts of the U.S. (196,313) as well as Belgium (40), the United Kingdom (20,148-150), and Denmark (2). Studies from Japan (329), in contrast, suggested that *E. faecium* was the dominant enterococcal species of poultry fecal flora whereas a Belgian study demonstrated a predominance of *E. cecorum* in older chickens (69).

The degree to which enterococcal populations from the food animal production environment enter the human microbiota is likely heavily influenced by the degree of contamination of the foods consumed. Recent surveys of U.S. retail meat products have demonstrated enterococci can be observed among 82-100% of samples (123,186). Contrary to the observations described in this study, *E. faecium* has been demonstrated as the largest enterococcal population among retail poultry and beef but not pork (123).

E. faecalis accounts for 80-90% of enterococcal isolates of clinical origin, with *E. faecium* the second most prevalent enterococcal species. Other species, such as *E. avium*,

E. durans, *E. gallinarum*, *E. casseliflavus*, *E. hirae*, *E. mundtii*, and *E. raffinosus* have been documented as causing disease, although infrequently, in humans (205).

Because of the apparent ubiquitous nature of enterococci in the environment, only a sufficiently large epidemiological study of multiple ecological niches would be robust enough to demonstrate the population dynamics within the ecosystem. It is important to note that the observed variances in species from the commercial poultry production environment observed in this study may be difficult to directly compare to other works due to the established effects of differences in isolation methodology (39) and the effect of medicated feed on the intestinal enterococcal microflora (18,20,149,196). Preliminary studies conducted in at the FDA (Center for Veterinary Medicine, Laurel, MD) suggest that the incubation temperature used during selective enrichment of samples may affect the recovery of various enterococcal species.

The rapid rise in antimicrobial resistance observed among human bacterial pathogens has brought into question the use of certain similar antimicrobials in both the human clinical and the food animal production environments. Analyses for antimicrobial resistance among targeted bacterial populations from these defined environments have often overlooked the more complex presentation of resistance to multiple antimicrobials. The isolated *Enterococcus* populations from the commercial poultry production environment of the eastern seaboard of the U.S. in this study were also examined for the occurrence of co-resistance among antimicrobials employed in agriculture and in human medicine.

Phenotypic grouping based on the susceptibility to multiple antimicrobials that are frequently used in the poultry production environment provided some important

observations. Most apparent is the magnitude of resistance to individual classes of antimicrobials across all isolated *Enterococcus* spp. with 98.5% resistant to the lincosamide, lincomycin; 78% resistant to the streptogramin, quinupristin-dalfopristin; 68% to tetracycline; 54% to the macrolide, erythromycin; and 27% to penicillin. Whereas the indeterminate nature of Group II and III isolates and the limited data sizes of *E. avium*, *E. casseliflavus*, and *E. durans* preclude generalizations, the diversity of observed antibiograms, especially among isolates of *E. faecium* and *E. hirae*, is striking given the larger population of *E. faecalis*. Interestingly, 63% of *E. faecalis* isolates demonstrated multiresistance to lincosamide, macrolide, streptogramin, and tetracycline classes of antimicrobials whereas the largest subset of *E. faecium* isolates demonstrated multiresistance to lincosamide, penicillin, streptogramin, and tetracycline antimicrobials. Acquired resistance elements that confer cross-resistance to macrolide-lincosamide-streptogramin antimicrobials have been well described among enterococci (240) and have been associated with co-resistance to tetracycline, chloramphenicol, and high-level gentamicin (331), tetracycline and chloramphenicol (224), or chloramphenicol (315). Isolates that express these resistance elements may be phenotypically characterized as resistant to macrolide and lincosamide classes with streptogramin resistance dependent upon the presence of other resistance elements (35). However, there were individual instances of isolated lincosamide and macrolide resistant phenotypes, as well as isolates that possessed co-resistance to lincosamide and streptogramin antimicrobials in the absence of macrolide resistance.

There are few published quantitative descriptions of multiply resistant phenotypes observed among *Enterococcus* spp. in the U.S., especially those from the poultry

production environment. Data from a Danish study illustrate the diversity of resistant phenotypes encountered among *E. faecalis* and *E. faecium* isolated from poultry as well as the frequent association of the resistance of macrolides and tetracycline with other antimicrobials (2). Streptogramin-resistant *E. faecium* from this study also appeared more likely to be resistant to tetracycline than the population of streptogramin-sensitive isolates, which is consistent with anecdotal descriptions of isolates from U.S. retail chicken, but were not more likely to be resistant to penicillin (186). Our results also suggest that streptogramin-sensitive isolates were more likely to be resistant to macrolides.

Consistent with poultry studies from Japan (329) and Denmark (2), high-level gentamicin resistance was only observed in this study among *E. faecalis* isolates, although a Belgian report has demonstrated higher rates among *E. faecium* (40). High-level aminoglycoside resistance is a comparatively more frequent occurrence among enterococci of clinical origin as compared with those from the general community in the U.S. (75). The observation of increased frequency of high-level gentamicin resistance among *E. faecalis* from this study has also been demonstrated among enterococci of clinical origin in the U.S. (75,308). Additionally, high-level gentamicin resistance was only found in isolates that were also resistant to high-level kanamycin which is consistent with studies of clinical enterococci (308,331). Similar to the observations of enterococci of poultry origin from Denmark, resistance to multiple aminoglycosides at high-levels was observed among the largest populations of this study with isolated high-level streptomycin resistance a predominant phenotype (2). A higher prevalence of high-level streptomycin resistance was also seen among *E. faecalis* and *E. faecium* isolates of

animal origin from the U.S. (288). Molecular studies of multiple high-level aminoglycoside resistance among *Enterococcus* spp. suggest that phenotypic antibiogram profiles belie the tremendous diversity of mechanisms that contribute to multiresistance (163). Indeed, enterococcal isolates resistant to multiple aminoglycosides at high levels account for the overwhelming majority of isolates of clinical origin that are also resistant to vancomycin (75).

Resistance to the production drug flavomycin, a bambermycin antimicrobial, has been previously described as an intrinsic characteristic among *E. faecium* from food or food production environments (40,79), whereas increased tolerance (MIC > 2 µg/ml) among *E. faecalis* isolates is rare (40,79). Whereas these observations are similar to results presented here, resistance among *E. faecium* from Norway, which has not used flavomycin, do not follow this accepted pattern, suggesting that resistance to flavomycin may be an acquired trait (6). Comparatively decreased susceptibility among *E. faecium* compared to *E. faecalis* to the bambermycin flavomycin has been previously ascribed to intrinsic resistance differences between the two species (40,78,79) although reduced tolerance among *E. faecium* from unexposed environments suggests otherwise (6).

Cephalosporins are rarely used to treat enterococcal infections, ostensibly due to their reported intrinsic resistance. Between 32 and 73% of environmental enterococcal isolates have been described as resistant to cefazolin and cephalothin (161,223). Similar to our results with isolates from the poultry production environment, a 1994 U.S. survey revealed that 91% of bloodstream infection isolates and 87% of urinary tract infection isolates were resistant to cefazolin (141). As a result, the use of cephalosporins has been shown to lead to superinfection or increased prevalence of *Enterococcus* spp. among the

fecal microflora (144). In contrast to the consensus opinion, however, the incidence of resistance among isolates from chicken, beef, and milk from Africa was observed to be present among 17 to 34% of enterococci (50). One explanation for this discrepancy may be the positive effect that exogenous sodium chloride in the suspension medium has upon MIC values of *E. faecalis*, thereby reducing the observed susceptibility of the isolates (179).

Although chloramphenicol is not commonly used in the food production environment, resistance is observed at levels of 5 to 10% of *E. faecalis* and 0 to 4% of *E. faecium* isolates from poultry in the U.S. (196). Prevalence of resistance in poultry production environments has been similarly observed at higher rates among *E. faecalis* than *E. faecium* in Japan (329) and Denmark (2). Chloramphenicol resistance of enterococci isolated from the healthy community of the U.S. has essentially not been observed (75). An increase in resistance has, however, been observed among clinical enterococci from the U.S. with 1% from 1953 to 1954 (15) to 7 to 14% today (194). Resistance rates to chloramphenicol among enterococci are also higher in clinical environments in the EU with resistance more frequent among isolates of *E. faecalis* than *E. faecium* (261). Although chloramphenicol resistance has been associated with multiple drug resistance among clinical isolates from the U.S. (331) and the EU (261), it has been shown to be a potentially effective therapy for treating resistant bacteremia (237). This suggests that the window of opportunity for treating resistant enterococcal infections with chloramphenicol might rapidly close following a more widespread clinical use of and subsequent resistance development to the antimicrobial.

Resistance to the fluoroquinolone ciprofloxacin has not previously been recognized among enterococci from the U.S. poultry production environment, ostensibly due to a lack of interest in non-mobile resistance, as the resistance mechanism is due to mutations in DNA gyrase or topoisomerase IV. These observations of ciprofloxacin resistance across all species (18%) is similar to that observed among enterococci of poultry origin from the Netherlands (28%) despite the striking differences in resistance among *E. faecium* (52%) and *E. faecalis* (2%) observed in this study (302). Resistance to ciprofloxacin is common among clinical isolates of enterococci (142,259) and more frequently observed in *E. faecium* than *E. faecalis* (259). Ciprofloxacin resistance in the clinical environment has also been shown to have increased from 1.4% in 1986 to 51% in 2000 (208,254). Multiple resistances to other antimicrobials have been observed, with a strong correlation to high-level aminoglycoside resistance (261), but no such association was observed in this study. Treatment of enterococcal infections with ciprofloxacin has been shown to reduce the gut enterococcal population (90) and select for *E. faecium* in studies among healthy volunteers (90), but has not been strongly endorsed as a course of therapy (98). Data on the resistance to lomefloxacin, norfloxacin, and ofloxacin is sparse, but the evidence suggests that resistance is similarly more frequently encountered in the clinical setting (75,161). Clinical resistance has been estimated among 98%, 27%, and 45 to 68% of clinical isolates to lomefloxacin, norfloxacin, and ofloxacin, respectively (129,141,161).

Vancomycin-resistant enterococci have not been isolated from food animal production environments in the U.S. (121,145,288,313), or processing environments (28), consistent with our observations. Similarly, VRE have not been isolated from domestic

retail meats (59,123,134,161,288). In contrast, the selection of resistant populations by the use of the glycopeptide avoparcin in food animal production environments in Europe (1,6,7,18,40,78,279,299) has resulted in the frequent isolation of VRE from retail meat products (22,156,157,160,169,233,303,311). The persistence of VRE on farms that have discontinued the use of avoparcin for growth promotion illustrates the impact posed by prior antimicrobial usage in food animal production environments (17,30,31,126,164). Most of these studies demonstrate an initial drastic decline in the prevalence of vancomycin-resistance followed by a stabilization at a constant low, but detectable level.

The observation of decreased susceptibility of *E. faecium* compared to *E. faecalis* isolates to the ionophore salinomycin seen in this study, especially those of poultry origin, is consistent with previous ionophore susceptibility results from production environments of Denmark (4), but differs from those of broiler origin from Japan (329) and Belgium (40). Salinomycin is widely used in the food animal production environment and this use is likely to increase as industry continues to limit the use of antibiotics in agriculture. The resulting increase in selective pressure may then result in the development of detectable resistance. As this antimicrobial agent is not used in human medicine, it is unlikely that clinical surveillance systems would monitor changes in susceptibility. A future observation of decreased susceptibility ($>8 \mu\text{g/ml}$) among clinical isolates would serve to support the contention that enterococci of poultry origin play a role in directly contributing to human disease. If, however, the prevalence of a phenotype of decreased susceptibility to salinomycin increases sufficiently in the poultry environment and no similar increase is observed among clinical isolates, the

independence in terms of colonization and carriage of the two populations would be supported.

Resistance to the lincosamide class of antimicrobials is common among enterococci and has been reported to be an intrinsic trait among enterococci (192). This type of resistance has been described as specific for *Enterococcus* spp. as well (79). More recent evidence has shown that resistance diminishes following the removal of lincosamide antimicrobials from the pork production environment (29), suggesting that resistance is an acquired trait. Among isolates from the poultry production environment, greater than 60% of *E. faecium* and 85% of *E. faecalis* have been observed to be resistant to clindamycin (329), whereas elevated lincomycin MICs were observed among 3 to 16% of enterococci from the UK production environment (148). Resistance to lincosamide antimicrobials has been observed at high rates in both *E. faecalis* and *E. faecium* from community (75) and clinical sources (75,194), with more homogeneous resistance among *E. faecalis* (75), which were consistent with our observations.

Congruent with the results of this study, others have reported that resistance to the macrolide antimicrobials erythromycin and tylosin is frequent among enterococci from the poultry production environments (2,7,148,302,329) and from those of clinical origin (2,75,186,194,261). The rapid development of erythromycin resistance in the poultry production environment as the result of medicated feed use (6,196) as well as exposure to tylosin (51) and virginiamycin (7) has been documented. The prevalence of macrolide resistance is higher among enterococci of clinical origin than among those isolates from the community (75) except those associated with the poultry production environment (302). Resistance is also more often observed in *E. faecium* than *E. faecalis* (75,261).

Whereas only 3% of U.S. clinical isolates were found to be resistant in the period between 1953 and 1954 (15), the rise was appreciable among isolates from 1968 to 1969 (290), and resistance continues to rise with rates presently approaching 100% (142). Little information is available regarding the susceptibility of enterococci to clarithromycin, but resistance appears to mirror that of erythromycin (170). Intestinal microflora studies have demonstrated that clarithromycin can increase the prevalence of fecal enterococci from humans (82). The association and possible horizontal transfer of macrolide resistance among enterococci, in conjunction with resistance to other antimicrobials, continues to be a source of concern (224,315).

In contrast to a study in 1986 of enterococci from U.S. poultry production environments in which penicillin resistance was reported among 18 to 33% of *E. faecium* and 5 to 11% of *E. faecalis* isolates, 71% of *E. faecium* and 0% of *E. faecalis* isolates from this study were resistant (196). Consistent with results from Japan (329), Denmark (2), and Belgium (40), between 14 and 78% of *E. faecium* from the U.S. poultry production environment were observed to be resistant to ampicillin whereas no resistance was observed among *E. faecalis* (313). Worldwide, *E. faecium* are usually more resistant to penicillin than *E. faecalis* in isolates of clinical origin (252), a pattern also seen with ampicillin (33,75,142). Resistance to oxacillin, in contrast, appears to be a trait common to all enterococci regardless of origin (161). Resistance to penicillin and its congeners has been increasingly observed among *E. faecium* isolates in clinical environments (33). Among healthy individuals, ampicillin-resistant enterococci have not been observed in the U.S. (75), whereas in the UK only 1% of isolates were resistant (115).

The prevalence of increased resistance to the peptide bacitracin among the predominant enterococci from this study is greater than those from poultry and pig operations in Denmark (2). It is notable that bacitracin resistance among *E. faecium* of poultry and porcine origin was much lower in Finland, where its use had been eliminated four years earlier (6). Although in vitro studies have suggested that bacitracin may select for vancomycin resistance by induction (167), no such association has been found in Danish poultry and pig production (18). Among clinical isolates in the U.S., an increase in resistance to bacitracin was observed between 1953 and 1968 (290).

Resistance to rifampin has been estimated at 16% of enterococci from raw meat products (223). Among environmental isolates, 28% of *E. faecalis* and 53% of *E. faecium* isolates were resistant to rifampin (161). Similarly, 36% of *E. faecalis* and 57% of *E. faecium* isolates of clinical origin from Lebanon were resistant (335). Rifampin resistance was observed at a higher prevalence among *E. faecium* from hospitalized patients (73%) than from those in the community (43%) whereas the prevalence was higher among *E. faecalis* from the community (35%) as compared with those of hospital origin (29%) (75).

Resistance to quinupristin-dalfopristin (Q-D) among food animal production environments in the U.S. is not surprising, given the use of the analog virginiamycin on farms since 1974 (124,313). *E. faecalis* isolates have been shown to be intrinsically resistant to streptogramins (271), however the recent observation of transferable resistance in this species may provide some insight into the resistance seen among other species (268). The incidence of resistance to the streptogramin has increased to 100% in older turkey flocks that are fed the analog virginiamycin (313) whereas the prevalence of

Q-D-resistant *E. faecium* (QDREF) from the U.S. chicken production environment has been estimated to be between 51 and 78% (124). The increased frequency of Q-D resistance among *E. faecium* from turkey compared to chicken might be related to the different periods of time that the flocks are exposed to antimicrobials prior to slaughter (124). Resistance has been observed among 70 to 79% of *E. faecium* from Denmark poultry farms (2,6) compared with 0 to 17 % in countries in which virginiamycin is not used (6) and mirrors changes in use on the farm (7). A study of isolates from chicken production environments of Japan has demonstrated resistance to the analog virginiamycin at 27% among *E. faecium* (329). The bimodal distribution of MIC values observed in that study is consistent with our observations.

The prevalence of resistance of isolates from marketed poultry meat has been estimated between 3 to 26% of *E. faecium* from raw chicken samples by U.S. surveillance studies (123,186). Estimates of human carriage of QDREF range from 0 to 1% in the U.S. (75,186) to 11% of those abroad (2). Virginiamycin use in the poultry industry has been closely linked to the increased prevalence of QDREF among those members of the community associated with the poultry production environment (302). Among clinical isolates, rates range from 8 to 19% from the U.S. (75) and 8 to 38% in the EU (261). It is notable that *E. faecium* that are resistant to vancomycin are more resistant to Q-D (56). Resistance to vancomycin and Q-D has been reported among isolates from British chicken and hospitalized persons (327) and co-localization of the resistance elements has been observed in a clinical strain from France (36).

Resistance to tetracycline among *Enterococcus* spp. is very common, especially among those of poultry origin in the U.S. (196,321) and abroad (40,329). Tetracycline

resistance also has been demonstrated to be linked closely to the poultry production environment (302), which is similar to the observations in this study. Among enterococci of clinical origin, the resistance rate has increased from 54% in 1954 (15) to 80% of those observed between 1964 and 1973 in the U.S. (194). Similar to observations of this study, a bimodal distribution has been reported elsewhere (325).

There is no previous measure of ampicillin/sulbactam (A/S) resistance outside of the clinical environment with which to compare our results. Estimates of clinical resistance to A/S suggest that 17 to 87% of *E. faecium* are resistant (182,189,225) compared with less than 1% of *E. faecalis* (182,225). The combination of A/S has seen increased interest as either a sole (171) or combination therapy (109,184) to treat enterococcal endocarditis and bacteremia (189). There is concern, however, that the use of A/S may increase the density of VRE among the fecal microflora of colonized humans due to the anti-anaerobic activity of this therapeutic combination (73). Given the disparate rates of A/S resistance among *E. faecium* from poultry and from clinical medicine, this suggests that little pressure is applied in the poultry production environment to select for this resistance trait. An alternative explanation may be that clinical surveys of *Enterococcus* spp. reflect a distinct population that has been disseminated widely in that environment and introduced a bias in the prevalence of A/S resistance.

The single isolate of *E. faecalis* that was observed to be resistant to trimethoprim/sulfamethoxazole (T/S) represents the first description of such a phenotype from the poultry production environment. By comparison, resistance has been estimated at 31% among isolates from raw meat in Italy (223). The interest in treatment of

enterococcal infections with T/S stems from the successful use of the antimicrobial combination to treat resistant infections (292). Enterococci are usually susceptible to low levels of T/S although *E. faecium* has been shown to be more resistant to T/S than *E. faecalis* among enterococci from the clinical environment (62). Comparisons to past work in the literature are complicated by the observation that in vitro susceptibility may be dramatically affected by media components and may not reflect the true in vivo activity of the antimicrobial combination (332).

CONCLUSIONS

The recent observations of enterococcal vancomycin resistance elements among U.S. clinical isolates of *S. aureus* suggest that alternative therapies, such as linezolid and Q-D, will be more frequently employed (45,46). As a result, resistant populations of enterococci that may have entered the human microflora through the consumption of contaminated retail meat products may be amplified following the inevitable increase in selective pressure in the clinical environment.

The results of this study illustrate that *Enterococcus* spp. are frequently resistant to multiple antimicrobials and that some of these patterns may arguably reflect the use of approved antimicrobials in poultry in the U.S. Considering some of the current estimates of the extent of antimicrobial use in the poultry production industry for growth enhancement, the increasing potential of such an intensive agricultural operation to effect antimicrobial resistance must be weighed against the reasonable risk that treatment of human bacterial infections may be compromised.

