MOLECULAR ANALYSIS OF *ENTEROCOCCUS* SPP. IN A POULTRY ENVIRONMENT

by

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(Under the Direction of David Suarez)

ABSTRACT

Enterococci may be reservoirs of antibiotic resistance, and it is important to characterize the strains isolated from animals and their environments. This study analyzed enterococci from four poultry houses for six growouts on a farm. Two houses on the farm were control houses and did not use any antimicrobials while two other houses on each farm used flavomycin, virginiamycin, and bacitracin during different poultry growouts. Litter, chick boxliners, feed, and poultry carcasses were obtained from each house and cultured for the presence of enterococci. Enterococci species were identified using a species-specific multiplex-PCR. Vitek, a commercial culture typing system, was also used a confirmatory procedure. Additionally, *Enterococcus faecium* isolates were further characterized using BOX-PCR and Pulsed- Field Gel Electrophoresis (PFGE). *Enterococcus faecalis* (n=763; 52%) and *E. faecium* (n=578; 40%), were isolated most often from the farm and houses regardless of antimicrobial treatment. *Enterococcus faecium* analyzed by BOX-PCR and PFGE appeared to be genetically different as few clusters were observed.

INDEX WORDS: Enterococcus, PFGE, BOX-PCR, virginiamycin, poultry

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DEDICATION

I would like to dedicate this to my parents, William and Louise Debnam, for 23 years of love, support, sacrifice, and prayers.

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TABLE OF CONTENTS

Chapter 1

Introduction

Literature Review

The genus *Enterococcus* is defined as gram-positive, facultative anaerobic organisms that are ovoid in shape and which may appear in short chains, pairs or as single cells. The enterococci were originally classified in the genus *Streptococcus* until the 1930's when they were placed into the Group D streptococci (51). The formal genus was not established until 1984 as a result of additional tests using nucleic acid relatedness. Schleifer and Kilpper-Bälz used DNA-DNA hybridization and DNA-rRNA hybridization studies to show that *Streptococcus faecalis* and *Streptococcus faecium* were so distantly related from other streptococci that they should be placed in their own genus (61). Furthermore, a joint effort of Colins, Jones, Kilpper-Bälz, and Schleifer examined more streptococci using nucleic acid techniques (51). The species were *Streptococcus avium*, *Streptococcus casseliflavus*, *Streptococcus durans*, *Streptococcus malodoratus*, and *Streptococcus gallinarum*. Results indicated that all the organisms were distinct enough for separate species, but should collectively be placed under the genus *Enterococcus* (11). The previously mentioned studies were published in the Bergey's manual in 1984 and subsequently paved the way for the incorporation of *Enterococcus* as a formal genus (51). Presently, DNA-DNA reassociation values, 16S rRNA gene sequencing, whole-cell protein analysis, and conventional phenotypic tests are the standards used to evaluate inclusion

into the genus *Enterococcus* (69). *Enterococcus faecium* and *Enterococcus faecalis* were the first species to be placed in the genus; since then, 25 other species have been proposed for inclusion (24). The majority of enterococci can be identified to species level by conventional identification techniques including morphological characteristics, motility, and pigmentation (51). Two species of enterococci are motile, *Enterococcus gallinarum* and *Enterococcus casseliflavus* (74). *Enterococcus casseliflavus* is also one of the two yellow- pigmented enterococci of clinical significance to humans; the other is *Enterococcus mundtii* (74). The other three yellow pigmented enterococci are *Enterococcus sulfureus*, *Enterococcus gilvus*, and *Enterococcus pallens*; the last two were recently identified in 2002 (74). Enterococci are ubiquitous as they are natural commensals of humans and animals. They are predominantly inhabitants of the gastrointestinal tract, but have also been found in the genitourinary tract and the oral cavity of healthy individuals (69). Furthermore, enterococci are also found in soil, water, and insects (51,69). Historically, their unique features have been taken advantage of by using them for fermentation of foods. Their role in ripening, flavor development, and metabolic traits have been proposed as part of a defined starter culture for different European cheeses (22). As probiotics, enterococci have been used in the management of gastrointestinal illness (20). Different sources are associated with certain species. For example, plants are usually associated with *E. casseliflavus*, *E. mundtii*, and *E. sulfureus,* while water and insects are associated with *E. faecium* and *E. faecalis* (24,46).

The optimum growth temperature for enterococci is 10 to 45 ^{0} Celsius, but enterococci have been documented to survive at 60^{0}C for 30 minutes (62,63). Furthermore, enterococci have the ability to hydrolyze esculin in the presence of 40% bile and grow in the presence of 6.5% NaCl at pH 9.6; the pH profile of enterococci is broad, ranging from pH 5 to 11 (36).

Enterococci have the ability to hydrolyze pyrrolidonyl-B-napthylamide (PYR) with the exception of *Enterococcus cecorum*, *Enterococcus saccharolyticus*, *Enterococcus columbae,* and *Enterococcus pallens* (24). Additionally, enterococci hydrolyze leucine-β–napthylamide by producing leucin aminopeptidase (LAPase) (51,69). Another feature that distinguishes enterococci is their resistance to desiccation and several antiseptics including carbolic acid and chloroform (25).

IDENTIFICATION

In a clinical setting, enterococci are identified to the genus level by a combination of morphological and culture characteristics. These characteristics include gram-positive cocci that are catalase-negative, hydrolyze pyrrolidonyl-B-napthylamide (PYR), will react with Group D antisera, have the ability to hydrolyze esculin in the presence of bile, and grow in the presence of 6.5% NaCl at 45^0C, pH 9.6 (51). However, identifying enterococci in this manner is becoming difficult because these characteristics cannot unequivocally distinguish enterococci from other gram-positive, catalase-negative, coccus-shaped bacteria. For instance, *E. faecalis* will produce a pseudocatalase and appear weakly catalase-positive (51). In addition, PYR hydrolysis is also characteristic of Group A streptococci (51). *Enterococcus asini* has phenotypic traits that are not consistent with any of the previously known species (12). *E. asini* does not grow in the presence of 6.5% NaCl, and does not exhibit some of known biochemical characteristics such as mannitol, sorbose, sorbitol, raffinose, and ribose fermentation. However, 16S rRNA gene sequence analysis placed this proposed organism into the *Enterococcus* genus being closely related to *E. faecium* and *E. faecalis* (12).

Increased difficulties exist when identifying enterococci to the species level. The traditional Facklam scheme of grouping *Enterococcus* separates them into five groups on the basis of acid formation in mannitol and sorbose broths and hydrolysis of arginine (24). Identification of species in this manner is not rapid and may require incubation of the tests up to 10 days, although most interpretations can be made after two days (51). Clinical laboratories also utilize commercially available kits such as API 20S (Analytab products, Plainview, NY), the GPI (gram-positive identification) system (Vitek Systems,Inc., Hazel Wood, MO) and the BBL Crystal Rapid Gram-Positive ID Kit (BD Bioscience, Cockeysville, MD) (80). These systems, which are also biochemically based, can give rise to errors in the identification of the enterococci and other bacterial species such as *Lactococcus garvieae, Lactococcus lactis*, and *Vagococcus fluvialis* (26). Although these are less frequently encountered bacteria, they have some of the same characteristics of enterococci and are commonly misidentified as enterococci (26). There are now at least 27 proposed *Enterococcus* species; some of the kits such as the Vitek 32 system (bioMerieux Vitek, Hazelwood, MO, USA) can only identify up to eight. To overcome these problems, molecular techniques based on the amplification of bacterial 16S rRNA has been proposed to establish genus identification. Deasy *et al*. used published 16S rRNA sequences to design genus-specific primers which, when used in separate PCR reactions, are capable of distinguishing all type strains of *Lactococcus* and *Enterococcus* from other genera (13).

Subsequently, more molecular based methods are being established in an attempt to develop more rapid and reliable species identification methods for laboratory use. Accuprobe (Geneprobe, San diego,CA) is a DNA-DNA hybridization based method. This probe is an improvement for processing time and accuracy when compared to conventional tests. However, *Vagococcus fluvialis* can also react with the probe (68). Whole- cell protein analysis is a method

that obtains patterns by a highly standardized procedure using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (69). This technique has provided complex and stable patterns that are easy to interpret and compare for grouping large numbers of closely related organisms, such as enterococci and other lactic acid bacteria (76). The major drawback of this method is the presence of distorted protein profiles or dense protein bands which makes visual interpretation difficult (76). Advances in DNA sequencing such as reading longer sequences faster and cheaper have made it a well-known and frequently used technique (18). Sequencing of genes has been explored as a way of distinguishing enterococci species from one another. Enterococci species specific sequencing methods have included sequencing of the 16S rRNA gene, *ddl*, and the manganese –dependent superoxide dismutase gene, *sodA* (24). 16S rRNA gene sequencing has virtually replaced DNA-DNA reassociation in the classification of the species (24). D-Ala:D-Ala ligase (*ddl*) genes are involved in peptidoglycan synthesis of the bacterial cell wall of the enterococci and are specific for each species (24). In addition, the manganese-dependent superoxide dismutase, *sodA,* gene has been shown to have unique sequences in 16 *Enterococcus* species and may become the "gold standard" technique for species identification (59). However, there are drawbacks to sequencing techniques also. The 16S rRNA sequence has strong similarities between some species such as *E. casseliflavus* and *E. gallinarum* such that sequencing would need to be used in conjunction with other tests for confirmation like WCP or phenotypic tests (24).

The most novel and promising methods are PCR-based methods. Combining information on genes that have been sequenced with PCR- based methods have allowed development of more rapid and reliable methods. A PCR based technique for rapid species identification of *E. faecalis*, *E. faecium*, and, *E. casseliflavus*, and *E. gallinarum* based on the amplification of the

ddl gene or portion of this gene in these species has been developed (16,73). However, this method only identifies four species and requires eight primers for the assay. A multiplex-PCR has recently been developed for the *sodA* gene (32). It has demonstrated the ability to provide an accurate and quick method for identification of 24 species of enterococci when compared to other tests (32).

PATHOGENESIS

Prior to the 1970's, enterococci were not classified as a human health concern with the exception of enterococci causing endocarditis (25). Macalum and Hastings initially addressed this pathogenecity when enterococci were isolated from a case of acute endocarditis, but were thought to be *Micrococcus zymogens* at the time (25). However, enterococci have been readily identified as a significant cause of nosocomial infections in the past two decades (70). Enterococci have been documented to cause infections including endocarditis, urinary tract infections, and bacteremia- accounting for 12% of all hospital-acquired infections (43). *E. faecalis* accounts for approximately 80% of all enterococcal nosocomial infections followed by *E. faecium* (25). Other enterococcal species cause human infections, but it is a rare occurrence. Because enterococci are opportunistic pathogens, they only cause infections with immunocompromised people which help to make them an ideal pathogen in a hospital environment (33). Treatment is often complicated by multi-drug resistance in enterococci.

Virulence determinants present in enterococcal pathogens are not fully understood. Research on enterococcal virulence genes is still being examined as they are not easily identified. Studies have shown that *E. faecalis* strains harbor significantly more virulence determinants than

do *E. faecium* (50). Virulence determinants that have been characterized include cytolysin, aggregation substance, gelatinase, enterococcocal surface protein, sex pheromones, and enterolysin A (17). Cytolysin is involved in toxin activities and has hemolytic activity by lysing a broad spectrum of cells including human, horse, and rabbit erythrocytes and has bactericidal activity (17). Aggregation substance is a pheromone-inducible surface protein of *E. faecalis* and is involved in mating aggregate formation during bacterial conjugation and also enhancing adherence to animal cells (17,24). Gelatinase, a protease, is thought to be involved in the inflammatory process, by hydrolyzing gelatin, collagen, casein, haemoglobin and other bioactive peptides (24). Enterococcal surface protein (Esp) also aids in cell surface adhesion and may contribute to the ability of *E. faecalis* to evade detection by the immune system (50). Additionally, sex pheromones are proteins expressed prior to conjugation which induce the plasmid of the donor strain to produce aggregation substance to encourage plasmid transfer (10). Other virulence traits are under investigation such as production of an enterolyisn A which is similar to other cell wall lytic enzymes that may contribute to the pathogenic potential of some enterococcal strains (30). Sequencing the whole enterococcal genome may bring better understanding of virulence traits and offer unique molecular targets which will aide in the development of new antimicrobials (70).

TYPING

Enterococcal infections were originally thought to arise from a patient's own gut flora. Recent studies have clearly demonstrated both intra-and interhospital spread of these organisms (71). Typing tools aid the identification of clonal outbreaks which is useful for further studies. Hence, efficient typing methods have become very important for epidemiologic investigations.

Classic methods for typing enterococci are phenotypic in nature. These methods include bacteriocin typing, phage typing, serotyping, biotyping, and antimicrobial susceptibility testing (49). While these methods still prove to be useful, they are generally time-consuming and difficult to reproduce or interpret (51).

More recently, molecular genetic techniques have been successfully applied to many species of bacteria. These techniques have greatly improved the ability of typing methods to discriminate enterococcal isolates and have helped with important data about outbreaks due to strains that exhibit clinically important antimicrobial resistance (24). One of the first genetic methods was plasmid profiling (24). Total plasmid content analysis is applicable to enterococci because enterococci do not have species-specific plasmids or plasmid patterns, therefore unrelated strains have different plasmid contents (51). However, problems arise with inconsistencies in plasmid yield and difficulty in interpretation (51). Technological advances have led to novel methods such as Pulsed-Field Gel Electrophoresis (PFGE), Repetitive-PCR (Rep-PCR), and ribotyping.

PFGE is currently considered the gold-standard for subtyping enterococci (72). The advantage of PFGE is its ability to separate large DNA fragments (from 10kb to 1.5 Mb) such as those generated by low-frequency-cleavage restriction endonucleases digestion (RED) of whole chromosomes (49). This technique yields RED patterns which are usually well separated and less ambiguous than patterns generated by other electrophoresis methods (49). A PFGE procedure for typing *Enterococcus* was developed in the early 1990's by Murray *et al*. (54). The procedure has evolved as an efficient tool for discriminating multi-drug resistant strains of enterococci. For example, vancomycin resistant *Enterococcus* (VRE) strains can be better distinguished in less time than before (72). General principles proposed for the interpretation of molecular typing

data, based on gradient differences, are usually applied to interpret PFGE profiles obtained for enterococcal strains. Conversly, PFGE can be a time-consuming procedure where expensive equipment is needed (67).

Rep-PCR offers a cheaper and quicker way of generating banding patterns to differentiate species; a form of Rep-PCR is BOX-PCR (4). BOX-PCR primers for *Enterococcus* were designed from highly conserved interspersed repetitive sequences that have been identified in *Streptococcus pneumoniae* (4). These sequences are termed boxA, boxB, and boxC and are 59, 45, and 50 basepairs in length, respectively. A study comparing BOX-PCR to PFGE was conducted in which PCR and PFGE patterns were generated using *E. faecalis* (45). Results indicated that reproducibility of the PCR patterns were found to be challenging, although when stricter criteria were used, the interpretation of Rep-PCR results were more similar to those obtained by PFGE (45).

Other novel methods including Multilocus Enzyme Electrophoresis (MLEE) and ribotyping have also been used for *Enterococcus*. MLEE compares differences in the net charge of housekeeping enzymes resulting from certain mutations in the genes (55). Ribotyping is based upon ribosomal DNA restriction fragment analysis (77). Studies performed with these methods have indicated that PFGE is at least comparable and may even be able to discriminate further than the other methods (27). PFGE in conjunction with a PCR-based method or PFGE using two different restriction enzymes is highly recommended as confirmatory steps (3). In addition to selection of a typing procedure, interpretation of molecular typing data is an ongoing problem (15). Currently, computer based analysis programs such as Bionumerics (Applied Maths, Sint-Martens-Latem, Belgium) are being utilized to interpret results from band-based methods (15). Software packages can perform sophisticated similarity calculations and cluster analyses of the

patterns in the database and from that generate analytical data in the form of dendrograms (23). These programs are now being used progressively more with epidemiological typing. These tools are helpful in establishing intralab and interlab similarity of interpretation (23).

ENTEROCOCCI AND ANTIMICROBIAL RESISTANCE

Antibiotic resistance is becoming less of a rarity and more of a widespread occurrence. The consequences of selection of resistance include prolonged illness, severe side effects to alternative drugs that may be more toxic, or death following complete treatment failure (53). Enterococci have both intrinsic and acquired resistance. Intrinsic resistance includes a number of antibiotics namely aminoglycosides, β-lactams, and quinolones (24).

β-lactam antibiotics are a diverse class of drugs that are the most commonly used antimicrobial agents (18). Beta-lactams act on penicillin binding proteins (PBP) which are involved in the production of the cell wall (24). In addition, while *E. faecalis* is known to cause the majority of infection-derived clinical isolates, *E. faecium* remains the species exhibiting a disproportionately greater resistance to multiple antibiotics especially β–lactams (70). High-level enterococci resistance to β-lactams occurs by two known mechanisms. The first is the overproduction of an altered penicillin-binding protein. It is thought this altered penicillin-binding protein arose as a result of a genetic mutation that confers a low affinity for penicillins or reduced susceptibility to inhibition by penicillins (24). The second mechanism is β–lactamase production by enterococci which is still considered rare (52). Hybridization studies have shown that the β–lactamase gene is highly homologous to that from *Staphylococcus aureus*, although differences exist in the expression of these enzymes (51).

Aminoglycosides are common antimicrobial agents used in the treatment of infections by both gram-negative and gram-positive organisms (18). All enterococci have intrinsic low-level resistance to aminoglycosides (9). Low-level resistance in enterococci resistance is attributed to low uptake of the drug aross the enterococcal cell membrane, although the uptake is markedly enhanced when combined with a cell wall synthesis inhibitor (51). High level resistance to aminoglycosides is mostly due to acquisition of genes encoding aminoglycoside modifying enzymes (38). These enzymes are prominent among several *Enterococcus* species. Genes associated with enterococci are acetyl transferase *-aac (6')-Ie+aph (2")*, aminoglycoside phosphotransferase, *-aph (3')-IIIa*, and aminoglycoside nucleotidyltransferase *-ant(4")-Ia* gene (9).

Quinolone antimicrobial agents act by inhibition of certain bacterial topoisomerase enzymes (18). DNA gyrase, which introduces negative supercoils into DNA, is composed of two subunits of GyrA and two of GyrB (24) . Both enzymes participate in maintaining an overall level of DNA supercoiling that is essential to chromosome integrity, cell physiology, and viability (29). Quinolones are mostly used for infections due to gram-negative bacteria. Therefore, quinolone resistance in enterococci has not been as well studied (24). However, a few studies have shown that alterations of target enzymes appear to be the most likely factor in enterococci resistance to quinolones. *E. faecium* and *E. faecalis* have shown mutations in the genes for these enzymes (24).