Figure 2. Sample collection and processing

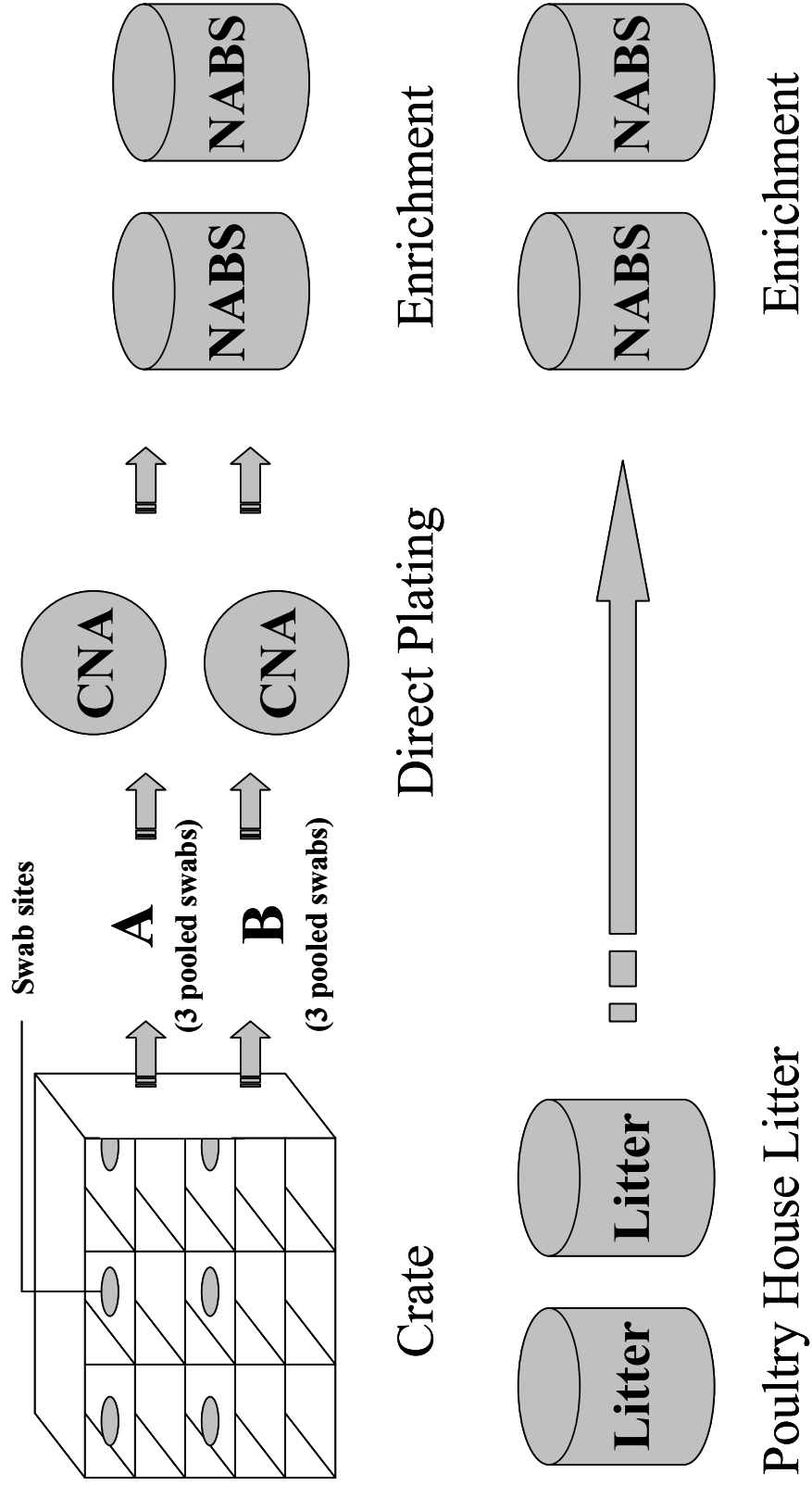


Figure 3. Location of sampled commercial poultry houses on the Delmarva Peninsula

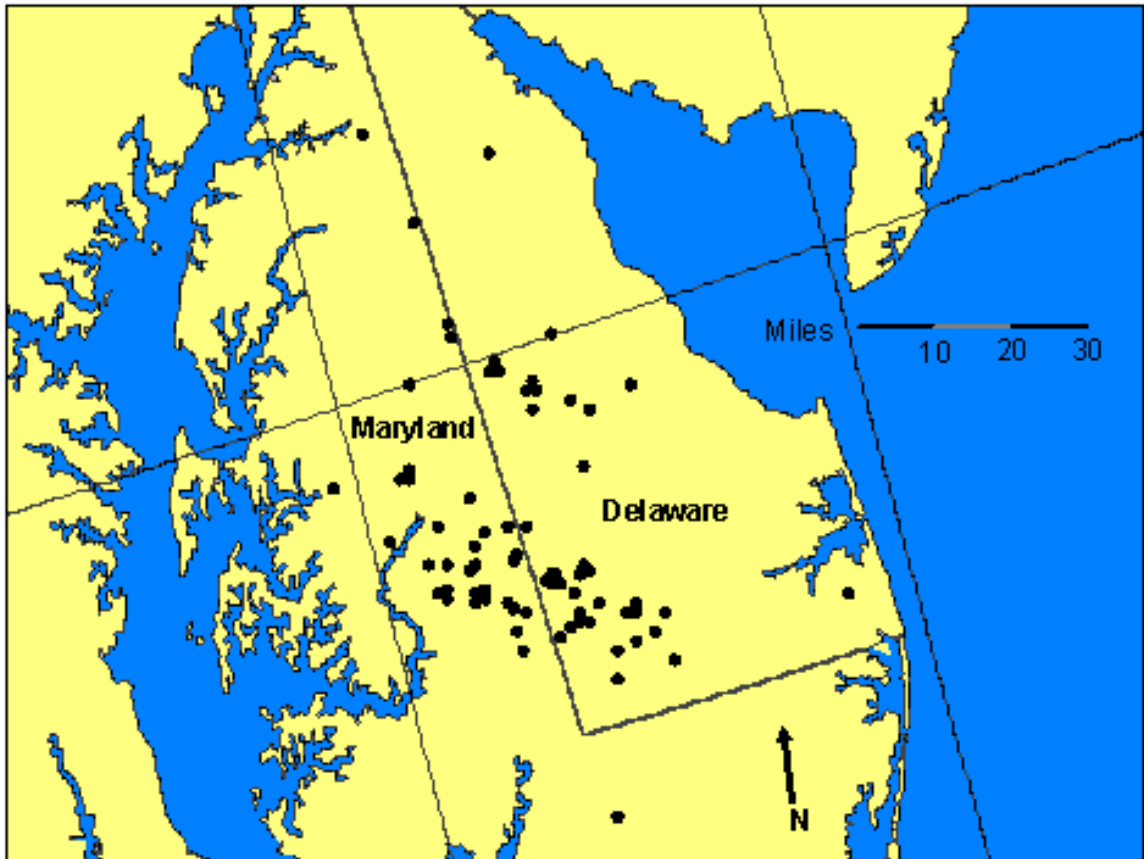


Figure 4. Photo of the Sensititre™ susceptibility testing system



Table 2. Antimicrobial susceptibility testing for *Enterococcus* spp. isolates

Antimicrobial class	Antimicrobial	Tested range ($\mu\text{g/ml}$)	Resistance breakpoint ^a
Aminoglycoside	Gentamicin	64 – 2048	>500
	Kanamycin	64 – 2048	>500
	Streptomycin	128 – 2048	>1000
Bambermycin	Flavomycin	0.5 – 32	>8 ^b
Cephalosporin	Cefazolin	2 – 16	>16
	Cephalothin	2 – 16	>16
Chloramphenicol	Chloramphenicol	2 – 64	>16
Fluoroquinolone	Ciprofloxacin	0.06 – 4	\geq 4
	Lomefloxacin	0.5 – 4	>4
	Norfloxacin	4 – 8	>8
	Ofloxacin	0.5 – 4	\geq 4
Glycopeptide	Vancomycin	0.5 – 32	>16
Ionophore	Salinomycin	1 – 32	>8 ^b
Lincosamide	Clindamycin	0.25 – 2	>2
	Lincomycin	1 – 32	NA ^c
Macrolide	Clarithromycin	0.12 – 4	>4
	Erythromycin	0.12 – 32	>4
	Tylosin	1 – 32	>8 ^b

Table 2. (Cont.)

Penicillin	Ampicillin	0.12 – 8	>8
	Oxacillin	0.25 – 2	>2
	Penicillin	0.03 – 32	>8
Peptide	Bacitracin ^d	8 – 256	>64 ^b
Rifamycin	Rifampin	0.5 – 2	>2
Streptogramin	Quinupristin- dalfopristin	0.5 – 32	≥4
	Virginiamycin	0.5 – 32	≥4 ^b
	Tetracycline	0.25 – 32	>8
Combination	Ampicillin/ Sulbactam	8/4 – 16/8	>16/8
	Trimethoprim/ Sulfamethoxazole	0.12/2.38 – 2/38	>2/38

^a All resistance breakpoints are those define by NCCLS unless otherwise noted (213).

^b Resistance breakpoints are those suggested for flavomycin and salinomycin (4) and tylosin and bacitracin (6).

^c NA, not applicable. No interpretive criteria have been established.

^d Expressed in international units (IU) per milliliter.

Table 3. Microtiter adaptation of biochemical panel for identification of *Enterococcus* spp.

Identification	Mannitol	Sorbose	Arabinose	Sorbitol	Arginine	Raffinose	MADG ^a	Sucrose	Inulin
Group I^b									
<i>E. avium</i>	+	+	+	+	-	-	-	+	-
<i>E. pseudoavium</i>	+	+	-	+	-	-	-	-	-
<i>E. raffinosus</i>	+	+	+	+	-	+	+	-	-
Group II									
<i>E. faecalis</i>	+	-	-	+	+	-	-	+	-
<i>E. faecium</i>	+	-	+	-	+	- (80%)	-	+	-
<i>E. casseliflavus^c</i>	+	-	+	-	+	+	+	+	-
<i>E. mundtii</i>	+	-	+	+	+	+	-	+	-
<i>E. gallinarum^c</i>	+	-	+	-	+	+	+	-	-
Group III									
<i>E. durans</i>	-	-	-	-	+	-	-	+	-
<i>E. hirae</i>	-	-	-	-	+	-	-	+	-
<i>E. dispar</i>	-	-	-	-	+	- (80%)	+	+	-
Group IV									
<i>E. sulfureus</i>	+	-	-	-	-	-	-	+	-
Group V									
<i>E. saccharolyticus</i>	+	+	+	+	-	+	+	+	-
<i>E. columbae</i>	+	-	+	+	-	+	-	+	-
<i>E. cecorum</i>	+	-	-	-	-	+	+	+	-

^a MADG, methyl- α -D-glucopyranoside.

^b As defined by Facklam and Collins. (93).

^c Denotes motile species.

Table 4. Distribution of *Enterococcus* spp. from the poultry production environment

Species identification	No. of isolates (% of total)	
	All isolates (<i>n</i> = 532)	Distinct isolates ^a (<i>n</i> = 331)
<i>E. avium</i>	1 (0.2)	1 (0.3)
<i>E. casseliflavus</i>	4 (0.8)	4 (1.2)
<i>E. durans</i>	5 (0.9)	5 (1.5)
<i>E. faecalis</i>	342 (64.2)	176 (53.2)
<i>E. faecium</i>	127 (23.9)	104 (31.4)
<i>E. gallinarum</i>	28 (5.3)	20 (6.0)
<i>E. hirae</i>	16 (6.0)	13 (3.9)
Group II ^b	6 (1.1)	5 (1.5)
Group III ^b	3 (0.6)	3 (0.9)

^a Isolates of the same species that came from the same farm were considered distinct if

MICs to one or more antimicrobial differed by more than two dilutions.

^b As defined by Facklam and Collins (93).

Table 5. Selected multiresistance phenotypes of *Enterococcus* spp. from the poultry production environment

Species	Percentage frequency of resistance phenotype ^b																				
	L	LM	LP	LS	LT	LMP	LMS	LMT	LPS	LPT	LST	LMPS	LMPT	LMST	LPST	LMPST	M	MP	P	PT	
<i>E. avium</i>		100																			
<i>E. casseliflavus</i>			25		25					25					25	25					
<i>E. durans</i>							80									20					
<i>E. faecalis</i>				6.8			2.3	4.0			24			63							
<i>E. faecium</i>	2.9	1.0	2.9		1.0	8.7		3.8	1.0	11	14	1.0	1.0	4.8	32	11	1.0	1.9	1.0	1.0	1.0
<i>E. gallinarum</i>					30									15	15	10					
<i>E. hirae</i>	7.7	23	23					7.7		7.7	7.7			7.7		15					
Group II ^a				20																	80
Group III ^a				66																	33
Total	1.8	2.7	0.9	5.4	2.4	2.7	1.2	5.1	0.3	3.9	19	0.3	0.3	36	11	5.1	0.3	0.6	0.3	0.3	0.3

^a As defined by Facklam and Collins (93).

^b Resistance phenotype as defined by ≥ 16 $\mu\text{g/ml}$ for lincomycin (L), >4 $\mu\text{g/ml}$ for erythromycin (M), >8 $\mu\text{g/ml}$ for penicillin (P), ≥ 4 $\mu\text{g/ml}$ for quinupristin-dalfopristin (S), and > 8 $\mu\text{g/ml}$ for tetracycline (T).

Table 6. Frequency of high-level aminoglycoside resistance patterns of *Enterococcus* spp. from the poultry production environment

Species	Frequency of resistance phenotype (% of species) ^a			
	HLS	HLK	HLS+HLK	HLK+HLG
<i>E. avium</i>	0	0	0	0
<i>E. casseliflavus</i>	1 (25)	0	1 (25)	0
<i>E. durans</i>	1 (20)	1 (20)	0	0
<i>E. faecalis</i>	62 (35)	15 (8.5)	5 (2.8)	13 (7.4)
<i>E. faecium</i>	29 (28)	28 (27)	13 (13)	0
<i>E. gallinarum</i>	7 (35)	1 (5.0)	1 (5.0)	0
<i>E. hirae</i>	1 (7.7)	2 (15)	1 (7.7)	0
Group II ^b	1 (20)	0	0	0
Group III ^b	2 (67)	0	0	0
Total	104 (31)	47 (14)	21 (6.3)	13 (3.9)

^a Resistance breakpoints for *Enterococcus* spp. were >1000 µg/ml for high-level streptomycin (HLS), >500 µg/ml for high-level kanamycin (HLK), and high-level gentamicin (HLG).

^b As defined by Facklam and Collins {14}.

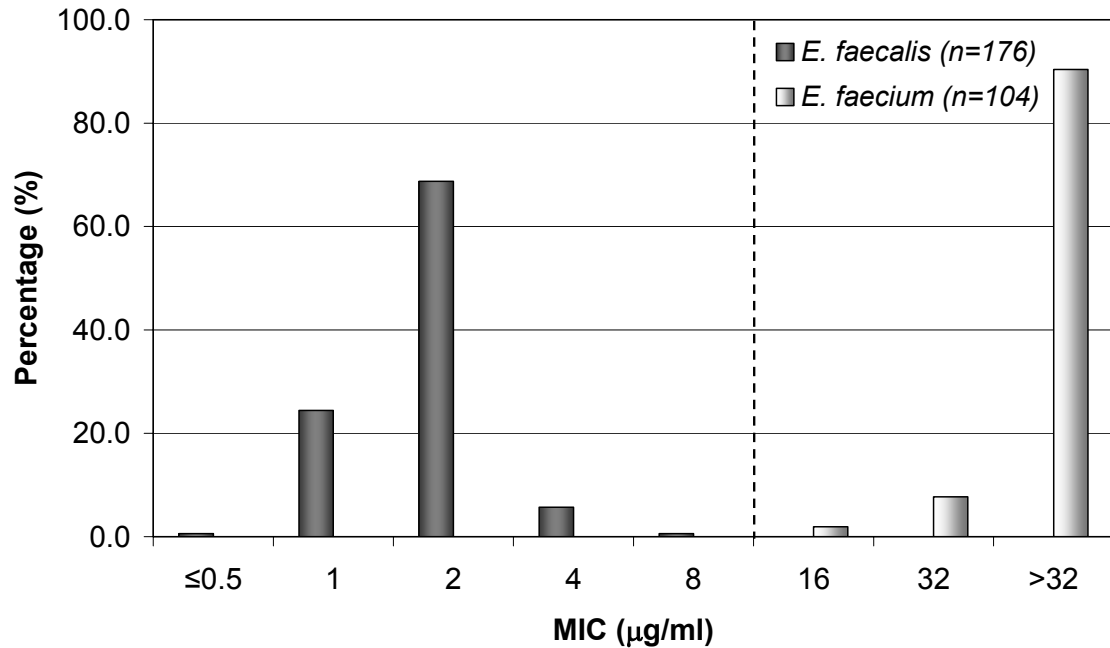


Figure 5. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **flavomycin**. Of the *E. faecalis* isolates, 0% were resistant (MIC₅₀ = 2 µg/ml, MIC₉₀ = 2 µg/ml, mode = 2 µg/ml) and 100% of *E. faecium* isolates were resistant (MIC₅₀ = >32 µg/ml, MIC₉₀ = >32 µg/ml, mode = >32 µg/ml). Dashed line denotes the defined breakpoint for resistance (>8 µg/ml) (4).

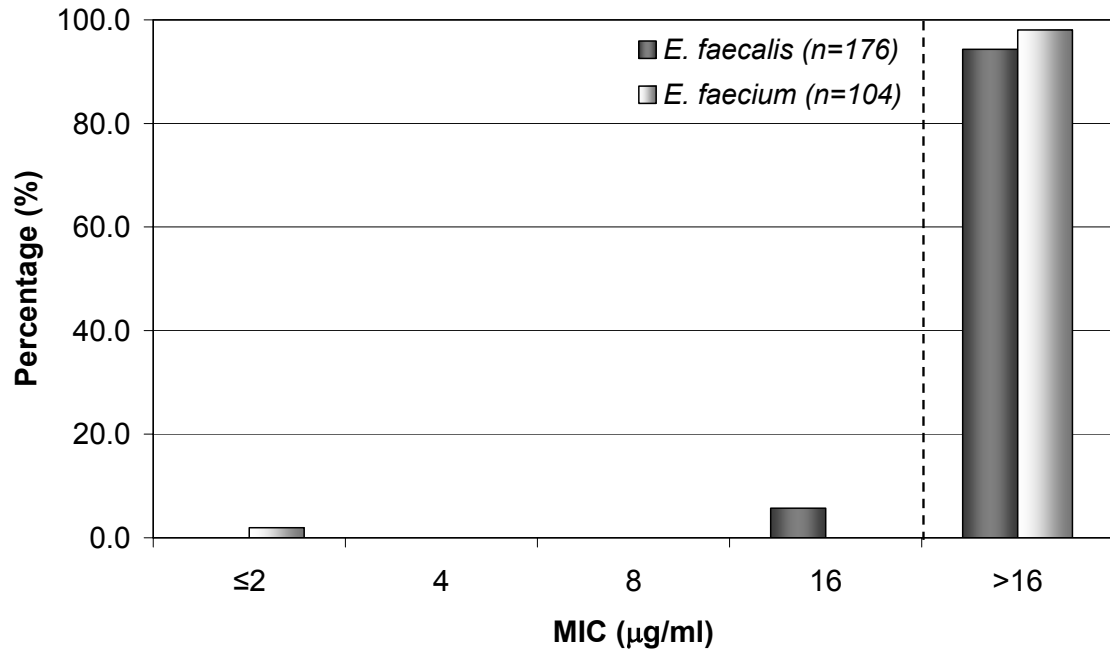


Figure 6. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **cefazolin**. Of the *E. faecalis* isolates, 94% were resistant (MIC₅₀ = >16 µg/ml, MIC₉₀ = >16 µg/ml, mode = > 16 µg/ml) and 98% of *E. faecium* isolates were resistant (MIC₅₀ = >16 µg/ml, MIC₉₀ = >16 µg/ml, mode = > 16µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>16 µg/ml) (213).

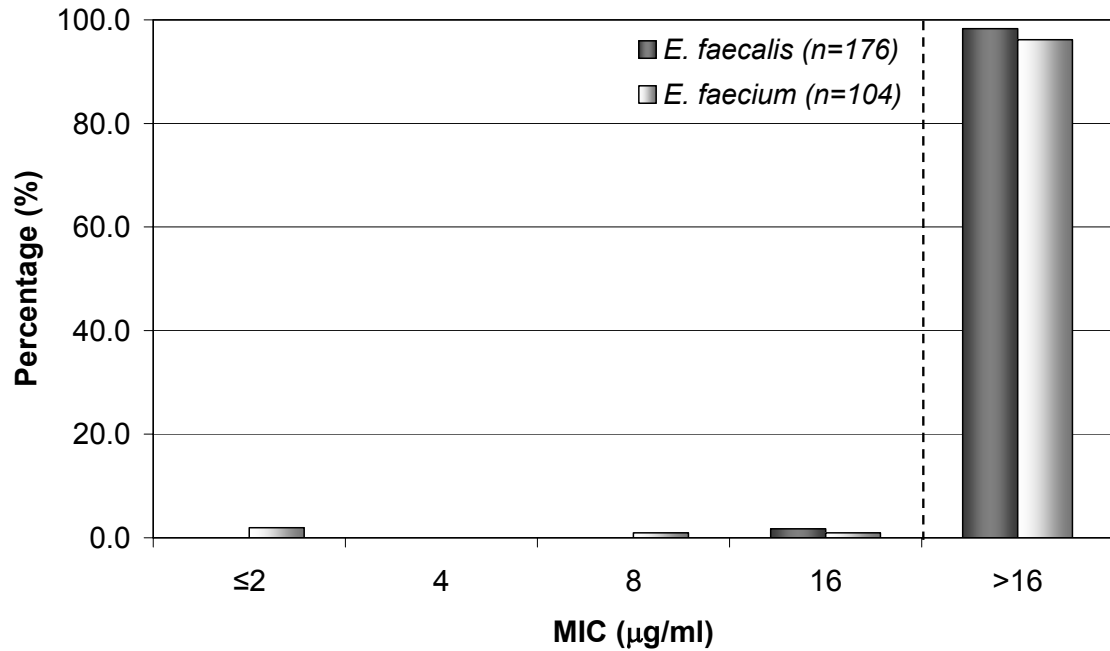


Figure 7. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **cephalothin**. Of the *E. faecalis* isolates, 98% were resistant (MIC₅₀ = >16 µg/ml, MIC₉₀ = >16 µg/ml, mode = >16 µg/ml) and 96% of *E. faecium* isolates were resistant (MIC₅₀ = >16 µg/ml, MIC₉₀ = >16 µg/ml, mode = >16 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>16 µg/ml) (213).

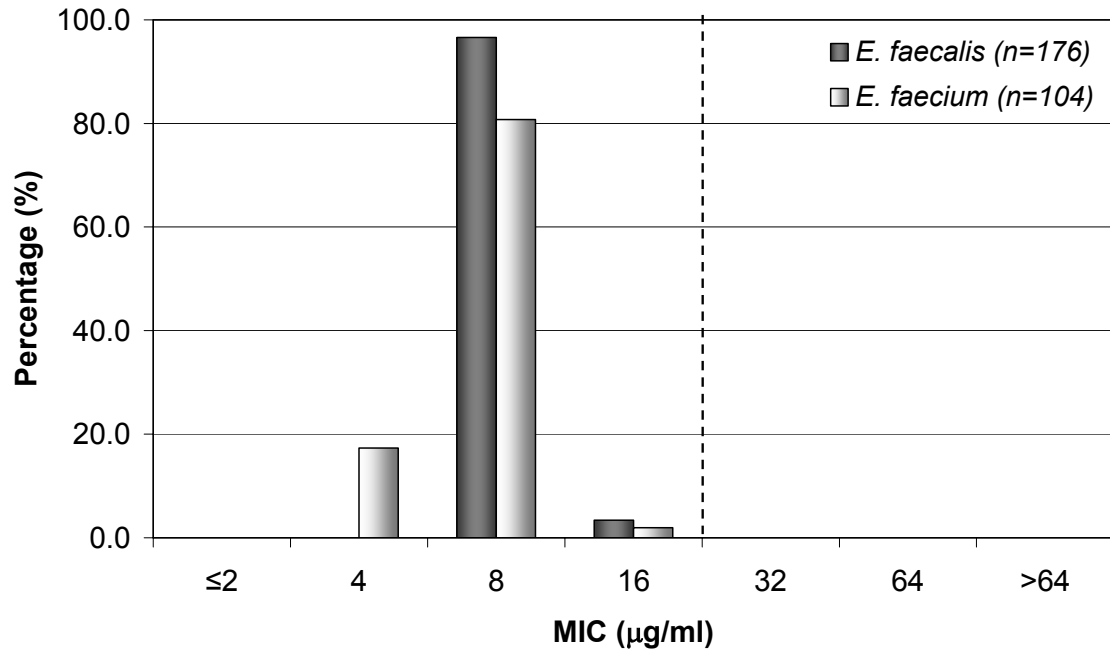


Figure 8. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **chloramphenicol**. Of the *E. faecalis* isolates, 0% were resistant (MIC₅₀ = 8 µg/ml, MIC₉₀ = 8 µg/ml, mode = 8 µg/ml) and 0% of *E. faecium* isolates were resistant (MIC₅₀ = 8 µg/ml, MIC₉₀ = 8 µg/ml, mode = 8 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>16 µg/ml) (213).

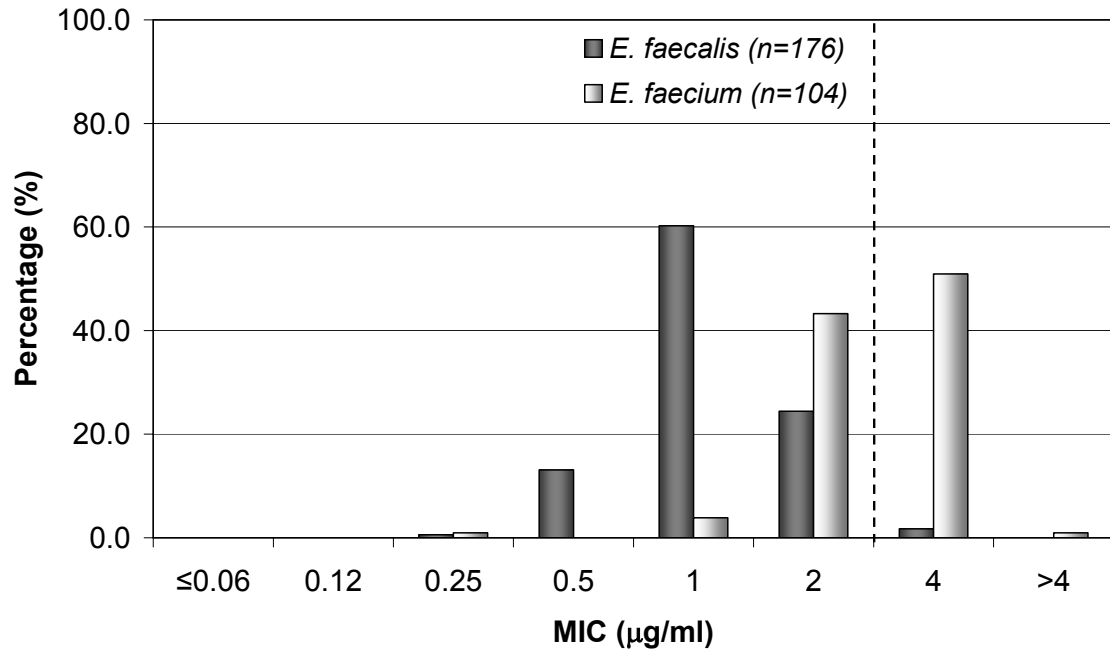


Figure 9. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **ciprofloxacin**. Of the *E. faecalis* isolates, 1.7% were resistant (MIC₅₀ = 1 µg/ml, MIC₉₀ = 2 µg/ml, mode = 1 µg/ml) and 52% of *E. faecium* isolates were resistant (MIC₅₀ = 4 µg/ml, MIC₉₀ = 4 µg/ml, mode = 4 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (≥ 4 µg/ml) (213).

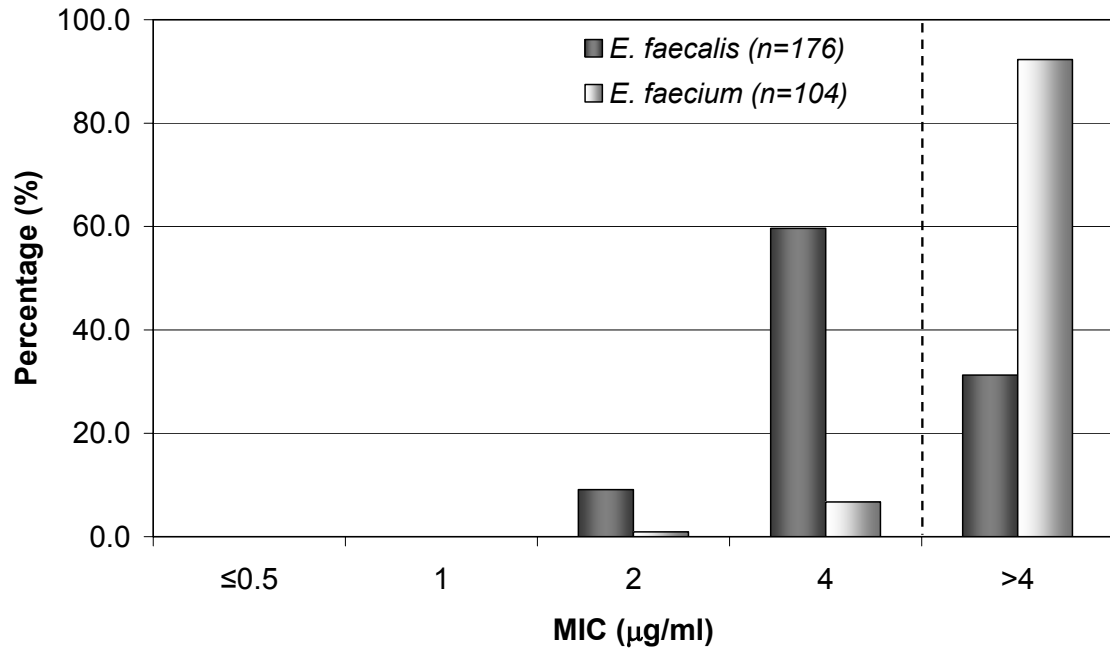


Figure 10. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **lomefloxacin**. Of the *E. faecalis* isolates, 31% were resistant (MIC₅₀ = 4 µg/ml, MIC₉₀ = >4 µg/ml, mode = 4 µg/ml) and 92% of *E. faecium* isolates were resistant (MIC₅₀ = >4 µg/ml, MIC₉₀ = >4 µg/ml, mode = >4 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>4 µg/ml) (213).

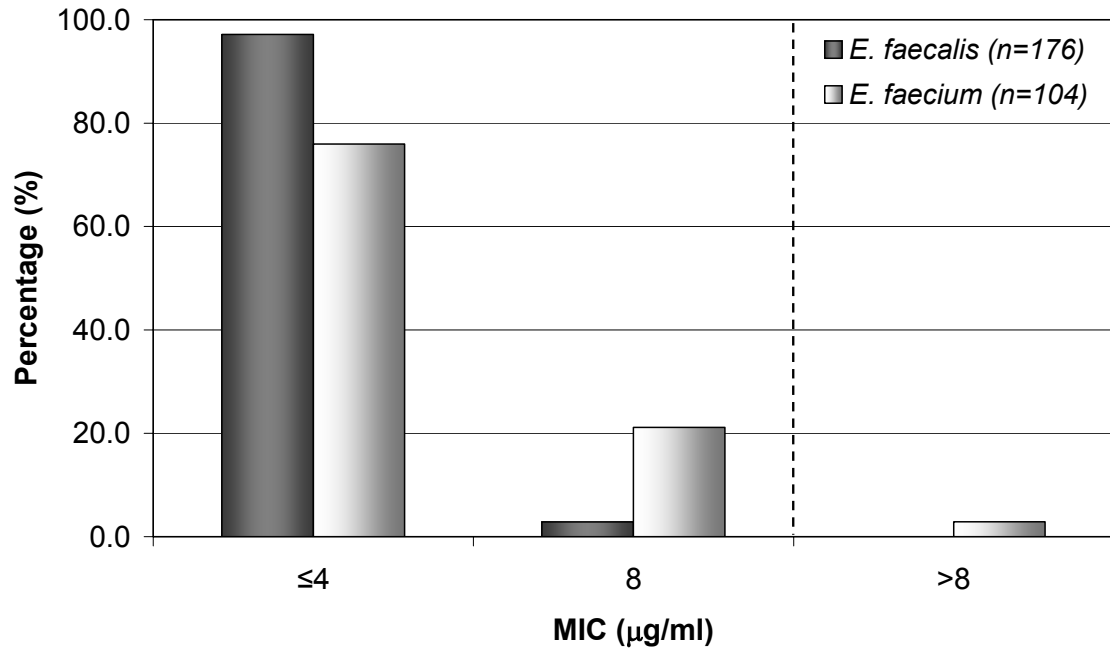


Figure 11. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **norfloxacin**. Of the *E. faecalis* isolates, 0% were resistant (MIC₅₀ = ≤4 µg/ml, MIC₉₀ = ≤4 µg/ml, mode = ≤4 µg/ml) and 2.9% of *E. faecium* isolates were resistant (MIC₅₀ = ≤4 µg/ml, MIC₉₀ = 8 µg/ml, mode = ≤4 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>8 µg/ml) (213).

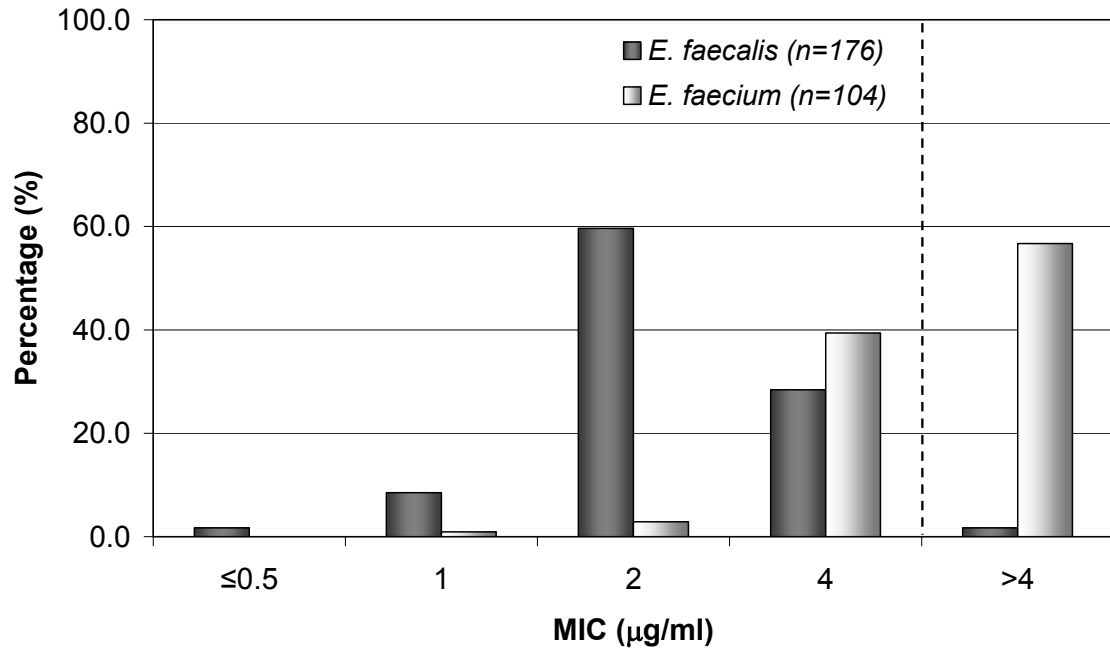


Figure 12. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **ofloxacin**. Of the *E. faecalis* isolates, 30.1% were resistant (MIC₅₀ = 2 µg/ml, MIC₉₀ = 4 µg/ml, mode = 2 µg/ml) and 96.2% of *E. faecium* isolates were resistant (MIC₅₀ = >4 µg/ml, MIC₉₀ = >4 µg/ml, mode = >4 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (≥ 4 µg/ml) (213).

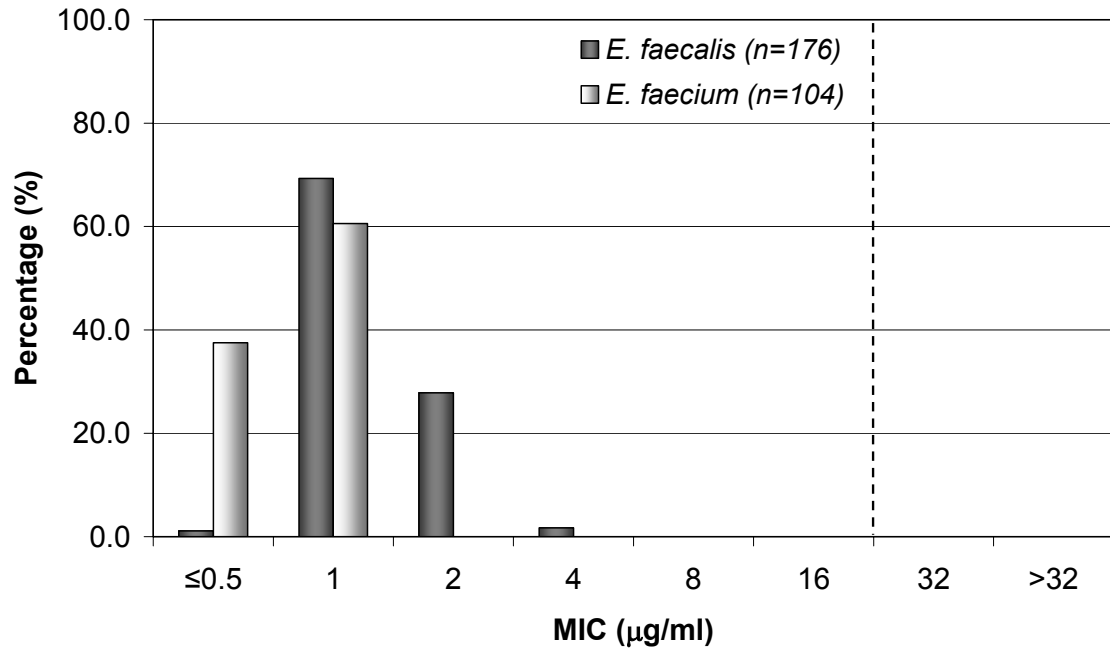


Figure 13. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **vancomycin**. Of the *E. faecalis* isolates, 0% were resistant (MIC₅₀ = 1 µg/ml, MIC₉₀ = 2 µg/ml, mode = 1 µg/ml) and 0% of *E. faecium* isolates were resistant (MIC₅₀ = 1 µg/ml, MIC₉₀ = 1 µg/ml, mode = 1 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>16 µg/ml) (213).

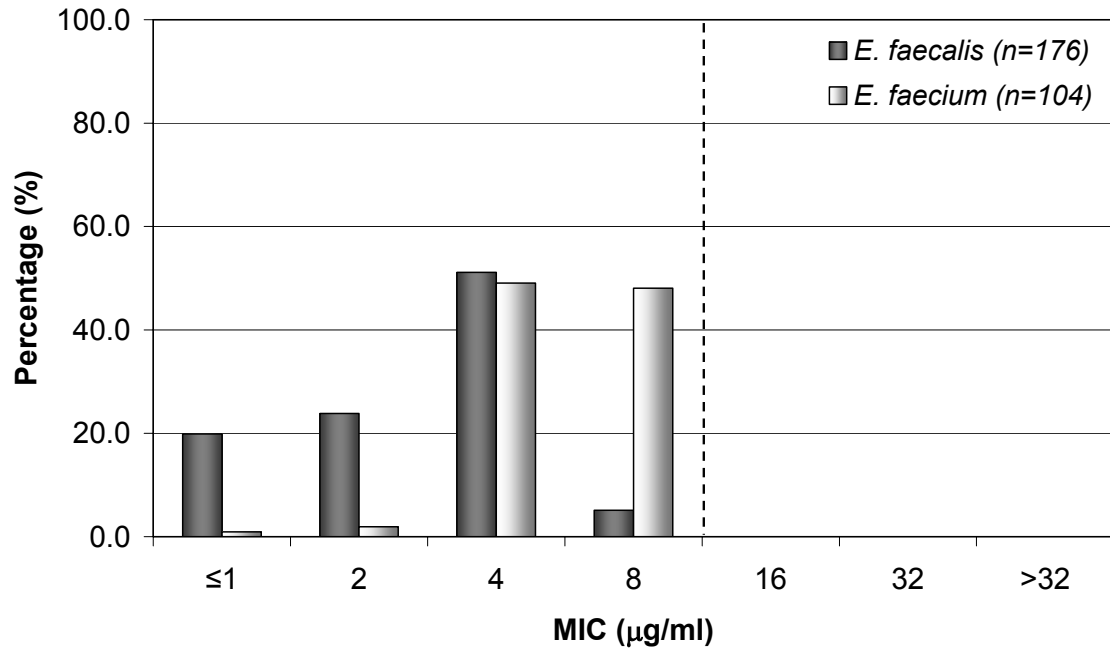


Figure 14. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **salinomycin**. Of the *E. faecalis* isolates, 0% were resistant (MIC₅₀ = 4 µg/ml, MIC₉₀ = 4 µg/ml, mode = 4 µg/ml) and 0% of *E. faecium* isolates were resistant (MIC₅₀ = 4 µg/ml, MIC₉₀ = 8 µg/ml, mode = 4 µg/ml). Dashed line denotes the defined breakpoint for resistance (>8 µg/ml) (4).