Enterococci are able to acquire resistance via mobility of the resistance genes on plasmids, transposons, and chromosomal exchange (44). In addition, transfer of genetic material usually occurs in the gastrointestinal tract of humans and animals, where other bacteria are harbored (44). These bacteria could potentially be the recipient of these genes. It is believed that

this capability has made enterococci resistant at high levels to tetracycline, chloramphenicol, and recently vancomycin (44).

Tetracycline antimicrobial agents act by binding to the 30S ribosomal subunit, resulting in the inhibition of protein synthesis (18). A growing number of bacteria have acquired resistance to the activity of tetracycline. There are 30 tetracycline (*tet*) determinants that have been identified to date (42). In a study, authors used DNA-DNA hybridization for screening a variety of organisms for the presence of *tet* genes (18). Results indicated that *E. faecium* and *E. faecalis* both had a prevalence of these *tet* genes. The *tet* genes that have been associated with *Enterococcus* include *tet*(K), *tet*(M) , *tet*(O), *tet*(L), and *tet*(U) (24). *tet*(K) and *tet*(L) confer resistance by efflux mechanisms and *tet* (O) and *tet* (M) by ribosmal protection (24). *tet*(U) has been described in *E. faecium,* but its mechanism of resistance has yet to be determined (24).

Chloramphenicol acts by binding to the 50 S ribosomal subunit and inhibits the peptidyl transferase step in protein synthesis (18). Enterococci resistance to chloramphenicol is generally due to inactivation by a chloramphenicol acetyl transferase (CAT) (24). The high prevalence of multi-drug resistant enterococci in many hospitals has led to interest in using chloramphenicol as an alternative therapeutic agent. However, 50% of clinical enterococci are resistant to chloramphenicol (24).

Glycopeptide antibiotics act on gram positive organisms by inhibiting cell wall biosynthesis (18). Vancomycin, a glycopeptide, was once thought to be the savior drug only prescribed in severe cases of infections where other drugs have failed in treatment (5). In 1986, the first vancomycin-resistant *Enterococcus* (VRE) was isolated from a patient in France (40). Since then, reports of VRE has been documented a number of times. VRE's have been isolated with increased frequency in all major medical centers in the United States, Canada, and Western

Europe most notably among the *E. faecalis* and *E. faecium* species (51,72). In 1998, over 20% of enterococcal isolates in the United States were resistant to vancomycin (24). This resistance has been found to occur through the acquisition of five different gene clusters (*vanA*, *vanB*, *vanC*, *vanG*, and *van E*) (19). *vanC* is a nontransferable chromosomal determinant which is an intrinsic property of motile enterococci- *E. casseliflavus* (*vanC-2*), *E. gallinarum* (*vanC-1*), and *Enterococcus flavescens* (*van C-3*) (18). *vanA* and *vanB* appear to be the most globally widespread (24). *vanE* has been described in *E. faecalis*, while *vanD* is associated with *E. faecium* (7). Complex clusters of these genes result in the production of peptidoglycan precursors which reduces binding affinity for glycopeptides which confers resistance (19). Molecular assays have been developed for several vancomycin resistant markers. For example, Kariyama *et al*., described primers for the detection of *vanA*, *vanB*, *vanC* and primer set that detected *van C-2*/C-3 in a multiplex PCR (37). The human health community is concerned about *Enterococcus* and multiple drug resistance due to the ability of these bacteria to persist in healthcare settings. The emergence of multi-drug resistant enterococci has stressed the importance for quick and reliable ways of determination of antimicrobial susceptibility of a clinical isolate, which is often crucial for the optimal antimicrobial therapy of infected patients (18).

GROWTH PROMOTANTS

The increasing frequency of enterococcal antibiotic resistance has become a serious concern. The agricultural industry has become a focal point of concern because of the usage of antimicrobials in livestock. Antimicrobial usage in the agricultural industry is chiefly for therapeutic and subtherapeutic growth promotant applications (48). All antimicrobials used in

animal production have withdrawal times intended to prevent harmful residues in meat, milk, and eggs (41). Therapeutic treatments are intended for animals that are diseased. In food animal production, it is often more efficient to treat entire groups by medicating feed or water (44). Certain mass-medication procedures, called metaphylaxis, aim to treat sick animals while medicating others in the group to prevent disease (44). In addition, coccidiostats, such as ionophores, and sulfonamides are antimicrobials that prevent coccidiosis, a common parasitic disease of poultry (48).

In 1951, the United States Food and Drug administration (FDA) approved the use of antibiotics in animal feeds without a veterinary prescription (34). Growth-promoting antibiotics are compounds which can be safely added into animal feed to improve the efficiency of food digestion in the animal's stomach (56). Growth-promoting antibiotics work directly on the animals gut microflora by controlling the numbers of undesirable bacteria in the intestine and allowing better conversion of feed into elements which can be absorbed through the gut wall (78). Farmers use growth promotants not only for nutritional reasons, but to decrease production costs in a very competitive business (48). The use of these antibiotics helps by shortening finishing times, improving feed conversion rates, improving performance by promoting better condition and vitality, reducing death rates and reducing the need for therapeutic treatment according to the National Office of Animal Health (56).

Recently, growth promotants have received much attention, although the antibiotics used in animals are not the same chemically as those used in human medicine. Some antimicrobials used in veterinary medicine are analogs of human medicine (34). Of the 32 antimicrobials approved for use in broiler feeds in the U.S. without a veterinary prescription, 11 are listed as growth promotants (34). These compounds include bacitracin, chlortetracycline, erythromycin,

lincomycin, novobiocin, oxytetracycline, penicillin and virginiamycin (34). This information has become vital to understanding multi-drug resistant bacteria. Consequently, research has increased on the contribution of this phenomenon. An effort to solve this problem was initialized in Europe when Denmark banned avoparcin in 1995 ,virginiamycin in 1998, and in 1999, producers decided to stop all usage of antibiotics for growth promotion (1). A Denmark study conducted from 1995 through 2000 was performed to obtain outcomes after the ban. Isolates included *E. faecium* and *E. faecalis* from swine together with some *E. faecium* isolates from broilers (1). Isolates were tested for susceptibility to four different antibiotics (avilamycin, erythromycin, vancomycin, and virginiamycin) as part of the Danish program of monitoring for antimicrobial resistance. The results indicated a decrease in the occurrence of antimicrobial resistance when the selective pressure was removed (1). However, some of the other effects of the removal were not addressed. Some issues include an increase in the use of therapeutic drugs once the growth-promotants were removed which could have adverse effects on production costs and speed (48). The Denmark study and some similar to it provide substantial data about the relationship between growth promotants conferring antibiotic resistance in enterococci in animals (1). Nonetheless, to date, no link has been found and proven between usage of growth-promoting antibiotics and resistance in human medicine. Approximately, 15% of all enterococci isolated in US hospitals are resistant to vancomycin (35). VRE issues in the U.S. remain to be addressed since neither vancomycin nor its agricultural analogue avoparcin have been used in animal production in the US (35).

The growth promotant virginiamycin, in particular, has been closely scrutinized. With the increasing prevalence of vancomycin-resistant *E. faecium* (VREF) over the past decade, the FDA approved the use of a quinupristin-dalfopristin drug, Synercid, for the treatment of VREF

in 1999 (64). Quinupristin /dalfopristin is a combination of streptogramin A and B antimicrobial agents (dalfopristin 70% , quinupristin 30%) (65). The two streptogramins work synergistically targeting the 50S bacterial ribosome to inhibit protein synthesis (65). Streptogramins are a part of the macrolide, lincosoamide, and streptogramin (MLS) antibiotics (24). All strains of *E. faecalis* are intrinsically resistant to quinupristin/dalfoprisitin (24). There has been numerous studies on *E. faecium* and potential resistance genes to streptogramins (66). Resistance can be due to the combined presence of a single gene that mediates resistance to streptogramin A or the combined presences of streptogramin A and streptogramin B resistance genes (18). The genes associated with *E. faecium* resistance are *vat*(D) and *vat*(E) (24).

Virginiamycin is an analog of Synercid and has been used in animal production for over two decades (28). Concerns about virginiamycin use are based on the theory that its use in animals has established a reservoir of streptogramin resistant bacteria in poultry and other food animals (65). A study from retail meats revealed that there is already a high-rate of recovery of quinupristin-dalfopristin resistant *E. faecium* from chickens in the United States (64). Another study concluded that resistance against antibiotics used solely for growth promotion was more prevalent in *E. faecium* strains than in *E. faecalis* strains (8). However, there is a low prevalence and low level of resistance of these strains in human stool specimens suggesting that the use of virginiamycin in animals has not yet had a substantial influence on enterococci resistance in humans (47).

There are some considered alternatives to growth promotants such as in-feed enzymes and competitive exclusion products. In-feed enzymes help break down certain components of the feed that the animal may have problems digesting (57). Competitive exclusion products are in-feed microbes consisting of a variety of species of bacteria that are marketed as being

“friendly” (57). These alternatives have pros and cons also. Radical ethicists believe that adding enzymes to animals merely shows that we think of them as "factory beasts” (57). Otherwise, in-feed enzymes are a promising alternative. Therefore, it is agreed that more studies investigating the link between growth promotants in agricultural and bacteria in humans containing antibiotic resistance needs to be performed (48). The basis for these studies should be the normal ecology of *Enterococcus* in animals.

ENTEROCOCCUS ECOLOGY

Understanding *Enterococcus* ecology is an ongoing process. It is has been established that *E. faecalis* and *E. faecium* are the predominant species that cause nosocomial infections (51). However, a study conducted at a hospital in France reported a high prevalence of *E. gallinarum* and *E. casseliflavus* (21). Research has explored the distribution of *Enterococcus* populations from different animal sources (58). One study examined the prevalence of different enterococci species from an integrated chicken-fish farms and control fish farms in Thailand (58). Results indicated that *E. faecium* and *E. faecalis* were the predominate species isolated from the integrated farm, whereas *E. casseliflavus* and *E. mundtii* isolates were most prevalent in traditional farms. It is now hypothesized that *Enterococcus* is an indigenous flora of the fish gut (58). Khun *et al*. conducted a study which aimed to generate knowledge of enteroccocal populations in the food chain (39). In the study, enterococci in different geographical regions and in different parts of the food chain were examined and samples were collected from healthy and hospitalized humans, animals (slaughter carcasses and farm animals), from the environment (pig farms, sewage, and surface water), and from four European countries –Sweden, Denmark, UK,

and Spain (39). Results indicated that enterococci were abundant in most parts of the food chain; human feaces, animal manure, farmland fertilized with manure, animal carcasses, sewage water, and surface water all had a considerable prevalence of enterococci. Collectively, *E. faecium*, *E. faecalis,* and *E. hirae* were the dominant species, respectively (39). The urban sewage was dominated by *E. faecium*. The broiler slaughterhouses had a dominance of *E. faecalis*, and cattle and pig slaughterhouses were dominated by *E. hirae* (39). Studies have been examining the enterococcal composition of poultry since the 50's. Barnes *et al*., described *E. faecalis* and *E. faecium* as the most frequently occurring enterococci in 12 -week old chicks with *E. faecalis* becoming more numerous as the birds grew older (2). Other species are found only occasionally or in distinct species or age groups. *E. faecalis* and *E. faecium* were equally dominant in the enterococcal gut flora of 1-day old chicks (24). *E. faecium* was most often identified in the samples from broilers age 3-4 weeks and *E. cecorum* from older birds (14). Other species occasionally present in chickens are *E. casseliflavus*, *E. gallinarum,* and *E. mundtii* (14). Species distribution within certain location of organs from chickens has also been examined. For example, *E. durans* and *E. hirae* are a part of the flora of the small intestine, whereas they are absent in the crop and ceca (24).

In addition to species distribution of enterococci between isolates, studies are also being conducted to understand the genetic relatedness of enterococci populations. Jackson *et al.,* conducted a study from 1999 to 2000 with 162 poultry carcass rinsates as part of the veterinary surveillance branch of the National Antimicrobial Resistance Monitoring System (NARMS) (31). The study examined prevalence as well as distribution of genes conferring resistance to several antibiotics. In addition, genetic relatedness of these isolates was evaluated. Results indicated that the predominant species were *E. faecium*, *E. faecalis*, and *E. durans* (31).

Phylogenetic analysis was conducted using PFGE. Clusters were examined by isolate, species, and aminoglycoside resistance profile, but there was no distinct clustering based solely upon these criteria (31). Another study conducted on 197 Norwegian vancomycin resistant *Enterococcus faecium* (VREF) poultry isolates examined the genetic relatedness of these isolates using amplified fragment length polymorphism analysis (AFLP) (6). Results indicated that the isolates consisted of a relatively homogeneous population of *E. faecium* and clustered according to source. Willems *et al*., genotypic study concluded that VREF strains are predominantly host-specific using AFLP (79). In addition, Quednau *et al*., found that strains from chicken, pork, and humans clearly divided into separate clusters with phylogenetic analysis conducted with restriction endonuclease analysis (REA) of total chromosomal DNA (60). However, host-specificity of enterococcal populations is still being investigated because data from a recent study demonstrated that no host specificity or correlation with the country of isolation was found with enterococci isolates from humans, animals, and foods (75).

The purpose of this study was to examine the prevalence of *Enterococcus* species among different sampling areas on a commercially integrated poultry farm from 2002 to 2003. Sampling areas included boxliners, litter, feed, and carcass rinses. This study examined the *Enterococcus* population throughout the total poultry processing up to slaughter. The effect of different growth promotants on the species was also determined, primarily with virginiamycin, but also flavomycin and bacitracin. Furthermore, phylogenetic analysis was conducted to examine the genetic relatedness of the isolates according to sampling area and growth promotant administered, if any. Enterococcal species were identified using a new multiplex PCR method (32) in conjunction with Vitek 32 (bioMerieux Vitek, Hazelwood, MO, USA) for confirmation. Phylogenetic analysis was conducted by BOX-PCR and PFGE to create genetic profiles. Finally,

the two molecular typing methods were evaluated to examine similarities and differences among clustering and efficiency of each method.

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CHAPTER 2

Relationship between *Enterococcus* spp. and growth promotants usage of a poultry farm[1]

[1]**Debnam, Antoinette, Charlene Jackson, Charles L. Hofacre. Submitted to Journal of Food Protection**.

Relationship between *Enterococcus* spp. and growth promotant usage on a poultry farm.

ABSTRACT

The use of antimicrobials in animals and the potential for transfer of antimicrobial resistant bacteria from animals to humans is cause for concern. Because commensal bacteria such as enterococci may be reservoirs of resistance, it is important to characterize the strains isolated from animals and their environments. This study analyzed enterococci from four poultry houses for six growouts on one farm. Two houses on the farm were control houses and did not use any antimicrobials, while two other houses on the farm used flavomycin, virginiamycin, and bacitracin during different poultry growouts. Litter, chick boxliners, feed, and poultry carcass rinses were obtained from each house and cultured for the presence of enterococci. Enterococci were identified to species using species specific multiplex-PCR and Vitek. Additionally, *Enterococcus faecium* isolates were further characterized using BOX-PCR and PFGE. *Enterococcus faecalis* (n=763; 52%) and *E. faecium* (n=578; 40%), were isolated most often from the farm and houses regardless of antimicrobial treatment. The control houses were dominated by *E. faecalis* (n=389; 50%) and *E. faecium* (n=295; 38.5%) as were treated houses *E. faecalis* (n=374; 53%) and *E. faecium* (n=283; 40%). There was a total of 753 (51%) *Enteroccocus* isolates from the control houses, whereas there were 725 (49%) *Enterococcus* isolates from the treated houses.

INTRODUCTION

Enterococci are gram-positive cocci that are normally found in the gastrointestinal tracts of humans and animals. They are resistant to heat, desiccation, high salt concentrations, certain disinfectants and multiple antibiotics (4). Consequently, enterococci have emerged as a leading cause of nosocomial infections. Two species of enterococci, *Enterococcus faecalis* and *Enterococcus faecium* account for a majority of hospital-acquired infections (5). The emergence of multi-drug resistant enterococci poses a serious threat and challenges the health care community to control the spread of this organism.

One source that is being explored as a possible contributor to multi-drug resistance is the use of growth promotants antibiotics in food animals. Some antibiotics used for growth promotion with farm animals have analogs to antibiotics used in human medicine (7). The use of antimicrobials in food animals may result in antimicrobial resistant bacteria which may be transmitted to humans through the food supply (13). Recently, the growth promotant virginiamycin has been the subject of much investigation. Virginiamycin is a streptogramin A and B antibiotic that has been used as a growth promotant for decades (14). Synercid, a human analog of virginiamycin, was recently approved for treatment of vancomycin resistant *E. faecium* (VREF) (4). The human health community is concerned as to whether usage of virginiamycin has established a reservoir of streptogramin resistant bacteria in poultry and other food animals conferring resistance to Synercid (14).

There are few studies that have investigated the normal population of *Enterococcus* in a poultry setting (4,9). Further knowledge about the activity of the growth-promoting agents on

the normal intestinal enterococcal species might lead to better understanding of the influence of growth promoting agents. In this study, the normal enterococcal population present on a

poultry farm with their current use of growth promotants (flavomycin, virginiamycin, and bacitracin) was investigated.

MATERIALS AND METHODS

Origin of samples: Between 2002 and 2003, samples were collected from a commercial four house broiler farm in North Georgia. Broiler chickens from six growouts from four different houses were studied. A grow-out period consisted of chickens ranging from a few days old to slaughter (eight weeks old). The farm was contracted to raise chickens for a single commercially integrated company. The company provided the farm with the chickens and feed. The four houses were designated A, B, C, and D; A and B were the control houses and C and D were the treatment houses. The treatment houses were administered a different antibiotic for the six growouts. The antibiotics used in the final feed before slaughter were flavomycin (2 grams per ton) for growout 1, virginiamycin (20 grams per ton) for growout 2, virginiamycin (20 grams per ton) for growout 3, virginiamycin (20 grams per ton) for growout 4, flavomycin (20 grams per ton) for growout 5, and bacitracin (20 grams per ton) for growout 6, respectively. Types of samples and methods of sampling and culturing were as follows:

Boxliners: Whole boxliners were collected after chicks were transported to the houses from the hatchery. Contents of the boxliners were sampled aseptically with swabs. Swab samples were placed into 50 ml conical tubes filled with 40 ml of 1X phosphate buffered saline (PBS, pH 7.2) and mixed with a shaker for ten minutes. Debris was removed by filtering with gauze into a new conical tube and supernatant was discarded. One hundred microliters was

removed for plating onto M- Enterococcus agar (Becton Dickinson Microbiology Systems, Sparks, MD) for isolation.

Litter: Wood shavings from softwoods were used as bedding, material commonly used in poultry houses in the southeastern United States. The litter remained unchanged in each house throughout the study period. Litter samples were a composite of five locations in the house and then pooled. Five of these samples were collected from each house. Five grams of chicken litter was weighed out in a 50 ml conical tube with 30 ml of 1X PBS, pH 7.2 and mixed with a wrist-action shaker for five minutes. Debris was removed by low speed centrifugation (600 rpm, 15 minutes). The bacteria were pelleted by high speed centrifugation (10,000 rpm, 15 min) and the supernatant was discarded. The resulting pellet was streaked onto M-Enterococcus agar (Becton Dickinson Microbiology Systems, Sparks, MD) for isolation and identification. Pre-litter samples were composed of samples taken from the last layer of bedding used before the study was conducted.

Feed: Heat-treated pelleted feed was fed to the chickens ad libitum. All feed was stored in steel storage tanks with no access to rodents or wild birds. The control houses received feed without antibiotics, while the treated houses received the above mentioned antibiotics. Ten grams of feed was collected at four and seven weeks per growout. Samples were collected as the feed flowed from the pipes which delivered feed to each feeder in the houses to eliminate contamination. Samples were taken aseptically with changing of latex gloves between each sample. The samples were processed in the same manner as litter samples.

Carcass rinses: Ten random chicken carcasses per house were selected immediately before the chickens entered the cold water chill-tank and placed in a container with ice for refrigeration. Each whole chicken was rinsed in 250 ml of peptone water in an automated carcass

shaker for one minute at the USDA-ARS-RRC. Forty-five milliliters of rinsate from the bag was transferred to a 50 ml conical tube and 100 µl were inoculated on M-Enterococcus agar (Becton Dickinson Microbiology Systems, Sparks, MD) for isolation.

Isolation and initial identification: Ten well isolated positive colonies from M-Enterococcus agar (Becton Dickinson Microbiology Systems, Sparks, MD) were subcultured onto blood agar and Enterococcosel agar (Becton Dickinson Microbiology Systems, Sparks, MD) and incubated for 24 h at 37^0C. Initial identification was performed using Gram staining, catalase test, bile-esculine test, and pyrrolidonyl-β-naphthylamide (PYR)(15). One colony per isolate was selected and placed into a 96-well plate containing bile-esculin agar from the different houses.

Statistical analysis: Differences in prevalence of species from different origins were analyzed by the Chi square test. Statistical significance was defined as probability of less than or equal to 0.05 ($P \leq 0.05$).

Identification of *Enterococcus* spp. by PCR and phenotypic testing: All isolates were tested in a multiplex, species–specific PCR for 24 species of *Enterococcus* as previously described by Jackson *et al*. (6) Since the method is novel, isolates were also identified with the automatic Vitek 32 system (bioMerieux Vitek, Hazelwood, MO, USA) Vitek Gram Positive Identification Card (GPI) (bioMérieux Vitek Inc., Hazelwood, Mich) according to the manufacturer's instructions.

RESULTS

A total number of 1478 isolates were collected from the farm, of which 432 (29%) were from litter samples, 36 (2.4%) from pre-litter samples, 105 (7%) from feed samples, 192 (13%) from boxliners, and 713 (48.6%) from carcass rinses (Table 1). Overall, the distribution of species from the samples collectively were *E. faecalis* (n=763; 52%), *E. faecium* (n=578; 40%), *E. casseliflavus* (n=51; 3.4%) *E. hirae* (n=37; 2.5%), *E. gallinarum* (n=26; 1.7%), *E. durans* (n=15; 1%), *E. avium* (n=4; 0.27%) *E. cecorum* (n=1;0.06%), *E. malodoratus* (n=1;0.06), and 2 (0.1%) remained unidentified (Fig. 1). Unidentified isolates were due to lack of growth.

Houses receiving no growth promotants (control) accounted for 753 (49.6%) of the total isolates. Houses receiving growth promotants (treated) accounted for 725 (50.4%) of the total isolates. Species distribution between control and treated houses was comparable (Fig. 2). The control houses species distribution was *E. faecalis* (n=389; 50%), *E. faecium* (n=295; 38.5%), *E. casseliflavus* (n=24; 3.8%), *E. gallinarum* (n=6; 1.9%), *E. hirae* (n=31; 4%), *E. durans* (n=3; 1.5%), *E. avium* (n= 4; 0.27%), *E. cecorum* (n=0), *E. malodoratus* (n=0), and 1(0.1%) remained unidentified . Whereas, in treated houses, species distribution was *E. faecalis* (n=374; 52.2%), *E. faecium* (n=283; 40%), *E. casseliflavus* (n=27; 3.0%), *E. hirae* (n=6; 0.94%), *E. gallinarum* (n=20; 1.6%), *E. durans* (n=12; 0.4%), *E. avium* (n= 0; 0.26%), *E. malodoratus* (n=1; 0.13%), and *E. cecorum* (n=1; 0.3%), and 1(.1%) remained unidentified.

Species distribution according to sampling area is shown in (Tables 2-7). *E. faecium* and *E. faecalis* were the prevailing species among all sampling areas in control and treated houses. The combined litter samples of prelitter, litter at 4 weeks, and litter at 7 weeks are shown in Table 1. The predominant species was *E. faecium* in the control (n=165; 70.2%) and treated groups (n=196; 84%). The litter samples also showed a wide range of different *Enterococcus*

species. Litter at 4 weeks and litter at 7 weeks were examined individually (Tables 2-3), the results from both sampling times are comparable with *E. faecium* being the predominant species at both time periods (n=174; 80.5%) and (n=164; 76%), respectively. The combined feed samples of feed at 4 weeks and feed at 7 weeks are shown in Table 1. The predominant species was *E. faecium* in the control (n=38; 70.3%) and treated groups (n=29; 56.4%), respectively. Control and treated houses showed no observable difference. The combined feed samples also showed a wide range of different *Enterococcus* species. When examining feed at 4 weeks and feed at 7 weeks individually (Tables 4-5), the results from both sampling times are comparable. *Enterococcus faecium* is the predominant species at both the time periods (n=27; 25.7%) and (n=40; 38%), respectively. The predominant species found in carcass rinsates was *E. faecalis*. Results were comparable for both control (n=227; 32%) and treated houses (n=264; 37%). Carcass rinsates showed the most diversity among species; at least one of every species examined was isolated (Table 6). Boxliners were dominated by *E. faecalis* (n=176; 92%), and isolates consisted of only *E. faecium* and *E. faecalis* (Table 7). Results were comparable in both control and treated houses.

If usage of virginiamycin shifted the species when compared to the other growth promotants, it would be observed in litter at 7 weeks and carcass rinses. The results from the six growouts from these two sampling areas are shown in Figures 3-4. Cross resistance between virginiamycin and Synercid is only pertinent to *E. faecium*, because *E. faecalis* is intrinsically resistant to Synercid (4). Therefore, *E. faecium* was monitored for any changes due to virginiamycin usage. Overall, trends were similar between the control and treated houses among the different growouts. In litter 7 weeks, *E. faecium* appears to be consistent as the growouts progressed (Fig 3.). The *E. faecium* population in carcass rinses seemed to be variable as the first

flavomycin growout had 10 isolates, the second virginiamycin growout had 12 isolates, the third virginiamycin growout had 6 isolates, the fourth virginiamycin growout had 4 isolates, the fifth virginiamycin growout had 12 isolates, and the sixth bacitracin growout had 4 isolates (Fig 4.). Using the Chi-square probability test, virginiamycin growouts for *E. faecium* for litter at 7 weeks and carcass rinsates were pairwise tested for possible significant differences from each other. Significance was defined as $P \leq 0.05$. Although significant differences were observed in some cases, there were no consistencies observed throughout by either treatment. (Table 8).

DISCUSSION

This investigation determined the prevalence of *Enterococcus* spp., obtained from sampling of boxliners, litter, feed, and carcass rinses from an integrated poultry farm in Northeast Georgia. Samples were evaluated based upon those that received antibiotic treatment (treated) and those that did not (control).

One of the goals with this study was to identify the most frequently occurring species in a poultry habitat. Previous studies have shown that *E. faecalis*, *E. faecium*, *E. hirae*, and *E. durans,* respectively, are the most prevalent enterococci in the intestinal flora of poultry (2). All of these species were present in this study, however, the most prevalent were *E. faecalis* and *E. faecium*, *E. casseliflavus*, and *E. hirae*, respectively. *Enterococcus casseliflavus* was considered a rare isolation from poultry and has been a species associated with plants (4). However, it is the third most prevalent species found in this poultry study. It is possible that growth conditions selected for certain species of *Enterococcus*. Other species which have been previously associated with poultry environments were also found in this study including *E. gallinarum*, *E. durans*, *E.*

cecorum, *E. malodoratus*, and *E. avium* (4). *Enterococcus mundtii* has also been previously associated with a poultry environment although none were isolated in this study (4). The *Enterococcus* spp. and their relative occurrence varied between different sample sources. Samples from boxliners had a high prevalence of *E. faecalis,* while in litter and feed more *E. faecium* dominated. Perhaps this is the same result found in earlier studies where age-progression had an effect on the enterococcal microflora (4,8). Young chicks have more *E. faecalis* (boxliner samples), while litter taken at 4 and 7 weeks of age is expected to have more *E. faecium*. Feed had a high prevalence of *E. faecium*. Feed and pre-litter samples were taken as an implication of preceding enterococcal contamination within the facility. *E. faecium* was apparently the existing predominant species already on the farm. Simjee *et al.*, found in a study that poultry litter may serve as a potential source of harboring enterococci carrying known and as yet unidentified streptogramin resistance genes in enterococci (13). There have been studies where litter has been found to be a possible means of transmission, and serve as a medium for the maintenance of the organism and its subsequent transmission to later flocks (12). The farm uses the common practice of placing a thin layer of new litter on top of the old between growouts. Most poultry farms remove poultry litter only once or twice a year, placing fresh litter shavings on top of the old litter, which may allow the microbial activity to sufficiently compost in the litter (10). *E. faecalis* was the most frequently occurring species from carcass rinses. Several published articles about *Enterococcus* spp. isolated from retail poultry meat indicate *E. faecium* to be the most prevalent (13). Carcass rinses directly from the slaughterhouse have not been examined as much, but a study found more prevalence of *E. faecalis* also (16). Perhaps, the effect of immersion chilling and refrigerated storage effect the number and types of *Enterococcus* bacteria recovered from broiler carcass.

Another aim of this study was to assess whether or not the usage of growth promotants had an effect on the enterococcal microflora of poultry. The inclusion of growth-promoting antibiotics, primarily virginiamycin, did not appear to be associated with a change of the species in the flora. Control and treated houses species distribution was comparable when compared as a whole. These results did not change when looking at individual sampling areas. Virginiamycin, although administered in three out of the six growouts did not seem to cause a shift of the species. The statistical data indicated significant difference among certain growouts. However, despite some areas being statistically different, these differences were inconsistent and seen within control groups also. The study was based on a real commercial industry which included all normal proceedings that happen in a poultry environment. Therefore, the experimental design consisted of administering the antibiotics to chickens at 4 $^{1/2}$ weeks of age. Perhaps at the time of administration the intestinal microflora was already mature making it difficult to shift. In a previous study, the chickens were fed growth promotants including virginiamycin as chicks and were compared against "undosed" chickens (9). In this laboratory based study, they observed significant differences between the two and concluded that growth promotants are "capable of altering the balance of species within the intestinal flora."

In conclusion, to our knowledge this is the first study to report the distribution of *Enterococcus* spp., encompassing the total broiler environment. The results demonstrated that *Enterococcus* spp. were widespread throughout the farm, particularly, *E. faecalis* and *E. faecium* were predominant in all sampling areas. This occurrence is interesting as these are the two most isolated species in a clinical setting (11). Growth promoting agents used with livestock are under a lot of scrutiny due to the possible correlation between these agents conferring antibiotic resistance to humans through consumption. Denmark has already banned all growth-promoting

agents and has had some adverse consequences on the production of broilers (1). Virginiamycin, as a growth promotant for decades, is being examined as a possible route of resistance to human streptogramin A and B antibiotics like Synercid. Other studies have shown that with virginiamycin there is no correlation between receiving virginiamycin and the presence of quinupristin /dalfopristin resistant strains (3). Usage did not seem to have an impact on the enterococcal microflora of poultry in this study. Additional studies will be needed to assess the overall risk and benefit of virginiamycin for growth promotion such as antimicrobial susceptibilities and genes of the isolates.

TABLE 1. Distribution of *Enterococcus spp.* from poultry sources

No. of isolates (%)

	Boxliners n=192		Litter n=468		Feed n=105		Carcass Rinses n=713	
Species	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
***E. faecium* n=578**	**6(3%)**	**10(5%)**	**165(35%)**	**196(42%)**	**38(36%)**	**29(28%)**	**86(12%)**	**48(7%)**
***E. faecalis* n=763**	**99(52%)**	**77(40%)**	**52(11%)**	**20(4%)**	**11(10%)**	**13(12%)**	**227(32%)**	**264(37%)**
***E. hirae* n=37**	**0(0)**	**0(0)**	**7(1.5%)**	**2(0.5%)**	**1(1%)**	**0(0)**	**23(3%)**	**4(0.6%)**
***E. durans* n=15**	**0(0)**	**0(0)**	**1(0.2%)**	**5(1%)**	**1(1%)**	**3(3%)**	**1(0.1%)**	**4(0.6%)**
***E. gallinarum* n=26**	**0(0)**	**0(0)**	**1(0.2%)**	**6(1.4%)**	**0(0)**	**2(2%)**	**5(0.7%)**	**12(1.7%)**
***E. casseliflavus* n=51**	**0(0)**	**0(0)**	**6(1.4%)**	**4(0.9%)**	**3(3%)**	**4(4%)**	**15(2%)**	**19(3%)**
***E. avium* n=4**	**0(0)**	**0(0)**	**2(0.5%)**	**0(0)**	**0(0)**	**0(0)**	**2(0.2%)**	**0(0)**
***E. cecorum* n=1**	**0(0)**	**0(0)**	**0(0)**	**0(0)**	**0(0)**	**0(0)**	**0(0)**	**1(0.1%)**
***E. malodoratus* n=1**	**0(0)**	**0(0)**	**0(0)**	**0(0)**	**0(0)**	**0(0)**	**0(0)**	**1(0.1%)**
Unidentified n=2	**0(0)**	**0(0)**	**0(0)**	**0(0)**	**0(0)**	**0(0)**	**1(0.1%)**	**1(0.1%)**
Total n=1478	**105(7.1%)**	**87(5.9%)**	**235(16%)**	**233(15%)**	**54(3.6%)**	**51(3.4%)**	**359(24.2%)**	**354(24%)**

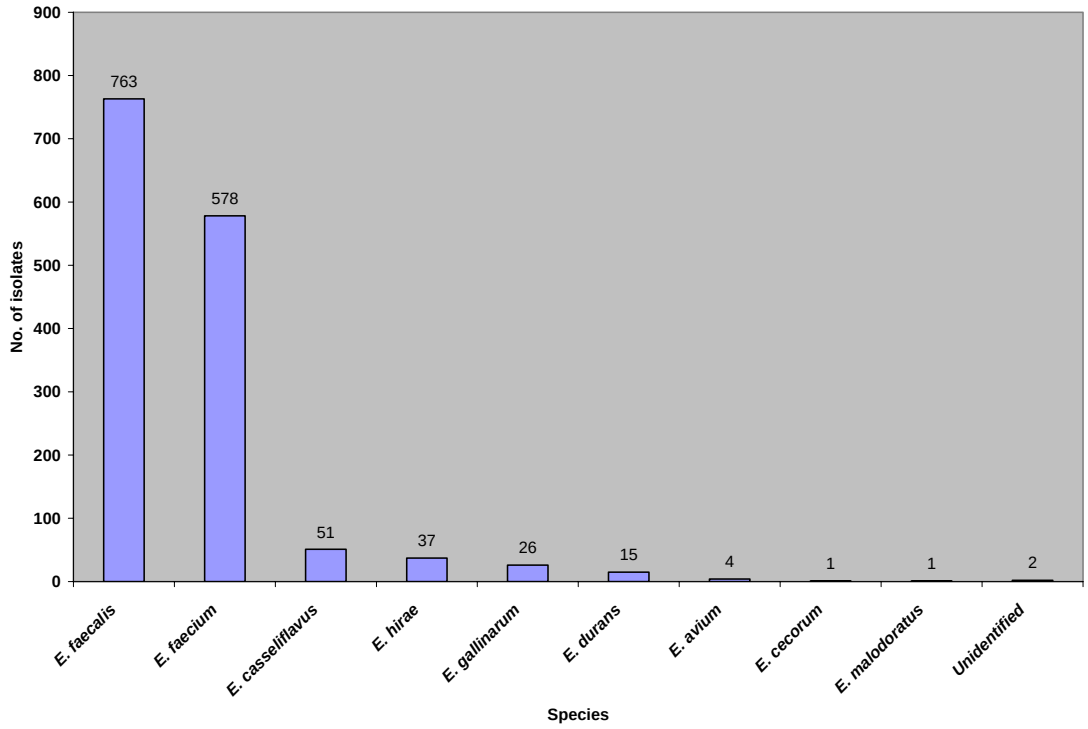

Fig. 1.Total distribution of *Enterococcus* species on poultry farm. *Enterococcus* spp. were isolated from boxliners, litter, feed, and carcass rinse samples.