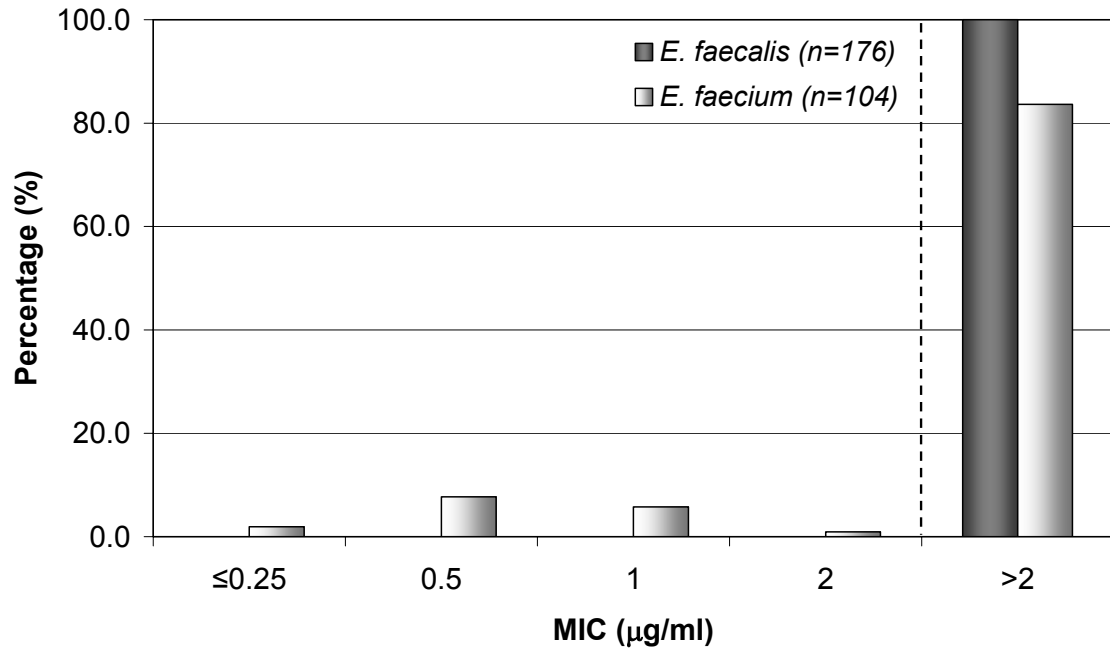


Figure 15. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **clindamycin**. Of the *E. faecalis* isolates, 100% were resistant (MIC₅₀ = >2 µg/ml, MIC₉₀ = >2 µg/ml, mode = >2 µg/ml) and 84% of *E. faecium* isolates were resistant (MIC₅₀ = >2 µg/ml, MIC₉₀ = >2 µg/ml, mode = >2 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>2 µg/ml) (213).

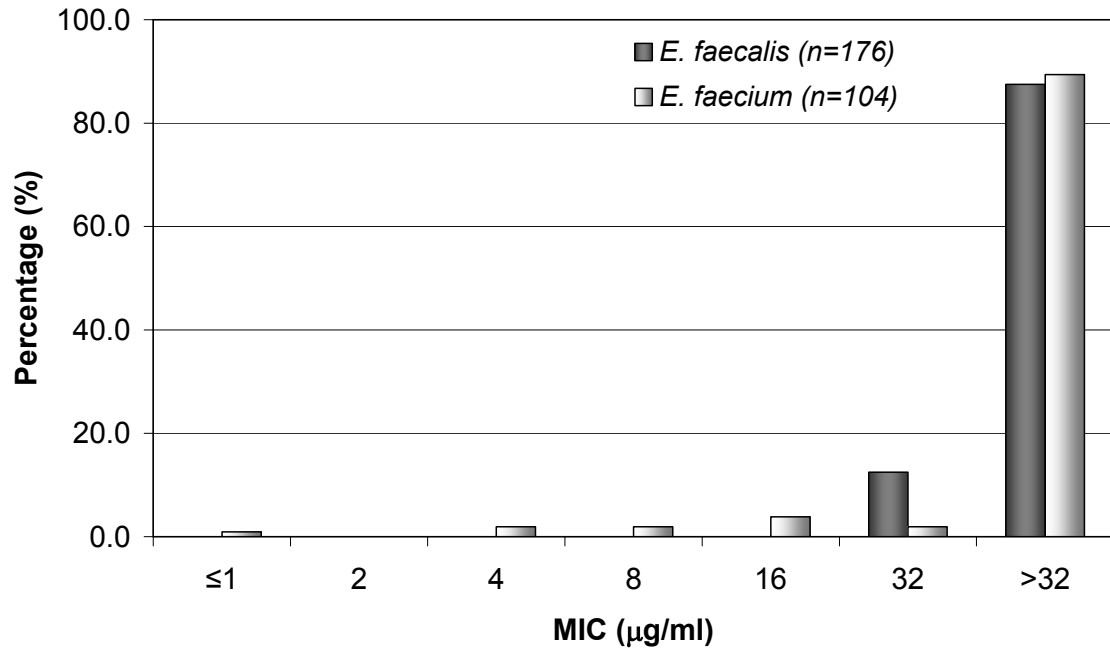


Figure 16. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **lincomycin**. Of the *E. faecalis* isolates, 88% were resistant (MIC₅₀ = >32 µg/ml, MIC₉₀ = >32 µg/ml, mode = >32 µg/ml) and 89% of *E. faecium* isolates were resistant (MIC₅₀ = >32 µg/ml, MIC₉₀ = >32 µg/ml, mode = >32 µg/ml) at >32 µg/ml. No interpretive criterion for resistance is available for this antimicrobial.

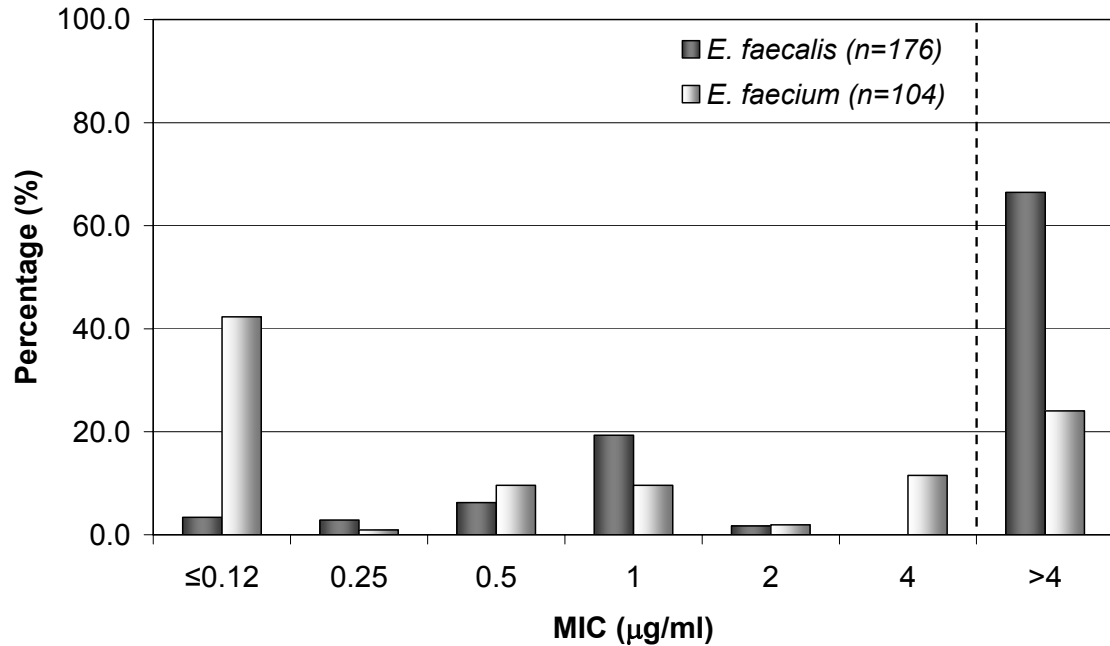


Figure 17. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **clarithromycin**. Of the *E. faecalis* isolates, 67% were resistant (MIC₅₀ = >4 µg/ml, MIC₉₀ = >4 µg/ml, modes = 1 and >4 µg/ml) and 24% of *E. faecium* isolates were resistant (MIC₅₀ = 0.5 µg/ml, MIC₉₀ = >4 µg/ml, modes = ≤0.12 and >4 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>4 µg/ml) (213).

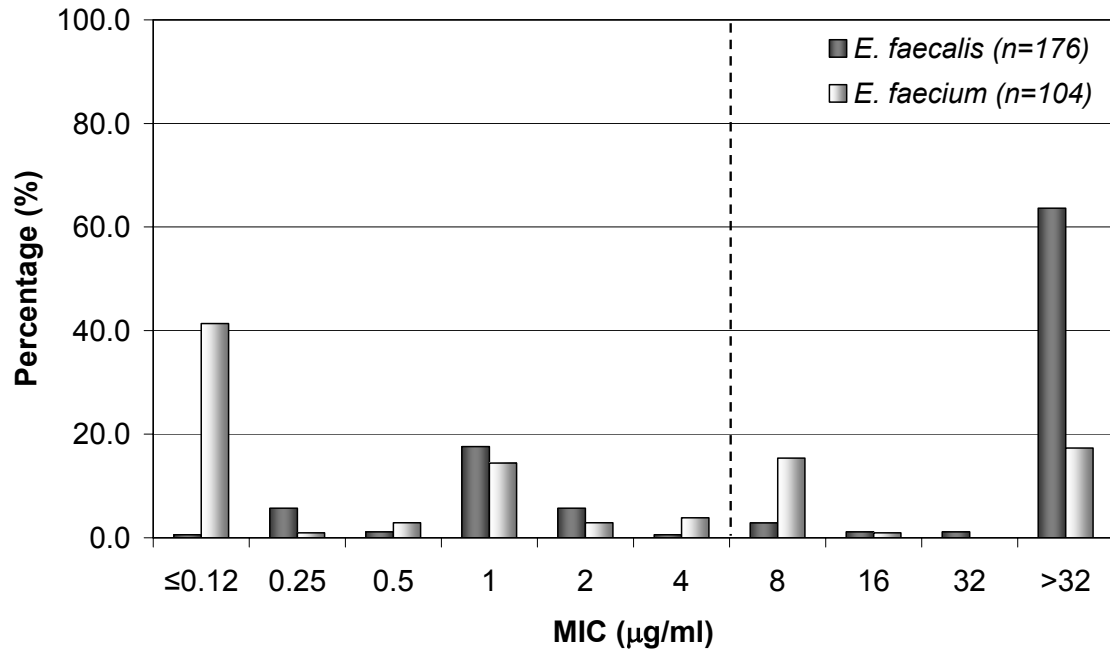


Figure 18. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **erythromycin**. Of the *E. faecalis* isolates, 69% were resistant (MIC₅₀ = >32 µg/ml, MIC₉₀ = >32 µg/ml, modes = 1 and >32 µg/ml) and 34% of *E. faecium* isolates were resistant (MIC₅₀ = 1 µg/ml, MIC₉₀ = >32 µg/ml, modes = ≤0.12, 1, 8, and >32 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (≥8 µg/ml) (213).

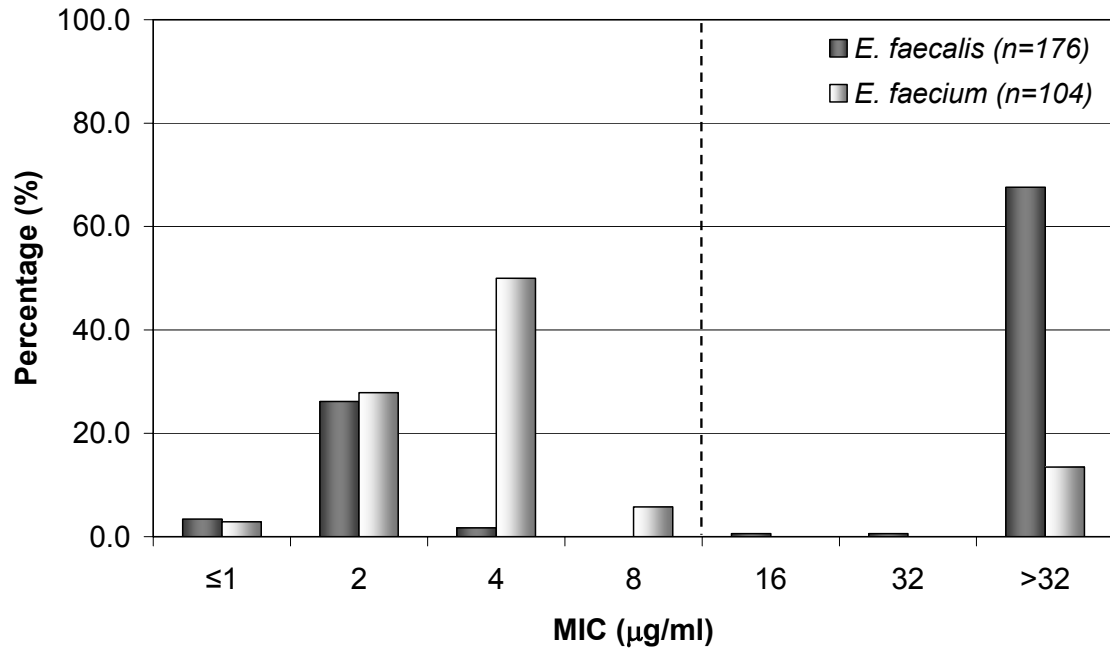


Figure 19. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **tylosin**. Of the *E. faecalis* isolates, 69% were resistant (MIC₅₀ = >32 µg/ml, MIC₉₀ = >32 µg/ml, modes = 2 and >32 µg/ml) and 13.5% of *E. faecium* isolates were resistant (MIC₅₀ = 4 µg/ml, MIC₉₀ = >32 µg/ml, modes = 4 and >32 µg/ml). Dashed line denotes the defined breakpoint for resistance (>8 µg/ml) (6).

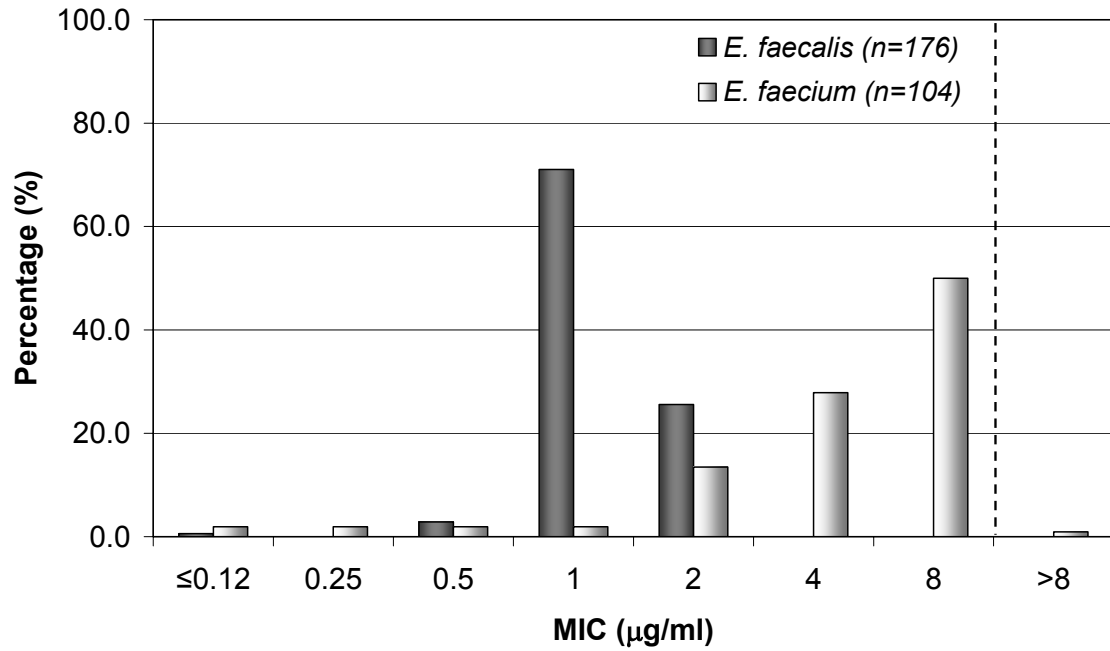


Figure 20. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **ampicillin**. Of the *E. faecalis* isolates, 0% were resistant (MIC₅₀ = 1 µg/ml, MIC₉₀ = 2 µg/ml, mode = 1 µg/ml) and 1% of *E. faecium* isolates were resistant (MIC₅₀ = 8 µg/ml, MIC₉₀ = 8 µg/ml, mode = 8 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>8 µg/ml) (213).

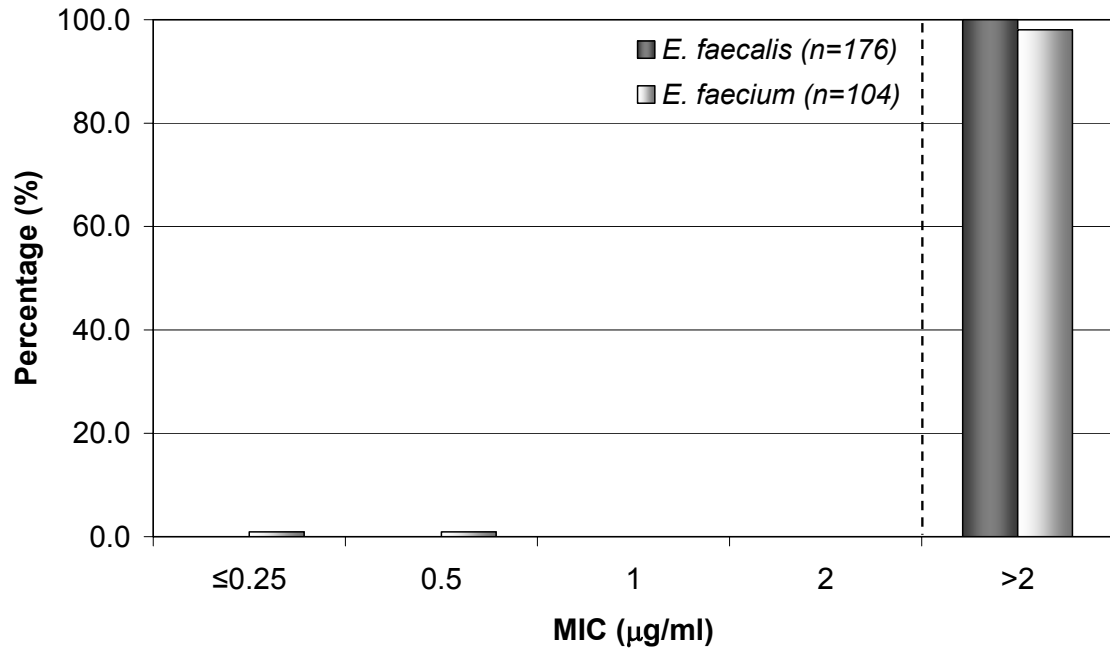


Figure 21. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **oxacillin**. Of the *E. faecalis* isolates, 100% were resistant (MIC₅₀ = >2 µg/ml, MIC₉₀ = >2 µg/ml, mode = >2 µg/ml) and 98% of *E. faecium* isolates were resistant (MIC₅₀ = >2 µg/ml, MIC₉₀ = >2 µg/ml, mode = >2 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>2 µg/ml) (213).