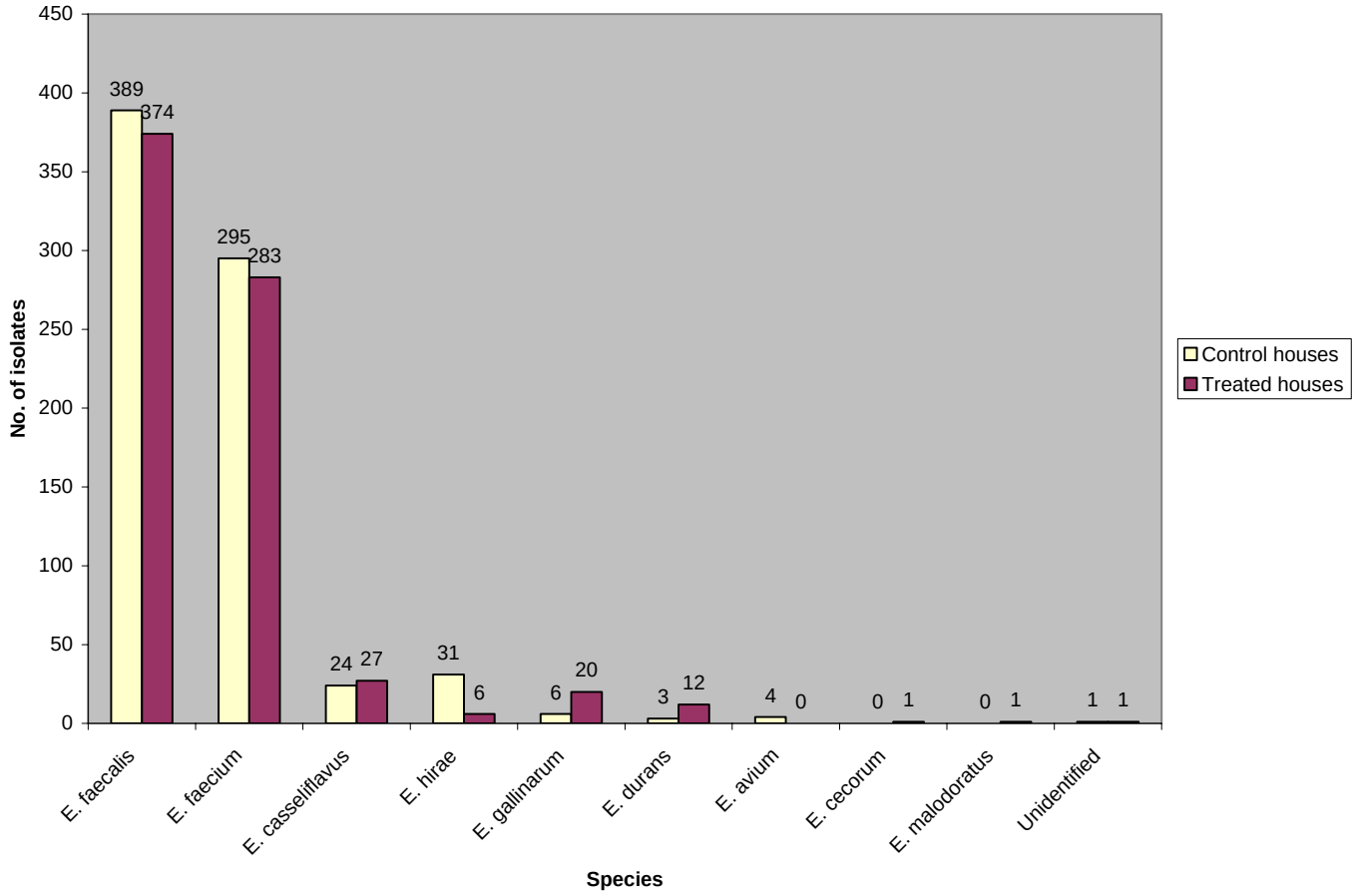

Fig. 2. Comparison of control vs. treated houses *Enterococcus* species distribution for all six growouts. No growth promotants were used in control houses, but were used in treated houses. Growth promotants used per growout were:1-flavomycin, 2-virginiamycin, 3-virginiamycin,4-virginiamycin, 5-flavomycin, and 6- bacitracin.

TABLE 2. Distribution of *Enterococcus* spp. from poultry litter at 4 weeks on farm

Litter at 4 weeks

No. of isolates (%)

	Flavomycin		Virginiamycin		Virginiamycin		Virginiamycin		Flavomycin		Bacitracin	
Species	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
E. faecium n=172	15(83%)	17(94%)	17(94%)	17(94%)	16(89%)	17(94%)	11(60%)	15(83%)	2(11%)	12(67%)	17(94%)	16(89%)
E. faecalis n=21	0(0)	0(0)	0(0)	0(0)	2(11%)	0(0)	3(17%)	0(0)	16(89%)	0(0)	0(0)	0(0)
E. hirae n=5	0(0)	0(0)	1(6%)	1(6%)	0(0)	0(0)	2(11%)	1(6%)	0(0)	0(0)	0(0)	0(0)
E. durans n=5	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	4(22%)	1(6%)	0(0)
E. gallinarum n=6	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(6%)	2(11%)	0(0)	2(11%)	0(0)	1(6%)
E. casseliflavus n=6	3(17%)	1(6%)	0(0)	0(0)	0(0)	1(6%)	0(0)	0(0)	0(0)	0(0)	0(0)	1(6%)
E. avium n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. cecorum n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. malodoratus n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Unidentified n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Total n=216	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)

TABLE 3. Distribution of *Enterococcus* spp. from poultry litter at 7 weeks on farm

Litter at 7 weeks

No. of isolates (%)

	Flavomycin		Virginiamycin		Virginiamycin		Virginiamycin		Flavomycin		Bacitracin	
Species	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
E. faecium n=162	9(50%)	8(44%)	8(44%)	18(100%)	18(100%)	15(83%)	15(83%)	15(83%)	12(67%)	14(78%)	14(78%)	16(88%)
E. faecalis n=43	6(33%)	10(56%)	9(50%)	0(0)	0(0)	3(17%)	2(11%)	2(11%)	6(33%)	4(22%)	1(6%)	0(0)
E. hirae n=3	2(11%)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(6%)	0(0)
E. durans n=1	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(6%)
E. gallinarum n=1	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(6%)	0(0)	0(0)	0(0)	0(0)
E. casseliflavus n=4	0(0)	0(0)	1(6%)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	2(11%)	1(6%)
E. avium n=2	1(5%)	0(0)	0(0)	0(0)	0(0)	0(0)	1(6%)	0(0)	0(0)	0(0)	0(0)	0(0)
E. cecorum n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. malodoratus n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Unidentified n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Total n=216	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)	18(8.3%)

TABLE 4. Distribution of *Enterococcus* spp. from poultry feed at 4 weeks on farm

Feed at 4 weeks

No. of isolates (%)

	Flavomycin		Virginiamycin		Virginiamycin		Virginiamycin		Flavomycin		Bacitracin	
Species	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
E. faecium n=27	6(100%)	2(33%)	4(67%)	3(50%)	3(100%)	0(0)	6(100%)	3(100%)	0(0)	0(0)	0(0)	0(0)
E. faecalis n=14	0(0)	0(0)	2(33%)	0(0)	0(0)	0(0)	0(0)	0(0)	6(100%)	6(100%)	0(0)	0(0)
E. hirae n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. durans n=3	0(0)	0(0)	0(0)	3(50%)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. gallinarum n=1	0(0)	1(17%)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. casseliflavus n=4	0(0)	3(50%)	0(0)	0(0)	0(0)	1(17/%)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. avium n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. cecorum n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. malodoratus n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Unidentified n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Total n=49	6(12.5%)	6(12.5%)	6(12.5%)	6(12.5%)	6(12.5%)	6(12.5%)	6(12.5%)	6(12.5%)	6(12.5%)	6(12.5%)	6(12.5%)	6(12.5%)

TABLE 5. Distribution of *Enterococcus* spp. from poultry feed at 7 weeks on farm

Feed at 7 weeks

No. of isolates (%)

	Flavomycin		Virginiamycin		Virginiamycin		Virginiamycin		Flavomycin		Bacitracin	
Species	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
E. faecium **n=40**	5(83%)	6(100%)	0(0)	2(67%)	2(33%)	3(100%)	3(100%)	4(66%)	3(50%)	1(17%)	6(100%)	5(83%)
E. faecalis **n=10**	0(0)	0(0)	0(0)	1(33%)	0(0)	0(0)	0(0)	0(0)	3(50%)	5(83%)	0(0)	1(17%)
E. hirae **n=1**	0(0)	0(0)	0(0)	0(0)	1(17%)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. durans **n=1**	0(0)	0(0)	0(0)	0(0)	1(17%)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. gallinarum **n=1**	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(17%)	0(0)	0(0)	0(0)	0(0)
E. casseliflavus **n=4**	1(17%)	0(0)	0(0)	0(0)	2(33%)	0(0)	0(0)	1(17%)	0(0)	0(0)	0(0)	0(0)
E. avium **n=0**	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. cecorum **n=0**	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. malodoratus **n=0**	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Unidentified n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Total n=56	6(10.5%)	6(10.5%)	0(0)	3(5.3%)	6(10.5%)	3(5.3%)	3(5.3%)	6(10.5%)	6(10.5%)	6(10.5%)	6(10.5%)	6(10.5%)

TABLE 6. Distribution of *Enterococcus* spp. from poultry carcass rinses on farm

Carcass rinses

No. of isolates (%)

	Flavomycin		Virginiamycin		Virginiamycin		Virginiamycin		Flavomycin		Bacitracin	
Species	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
***E. faecium* n=134**	37(62%)	10(16%)	20(33%)	12(20%)	6(10%)	6(10%)	0(0)	4(7%)	13(21%)	13(23%)	10(17%)	4(7%)
***E. faecalis* n=491**	11(18%)	33(55%)	30(50%)	42(70%)	53(88%)	48(80%)	60(100%)	45(78%)	46(77%)	44(77%)	27(45%)	52(86%)
***E. hirae* n=26**	0(0)	1(2%)	1(2%)	0(0)	0(0)	2(3%)	0(0)	1(2%)	0(0)	0(0)	22(37%)	0(0)
***E. durans* n=5**	0(0)	1(2%)	1(2%)	0(0)	0(0)	1(2%)	0(0)	0(0)	0(0)	0(0)	0(0)	2(3%)
***E. gallinarum* n=17**	3(5%)	4(7%)	2(3%)	4(7%)	0(0)	1(2%)	0(0)	3(5%)	0(0)	0(0)	0(0)	0(0)
***E. casseliflavus* n=34**	8(13%)	10(16%)	4(7%)	2(3%)	1(2%)	2(3%)	0(0)	4(7%)	1(2%)	0(0)	1(2%)	1(2%)
***E. avium* n=2**	1(2%)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(2%)
***E. cecorum* n=1**	0(0)	1(2%)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
***E. malodoratus* n=1**	0(0)	1(2%)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Unidentified n=2	0(0)	0(0)	1(1.6%)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(2%)
Total n=713	60(8.4%)	60(8.4%)	59(8.3%)	60(8.4%)	60(8.4%)	60(8.4%)	60(8.4%)	57(8%)	60(8.4%)	57(8%)	60(8.4%)	60(8.4%)

TABLE 7. Distribution of *Enterococcus* spp. from poultry boxliners on farm

Boxliners

No. of isolates (%)

	Flavomycin		Virginiamycin		Virginiamycin		Virginiamycin		Flavomycin		Bacitracin	
Species	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
E. faecium n=16	3(5%)	3(10%)	2(22%)	3(14%)	0(0)	0(0)	1(8%)	0(0)	0(0)	2(22%)	0(0)	2(33%)
E. faecalis n=175	57(95%)	27(90%)	7(78%)	18(86%)	6(100%)	6(100%)	11(92%)	15(100%)	12(100%)	7(78%)	6(100%)	4(67%)
E. hirae n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. durans n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. gallinarum n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. casseliflavus n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. avium n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. cecorum n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
E. malodoratus n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Unidentified n=0	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Total n=192	60(31%)	30(16%)	9(5%)	21(11%)	6(3%)	6(3%)	12(6%)	15(8%)	12(6%)	9(5%)	6(3%)	6(3%)

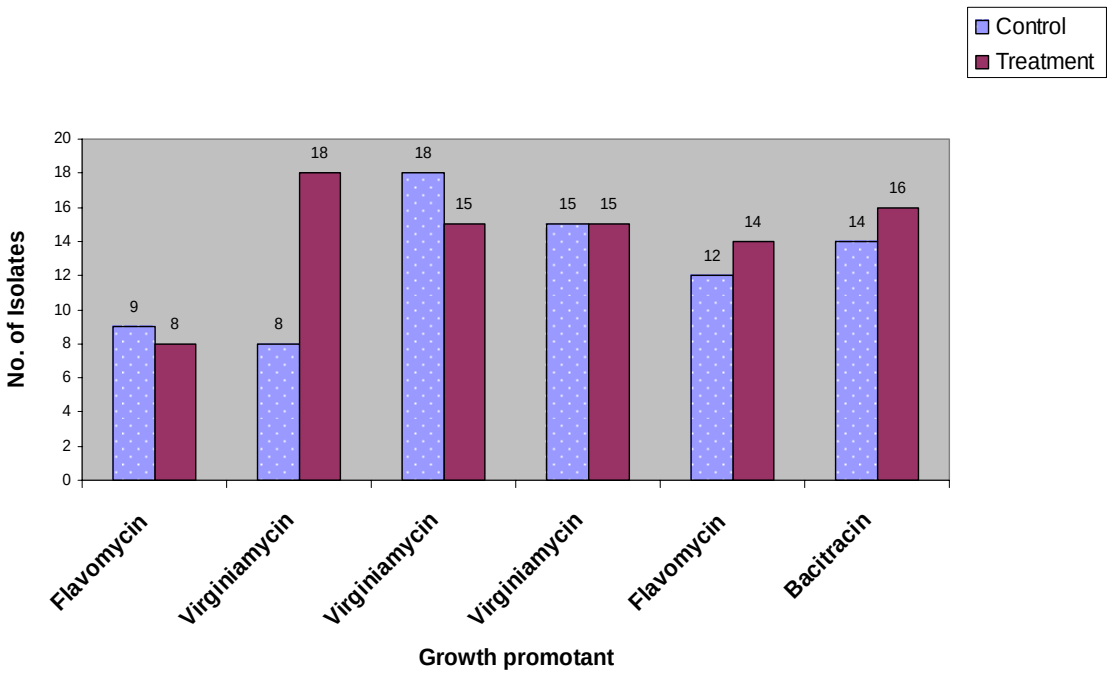

Fig. 3. Comparison of control vs. treated houses *Enterococcus faecium* species distribution in litter at 7 weeks. No growth promotants were used in control houses, but were used in treated houses. Growth promotants used per growout were: 1-flavomycin, 2-virginiamycin, 3-virginiamycin, 4-virginiamycin, 5-flavomycin, and 6- bacitracin.

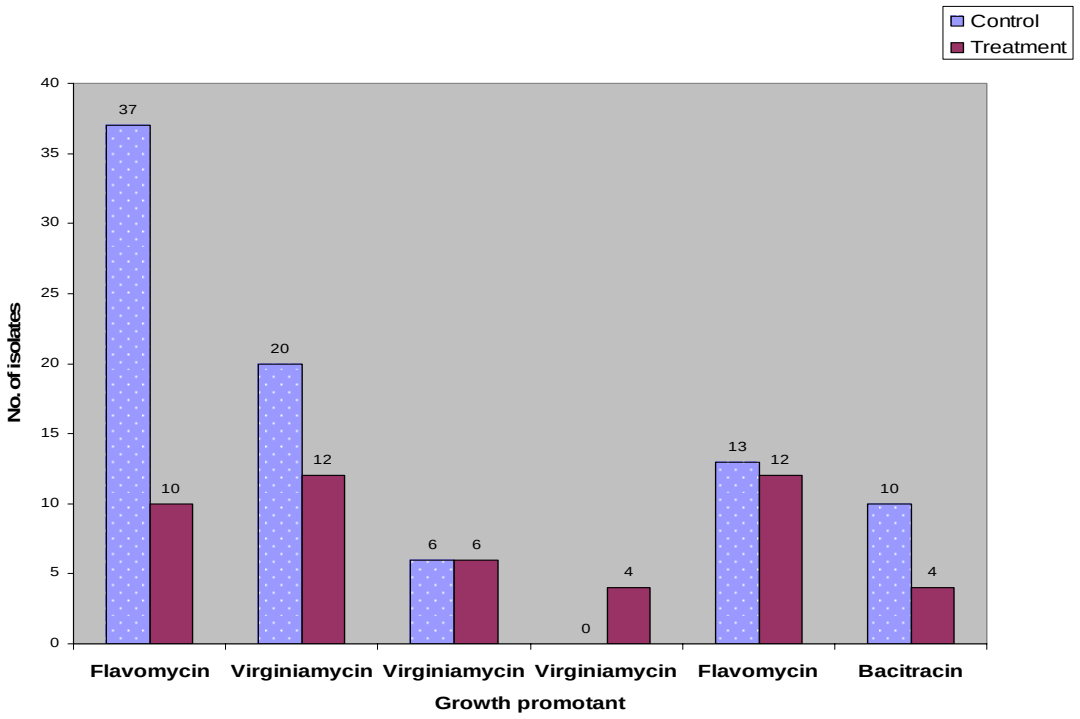

Fig. 4. Comparison of control vs. treated houses *Enterococcus faecium* species distribution in carcass rinses. No growth promotants were used in control houses, but were used in treated houses. Growth promotants used per growout were: 1-flavomycin, 2-virginiamycin, 3-virginiamycin,4-virginiamycin, 5-flavomycin, and 6- bacitracin

Table 8. Statistical analysis using Chi-square probability of *E. faecium* from litter 7 weeks and carcass rinses [a]

Source	Virginiamycin vs. Virginiamycin growouts	1st Flavomycin vs. Virginiamycin growouts	1st Flavomycin vs. all Virginiamycin growouts
Litter 7 weeks	2 vs. 3	1 vs. 3	1 vs. 3,4,5*
	3 vs. 4	1 vs. 4	
	2 vs. 4	1 vs. 2*	
Carcass Rinses			
	2 vs. 3	1 vs. 3	1 vs. 3,4,5
	3 vs. 4*	1 vs. 4*	
	2 vs. 4*	1 vs. 2	

[a]- Growouts are as follows: 1-flavomycin, 2-virginiamycin, 3-virginiamycin, 4-virginiamycin, 5-flavomycin, 6-bacitracin.
*significant different ($P \leq 0.05$)

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CHAPTER 3

Genetic relatedness of *Enterococcus faecium* on a poultry farm[1]

[1]**Debnam, Antoinette, Charlene Jackson, Charles L. Hofacre. Submitted to Journal of Food Protection**.

Genetic relatedness of *Enterococcus faecium* from a poultry farm

ABSTRACT

With the increasing prevalence of vancomycin-resistant *Enterococcus faecium* (VREF) over the past decade, the FDA approved the use of a quinupristin-dalfopristin drug, Synercid, for the treatment of VREF in 1999. Quinupristin /dalfopristin is a combination of streptogramin A and B antimicrobial agents. Virginiamycin, a streptogramin A and B combination, is a growth promotant that has been used in the agricultural industry for decades. The human health community is concerned about virginiamycin, based on the theory that its use in animals has established a reservoir of streptogramin resistant bacteria in poultry and other food animals. All strains of *E. faecalis* are intrinsically resistant to quinupristin/dalfoprisitin. This study examines whether growth promotants usage, primarily virginiamycin, affects the population of *E. faecium* on a genetic level. Phylogenetic analysis was performed of *E. faecium* from four poultry houses on a farm. Two houses on the farm were control houses and did not use any antimicrobials while two other houses on each farm used flavomycin, virginiamycin, and bacitracin during different poultry growouts. Litter, chick boxliners, feed, and poultry carcasses were obtained from each house and cultured for the presence of enterococci. *E. faecium* isolates were characterized using BOX-PCR and Pulsed-Field Gel Electrophoresis (PFGE). Results, with both method, idicated that the *E.faecium* strains have a high degree of genetic diversity.

INTRODUCTION

Enterococci are a frequent source of nosocomial infections, ranging from urinary tract infections, endocarditis, surgical wounds, to bacteremia (15). Two species of enterococci, *Enterococcus faecalis* and *Enterococcus faecium* account for a majority of hospital-acquired infections (6). In addition, enterococci are intrinsically resistant to an array of antibiotics, and have the ability to acquire resistance (10). Therefore, the human health community is concerned about the future treatment of enterococcal infections. With the increasing prevalence of vancomycin-resistant *Enterococcus faecium* (VREF) over the past decade, the FDA has approved the use of quinupristin/dalfopristin for the treatment of VREF in 1999 (12). Quinupristin /dalfopristin are a combination of streptogramin A and B antimicrobial agents (dalfopristin 70% , quinupristin 30%), the two streptogramins work synergistically targeting the 50S bacterial ribosome to inhibit protein synthesis (13). Streptogramins are a part of the macrolide, lincosoamide, and streptogramin (MLS) antibiotics. Virginiamycin, a streptogramin A and B combination, is a growth promotant that has been used in agriculture for decades (13). Although the majority of strains of *E. faecalis* are intrinsically resistant to quinupristin/dalfopristin, *E. faecium* strains are not (5). The human health community is concerned as to whether usage of virginiamycin as a growth promotant for decades is responsible for a reservoir of resistance genes to Synercid in human enterococci (4).

Growth promotant usage affect on *E. faecium* antimicrobial susceptibilities has been examined previously (1) . In this study, the *E. faecium* population present on a poultry farm was

examined in order to determined if usage of growth promotants (flavomycin, virginiamycin, and bacitracin) causes a shift in genetic relatedness. Furthermore, although a large number of phenotypic and genotypic typing methods have been applied to phylogenetic analysis of *E. faecium*, pulsed-field gel electrophoresis (PFGE), is considered the gold-standard for epidemiological analysis of enterococci (10). However, Malathum *et al.* described a BOX-PCR which is a faster and cheaper method for typing clinical isolates of *E. faecalis* (8). The purpose of this study was to evaluate the efficiency of these two typing methods for determination of genetic relatedness of *E. faecium* from poultry environment samples.

MATERIALS AND METHODS

Origin of samples: Between 2002 and 2003, samples were collected from a commercial four house broiler farm in North Georgia. Broiler chickens from six growouts from four different houses were studied. A grow-out period consisted of chickens ranging from a few days old to slaughter (eight weeks old). The farm was contracted to raise chickens for a single commercially integrated company. The company provided the farm with the chickens and feed. The four houses were designated A, B, C, and D; A and B were the control houses and C and D were the treatment houses. The treatment houses were administered a different antibiotic for the six growouts. The antibiotics used in the final feed before slaughter were flavomycin (2 grams per ton) for growout 1, virginiamycin (20 grams per ton) for growout 2, virginiamycin (20 grams per ton) for growout 3, virginiamycin (20 grams per ton) for growout 4, flavomycin (20 grams per ton) for growout 5, and bacitracin (20 grams per ton) for growout 6, respectively. Types of samples and methods of sampling and culturing were as follows:

Boxliners: Whole boxliners were collected after chicks were transported to the houses from the hatchery. Contents of the boxliners were sampled aseptically with swabs. Swab samples were placed into 50 ml conical tubes filled with 40 ml of 1X phosphate buffered saline (PBS, pH 7.2) and mixed with a shaker for ten minutes. Debris was removed by filtering with gauze into a new conical tube and supernatant was discarded. One hundred microliters was removed for plating onto M- Enterococcus agar (Becton Dickinson Microbiology Systems, Sparks, MD) for isolation.

Litter: Wood shavings from softwoods were used as bedding, material commonly used in poultry houses in the southeastern United States. The litter remained unchanged in each houses throughout the study period. Litter samples were a composite of five locations in the house and then pooled. Five of these samples were collected from each house. Five grams of chicken litter was weighed out in a 50 ml conical tube with 30 ml of 1X PBS, pH 7.2 and mixed with a wrist-action shaker for five minutes. Debris was removed by low speed centrifugation (600 rpm, 15 minutes). The bacteria were pelleted by high speed centrifugation (10,000 rpm, 15 min) and the supernatant was discarded. The resulting pellet was streaked onto M-Enterococcus agar (Becton Dickinson Microbiology Systems, Sparks, MD) for isolation and identification. Pre-litter samples were composed of samples taken from the last layer of bedding used before the study was conducted.

Feed: Heat-treated pelleted feed was fed to the chickens ad libitum. All feed was stored in steel storage tanks with no access to rodents or wild birds. The control houses received feed without antibiotics, while the treated houses received the above mentioned antibiotics. Ten grams of feed was collected at four and seven weeks per growout. Samples were collected as the feed flowed from the pipes which delivered feed to each feeder in the houses to eliminate

contamination. Samples were taken aseptically with changing of latex gloves between each sample. The samples were processed in the same manner as litter samples.

Carcass rinses: Ten random chicken carcasses per house were selected immediately before the chickens entered the cold water chill-tank and placed in a container with ice for refrigeration. Each whole chicken was rinsed in 250 ml of peptone water in an automated carcass shaker for one minute at the USDA-ARS-RRC. Forty-five milliliters of rinsate from the bag was transferred to a 50 ml conical tube and 100 µl were inoculated on M-Enterococcus agar (Becton Dickinson Microbiology Systems, Sparks, MD) for isolation.

Isolation and initial identification: Ten well isolated positive colonies from M-Enterococcus agar (Becton Dickinson Microbiology Systems, Sparks, MD) were subcultured onto blood agar and Enterococcosel agar (Becton Dickinson Microbiology Systems, Sparks, MD) and incubated for 24 h at 37^0C. Initial identification was performed using Gram staining, catalase test, bile-esculine test, and pyrrolidonyl-β-naphthylamide (PYR)(16). One colony per isolate was selected and placed into a 96-well plate containing bile-esculin agar from the different houses.

Identification of *Enterococcus* spp. by PCR and phenotypic testing: All isolates were tested in a multiplex, species–specific PCR for 24 species of *Enterococcus* as previously described by Jackson *et al*. (7) Since the method is novel, isolates were also identified with the automatic Vitek 32 system (bioMerieux Vitek, Hazelwood, MO, USA) Vitek Gram Positive Identification Card (GPI) (bioMérieux Vitek Inc., Hazelwood, Mich) according to the manufacturer's instructions.

BOX-PCR: BOX-PCR was performed on all *E. faecium* isolates as previously described by Malathum *et al*. with the following modifications (8). Whole cell template was prepared with

a well isolated colony inoculated in 50 µl ddH_20. The base master mix consisted of 5 µl of 20mM $MgCl_2$ (with ficol and tartrazine) (Idaho Technology, Salt Lake City, UT) , 2.5µl of the BOXA2R primer (1.25mM) (Roche, Indianapolis, IN), 0.5µl of a 10mM dNTP mix (Roche, Indianapolis, IN), 2.5 µl of a 10% dimethyl sulfoxide (Sigma, St. Louis, MO), 11.5 µl of ddH_20, and 0.5 µl of a 250 U *Taq* DNA polymerase (Roche, Indianapolis, IN). PCR reactions were performed in a final volume of 25 µl consisting of 22.5 µl of master mix and 2.5 µl of whole cell template. Ten microliters of product was electrophoresed on a 1.5% 1 X TAE agarose gel containing 2 ug/ml ethidium bromide. DNA molecular weight marker XVII (500 bp, Roche, Indianapolis, IN was used as the standard. Electrophoretic separation was at 100 V for 85 min.

Characterization by PFGE: PFGE on all the *E. faecium* strains was performed as previously described by Turabelidze *et al*. with the following modifications (17). Five hundred microliters of Brain Heart Infusion (BHI) (DIFCO, Detroit, MI) was inoculated with a single *Enterococcus* isolate and incubated 16-18 h at 37^0C with shaking. A pellet was formed by centrifugation at 4900 RPM for five minutes. The pellet was resuspended in one ml 1XTris-EDTA (TE,pH 8.0) buffer (Sigma, St. Louis , MO). The resulting pellet was washed two more times by centrifuging at 4900 RPM for two minutes and then resuspended in one ml TE buffer (Sigma, St. Louis , MO). Optical density (2.5 X 10^9 CFU/ml) was performed using a Dade turbidity meter. Two hundred microliters of the suspension was added to 0.2 ml of the lysis solution consisting of 50 mM Tris-HCl ,pH 8.0, 50 mM EDTA, 1,250 U/ml mutanolysin, 2.5 mg/ml lysozyme, and 1.5 mg/ml proteinase K as previously described (17); Cells were lysed 10 min at 37°C. Four ml of molten 1.2% Seakem Gold Agarose in TE buffer was added, gently mixed by pipetting up and down and immediately dispensed into a reusable plug mold (Biorad, Hercules, CA). Agar was solidified by placing at –20 ^{0} C for five minutes. Plugs were transferred

into 50 ml polypropylene screw cap tubes containing five ml of the proteolysis solution consisting of 0.5 M EDTA, 1% sarcosyl, and 400 µg of proteinase K/ml; 2 h at 55°C as described previously (17). Tubes were incubated in a 54^0C shaking water bath for two hours. Plugs were then removed and proteolysis solution was decanted. Plugs were washed using a Tube Plug washer as follows: One liter of sterile water was prewarmed to 54^0 C and circulated through the Tube Plug washer for ten minutes. One liter of fresh sterile water was flushed through the Tube Plug washer and, upon completion, water was drained from the tube. One liter of prewarmed to 54^0C TE buffer was then circulated through the Tube Plug washer for ten minutes. Finally, the plugs were flushed with three liters of prewarmed (54^0C) TE buffer. Plugs were digested using 30 U of *SmaI* at 25^0C for two hours. *Salmonella* serotype Braenderup H9812 reference standard was used as the molecular standard. Electrophoresis was performed at 6 V/cm for 18 h with initial and final switch times of 5 s and 30 s, respectively. Gels were stained and destained as previously described (17).

Phylogenetic analyis: Dendrograms of PCR and PFGE results were generated using Bionumerics software program (Applied Maths, Sint-Martens-Latem, Belgium) using Dice coefficient and the unweighted pair group method (UPGMA). Optimization settings for both BOX-PCR and PFGE dendrograms were 1.06% and a band tolerance of 1%.

RESULTS

Phylogenetic analysis was conducted on all the *E. faecium* isolates (n=554) recovered in this study, in order to identify any genetic relatedness among sampling areas, houses (treated vs. control), and antibiotic administered. Both methods indicated that distinct clustering could not be

defined upon these criteria. Due to the large total number of isolates, separation into smaller groups by sampling areas was explored. Consequently, a high degree of genetic diversity was observed by this approach also. *E. faecium* on this farm appeared to represent a heterogeneous population. In order, to visualize the results presented below, isolates were further separated by not only sampling area, but also houses [A,B (control); C,D (treated); Fig.1-30].

Boxliners: PCR products generated with BOXA2R primers yielded 7 to 11 bands. The amplification products were mainly in the range of 300-2600 bp. Isolates with patterns with two or more band difference were observed. By this criterion, 16 different patterns among the 17 isolates were observed. Only two isolates shared the same pattern and they were from the same house and growout (Fig.1). The genetic relationships between the 17 isolates of boxliners exhibited 43% similarity (Fig1.). Although clusters were examined by isolate, house (treated vs. control) and growth promotant administered, no distinct clustering could be defined based solely upon the criteria. PFGE patterns after restriction with *Sma I* were characterized by up to 10 to 18 bands in 02-1100 kb size range. Among the 17 isolates, there were 17 different PFGE patterns. The genetic relationships between the 17 isolates of boxliners exhibited 70% similarity (Fig. 2). Clusters were examined by isolate, house (treated vs. control), and growth promotant administered, distinct clustering could not be defined based solely upon these criteria.

Feed: PCR products generated with BOXA2R primers yielded 5 to 15 bands in isolates from feed at 4 weeks and 4 to 14 bands in feed at 7 weeks. Similar to boxliner samples, the amplification products from both feed time periods were mainly in the range of 300- 2600 bp. Feed at 4 weeks exhibited 27 different patterns among the 27 isolates, (Fig.3) while there were 39 different patterns among 40 isolates for feed at 7 weeks (Fig 5). The genetic relationships of feed at 4 weeks and feed at 7 weeks were 41.83% and 43% similarity, respectively (Fig. 3 and 5).

Although clusters were examined by isolate, house (treated vs. control), and growth promotant administered, no distinct clustering could be defined based solely upon these criteria from either feed time periods. PFGE patterns after restriction with *Sma I* were characterized by up to 10 to 18 bands in size range 20- 1100 kb in feed at 4 weeks, while feed at 7 weeks were characterized by up to 10 to 20 within the same size range. Among the 27 isolates, there were 27 different PFGE patterns in feed at 4 weeks (Fig 4). There were 40 different PFGE patterns among the 40 isolates from feed at 7 weeks (Fig 6). The genetic relationships between the isolates of feed at 4 weeks exhibited 75% similarity, while feed at 7 weeks isolates exhibited 70% similarity, respectively (Fig. 4 and 6). Clusters were examined by isolate, and houses (treated vs. control), and growth promotant administered, no distinct clustering could be defined solely upon these criteria from either feed time periods for BOX-PCR or PFGE.

Litter: PCR products generated with BOXA2R primer yielded 5 to 13 bands from litter 4 weeks isolates, and 6 to 15 bands in isolates from litter at 7 weeks. The amplification products for both litter time periods were mainly in the range of 300-2600bp. Litter at 4 weeks contained a total of 174 isolates. 154 of these isolates had 154 different patterns. The remaining 20 isolates shared nine other patterns. Two of these isolates were grouped according to the same house and growout (house D, growout three) , six isolates grouped according to the same growout (two isolates grouped for growout one, two isolates grouped for growout four, and two isolates grouped for growout two) ,and the other 12 isolates grouped without any of the same criteria (Fig.7-10). In litter at 7 weeks there were a total of 162 isolates. 144 of these isolates had 144 different patterns. The remaining 18 isolates shared eight other patterns. Four of these isolates were grouped according to the same house and growout (two isolates grouped according to house A, growout 4; two isolates grouped according to house A, growout six) , two isolates

grouped according to the same growout (growout five) and the other 12 isolates grouped without any of the same criteria (Fig. 15-18). The genetic relationships between the isolates of litter at 4 weeks exhibited 35% similarity, while the isolates of litter at 7 weeks exhibited 39.68% similairity. Although Clusters were examined by isolate, house (treated vs. control) and growth promotant administered, no distinct clustering could be defined based solely upon these criteria from either litter time periods. PFGE patterns were characterized by up to 8 to 20 bands in size range 20-1100kb in litter at 4 weeks isolates (Fig. 11-14), while litter at 7 weeks isolates were characterized by 7 to 20 bands in the same size range (Fig. 19-22). Among the 174 litter at 4 weeks isolates, there were 174 different PFGE patterns. Among the 162 litter at 7 weeks isolates, there were 162 different PFGE patterns. The genetic relationships between isolates from litter at 4 weeks and litter at 7 weeks exhibited 65.71% and 60.53% similarity, respectively. Clusters were examined by isolate, houses (treated vs. control) and growth promotant administered, no distinct clustering could be defined solely upon these criteria from either litter time periods for BOX-PCR or PFGE.

Carcass rinses: BOX-PCR products yielded 6 to 15 bands and were mainly in the range of 300-2600 bp. There were a total of 134 isolates. 125 isolates had 125 different patterns. The remaining nine isolates shared three other patterns. Three of these isolates grouped according to the same house and growout (house A, growout 1). The other six isolates grouped without any of the set criterion. The genetic relationships between the 134 isolates of carcass rinsates exhibited 35.38% similarity (Fig 23-26.). Although Clusters were examined by isolate, house (treated vs. control) and growth promotant administered, distinct clustering could not be defined based solely upon these criteria. PFGE patterns were characterized by 7 to 20 bands in size range 20-1100kb. Among the 134 isolates, there were 134 different PFGE patterns. The genetic relationships

between the 134 isolates of carcass rinsates exhibited 60.53% similarity (Fig. 27-30). Clusters were examined by isolate, houses (treated vs. control), and growth promotant administered, distinct clustering could not be defined solely upon these criteria for either BOX-PCR or PFGE.

Comparison of techniques: Band differences, including size and appearance (whether a band was there or not) was used to evaluate the isolates genetic relatedness. Most isolates which were classified as different by two bands or more with PCR were also different by PFGE. However, PFGE in all cases of sampling areas had no defined patterns that were the same among the isolates, whereas BOX-PCR found identical isolates in the case of feed at 7 weeks, litter at 4 weeks, litter at 7 weeks, and carcass rinsates. No clonality was found for the isolates using either method. Isolates according to both methods did not cluster based upon the criterion of house (treated vs. control), or growth promotant administered. Overall genetic relatedness among the *E. faecium* isolates strains was inferred from the numerical analysis by performing a UPGMA cluster analysis. In the cluster analysis from the BOX-PCR methods, overall similarity ranged from 35-45%, while PFGE overall similarities ranged 60-75%.

DISCUSSION

Molecular techniques provide genotypic characteristic of bacteria without the drawbacks of phenotypic methods. The purpose of using molecular typing in this ecology study was to evaluate *E. faecium* interspecies diversity among different sampling areas in the poultry environment. In addition, effects of antibiotics used as growth promotants on the *E. faecium* population on a genetic level was explored. In order to effectively examine the genetic relatedness of *E. faecium*, two different molecular typing methods, a rep-PCR (BOX-PCR) and

PFGE were used. It has been reported that a BOX-PCR technique could be directly applied for typing *E. faecalis* DNA as described by Malathum *et al. (8)* Although Pulsed-Field Gel Electrophoresis is considered the gold standard for typing *Enterococcus*, both methods were successfully used in this study to fingerprint *E. faecium* from poultry environment isolates. The results with both the PFGE and BOX-PCR analysis indicated a high degree of genetic diversity within *E. faecium*, irrespective of the sampling area, houses (treated or control), or growout.

The combination of litter 4 weeks, litter 7 weeks, feed 4 weeks, feed 7 weeks, carcass rinses, and boxliners produced dendrograms, that showed a high degree of genetic variation (data not shown), as there was no distinct clustering according to sampling area, between the control and treated houses, or the type of growth promotant administered. Due to the large number of isolates, individual dendrograms were created based upon sampling area. Once more, individual dendrograms indicated that there was no distinct clustering according to any criterion. Interestingly, when comparing the individual sampling area dendrograms there were some similarities. BOX-PCR generated dendrograms indicated an overall homology from boxliners, feed at 4 weeks, feed at 7 weeks, litter at 4 weeks, litter at 7 weeks, and carcass rinses to be 43%, 41.83%, 45%, 35%, 39.68%, and 35.38%, respectively. The overall homology of boxliners, feed at 4 weeks, feed at 7weeks, litter at 4 weeks, litter at 7 weeks, and carcass rinses from PFGE generated dendrograms were 70%,75%, 70%, 65.7%, 60.53%, and 60.53% respectively. The range of overall similarities from both methods was not broad. Furthermore, there were similarities seen between the different time periods from which samples were taken. For example, feed at 4 weeks compared to feed at 7 weeks from PFGE had overall similarities of 75% and 70%, respectively. Litter at 4 weeks and litter at 7 weeks had 65.71% and 60.53% overall similarities, respectively, by PFGE. Also, the number of bands produced and the size

range of the bands produced by each typing method were similar between the different sampling areas. Therefore, it seems that both methods indicate that sampling areas are quite similar to each other, indicating they may be a common gene pool of *E. faecium* shared on the farm. This hypothesis would explain why no distinct clustering was observed. Wegner *et al*. explored the hypothesis of a common *E. faecium* gene pool when comparing 84 isolates of *E. faecium* from swine, chickens, and humans in Denmark by *SmaI* generated macrorestriction profiles and *EcoRI* ribotyping (19). Similarity analysis by unweighted pair group method with arithmetic averages–derived dendrograms did not indicate a higher degree of similarity among *E. faecium* isolates (VRE as well as non-VRE) from humans than from animals. He concluded that this finding indirectly supports the hypothesis that *E. faecium* from different food animals and humans are not discrete populations, but belong to a common pool of *E. faecium* shared by animals and humans (19).