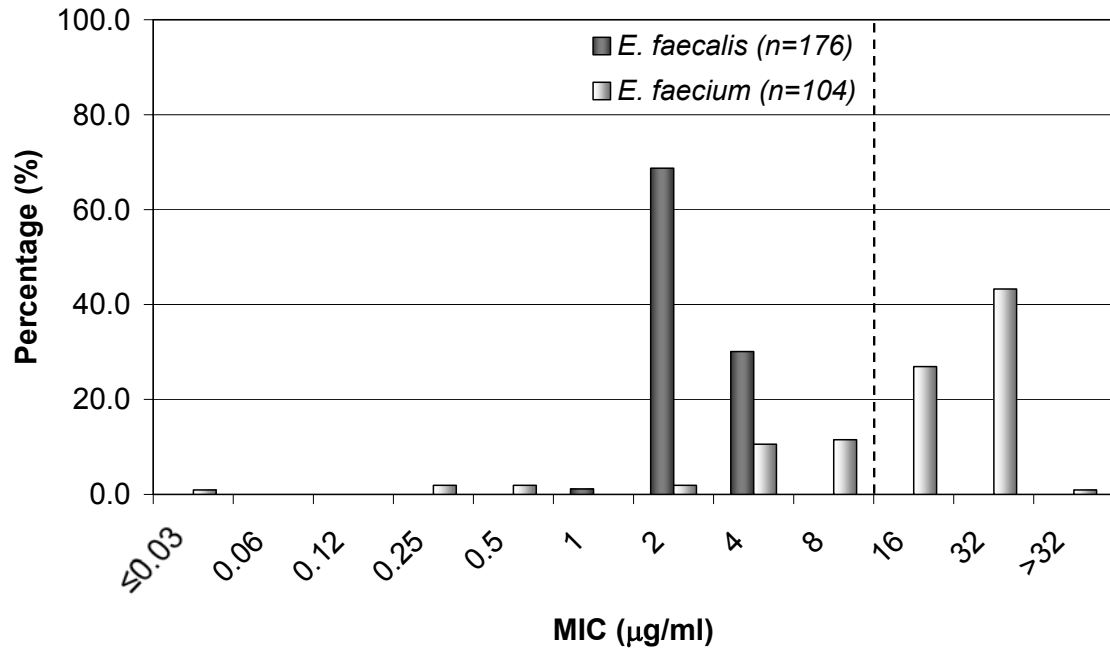


Figure 22. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **penicillin**. Of the *E. faecalis* isolates, 0% were resistant (MIC₅₀ = 2 µg/ml, MIC₉₀ = 4 µg/ml, mode = 2 µg/ml) and 71% of *E. faecium* isolates were resistant (MIC₅₀ = 16 µg/ml, MIC₉₀ = 32 µg/ml, mode = 32 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>8 µg/ml) (213).

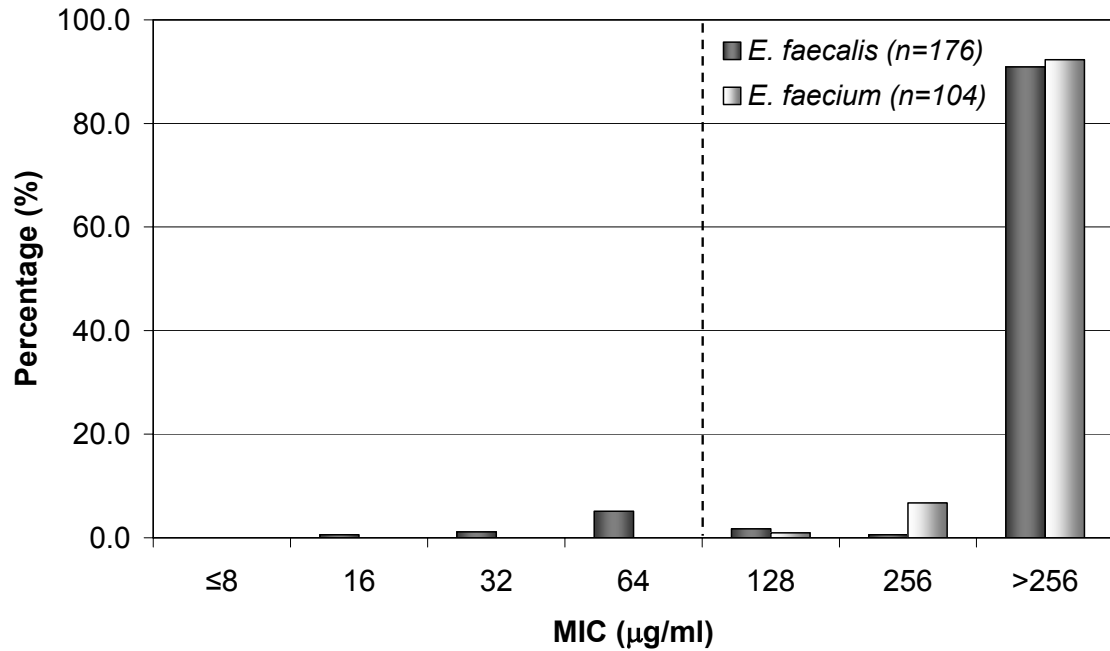


Figure 23. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **bacitracin**. Of the *E. faecalis* isolates, 93% were resistant (MIC50 = >256 IU/ml, MIC90 = >256 IU/ml, modes = 64 and >256 IU/ml) and 100% of *E. faecium* isolates were resistant (MIC50 = >256 IU/ml, MIC90 = >256 IU/ml, mode = >256 IU/ml). Dashed line denotes the defined breakpoint for resistance (>64 IU/ml) (6). IU stands for International Units.

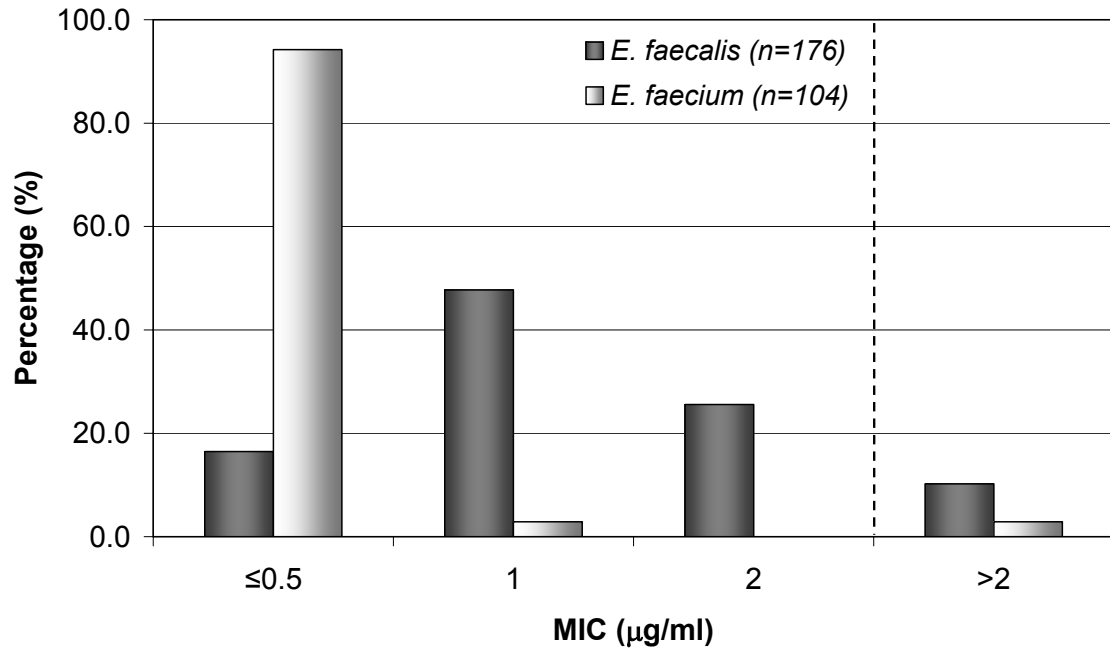


Figure 24. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **rifampin**. Of the *E. faecalis* isolates, 10.2% were resistant (MIC₅₀ = 1 µg/ml, MIC₉₀ = >2 µg/ml, mode = 1 µg/ml) and 2.9% of *E. faecium* isolates were resistant (MIC₅₀ = ≤0.5 µg/ml, MIC₉₀ = ≤0.5 µg/ml, mode = ≤0.5 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>2 µg/ml) (213).

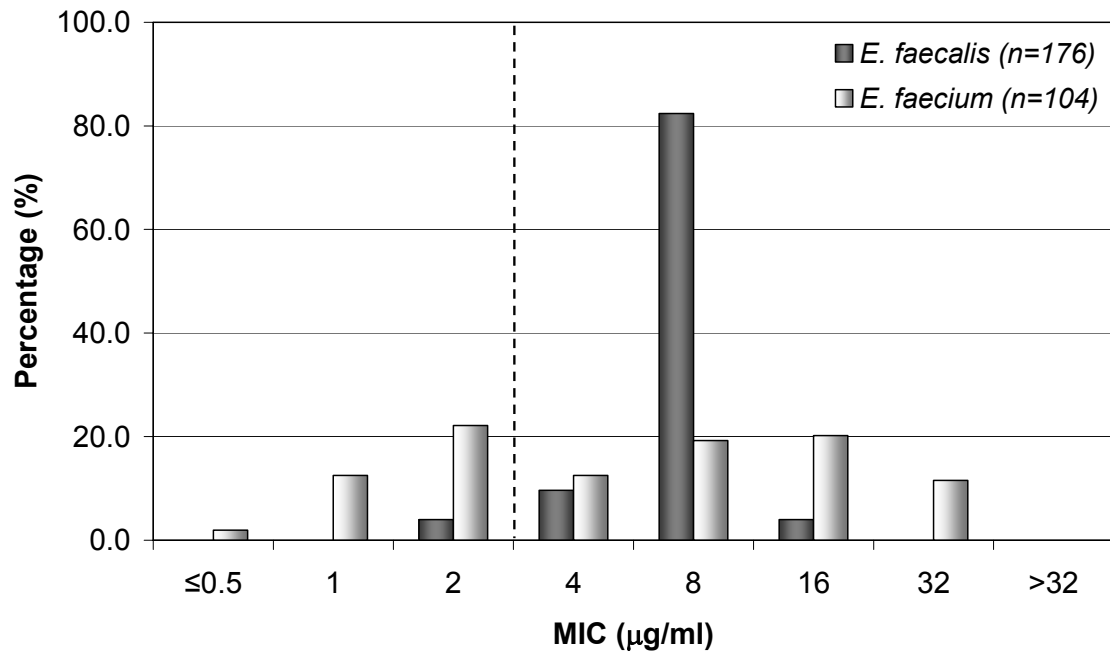


Figure 25. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **quinupristin-dalfopristin**. Of the *E. faecalis* isolates, 96% were resistant (MIC₅₀ = 8 µg/ml, MIC₉₀ = 8 µg/ml, mode = 8 µg/ml) and 64% of *E. faecium* isolates were resistant (MIC₅₀ = 8 µg/ml, MIC₉₀ = 32 µg/ml, modes = 2 and 16 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (≥ 4 µg/ml) (213).

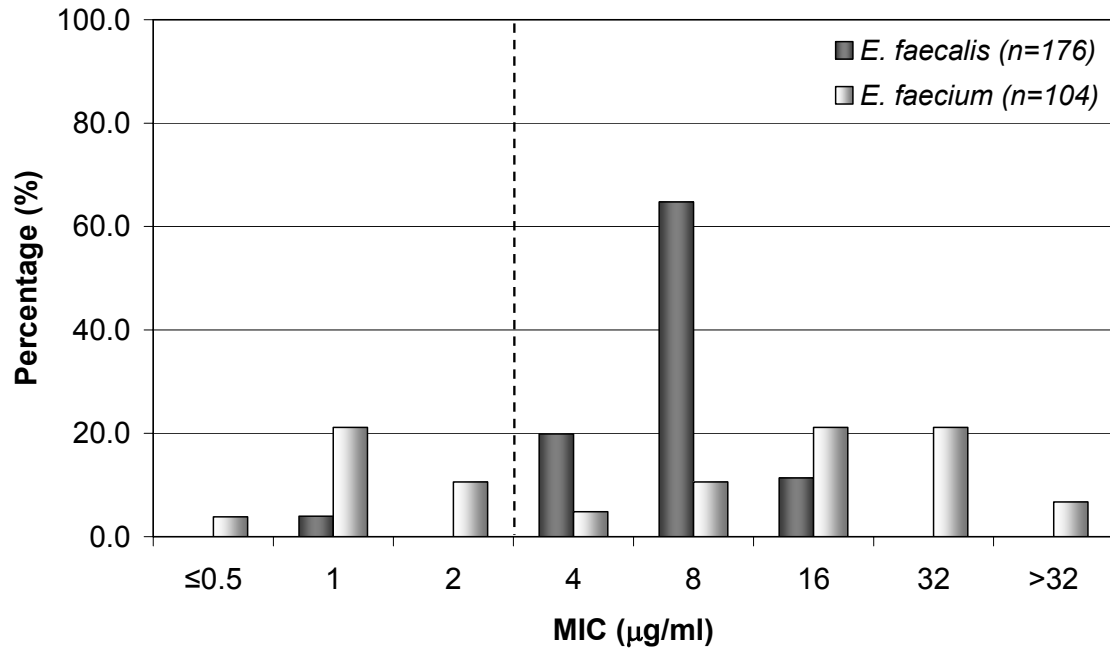


Figure 26. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **virginiamycin**. Of the *E. faecalis* isolates, 96% were resistant (MIC₅₀ = 8 µg/ml, MIC₉₀ = 16 µg/ml, mode = 8 µg/ml) and 64% of *E. faecium* isolates were resistant (MIC₅₀ = 8 µg/ml, MIC₉₀ = 32 µg/ml, modes = 1, 16, and 32 µg/ml). Resistance was defined as an MIC ≥4 µg/ml.

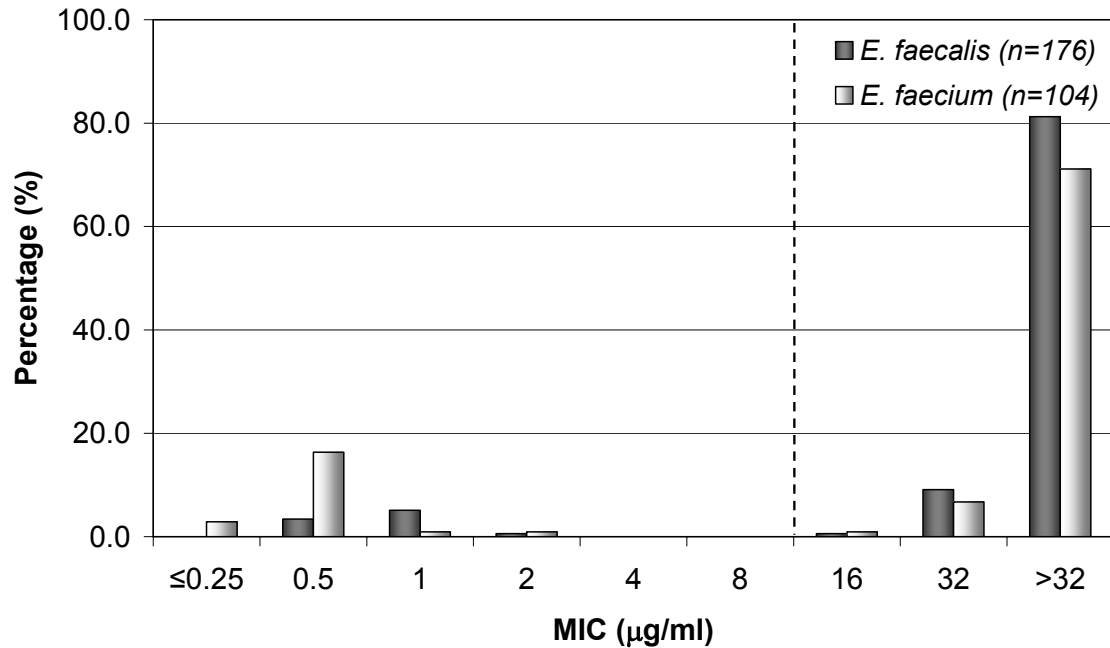


Figure 27. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **tetracycline**. Of the *E. faecalis* isolates, 91% were resistant (MIC₅₀ = >32 µg/ml, MIC₉₀ = >32 µg/ml, modes = 1 and >32 µg/ml) and 79% of *E. faecium* isolates were resistant (MIC₅₀ = >32 µg/ml, MIC₉₀ = >32 µg/ml, modes = 0.5 and >32 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>8 µg/ml) (213).

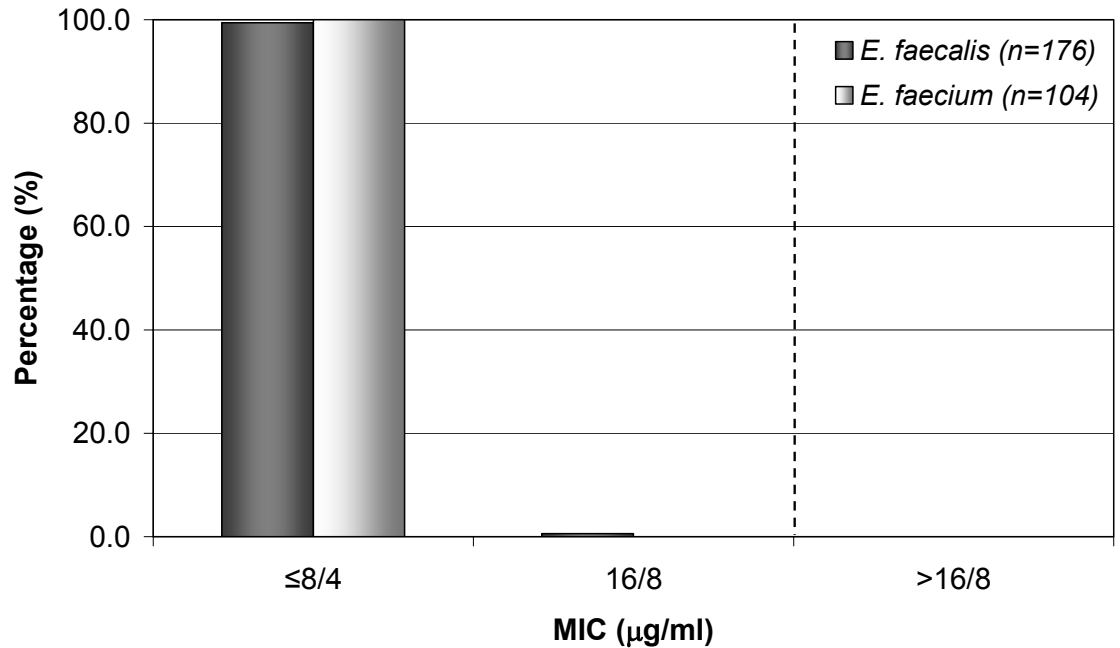


Figure 28. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **ampicillin/sulbactam**. Of the *E. faecalis* isolates, 0% were resistant (MIC₅₀ = ≤8/4 µg/ml, MIC₉₀ = ≤8/4 µg/ml, mode = ≤8/4 µg/ml) and 0% of *E. faecium* isolates were resistant (MIC₅₀ = ≤8/4 µg/ml, MIC₉₀ = ≤8/4 µg/ml, mode = ≤8/4 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>16/8 µg/ml) (213).

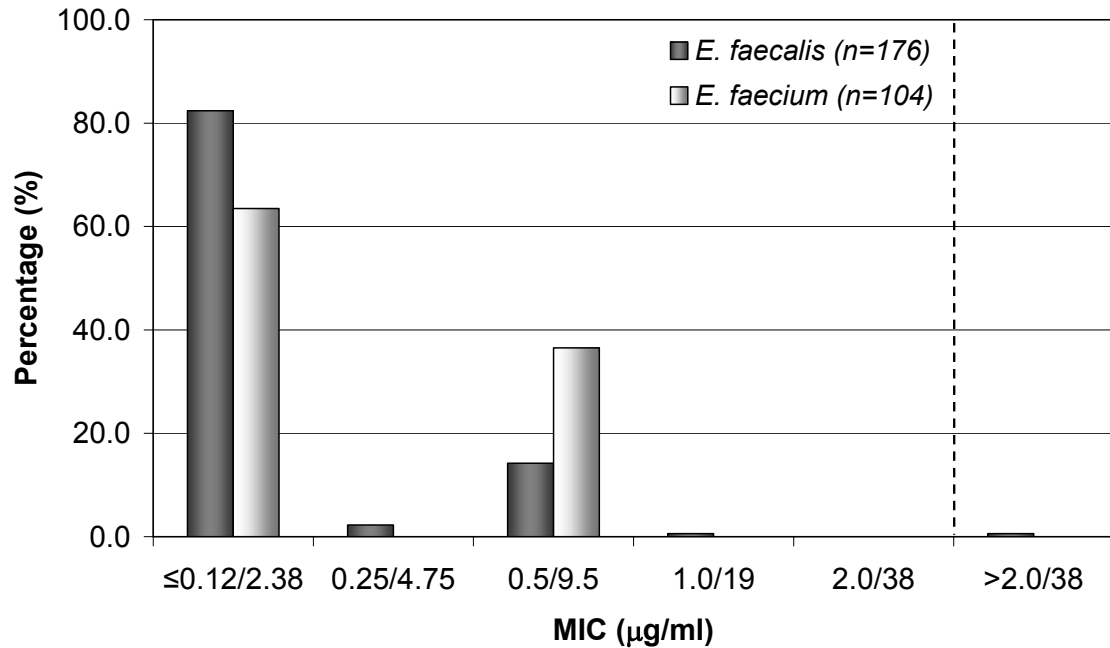


Figure 29. Susceptibility profiles of *E. faecalis* and *E. faecium* from the poultry production environment to **trimethoprim/sulfamethoxazole**. Of the *E. faecalis* isolates, 0.6% were resistant (MIC₅₀ = ≤0.12/2.38 µg/ml, MIC₉₀ = 0.5/9.5 µg/ml, modes = ≤0.12/2.38 and 0.5/9.5 µg/ml) and 0% of *E. faecium* isolates were resistant (MIC₅₀ = ≤0.12/2.38 µg/ml, MIC₉₀ = 0.5/9.5 µg/ml, modes = ≤0.12/2.38 and 0.5/9.5 µg/ml). Dashed line denotes the NCCLS-defined breakpoint for resistance (>2/38 µg/ml) (213).

CHAPTER 2

Characterization of the Macrolide-Lincosamide-Streptogramin Resistance Phenotype of *Enterococcus faecium* from the Poultry Production Environment

ABSTRACT

The impact of agricultural use of antimicrobials on the future efficacy of therapeutic drugs in human medicine is a growing public concern. Quinupristin-dalfopristin (Q-D) has been approved to treat human disease due to vancomycin-resistant *Enterococcus faecium* and is related to virginiamycin, a streptogramin that has long been used in U.S. agriculture poultry production. The magnitude of transferable resistance elements selected by the agricultural use of streptogramin antimicrobials is largely unquantified within the poultry production environment of the U.S.

Streptogramin-resistant isolates of *E. faecium* from poultry production environments from the eastern seaboard were recovered without selection for streptogramin resistance and subjected to ribotyping to evaluate clonal bias. Colony PCR screening for the streptogramin resistance determinants *erm(A)*, *erm(B)*, *msr(C)*, *mef(A)*, *lnu(B)*, *vgb(A)*, *vat(D)*, and *vat(E)* was performed to determine the prevalence of previously described macrolide-lincosamide-streptogramin (MLS) resistance mechanisms.

The collection of *E. faecium* were unevenly distributed among 28 ribogroups that did not represent geographic clustering. The most prevalent ribogroups were composed of isolates that possessed diverse antimicrobial resistance profiles. Of these 127 isolates, 63% were Q-D-resistant. The resistance determinants *erm(A)*, *erm(B)*, and *mef(A)* were

observed among 15%, 30%, and 11%, respectively, of macrolide-resistant isolates. *Msr(C)* was isolated in a single isolate that was resistant to macrolide and lincosamide antimicrobials. The streptogramin B hydrolase *vgb(A)* and the streptogramin A acetyltransferases genes *vat(D)* and *vat(E)* were not detected in any of the *E. faecium* isolates. These results indicate that resistance to MLS antimicrobials is widespread among *E. faecium* from the poultry production environment and that the mechanisms of resistance within this population remain largely uncharacterized.

INTRODUCTION

Enterococci, particularly *E. faecalis* and *E. faecium*, present serious challenges to the control of antimicrobial resistance as they are the third leading cause of nosocomial infections in intensive care units in the United States (44). In addition, they are known to be intrinsically resistant to several antibiotics and perhaps more importantly, are adept in acquiring and transferring elements that confer resistance to antimicrobials. As a result, therapeutic options are increasingly limited for the treatment of enterococcal infections (205).

In 1980 the reported development and subsequent increase in resistance to the glycopeptide vancomycin among clinical isolates of *Enterococcus* spp. was followed by a flurry of research into new antimicrobials for alternative therapy. The 1999 Food and Drug Administration approval of the streptogramin, quinupristin-dalfopristin (Q-D or Synercid[®]), to treat vancomycin-resistant *E. faecium* infections came after more than 20 years of widespread use of a streptogramin analogue, virginiamycin, in animal production in the U.S. This has revived concerns that use of antimicrobials in food animal production might compromise the efficacy of related drugs in human clinical medicine through selection of resistant populations and their subsequent transfer through the food supply (112).

Previous work has demonstrated that single resistance elements can confer resistance to macrolide-lincosamide-streptogramin B (MLS_B) antimicrobials whereas resistance to a streptogramin combination requires resistance to both A and B components (35). We have previously described the frequent occurrence of streptogramin resistance among *E. faecium* from a poultry production environment of the

U.S. (124), likely due to the continued use of macrolide and lincosamide antimicrobials, specifically tylosin and lincomycin, as well as the streptogramin virginiamycin. This work describes the diversity and genetic mechanisms that contribute to the prevalence of MLS resistance phenotypes among *E. faecium* from the poultry production environment of a geographically defined region of the U.S. that is an area of intensive poultry production.

MATERIALS AND METHODS

Ribotyping of *Enterococcus faecium* isolates. Automated ribotyping of the 127 isolates of *E. faecium* was accomplished using the RiboPrinter® microbial characterization system (Dupont Qualicon United States, Wilmington, DE) performed under the conditions recommended by the manufacturer of as described previously (Figure 30) (38). In brief, this automated process included the lysis of a bacterial cell suspension through the use of a ten minute heat treatment at 80°C and lysis reagents. The extracted DNA was then cleaved by the activity of the restriction endonuclease *EcoRI*. DNA fragments were separated by size using electrophoresis through a 1% agarose gel and transferred to a moving nylon membrane by direct blot electrophoresis. The DNA fragments were then hybridized with a labeled rRNA operon probe derived from *Escherichia coli*, washed and treated with blocking buffer and an antisulfonated DNA antibody/alkaline phosphatase conjugate. Unbound conjugate was removed and the bands were detected using a chemiluminescent substrate. An image was captured using a customized charge-coupled device camera and was then electronically transferred to the system's computer. The analysis software uses the position and intensity of the five well characterized marker fragments (1, 3.2, 6.5, 9.6, and 48 kb), which were run alongside the samples, to normalize the resulting output data. The positions of these standard marker bands are used to correct for lane to lane and membrane-to-membrane variations in band position. In addition, the following ATCC quality control organisms were run with every fifth batch: *E. coli* 51739, *S. aureus* 51740, *Salmonella* ser. Infantis 51741, and *Listeria innocua* 51742.

Cluster Analysis. Normalized patterns were imported into the Bionumerics version 3.0 software (Applied Maths) using an import script provided by DuPont Qualicon. Band analysis was performed following an automatic band search by using the following parameters: minimum profiling parameter of 3.0% and gray zone parameter of 0% relative to the maximal value, a minimum area parameter of 0%, and a shoulder sensitivity parameter of zero. Clustering was performed by using the unweighted pair group method with average (UPGMA) based on the Dice similarity index with a 1.5% band position tolerance and 1.5% optimization coefficient to determine isolate similarity.

Preparation of DNA from *Enterococcus faecium* isolates. DNA template was prepared by boiling 4-5 colonies in 200 μ l of sterile water for ten minutes in MicroAmp® 96-well plates (Applied Biosystems, Foster City, CA). Plates were centrifuged at 2500 rpm for three minutes in a Centra®-CL3 centrifuge (Thermo IEC, Needham Heights, MA) and the supernatant was transferred to another plate for storage at -20°C.

Alternative means of DNA isolation were employed when positive control PCR reactions did not result in amplification of product. The method of Vakulenko et al. involved the emulsification of 3-5 colonies in 25 μ l of a 0.25% sodium-docecyl-sulfate/0.05 N NaOH solution (296). The suspension was then boiled in a water bath for 15 minutes and 200 μ l of deionized water were added to generate a working stock of template DNA. The method of De Azavedo involved suspending a loopful of overnight bacterial growth taken from sheep blood agar in 100 μ l of a lysis buffer (100 mM NaCl, 10 mM Tris HCl [pH 8.0], 1% Triton X-100) and boiling for 10 minutes (67). The suspension was then centrifuged briefly and the supernatant was used as template.

PCR screening for MLS resistance determinants. Ten microliters of template DNA was used in all PCR reactions. All PCR reactions were carried out using a 96-well GeneAmp® PCR System 9700 thermocycler (Applied Biosystems) in a 50 µl volume. Fifty pmol of each primer were used in resistance screening and are listed in Table 7. Primer sequences for *msr(C)*, *mef(A)*, and *vgb(A)* were determined using the Vector *NTI*TM software package (Informax, Frederick, MD). Two primer sets were used for the *lnu(B)* PCR reactions; *lnuB1*, 5'-GCA AAT GGT GTA GGT AAG ACA ACT-3' and *lnuB2*, 5'-ATC ATG TGA TGT AAA CAA AAT-3' as published previously (270); *linB1*, 5'- GCA AAC GTT AAG AAT CTT AC-3' and *linB2* 5'-GCA TTT TCT TCA GTA AGT CT-3' were determined using the Vector *NTI*TM software of the GenBank accession AJ238249 (34).

PCR reactions for each of the previously described streptogramin resistance determinants [*erm(A)*, *erm(B)*, *msr(C)*, *vgb(A)*, *vat(D)*, and *vat(E)*] as well as the *E. faecium*-specific gene D-alanine:D-alanine ligase (*ddl*) was carried out using AmpliTaq Gold® (Applied Biosystems) according to the manufacturer's suggestions. The PCR conditions used for each reaction are listed in Table 8. PCR screening was performed in triplicate among all isolates that produced an amplicon and among an equal number of PCR-negative isolates.

PCR reactions were visualized using the 2% agarose E-Gel® 96 High-Throughput Agarose Electrophoresis System (Invitrogen, Carlsbad, CA). Positive control strains are listed in Table 9. *E. faecium* TX1330 SE34 was kindly provided by Dr. B. Murray, *E. faecalis* JH2-2/1025 and *E. coli* DH10B/pVMM25 from Dr. R. Leclercq, *S. aureus*

BM3093/pIP680 from Dr. N. El Solh, and Group B Streptococcus R132 from Dr. J. de Azavedo. Additional CVM control strains were provided by Dr. S. Simjee.

RESULTS

Diversity and dispersion of *Enterococcus faecium* isolates from the Delmarva Peninsula. The collection of 127 *E. faecium* from the poultry production environment was distributed among 28 distinct ribogroups (Figure 31). Five of the isolates did not generate a ribotype pattern despite repeated testing. Sixteen ribotypes were rarely encountered and were represented by single isolates of *E. faecium*. Interestingly, a single ribotype was observed among 33% of the isolates whereas the four most prevalent ribotypes constituted 82/127 (65%) of the observed population, but displayed inconsonant antibiograms within each ribotype.

The geographical distribution of the encountered ribotypes of *E. faecium* isolates is presented in Figure 32. *E. faecium* was not isolated from twelve sampled farms and a farm location was not provided for 26 (20%) of the recovered isolates. The greatest diversity of ribotype patterns from a given region was directly related to the number of farms sampled.

Analysis of the geographic distribution of *E. faecium* ribogroups was confounded by the predominance of three ribogroups and the infrequent observation of a majority of other ribogroups. The four most prevalent ribogroups were observed to be widely distributed across the sampling region with no obvious clustering.

Geographic dispersion of MLS phenotypes of *E. faecium* from the Delmarva Peninsula. Phenotypic resistance to MLS antimicrobials was heterogeneous among the *E. faecium* isolated. The most prevalent phenotype observed was resistance to lincosamide and streptogramin antimicrobials (47%) (Table 10). Macrolide-lincosamide-

streptogramin resistance was observed among 17% of isolates as well as resistance to macrolide and lincosamide antimicrobials and isolated lincosamide resistance. Four isolates (3%) were observed to possess resistance to macrolide antimicrobials and a single isolate (0.8%) was sensitive to all MLS antimicrobials. Whereas increased tolerance to lincomycin was observed independent of resistance to other antimicrobials, only a small percentage of isolates were resistant to macrolide antimicrobials and no isolates were observed to be resistant to streptogramin antimicrobials in the absence of macrolide or lincosamide resistance.

Many diverse MLS phenotypes were observed across the sampled region (Figure 33). Lincosamide resistance was observed to be a nearly ubiquitous trait among the isolates as well as geographically over the sampled region. This phenotype was observed to be frequently accompanied by resistance to streptogramin antimicrobials in the absence of resistance to macrolides. Isolates that were observed to be resistant to macrolide, lincosamide, and streptogramin antimicrobials were somewhat concentrated in the interior of the sampled region, most often from areas that were heavily surveyed. Within this region, however, isolates were also recovered that were not observed to be resistant to more than a single antimicrobial. It is important to note that the analysis of the geographic dispersion of MLS phenotypes among isolated *E. faecium* suffers the same limitations as the previously mentioned ribogroup pattern analysis, i.e., the lack of isolation of *E. faecium* from twelve sampled farms and no farm location for 26 (20%) of the recovered isolates.

Distribution of MLS resistance elements among *E. faecium*. PCR screening for MLS resistance determinants revealed the presence of the rRNA methylase genes *erm(A)* and *erm(B)* in 6.3 and 14.2% of all isolates (Table 10). The prevalences of the individual genes were predictably highest among macrolide-resistant isolates of with 15% with *erm(A)* and 30% with *erm(B)*. The *mef(A)* gene was detected among 5% and the *msr(C)* gene in 1% of all isolates. Of particular interest was the detection of *erm(A)*, *erm(B)*, and *mef(A)* among isolates that were not phenotypically resistant to macrolide antimicrobials. Additionally, no macrolide resistance element was detected among 23/46 isolates that were phenotypically resistant to macrolides.

Resistance to streptogramin antimicrobials was largely unaccounted for by PCR screening for specific resistance determinants. The genes *vgb(A)*, *vat(D)*, and *vat(E)* were not detected among the collection of *E. faecium* isolates. The genes *erm(A)* and *erm(B)* were detected in only 6% and 10%, respectively, of isolates phenotypically resistant to streptogramins.

Detection of the gene *lnu(B)* was not possible due to negative PCR reactions from three different control strains. The use of two alternative methods of preparing template DNA did not affect the PCR screen despite the use of alternative primer sequences and reaction conditions.

DISCUSSION

Although enterococci from the food animal production environment have not been conclusively linked as direct causes of clinical infections, the potential risk of the agricultural usage of antimicrobials in food animal production is increasingly an issue of public concern. Of the numerous methods currently employed to type bacterial collections, pulsed-field gel electrophoresis (PFGE) is considered the “gold-standard” typing method for *E. faecium* of clinical origin (190). Newer methods that have been employed in this fashion also include amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST) analysis (14,128,324). Most typing methods suffer from the dual limitations of a lack of standardization and the need for a significant amount of skilled personnel time. The application of automated rRNA gene restriction fragment analysis, or ribotyping, is a highly reproducible method for clonal analysis. Because rRNA operons are the most highly conserved region of the bacterial genome, ribotyping offers the advantage of eliminating most variability and has been previously applied in studies of *E. faecium* from a variety of environments (37,116).

Although ribotyping has been previously described as a less discriminatory tool than PFGE, it offers the advantages of facility, speed, and standardization (116). Additionally, ribotyping has been demonstrated to be as capable as PFGE of detecting identical “types” of vancomycin-resistant *E. faecium* of human and animal origin in Denmark (116). Despite the comparatively reduced “power” of ribotyping, the method has been shown to discriminate between strains of *E. faecium* that were considered identical by AFLP typing and/or MLST (37).

The results of this study suggest that common lineages of *E. faecium* can be isolated from poultry production environments that are geographically widely distributed. Whereas several ribogroup clusters were predominant, the analysis indicates that many less common sub-populations can also be observed. These observations are supported by other studies which illustrate the diversity of enterococcal populations within distinct environments (158,299,302,313).

The relative predominance of increased tolerance of isolates of *E. faecium* to lincosamide antimicrobials that we have observed has been similarly described in studies of human (75) and animal production environments (4). Given the use of the analog virginiamycin in food animal production environments in the U.S. since 1974, resistance to quinupristin-dalfopristin among *E. faecium* of poultry origin is not surprising (124,313). Our observations of the magnitude of resistance are generally comparable to those of poultry origin from Denmark (2,6), however our observation of macrolide resistance among 36% of *E. faecium* is much lower than previous reported observations from Denmark and Japan (2,329).

The heterogeneous nature of MLS resistance phenotypes observed among *E. faecium* belonging to a distinct ribogroup pattern suggests that acquisition of resistance to these antimicrobials is dynamic. Despite the long-term approval and presumed industrial usage of MLS antimicrobials in this region, this diversity is quite striking. Whereas the decreased use of these antimicrobials during production has been shown to parallel the drop in the prevalence of resistance to MLS antimicrobials among *E. faecium* (7), other factors may help to explain the absence of uniform resistance to all MLS antimicrobials. The regular entry of a sizeable population of susceptible *E. faecium* into the poultry

production environment from sources such as feed commodities and the natural flora of incoming flocks of unmedicated chickens are likely sources. Resistant populations may be present at an undetectable density due to the comparative sizes of resistant and susceptible populations and may preclude the observation of phenotypic resistance through the use of a non-selective cultural method. In this situation, the small, resistant population of *E. faecium* may persist in the production environment until such time that it is selectively amplified by the use of MLS antimicrobials, whereupon MLS-resistant *E. faecium* could rapidly become predominant.

Alternative selective pressure may be applied through the periodic use of other antimicrobials that do not select for MLS-resistant isolates, which would lead to a decreased prevalence of observed resistance. A recent prospective study in Denmark suggests that other environmental influences may also affect the dynamics of MLS resistance in the animal production environment. In that study, researchers demonstrated that the use of copper sulfate may co-select for antimicrobial resistance in animal production, with the strongest interactions in production environments that heavily use the additive (122).

The relative prevalence of previously described rRNA methylases that confer resistance to macrolide, lincosamide, and streptogramin B antimicrobials among *E. faecium* from this study was conserved, but the frequency differed. The prevalence of *erm(A)* among macrolide-resistant isolates of *E. faecium* seen in this study (15%) is somewhat larger than that observed in previous studies. Estimates of *erm(A)* prevalence among macrolide-resistant isolates reveal that it is infrequent (229) with an observed prevalence of 1% of poultry and porcine isolates from Denmark, Finland, and Norway (6)

and 5% of EU clinical isolates (258). The prevalence is similar to that reported in streptogramin-resistant enterococcal isolates from Danish poultry (138).

The results from this study differ markedly from the prevalence of *erm*(B) in the EU which is estimated to be up to 88% of macrolide-resistant isolates of poultry and porcine origin (5,6). The high prevalence of *erm*(B) is similarly observed among clinical isolates resistant to macrolides from European countries (229,258). Analogous to the situation with *erm*(A), the prevalence of *erm*(B) is similar among streptogramin-resistant isolates from Danish poultry and pigs (138), as well as those of clinical origin (35,273). Less resistance was observed in a U.S. study of retail poultry products in which *erm*(B) was observed in 41% of streptogramin-resistant isolates (267).

The prevalence of the efflux pump *mef*(A), estimated as high as 22% of U.S. clinical isolates of *Enterococcus* spp. (176), is in sharp contrast to with the absence of *mef*(A) among enterococci isolated from Belgian pork production environments (183). A *mef*-like gene has been observed in a single clinical isolate from Spain (229). The observation that most of the infrequently-encountered *mef*(A)-positive isolates in this study were not phenotypically resistant to macrolide antimicrobials suggests that the gene may not be constitutively expressed.

The *msr*(C) gene was initially described among clinical isolates of *E. faecium* as a likely intrinsic feature of the species (229). Subsequent studies of *msr*(C) confirmed the previous observation that *msr*(C) was conserved among *E. faecium* of clinical origin (270). The gene contributed to a rise in erythromycin MIC by six-fold, but was not solely responsible for macrolide resistance. The gene also did not markedly affect the MIC for lincosamide antimicrobials (270). A study of *E. faecium* from multiple sources in

Germany revealed that the *msr(C)* gene could not be detected in 42% of isolates (315) with a subsequent study indicating its absence in 31% of isolates from poultry (316). The results of the present study (0.8%), in contrast, suggest that the gene is infrequent among isolates from U.S. poultry production environments.

The nucleotidyltransferase *Inu(B)* [previously known as *lin(B)*] was initially described in a clinical isolate of *E. faecium* from France. This genetic element was demonstrated to be on a 240-kb plasmid and parenthetically described as present among all 14 isolates of *E. faecium* of clinical origin that inactivated lincosamides (34). In the time since the publication of this observation, *Inu(B)* has been described in only a single, lincosamide-resistant isolate of group B Streptococcus of clinical origin from Canada (66). The inability to amplify this gene from isolates received by either research group despite exhaustive efforts precludes a description of its frequency among isolates from this study. Future research will hopefully shed light on the validity of these observations.

The initial description of the streptogramin B hydrolase *vgb(A)* among enterococci was in a clinical isolate of streptogramin-resistant *E. faecium* from The Netherlands (137). Since its description, it has been identified in only one other instance; a clinical isolate from France (36). Its absence among isolates in this study is consistent with previous studies that indicate the rarity of this gene among *E. faecium* from diverse environmental sources (138,241,267,315,317).