BOX-PCR is not commonly used as a technique for investigating *Enterococcus* genomes for strain fingerprinting, whereas PFGE is well established and considered the gold-standard (8). PFGE in other studies has proven to be reliable in generating DNA fragments that can be easily compared with isolates obtained from the DNA of other isolates (9). The major drawback of PFGE is the expensive equipment needed and time consumption (14). BOX-PCR offers a highly reproducible and simple method to distinguish closely related organisms that is less expensive than PFGE (3). This study utilized both BOX-PCR and PFGE for genotyping *E. faecium*. It was evident from the results that both methods were applicable to all strains and provided comparable levels of discrimination. The two methods indicated no clustering according to any criterion, comparable overall similarities between the different sampling areas, and both produced a range of band products and sizes that were comparable among the different sampling areas. The one

apparent difference between the methods were that BOX-PCR indicated some patterns as being the same among the isolates in boxliners, feed at 7 weeks, litter 4 weeks, litter 7 weeks , and carcass rinses, where as PFGE patterns for the isolates in these sampling areas were all different. Olive *et al.* explored different molecular typing methods and found that several studies have shown Rep-PCR to have good correlation with PFGE results but, in general, with slightly less discriminatory power (11). Nonetheless, since BOX-PCR is significantly faster and cheaper to perform than PFGE, it should prove to be an additional valuable tool for understanding the epidemiology of this species.

In conclusion, another aim of this study was to investigate whether usage of growth promotants, primarily virginiamycin would cause a change in the *E. faecium* population on a genetic level. Clusters from individual sampling areas were examined based upon whether they were administered growth promotant (treatment) or not (control), and the type of growth promotant received in the finisher diet including flavomycin, virginiamycin, and bacitracin. It has already been stated that there was no distinct clustering based upon any of these criteria. Control house isolates and treatment house isolates tended to type comparable to each other. Although, it was assumed that there would be some definitive factors influencing the genetic relatedness of these strains, results from this study indicated otherwise. However, throughout the literature there are examples of heterogeneous populations of *E. faecium*. Strains seem to be influenced by their resistance susceptibilities. For example, vancomycin resistant enterococci (VRE) have shown genetic diversity (2). *E. faecium* is thought to be distinctive based upon host-specificity (20). However, other studies have data showing non-host specific strains of *E. faecium* (18). It is apparent from these contradictory information that enterococci population dynamics need to be further investigated.

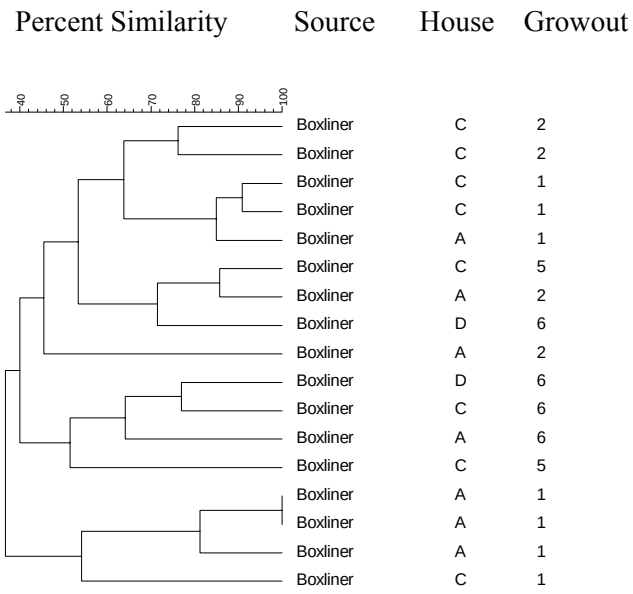

FIG. 1. Cluster analysis of *E. faecium* from poultry boxliners using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

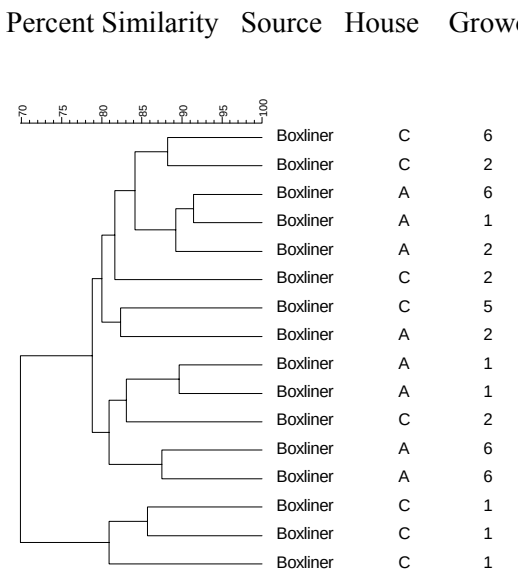

FIG. 2. Cluster analysis of *E. faecium* from poultry boxliners using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

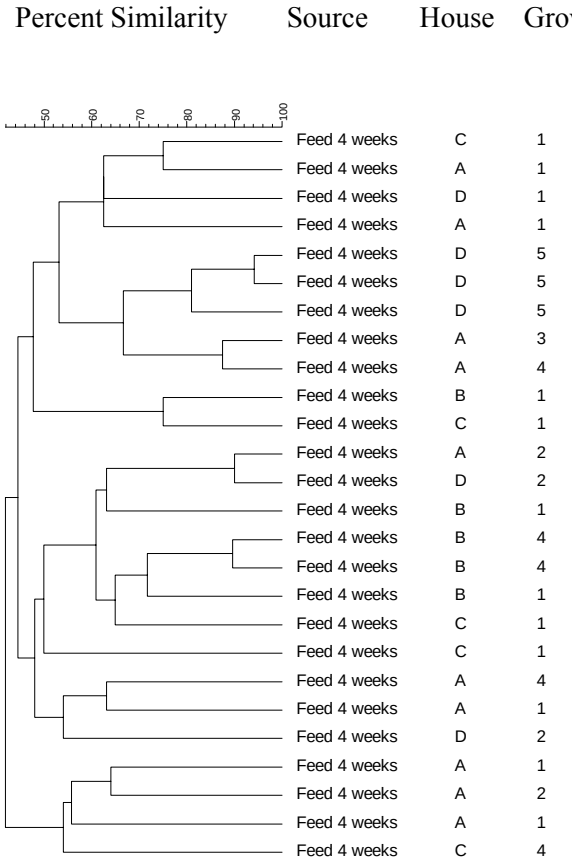

FIG 3. Cluster analysis of *E. faecium* from poultry feed 4 weeks using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

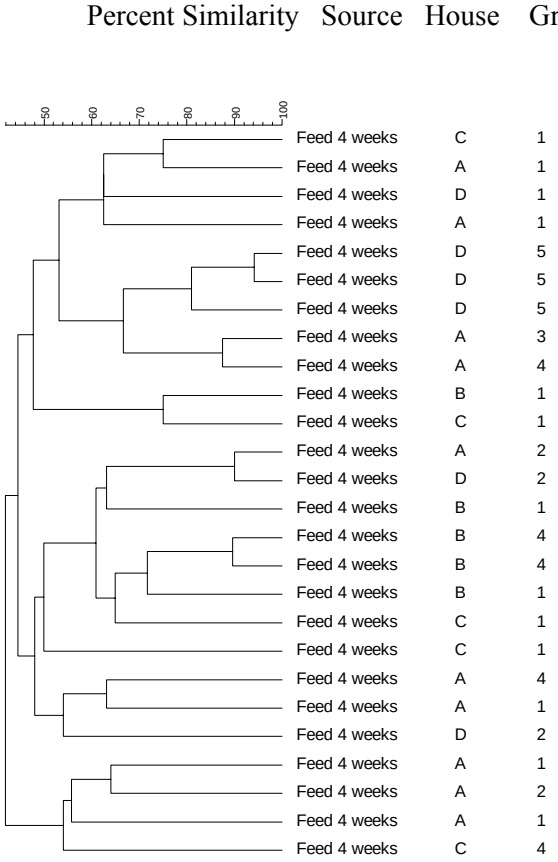

FIG. 4. Cluster analysis of *E. faecium* from poultry feed 4 weeks using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

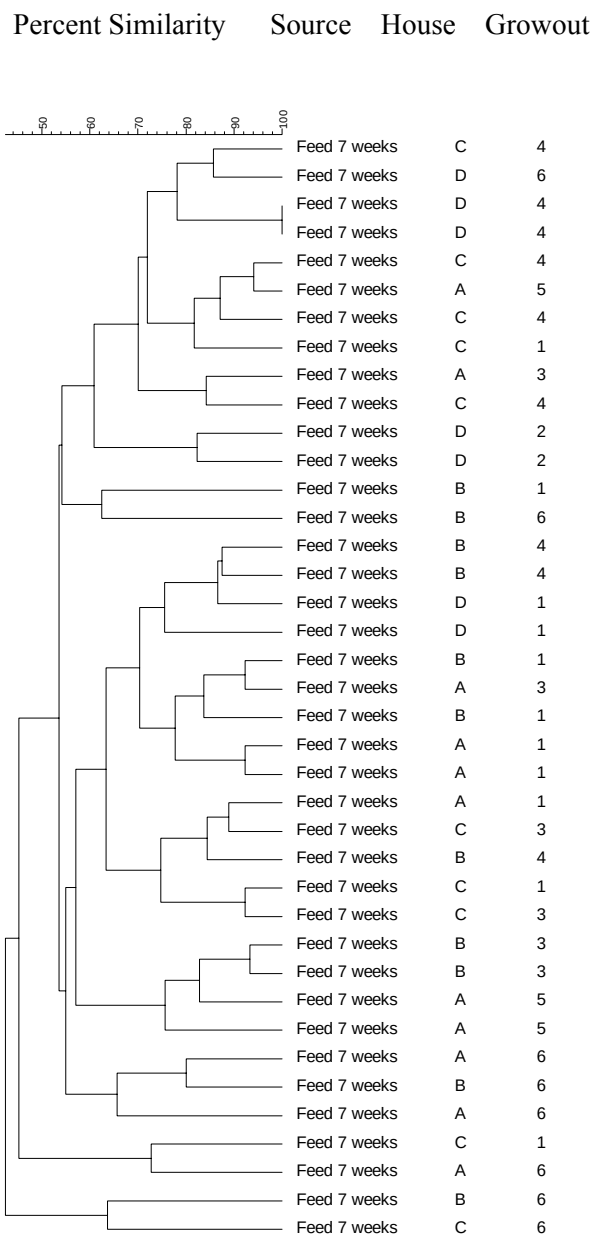

Fig. 5. Cluster analysis of *E. faecium* from poultry feed 7 weeks using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

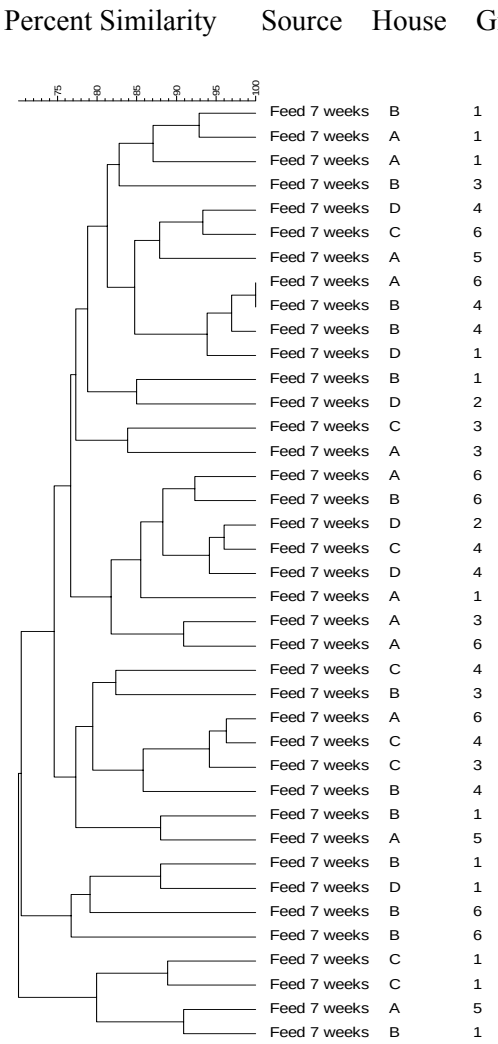

Fig 6. Cluster analysis of *E. faecium* from poultry feed 7 weeks using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

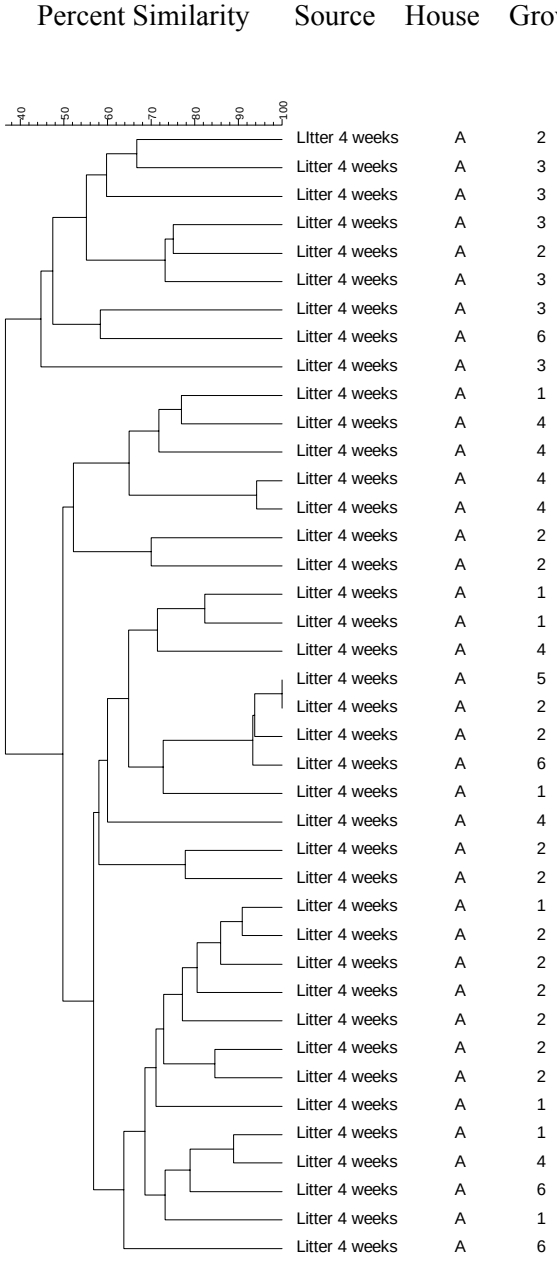

Fig. 7. Cluster analysis of *E. faecium* from poultry litter 4 weeks using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

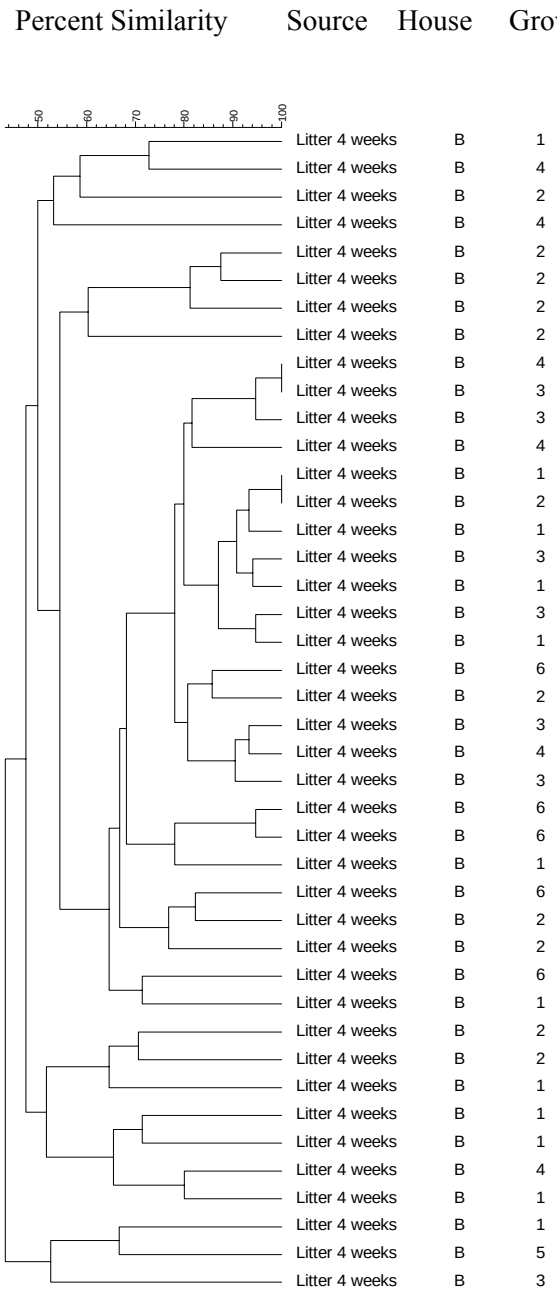

FIG.8. Cluster analysis of *E. faecium* from poultry litter 4 weeks using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