Known resistance elements that confer streptogramin A resistance to *E. faecium* include the acetyltransferases encoded by the genes *vat(D)* and *vat(E)*. The prevalence of *vat(D)* among isolates of poultry origin from Europe is estimated between 10 to 13% (6,138). The gene has not been detected in a recent survey of resistant isolates from U.S.

retail poultry (267), which is consistent with the results of this study. Among resistant isolates from pigs in European production operations, *vat(D)* has been found among 2-7% of isolates (5,6,138). Comparative studies suggest that in addition to its more frequent isolation from pigs, *vat(D)* also is found more frequently among isolates of human origin (137,273,317). A survey of clinical isolates from Spain, however, suggests that the predominant resistance mechanism may vary by region (241).

Vat(E) has been observed to be more frequently encountered among isolates of poultry origin and accounts for 72 to 100% of streptogramin-resistant poultry isolates from Europe (6,120,138,317). In contrast, *vat(E)* is estimated to be present among only 4 to 7% of resistant-isolates from pigs (5,138). Among resistant isolates from U.S. retail poultry, *vat(E)* was observed in 37% of resistant isolates (267).

The absence of streptogramin A resistance elements previously described among isolates of *E. faecium* has been observed previously. A UK study revealed that neither *vat(D)* nor *vat(E)* could be amplified from streptogramin-resistant isolates from humans and seawater (274). This was similarly observed among resistant isolates from healthy and hospitalized subjects from the U.S (75) and clinical isolates from France (35).

Unidentified mechanisms are also thought to be present in 3 to 29% of isolates from different ecological origins (138,273,317) and 86% of isolates from Danish pigs (138).

Whereas the negative screening results from this study for known acetyltransferases does not exclude the possibility that other acetyltransferases may exist in this collection of *E. faecium*, other studies do not support the contention of acetyltransferase genes similar in sequence to *vat(D)* and *vat(E)* (273). This work did not examine the possibility that the ATP-binding cassette transporter mechanisms that can

confer streptogramin A resistance in staphylococci by active efflux [*vga(A)* and *vga(B)*] may be present in these isolates (10,11). Previous surveys of resistance mechanisms among streptogramin-resistant *E. faecium* that have sought to identify these mechanisms have not proven successful (35,267,273).

The seminal work by Bozdogan and Leclercq suggests that in an isolate resistant to lincosamide and streptogramin A components by an unknown mechanism can be conferred resistance by complementation with streptogramin B [*vgb(A)*] streptogramin A [*vat(D)*] resistance elements (35). It is noteworthy that the quinupristin-dalfopristin MIC was unaffected when this strain was complemented with the MLSb resistance determinant *erm(B)*. In the case of complementation studies with the *vgb(A)*, the mechanism is straightforward. Complementation studies with the acetyltransferase *vat(D)*, however, would suggest that the Vat(D) enzyme, in the presence of an unidentified mechanism, might have some measure of activity against streptogramin B antimicrobials (35). Although the original description of *vat(D)* in *E. faecium* illustrates the high activity of the product against streptogramin A components, the enzyme was also characterized as having a broader spectrum of activity (236).

The recent observations of vancomycin resistance elements of enterococcal origin in U.S. clinical isolates of *S. aureus* suggest that alternative therapies, such as linezolid and Q-D, will be more frequently employed (45,46). Resistance to linezolid has already been observed (16,21,111,139,209,247) and at least in one case without prior exposure (140) which raises concerns about the long-term efficacy of alternatives to antimicrobial chemotherapy. The resistance elements for vancomycin and streptogramin resistance have been reported to be co-localized on the same plasmid of clinical isolates from

France and Germany (36,318). It is troubling that in at least one study that these resistant strains have been found to be genetically identical to susceptible strains that are endemic in the hospital environment and have spread similarly (128,318), possibly due to the documented *in vivo* transfer of resistance in the mammalian gastrointestinal tract (117).

Current estimates of Q-D resistance among *E. faecium* in the U.S. community (75,186) do not currently mirror the European experience of the vancomycin-analogue avoparcin in food animal production establishing a base of resistance in the community (107,127,156,157,260,279,299,300). That small population, however, may be amplified as a result of an increase in selective pressure in the clinical environment.

Although existing evidence does not suggest that enterococci of foodborne origin should be regarded as bacterial pathogens, they could serve as potential reservoirs of virulence and antimicrobial resistance genes to host-adapted strains. The observations of this study suggest that resistant isolates of *Enterococcus faecium* are commonly found within poultry production environments and ostensibly, can contaminate retail meat products. Effective control strategies aimed at reducing the size of the resistant population to which consumers are exposed may become even more significant in the future with the potential increase in the frequency of resistant isolates as human opportunistic pathogens.

Table 7. Primer sequences used for resistance screening

Gene	Primer	Sequence of primer	Reference
<i>erm(A)</i>	ERMAF	TCTAAAAAGCATGTAAAAGAA	(281)
	ERMAR	CTTCGATAGTTTATTAATATTAGT	
<i>erm(B)</i>	ERMBF	CATTTAACGACGAAACTGGC	(273)
	ERMBR	GGAACATCTGTGGTATGGCG	
<i>msr(C)</i>	MSRCF	TATAACAAACCTGCAAGTTC	This study
	MSRCR	CTTCAATTAGTCGATCCATA	
<i>mef(A)</i>	MEFAF	GTTTTATACAATATGGGCAG	This study
	MEFAR	ATTCGTAAGCTGTTCTTCT	
<i>vgb(A)</i>	VGBF	ACCATATGGTATAACCGTTT	This study
	VGBR	GTATTCACGAATTTTACCGT	
<i>lnu(B)</i>	LNUBF	CCTACCTATTGTTTGTGGAA	(34)
	LNUBR	ATAACGTTACTCTCCATTC	
<i>vat(D)</i>	VATDF	GCTCAATAGGACCAGGTGTA	(273)
	VATDR	TCCAGCTAACATGTATGGCG	
<i>vat(E)</i>	VATEF	ACTATACCTGACGCAAATGC	(273)
	VATER	GGTTCAAATCTTGGTCCG	

Table 8. PCR reactions used for resistance screening

Gene	# Cycles	Temperature (°C)	Time (min.)	Reference
<i>erm(A)</i>	1	95	10 min	(281)
	35	94	1	
		52	1	
		72	1	
		72	5	
<i>erm(B)</i>	1	95	10 min	(273)
	25	94	1	
		52	1	
		72	1	
		72	10	
<i>msr(C)</i>	1	95	10 min	This study
	30	94	1	
		52	1	
		72	1	
		72	72	
<i>mef(A)</i>	1	95	10 min	This study
	30	94	1	
		52	1	
		72	1	
		72	10	
<i>vgb(A)</i>	1	95	10 min	This study
	30	94	1	
		52	1	
		72	1	
		72	10	
<i>vat(D)</i>	1	95	10 min	(273)
	35	94	1	
		55	1	
		72	1	
		72	10	
<i>vat(E)</i>	1	95	10	(273)
	30	94	25 sec	
		52	40 sec	
		72	50 sec	
		72	6	

Table 9. Control strains used for resistance screening

Control Strain	Gene ^a	Source
<i>E. faecium</i> CVM3480	<i>erm(A)</i>	Retail chicken rinse
<i>E. faecium</i> CVM3002	<i>erm(B)</i> , <i>vat(E)</i>	Human stool
<i>E. faecium</i> TX1330 SE34	<i>msr(C)</i>	Human clinical
<i>E. coli</i> Sp970264/pMR970	<i>mef(A)</i>	Human clinical ^b
<i>S. aureus</i> BM3093/pIP680	<i>vgb(A)</i>	Human clinical
<i>E. faecalis</i> JH2-2/1025	<i>lnu(B)</i>	Human clinical ^c
<i>E. coli</i> DH10B/pVMM25	<i>lnu(B)</i>	Human clinical ^c
Group B Streptococcus R132	<i>lnu(B)</i>	Human clinical
<i>E. faecium</i> CVM3001	<i>vat(D)</i>	Human stool
<i>E. faecium</i> CVM3475	<i>erm(A)</i> , <i>vat(E)</i>	Retail Chicken Rinse
<i>E. faecium</i> CVM3976	<i>erm(B)</i>	Retail Chicken Rinse
<i>E. faecium</i> CVM4761	<i>erm(B)</i> , <i>msr(C)</i>	Human stool – hospitalized patient
<i>E. faecium</i> CVM18769	<i>msr(C)</i>	Human stool

^a The presence of all indicated genes was confirmed with PCR with the exception of *lnu(B)* positive control organisms.

^b Transconjugant of original strain *Enterococcus* spp. 102.

^c Transconjugant of original strain *Enterococcus faecium* HM1025.

Figure 30. Photo of the Riboprinter® microbial characterization system



Figure 31. Ribogroups of *E. faecium* from the poultry production environment

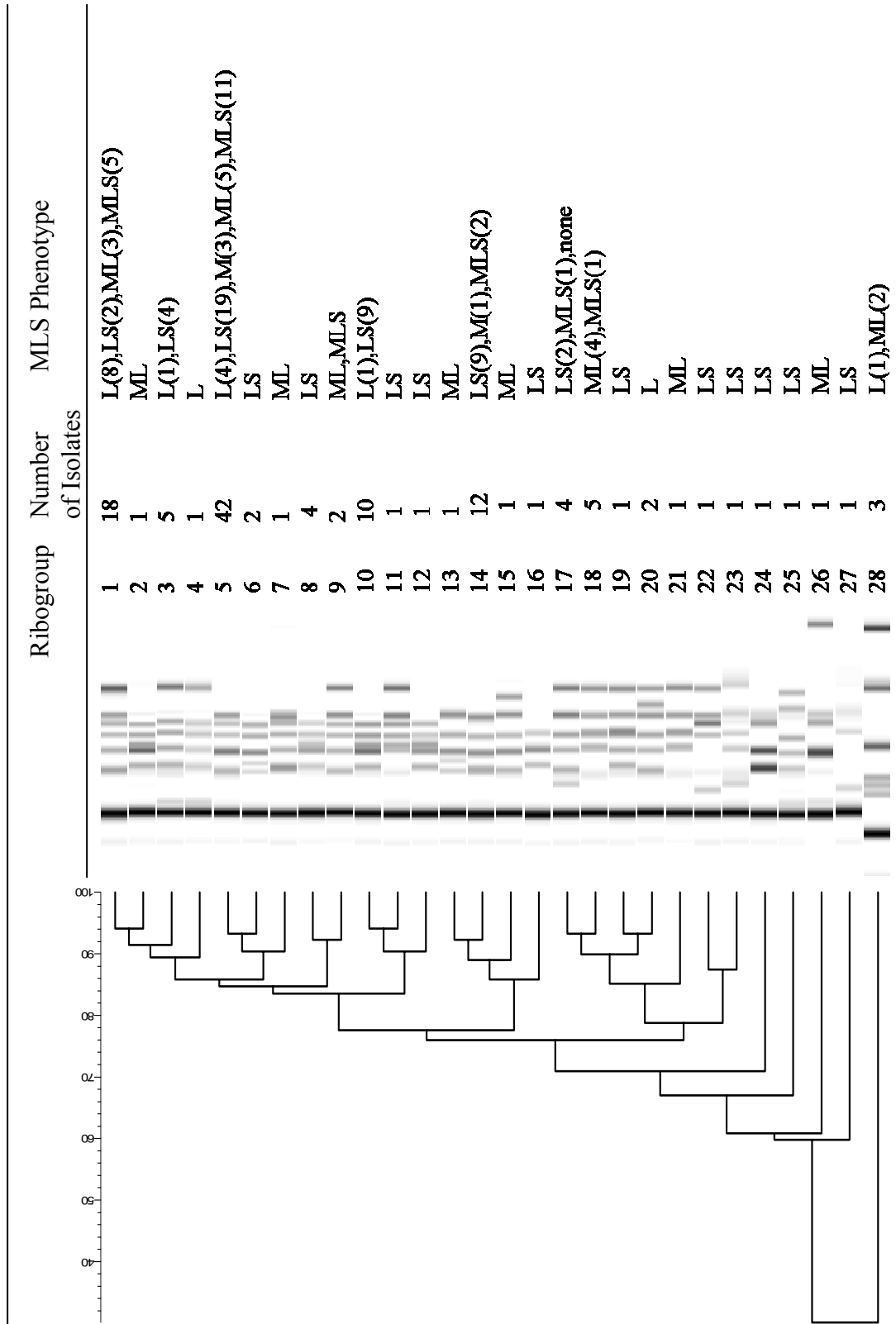
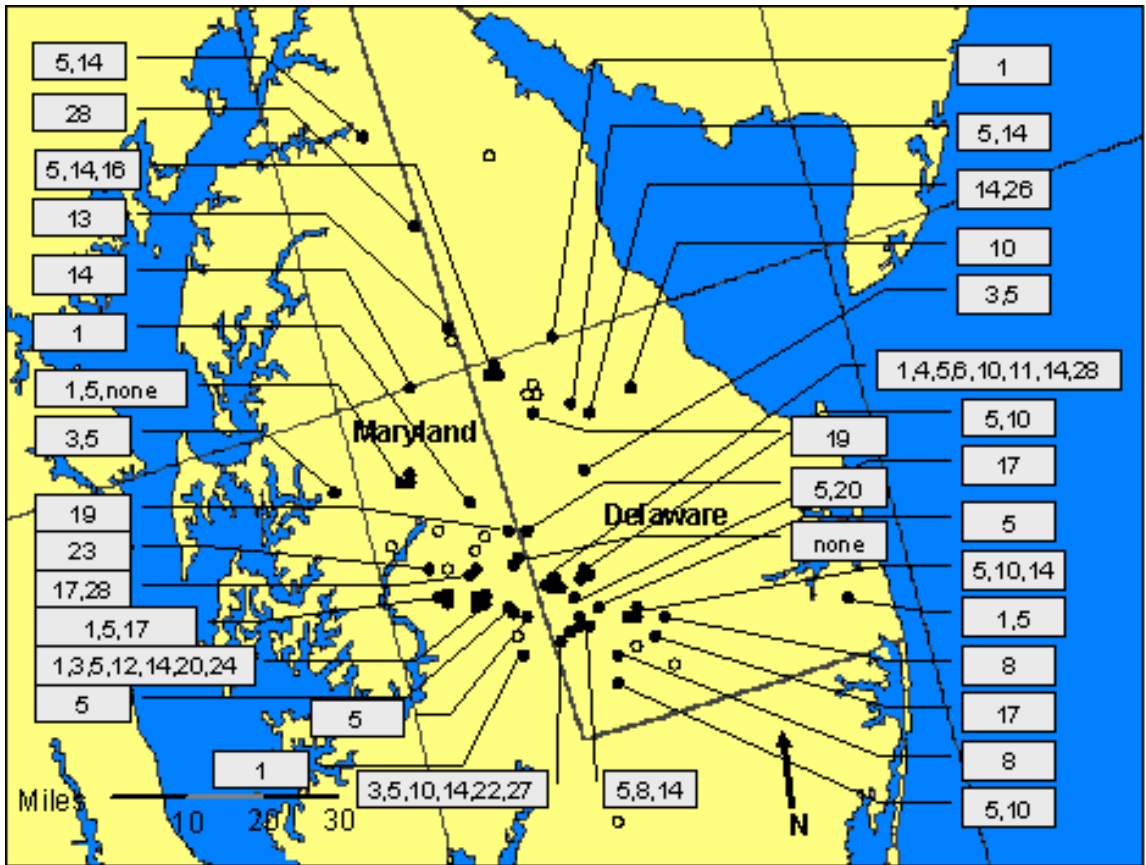
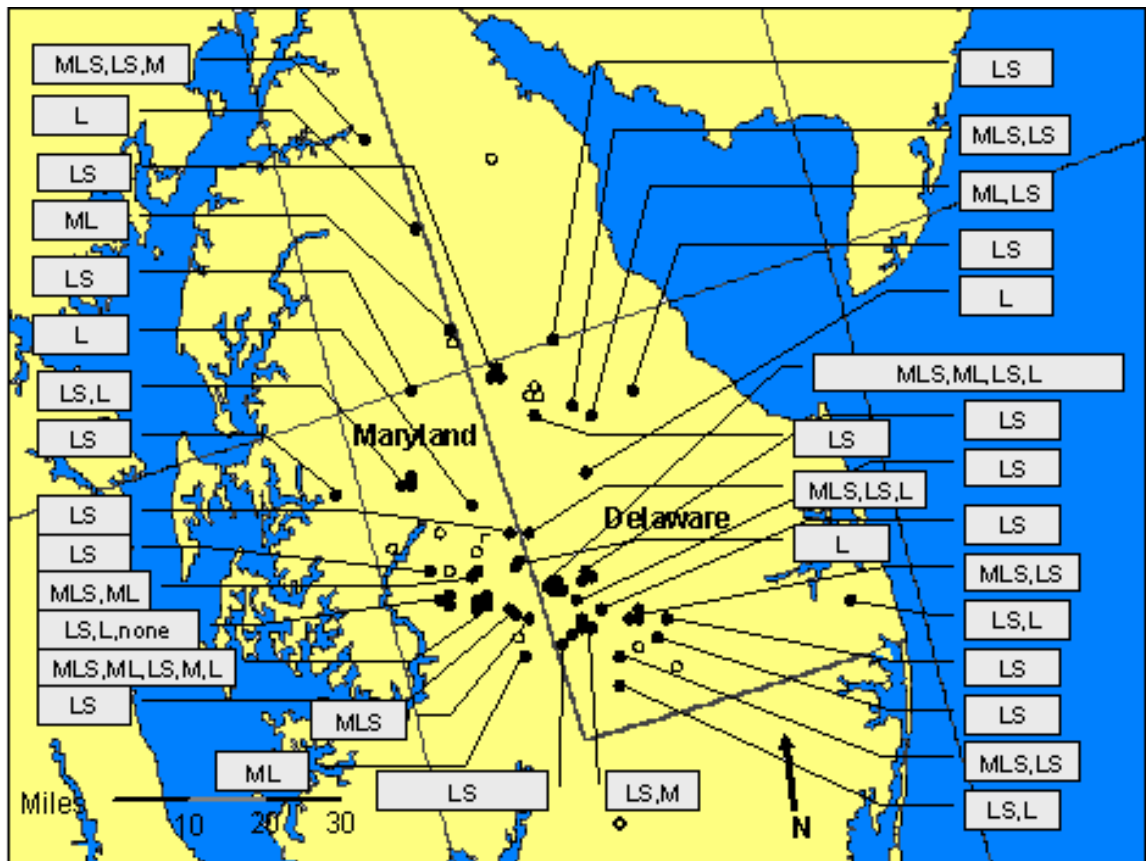


Figure 32. Dispersion of ribogroups of *E. faecium* among sampled commercial poultry houses on the Delmarva Peninsula



Open circles represent sampled houses whose samples did not yield *E. faecium*.

Figure 33. Dispersion of MLS resistance phenotypes of *E. faecium* among sampled commercial poultry houses on the Delmarva Peninsula



Open circles represent sampled houses whose samples did not yield *E. faecium*.

Table 10. MLS phenotypic and molecular profiles of *E. faecium* from the poultry production environment

Resistance phenotype ^a	Number of isolates (%)	Number of isolates (%) with the resistance element ^b						Riboprint patterns	
		<i>erm(A)</i>	<i>erm(B)</i>	<i>mst(C)</i>	<i>mef(A)</i>	<i>vgb(A)</i>	<i>vat(D)</i>		<i>vat(E)</i>
None	1 (0.8)	0	0	0	0	0	0	0	17
M	4 (3.1)	2 (1.6)	0	0	0	0	0	0	5,14
L	21 (16.5)	0	0	0	1 (0.8)	0	0	0	1,3,4,5,10,20,28,none
S	0	-	-	-	-	-	-	-	-
ML	21 (16.5)	1 (0.8)	10 (48)	1 (0.8)	0	0	0	0	1,2,5,7,9,13,15,18,21,26,28
MS	0	-	-	-	-	-	-	-	-
LS	59 (46.5)	1 (0.8)	4 (3.1)	0	3 (2.4)	0	0	0	1,3,5,6,10-12,14,16,17,19,
MLS	21 (16.5)	4 (3.1)	4 (3.1)	0	2 (1.6)	0	0	0	22-25,27,none
Total	127 (100)	8 (6.3)	18 (14.2)	1 (0.8)	6 (4.7)	0	0	0	

^a Resistance phenotype was defined as >4 µg/ml for erythromycin (M), ≥16 µg/ml for lincomycin (L), and ≥4 µg/ml for quinupristin-dalfopristin (S). With the exception of lincomycin, NCCLS interpretive criteria were used (44).

^b No isolate was observed to harbor more than a single resistance element. QC strains (Table 9) performed as expected.

GENERAL DISCUSSION

The clinical significance of enterococci is sometimes difficult to interpret because, in this setting, they are often part of a mixed microbiota in a number of pathologic conditions, and often, they are considered contaminants rather than true pathogens. However, the increased prevalence of nosocomial urinary tract infections and bacteremia in recent times has clearly demonstrated that these organisms are unmistakably pathogenic in these environments. The most important clinical feature of enterococcal infections is the high prevalence of resistance to numerous antimicrobials. Enterococci are known to be intrinsically resistant or tolerant to many antimicrobials, with *E. faecium* more often resistant than *E. faecalis* (193,286). Resistance to high-level aminoglycosides in the 1970s was soon followed by resistance to β -lactam antimicrobials, narrowing the therapeutic choices for treating enterococcal infections. This restriction of treatment options resulted in the increased and frequently unnecessary reliance upon the vanguard of formulary, the glycopeptide vancomycin. The initial description of vancomycin-resistant enterococci (VRE) infections in 1986 was followed by a twenty-fold increase in VRE infections from 1989 to 1993 in the U.S. (43). Currently VRE constitute 26% of nosocomial enterococcal isolates from U.S. intensive care units (44).

Whereas the transmission of enterococci in the clinical environment stems from fecal contamination of hospital surfaces, the most common vehicle of exposure of healthy persons is thought to be the consumption of contaminated food. Increased concern about the continued efficacy of current antimicrobials has led to a tremendous amount of

interest in the impact of the use of antimicrobials in food animal production on the future efficacy of treating enterococcal infections. One of the major limitations in assessing the risk of enterococci from food animals has been the understanding the species diversity present in production and processing environments. Among these species, the magnitude of antimicrobial resistance to drugs used in agriculture and clinical environments in *Enterococcus* spp. in the U.S. is largely unknown. Whereas few studies have estimated the degree of resistance beyond a single farm, it is critically important to sample a wider geographic area in an attempt to evaluate the prevalence of resistance. Public concern over the use of the streptogramin antimicrobial virginiamycin in poultry and other food animal production environments following the approval of quinupristin-dalfopristin to treat resistant human *E. faecium* infections underscores the importance in understanding the genetic background behind the phenotypic resistance observed to MLS antimicrobials.

The first area of study in this dissertation focused on the isolation and identification of *Enterococcus* spp. from the poultry production and processing environments. Using an experimental medium designed to isolate staphylococci as well as enterococci, isolates were successfully recovered from poultry environments on the Delmarva Peninsula. This work demonstrated that a more selective protocol would reduce the amount of time and resources spent working on non-enterococcal isolates as well as increasing the successful yield of enterococci from collected samples. Previous work has suggested that a resuscitation step in the isolation procedure followed by the use of an elevated incubation temperature may improve the recovery of stressed or injured enterococci from the environment (92). This modification would improve the recovery

of enterococci from diverse environmental samples through the suppression of other resident microflora. One disadvantage to this application is that despite the description of members of *Enterococcus* spp. as being able to grow at temperatures of 45°C, such thermotolerance may not be a consistent trait among all species of enterococci, especially in combination with a stressful selective medium. Both observations have been observed in preparatory studies conducted at the U.S. FDA Center for Veterinary Medicine, Laurel, MD. A recent survey has demonstrated that the incorporation of these changes in isolation procedures demonstrates the nearly ubiquitous distribution of enterococci from retail meat products (123). This work has detailed the similar presence of *Enterococcus* spp. from the production and processing environments across a wide geographic area. One proven disadvantage of the drive to develop methods to quickly screen for unique enterococci is that the species diversity from a given sample may be underestimated. Following growth on a solid selective medium, bacterial colonies that appear to be homogeneous may, after further incubation for up to 72h at room temperature, develop distinctive colonial morphotypes (231). This modification has also been demonstrated to have clinical relevance as up to 15% of VRE may be unrecognized with a shorter term of incubation (248).

Essential to the characterization of isolates was the adaptation of a biochemical methodology for identification of *Enterococcus* to the species level. This test was demonstrated to be equally capable as traditional biochemical assays, utilizing the most discriminatory assays of carbohydrate and amino acid utilization. This had the effect of reducing the total number of biochemical assays needed to achieve reasonable certainty of identification based on the knowledge of current species. The relative prevalence of

Enterococcus spp. described in this work confirms observations made in other countries as well as limited studies that have been conducted in the U.S. The predominance of *E. faecalis* and *E. faecium* species of the enterococcal microflora from poultry production and processing environments described in this work are notably the same species that are responsible for most enterococcal disease in humans.

Since the conclusion of this phase of the project, a number of new species of enterococci have been described. Given the similarity of these newly described species to more well-known members of the genus, confident identification to the species level may be questionable without confirmatory molecular screening (71). The increased use of molecular techniques to characterize the microbial ecosystems have demonstrated their utility in identifying small, distinct populations of enterococci that would otherwise not have been isolated without a series of species-optimized isolation protocols (242). In fact, enterococci have been estimated to account for only 7% of the microflora of poultry litter through the application of 16S rDNA sequence analysis (175). Unfortunately, a snapshot of the microbial diversity of a given sample, while valuable, provides a limited amount of information compared with the questions that can be sated by the analysis of a collection of bacterial isolates.

The second area of study focused on the susceptibility of the recovered enterococcal population to a number of antimicrobial agents. This work demonstrated the magnitude of resistance to a number of antimicrobials that are used in clinical medicine or in agriculture. Among the isolated enterococcal species, resistance was observed to multiple antimicrobials among a significant proportion of isolates. The prevalence of resistance to production drugs was predictably higher among *E. faecalis*

and *E. faecium* from poultry production and processing environment across the geographic area of the Delmarva Peninsula. The relative paucity of resistance among enterococci from this environment to clinical drugs that have been approved in veterinary medicine should be reassuring, however, the detection of any resistance in this environment is unsettling. The fact that the enterococci from this work were not isolated with the addition of selective pressure from an incorporated antimicrobial suggests that an increase in resistance prevalence would be observed. The presence of a reservoir of antimicrobial resistance, such as antibiotic-resistant enterococci evokes concern, given the renowned ability of enterococci to acquire genetic elements that confer resistance to antimicrobials and to spread those elements to other genera (287).

Of significant relief to both opponents and advocates of antimicrobial use in food animal production was the absence of resistance to vancomycin. This observation strengthens the argument that enterococcal resistance to vancomycin in the clinical environment is due to the use of vancomycin in that environment. On the other hand, resistance to quinupristin-dalfopristin among isolates of *E. faecium* of food animal origin is widely prevalent, especially among poultry, which is reflected in this work. An increased prevalence is of no surprise given the length of time that the analogue virginiamycin has been used in poultry production. The high prevalence of this phenotype among *E. faecium* from poultry would suggest that the community is likely to already be seeded with resistant isolates (123). Glycopeptide or streptogramin-resistant *E. faecium* from meat sources have been demonstrated to survive gastric passage and multiply, with subsequent isolation from feces up to two weeks following ingestion by healthy human volunteers (275). Reports from German health agencies, however,

suggest that human carriage of Q-D-resistant *E. faecium* occurs at a rate of 14% (317) which is significantly higher than the estimates of 0 to 1% in the U.S. (186).

The discrepancy in the between European and U.S. observations of the prevalence of resistance to this antimicrobial may be due to several factors. One possibility is that the resistant *E. faecium* strains in Europe have also acquired virulence determinants that allow them to be better adapted to a human host, whereas adapted U.S. strains have prevented detectable colonization of the gut. Another possibility is that the outpatient populations sampled between the two studies had significantly different eating habits and presumably different levels of exposure to resistant strains. This suggestion has some credence, because diet has been shown to greatly impact the prevalence of resistant enterococci in the intestines of humans whose food consumption varies between carnivorous and vegetarian diets (260). Differences in isolation methodology make the comparison of the two studies difficult and may have also affected the observed prevalence of comparative resistance between the two studies. In particular, the choice of selective media, incubation temperature, and number of isolates may have prevented a better estimate of the true prevalence of QDREF in feces from healthy members of the community.

Few studies have documented that Q-D resistance among vancomycin-resistant *E. faecium* isolates is a problem in the clinical setting, however if the lessons with VRE are not heeded with newer antimicrobials, streptogramin-resistant *E. faecium* may be poised to emerge as a significant nosocomial pathogen. Increased usage of Q-D in the hospital setting to treat VREF would prompt the emergence of clones of streptogramin-resistant *E. faecium* and would only spur the increased use of vancomycin, in turn further

shortening the long-term efficacy of both drugs. For these reasons, the U.S. medical community may be near the brink of losing another drug in their arsenal due to two risk factors: increase in community carriage rates from protracted use in agriculture and the standard overuse of antimicrobial agents in U.S. hospitals. The results of this work establish a comparative baseline of resistance among *Enterococcus* spp. from poultry environments to many antimicrobials of use in human and veterinary medicine. This information is vital for comparison in future studies that monitor the dynamics of resistance in or from this environment.

The medical significance of antimicrobial resistance upon the virulence of enterococci is largely unknown. Whereas increased mortality has been established among VREF compared with susceptible isolates in cases of similar severity (280), other studies have not confirmed these findings (108,168). This variation may be due to differences in compensatory mechanisms among strains responsible for outbreaks that reduce the fitness cost of resistance to the bacterial host (60). Given that some virulence and resistance mechanisms in enterococci have been demonstrated to be similarly regulated, the regulatory mechanisms may hold some promise as therapeutic targets (277). Until demonstrated otherwise, the question will remain as to whether the acquisition of a resistant bacterium increases the severity of a patient's illness or whether the severity of an illness predisposes a patient to the acquisition of a resistant bacterium.

The third area of this study focused on the genetic background behind the observed resistance to macrolide, lincosamide, and streptogramin antimicrobials. These three classes of antimicrobials are widely used in poultry production in the U.S. and present a unique situation with regard to antimicrobial resistance among enterococci, i.e.,

the use of any one of these drugs can select for resistance to the others in animal agriculture. This work is the first to examine in a single study all known MLS resistance mechanisms that have been described to date. This information is critical to begin to evaluate the potential risk that the use of related antimicrobials in animal agriculture poses to the treatment of disease among the human population. Among isolates phenotypically resistant to macrolide, lincosamide, or streptogramin antimicrobials, it was demonstrated in this work that a significant proportion possess uncharacterized mechanisms of resistance. As previously discussed, the absence of these resistance elements has been previously described among isolates of *E. faecium*. These results are at variance with the observations from Denmark, in which the presence of the acetyltransferases *vat(D)* and *vat(E)* accounted for most Q-D resistance in poultry, but is similar to the predominance of observations among isolates with unknown mechanisms from pigs (138). Thus, this work should be seen in the context of the urgent need for science-based risk assessments, focusing on the relative risks concerning use of antimicrobials in animal husbandry in the U.S.

Based on the current understanding of how MLS antimicrobials interact with the A2058 residue of 23S rRNA of the 50S subunit as described earlier, it is likely that the mechanism(s) that confer resistance among the 48% of isolates that had a ML resistance phenotype in this work do so by target site modification, most likely due to methylation of that site by an unknown mechanism. The comparatively high prevalence of lincosamide resistance may be due to the activity of an undetermined efflux pump or major facilitator protein that has specificity for such moieties. For example, Lmr(A) is an efflux pump that confers resistance to lincosamide antimicrobials, but has only been

isolated from *Streptomyces lincolnensis* (334). Major facilitator mechanisms like Msr(A) demonstrate macrolide specificity and confers resistance to 14-member macrolides and streptogramin B components, but not lincosamide and 16-member macrolides among *S. epidermidis* (245). Given this resistance profile, the presence of *msr*(A) among isolates in this work is possible in 3% (4/127) of *E. faecium*, assuming that the transporter behaves similarly among *Enterococcus* spp. Alternatively, lincosamide resistance may be due to inactivation of the antimicrobial, possibly due to the activity of a nucleotidyltransferase. Resistance to streptogramin antimicrobials may be similarly mediated by efflux or inactivation mechanisms. The results of prior surveys suggest that resistance to streptogramin A components is unlikely due to the activity of acetyltransferases that are common among *Staphylococcus* spp., but cannot be excluded as a possibility (267,273). Unpublished reports have suggested that these alternative mechanisms of streptogramin resistance are mobile in nature (319).

Mutation of the target site of MLS antimicrobials may be a possible mechanism of resistance among these isolates. Isolates that possess this or closely related mutations may not be phenotypically resistant to MLS antimicrobials until induced. It remains entirely possible that a number of *E. faecium* isolates from this work may possess this mutation and remain inducibly resistant, however this phenotype quickly becomes constitutive after exposure to inducing antimicrobials. The presumed frequency of use of inducing antimicrobials in the U.S. poultry industry would likely be sufficient to facilitate this switch. Because only 17% of *E. faecium* isolates in this study possessed a MLS resistance phenotype and were widely distributed across the sampling area, it is unlikely that this is a common trait.

This work also demonstrated the utility of ribotyping in helping to distinguish genetically similar clusters of *E. faecium*. Whereas the method is not as robust as pulsed-field gel electrophoresis (PFGE), it did successfully illustrate the diversity of recovered *E. faecium* isolates with relation to their phenotypic resistance to MLS antimicrobials. Ribotype analysis also illustrated the predominance of a few related ribotypes across the entire geographic region sampled. This would suggest that these bacterial populations are common among farms because these populations of *E. faecium* are either naturally predominant in poultry or that the strains have been disseminated widely across this region through the operation of the widely integrated poultry industry. Ribotyping could be very useful as a rapid screening method for investigating genetic relationships among isolates from diverse environments and for identifying strains of particular epidemiologic relevance. Databases of important ribotypes could be constructed to allow remote identification and tracking of these clones longitudinally and on a worldwide scale.

Future work should continue to investigate the mechanisms behind the large degree of uncharacterized resistance to MLS antimicrobials. The degree to which these mechanisms exist among resistant isolates from community and clinical sources remains an important question. These mechanisms may very well persist in streptogramin-resistant isolates that also possess acetyltransferases due to *vat(D)* or *vat(E)* genes. Given the nature of the spread of antimicrobial resistance among gram-positive organisms that are widely distributed in the environment, the presence of multiple mechanisms may very well pose multiple threats to the long-term efficacy of streptogramin antimicrobials. The potential spread of these mechanisms from poultry or other production environments through consumption of contaminated meat may establish a reservoir of resistance in the

community. Whereas this resistant population or resistance elements may not rise to a measurable level, the stage may be set for the emergence of resistance following the application of selective pressure. This would be especially important if the trends of Q-D usage increase due to the diminished effectiveness of alternative therapeutics.

Molecular technologies to monitor antimicrobial resistance will be critical tools to monitor the epidemiology of specific resistance elements. Microelectronic chip technology has proved to be a promising venue for such applications and has been shown to possess advantages over traditional procedures in terms of time and cost savings in the identification of bacteria in addition to the characterization of molecular resistance determinants (320). This technology can also be adapted for use in metagenomics to establish the reservoirs of resistance genes among the fecal bacterial microflora that have heretofore been marginalized by either their low prevalence or virulence in animal or human hosts. This knowledge could conceivably be utilized to model and direct intervention strategies against these carrier populations with the intent to minimize the development of an unfavorable population dynamic that would favor the emergence of resistance.

Experiences of other countries yield important information about what changes might be expected with a restricted use of antibiotics. Models for monitoring the effect of the withdrawal of antimicrobials used as growth promotants strongly link the ban of these drugs to the decrease in prevalence of erythromycin and virginiamycin resistance among *E. faecium* isolates from poultry meat in Denmark, as well as Germany (87,156). Despite the optimism that the removal of production drugs like avoparcin from animal agriculture will eradicate the presence of resistant isolates in animals, animal products,

and the healthy human population (298), observations of a decreased but stable prevalence of resistant isolates among bacterial populations suggests that resistance may persist, albeit in lower numbers (7,250). Among isolates of *E. faecium* from Danish broilers, the prevalence of resistance has moderated with stabilization of resistance at 13% for erythromycin and 34% for virginiamycin (7,17). Vancomycin resistance was observed to quickly decrease from 73% among broiler isolates before the avoparcin ban to 43% in the first year without the use of avoparcin. The prevalence of VREF from these environments has apparently stabilized at 6%.

Amid a storm of public controversy concerning the use of antimicrobials in agriculture, the EU in 1999 banned the use of all antimicrobials employed as growth promotants. Whereas a consistent picture of the effect of this ban has yet to emerge, there is evidence to suggest that food animal welfare has suffered. In pigs, increased morbidity and mortality attributable to enteric infections have been increasingly frequent (42). The result of the increased frequency of diarrhea as well as chronic infections due to *Lawsonia intracellularis* in Denmark and Spain has been a decrease in weight gain and increased mortality. Necrotic enteritis due to clostridial infections have increased among poultry from Denmark and France. In both environments, the use of therapeutic antimicrobials to treat food animals has substantially increased (200,228). A pilot study of organic farm practices in Denmark suggest that an increase in the carriage rate of human pathogens among food animals may be an unintended consequence of removing antimicrobials from food animal production (125). Recent reports from Denmark have observed the opposite response with a decrease in *Campylobacter* and *Salmonella* spp. in poultry and pork products (91). The potential for increased bacterial contamination of

meat products by human pathogens is also of concern in the processing of the food animal, owing largely to a more diverse range of sizes and weights of incoming animals for slaughter. This can realistically be expected to occur at the processing facility due to the high speed and largely automated sequence of animal processing and the increased heterogeneity of food animals raised without the use of antimicrobial agents to prophylactically treat animal disease. Thus, a balance between the interests of the management of resistant human food-borne infections and the frequency of infections may be required.

Improvements in farm management practices are increasingly important areas of research in order to compensate for the potential removal of antimicrobials to control food-animal infections (74). Improvements in sanitation, pest control, and litter management would help to reduce the exposure of food animals to pathogens. Enhancements such as the use and proper maintenance of watering and air handling systems and improved temperature control are also important. Development of methods to augment the immune response of food animals are also very viable means of enhancing the resistance of food animals to disease. This is likely to come in the form of vaccines and improved vaccine delivery vehicles as well as modulation of the immune response by the application of specific antibodies and augmenting cytokine response of food animals. The most robust area of research is currently in the modification of nutritional regimens and additives. The most active areas of research include the addition of enzymes to assist in feed digestion and organic acids to control intestinal pathogens, as well as other means of supplementation or modification of feedstuffs. Other strategies that have yet to see widespread acceptance and use include supplementation with

probiotic and bacteriophage preparations. It is expected that a combination of efforts would be required by food animal production industries to compensate for the losses of weight gain and feed efficiency that antimicrobials currently provide.

Despite the frequency of antimicrobial resistance observed among enterococci from this work, the burden of managing the problem should not fall solely on the shoulders of agricultural industry. Significant improvements in the prudent use of antimicrobials in the clinical environments can and should be made. These include the proper prescription of antimicrobial therapy to treat a bacterial infection, improved sanitation in medical facilities, and the proper education of consumers of medical services. This is especially relevant with the control of VRE in hospital settings, as the use of vancomycin is a strong predictor for the emergence of resistance (32,147,172,246).

A rather neglected area of research is the downstream effects of antimicrobial usage in animal agriculture as well as human medicine. Many antimicrobials used in the food animal production environment are poorly absorbed in the gut. This property makes them excellent additives to animal feed for the sake of avoiding detectable tissue residues, but this also means that a significant portion of the active agent is excreted into the environment with as much as 75% of antibiotics administered in feedlot animals found unaltered in feces (85). Given the variability in production practices, specifically litter management, the concentration of antimicrobials in litter from successive flocks being fed supplemented feed rations may accumulate to a significant level (166). Virginiamycin, in particular, possesses a long biodegradation half-life in different soils (309). Use of litter as fertilizer for produce could potentially create selective pressure that might affect the bacterial flora of the product. Indeed, the influence of antimicrobial

usage in integrated fish farming has been significant, with the increased prevalence of species from broilers and their resistance profiles found among overseas integrated fish operations (226,227). Alternatively, the antimicrobials, bacteria, and resistance determinants may be transported from the surface application of litter and find their way into various environments through groundwater (49,166). The comparison of results from areas proximate to poultry production environments to those established in this work would be of great interest.

Rising levels of resistance to multiple antimicrobials dictates the frequent and close monitoring of resistance in bacterial pathogens in both clinical and agricultural environments in the U.S. and abroad. Without this measure of surveillance, the management of this problem on a piecemeal basis could very well result in further waning of the effectiveness of antimicrobials and additionally lead to the reduction of the numbers of antimicrobials available to treat human infections. Additionally, surveillance is valuable to closely monitor the response of microbial populations to drugs that recently have or are soon to be introduced into clinical medicine (41,153,234) as well as the effectiveness of new antimicrobial regimens (276). The increase in public concern has led to the ban of growth promoting antimicrobials in the EU based on the perceived risk rather than clear scientific evidence (230). Failure to exercise continuing, efficient and sound scientific judgment in search of a means to reduce antibiotic resistance could, perhaps unalterably and regrettably, lead to implementation of a similar policy in the U.S.

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