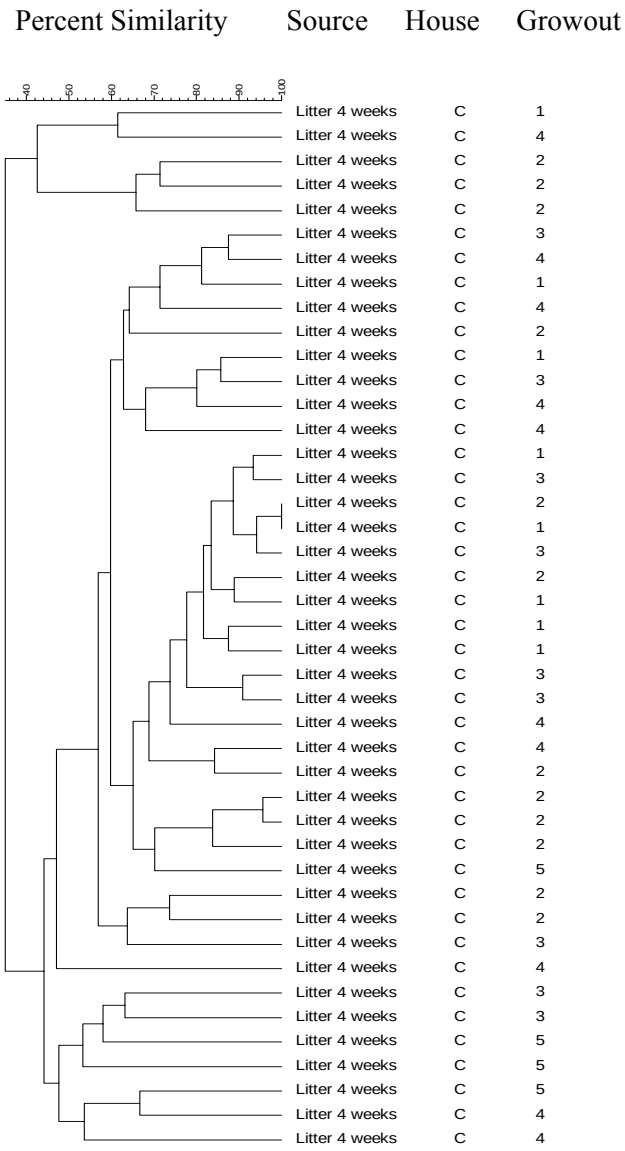

FIG.9.. Cluster analysis of *E. faecium* from poultry litter 4 weeks using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

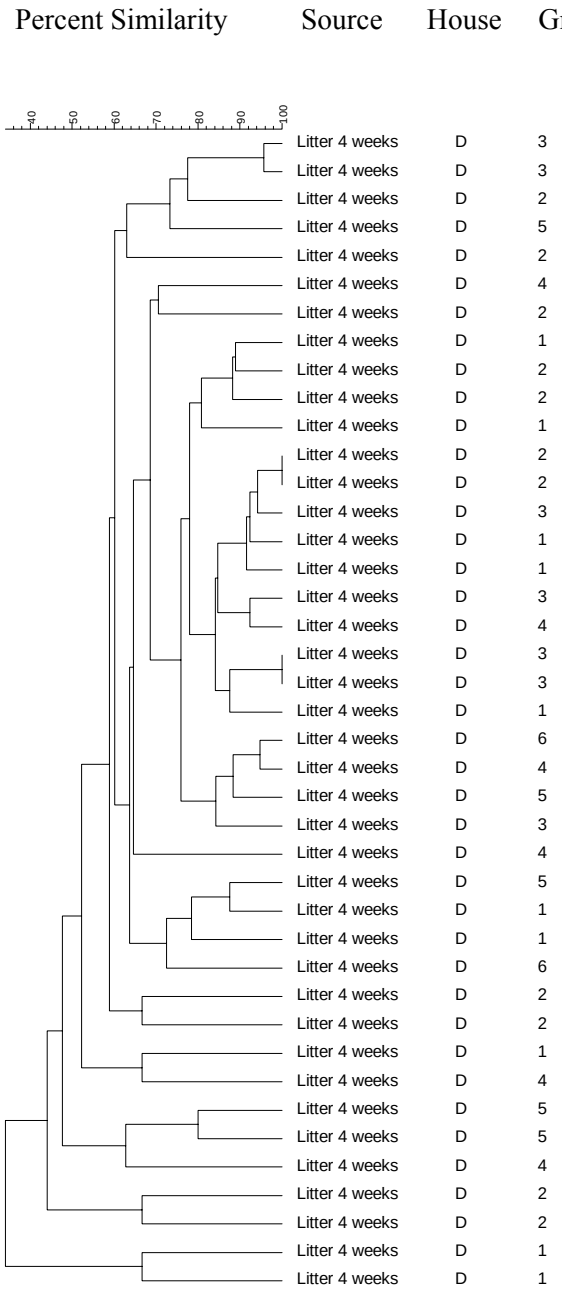

FIG.10.Cluster analysis of *E. faecium* from poultry litter 4 weeks using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

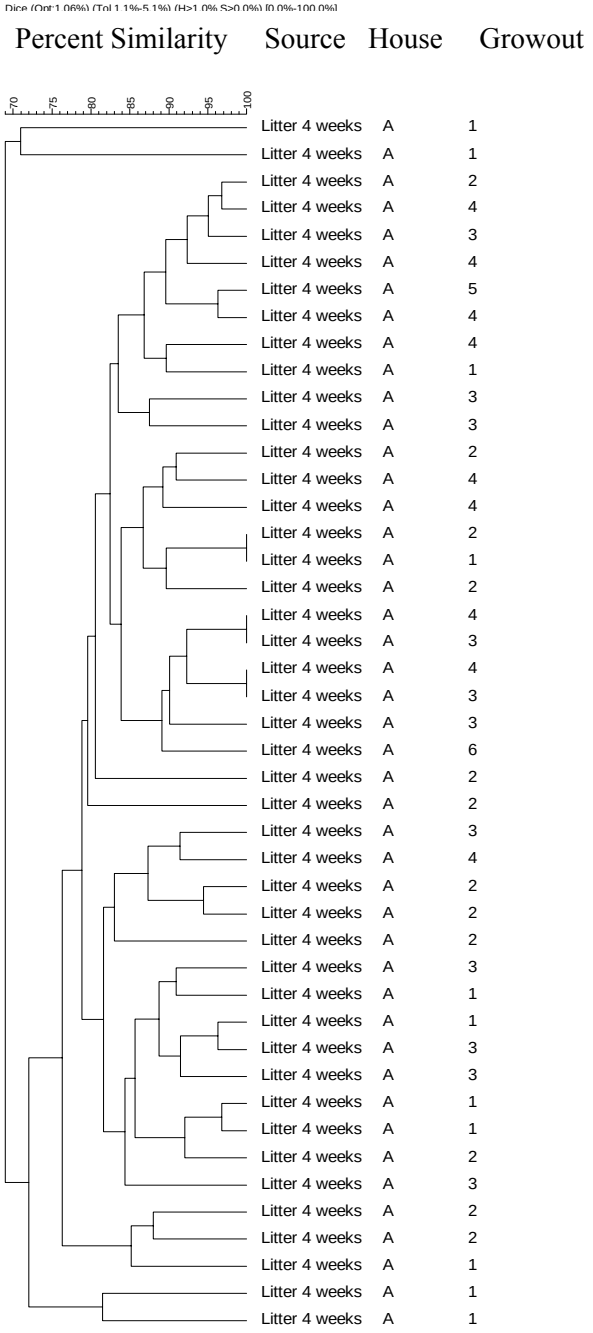

FIG.11. Cluster analysis of *E. faecium* from poultry litter 4 weeks using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

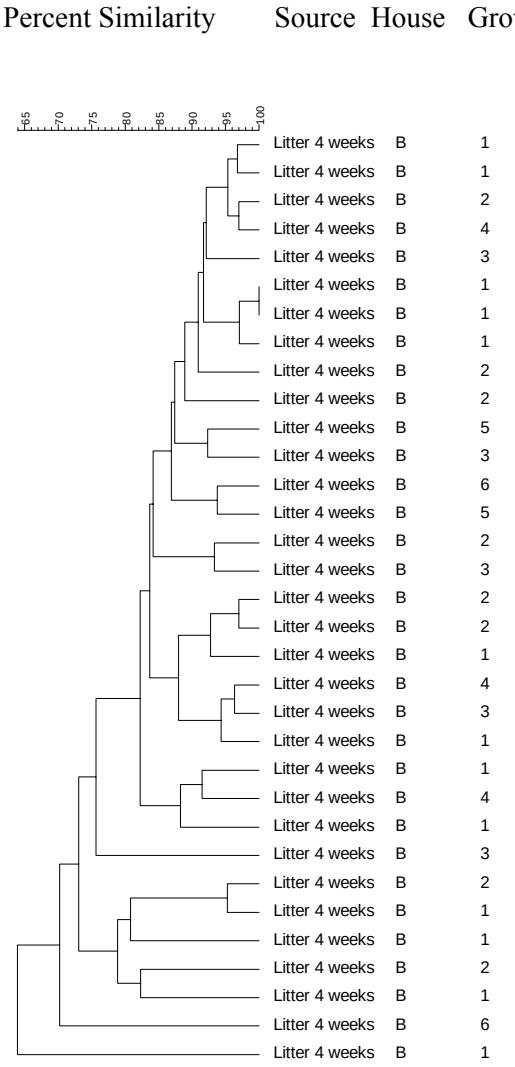

FIG. 12. Cluster analysis of *E. faecium* from litter 4 weeks using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

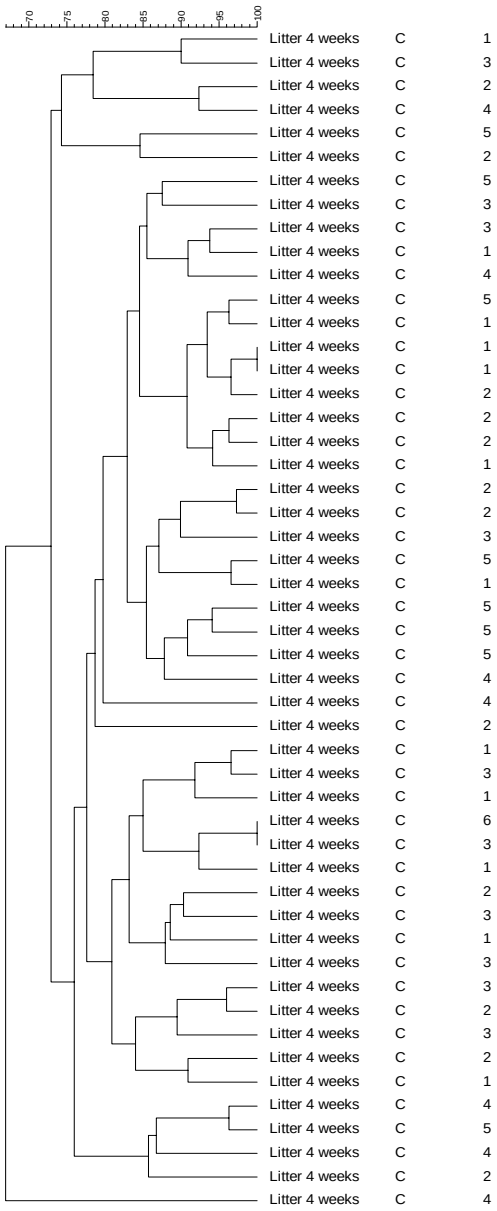

FIG. 13. Cluster analysis of *E. faecium* from poultry litter 4 weeks using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

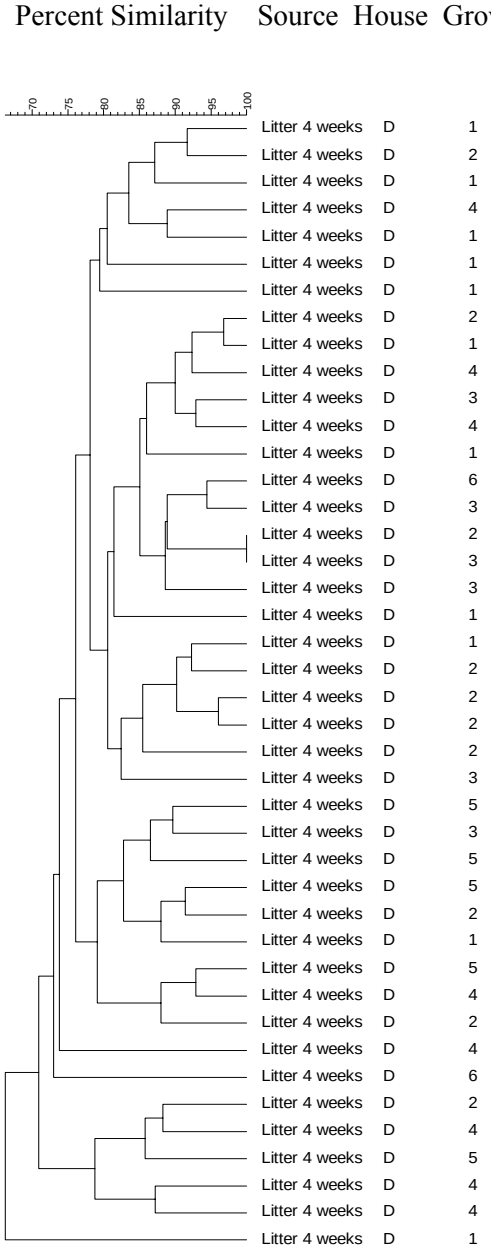

FIG. 14. Cluster analysis of *E. faecium* from poultry litter 4 weeks using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

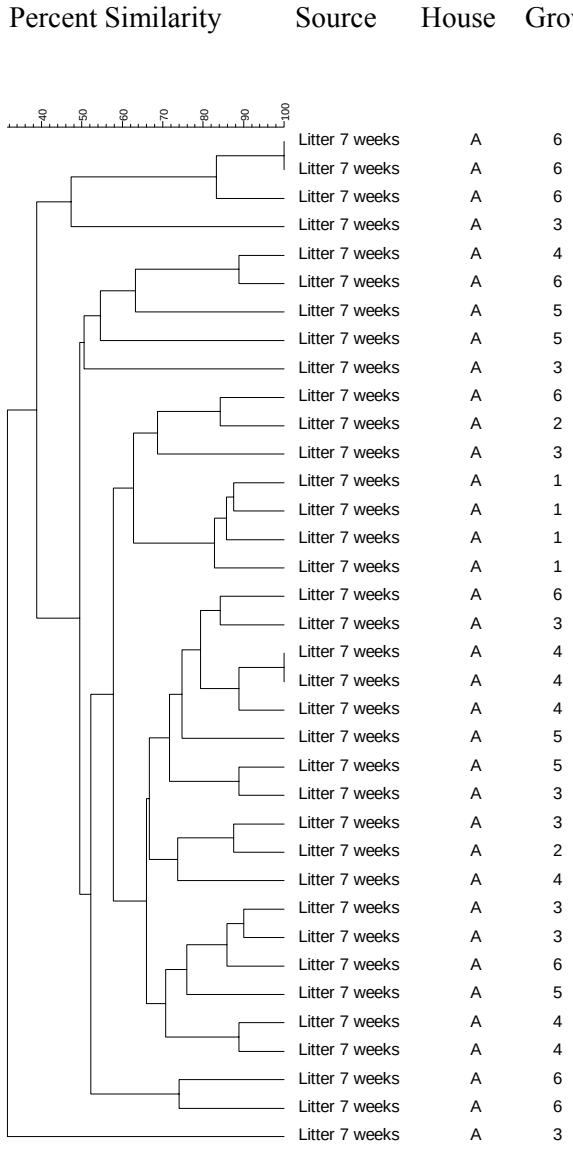

FIG. 15. Cluster analysis of *E. faecium* from poultry litter 7 weeks using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

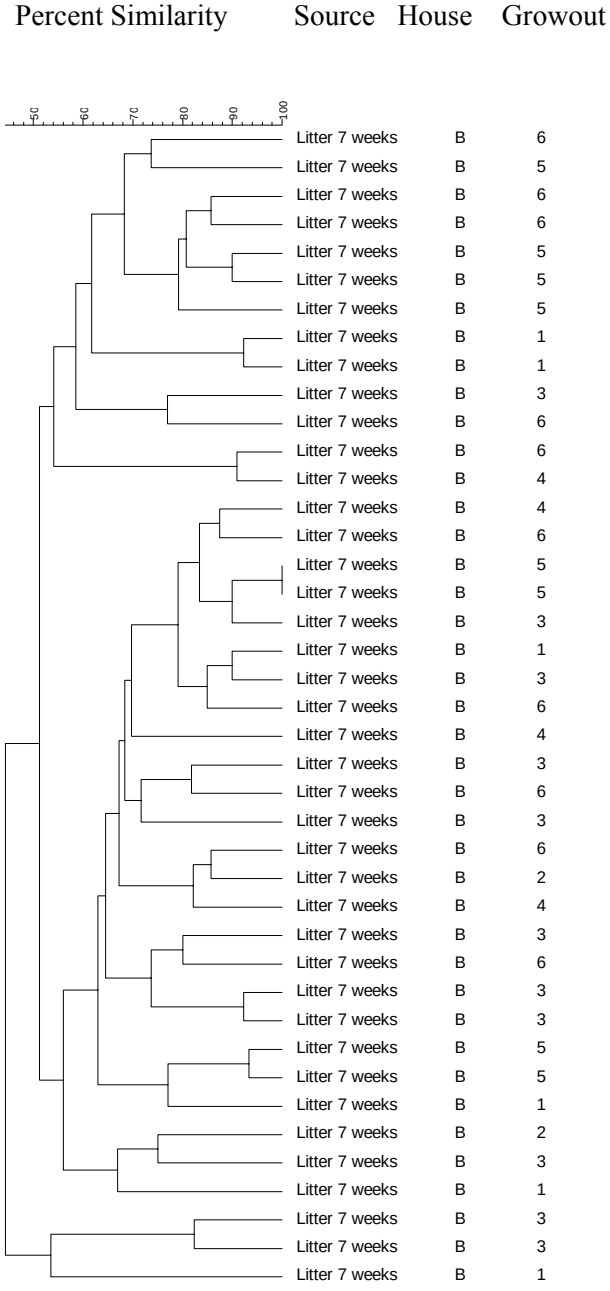

FIG. 16. Cluster analysis of *E. faecium* from poultry litter 7 weeks using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

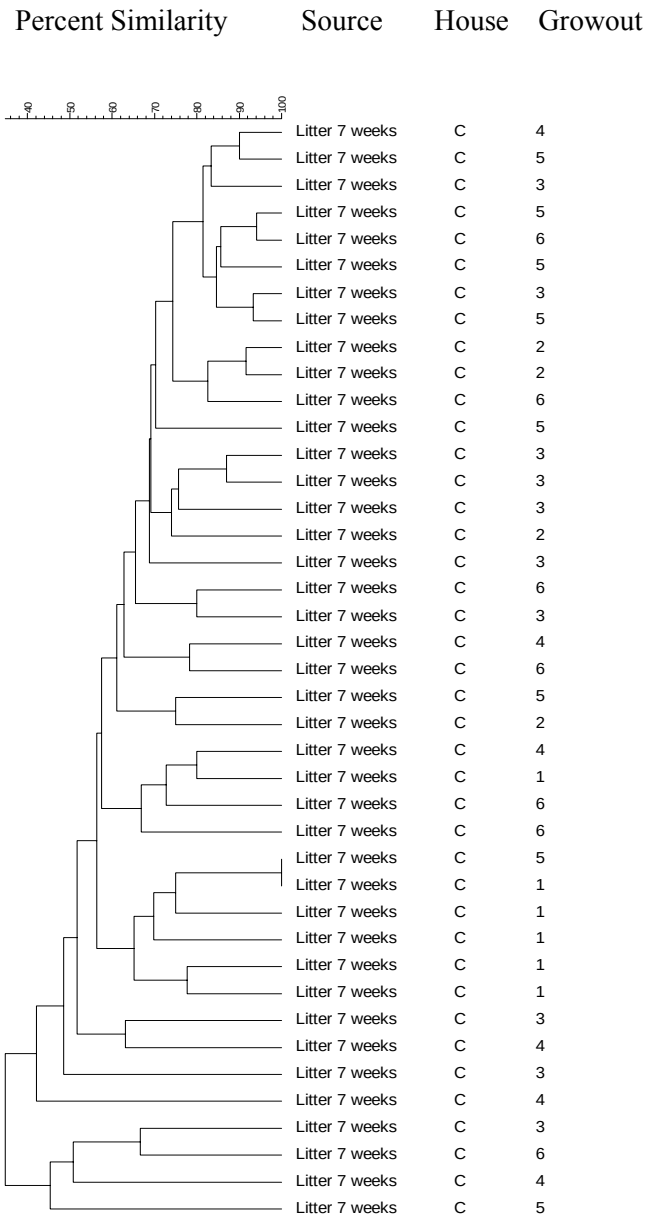

FIG. 17. Cluster analysis of *E. faecium* from poultry litter 7 weeks using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

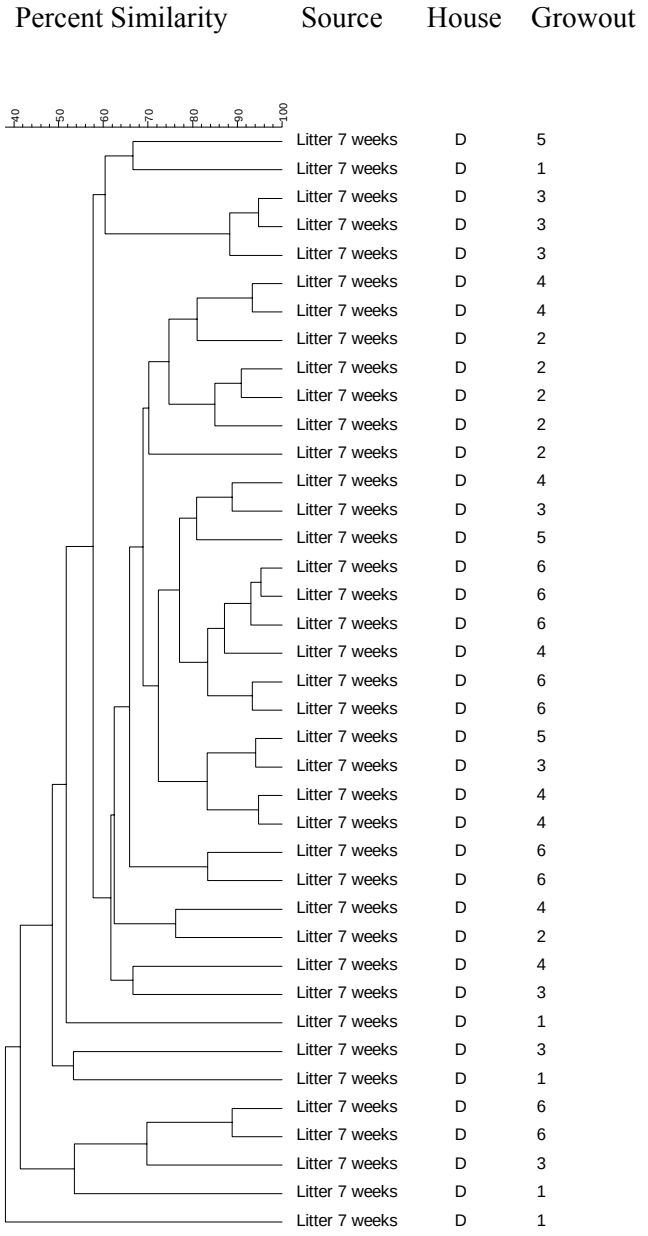

FIG. 18. Cluster analysis of *E. faecium* from poultry litter 7 weeks using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

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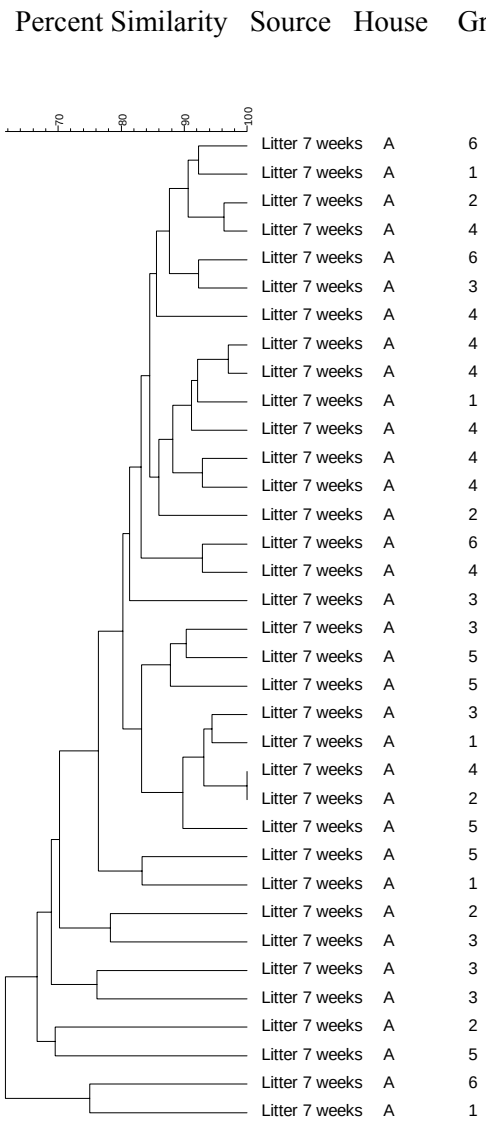

FIG. 19. Cluster analysis of *E. faecium* from poultry litter 7 weeks using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

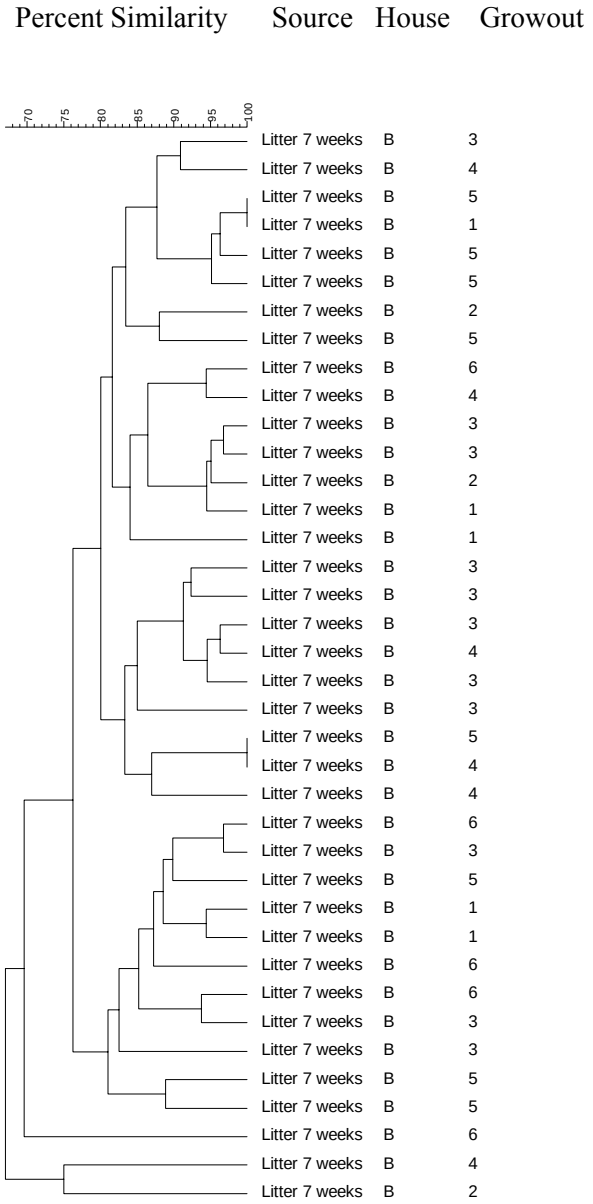

FIG. 20. Cluster analysis of *E. faecium* from poultry litter 7 weeks using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

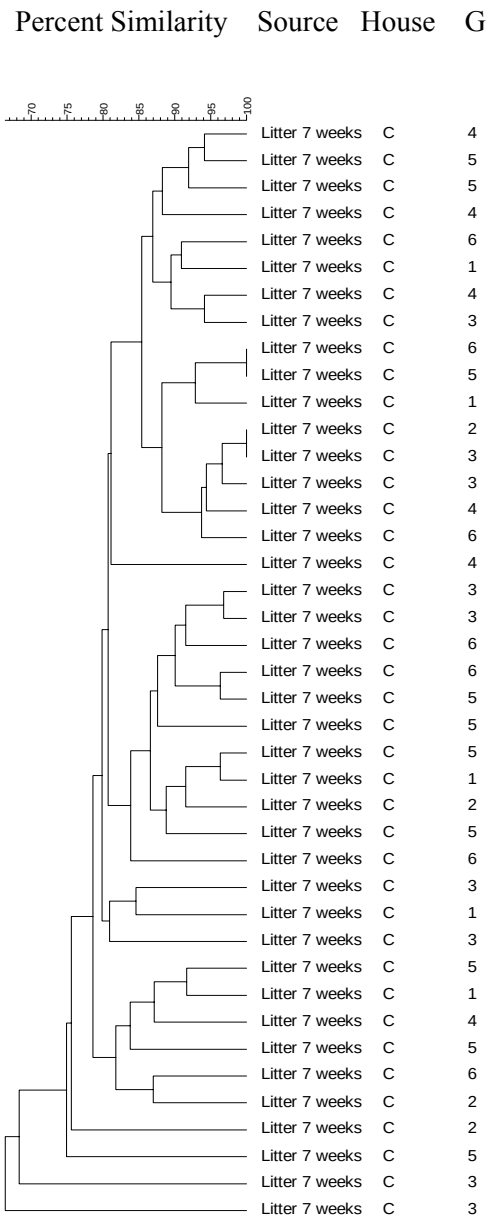

FIG. 21. Cluster analysis of *E. faecium* from litter 7 weeks using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

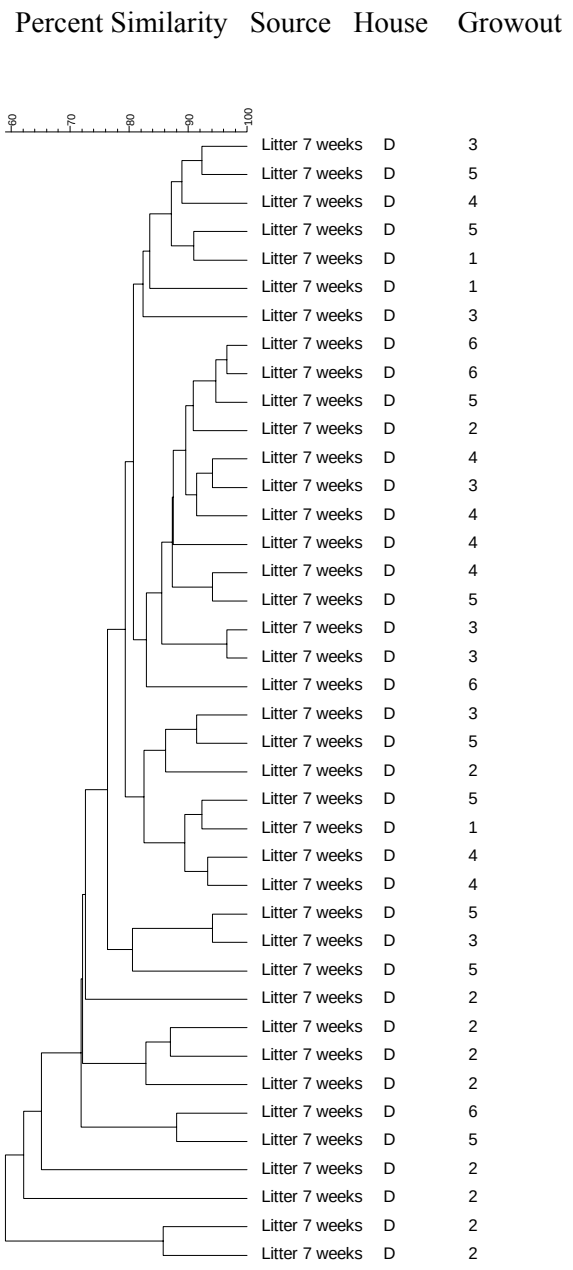

FIG. 22.. Cluster analysis of *E. faecium* from poultry litter 7 weeks using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

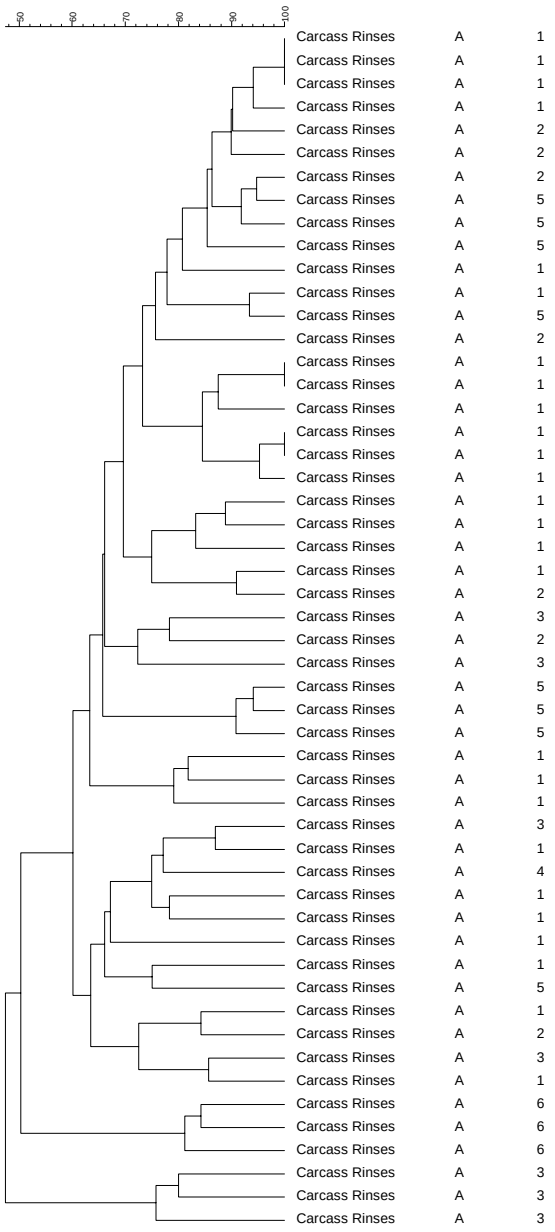

FIG. 23. Cluster analysis of *E. faecium* from poultry carcass rinses using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

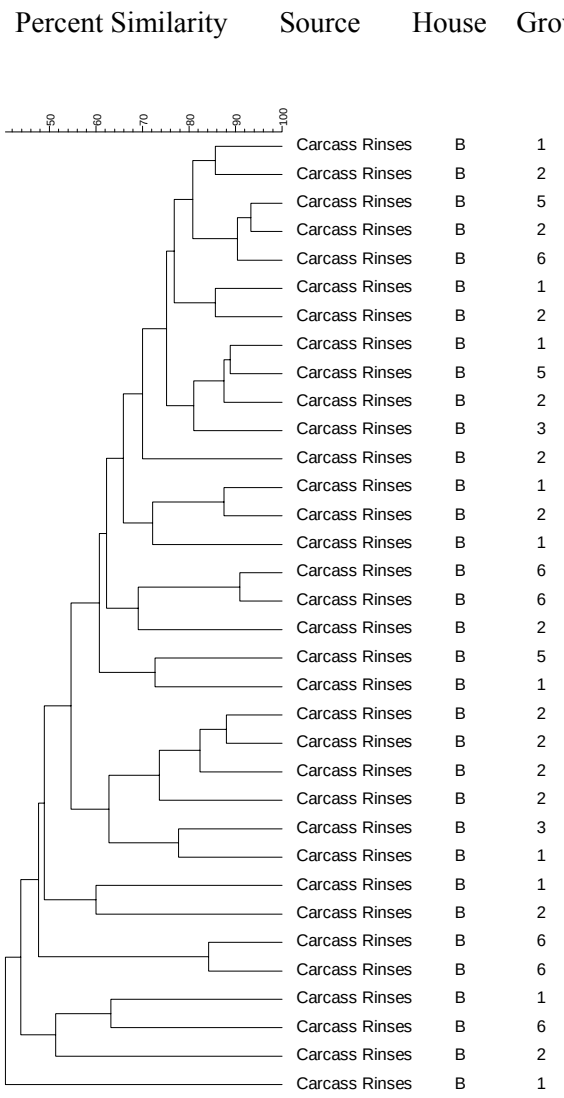

FIG. 24. Cluster analysis of *E. faecium* from poultry carcass rinses using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

.

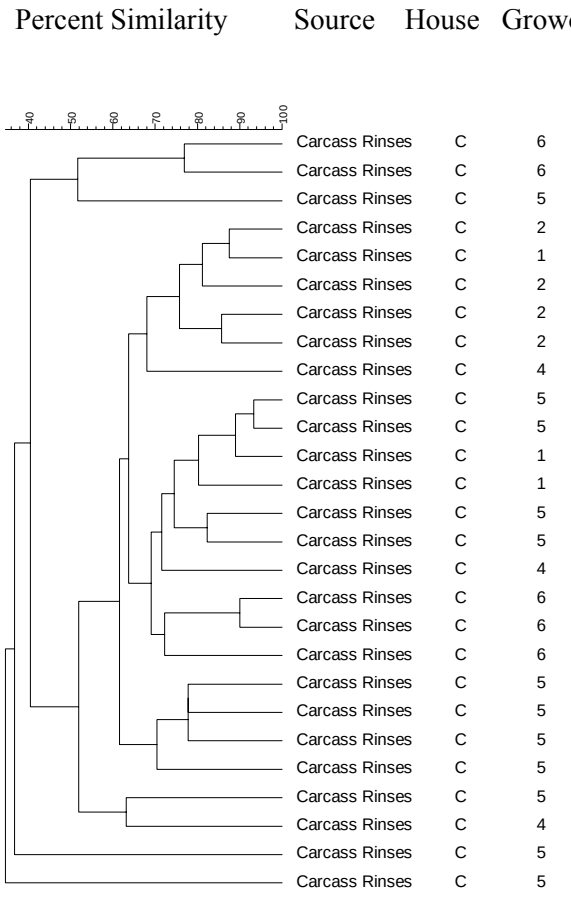

FIG. 25. Cluster analysis of *E. faecium* from poultry carcass rinses using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

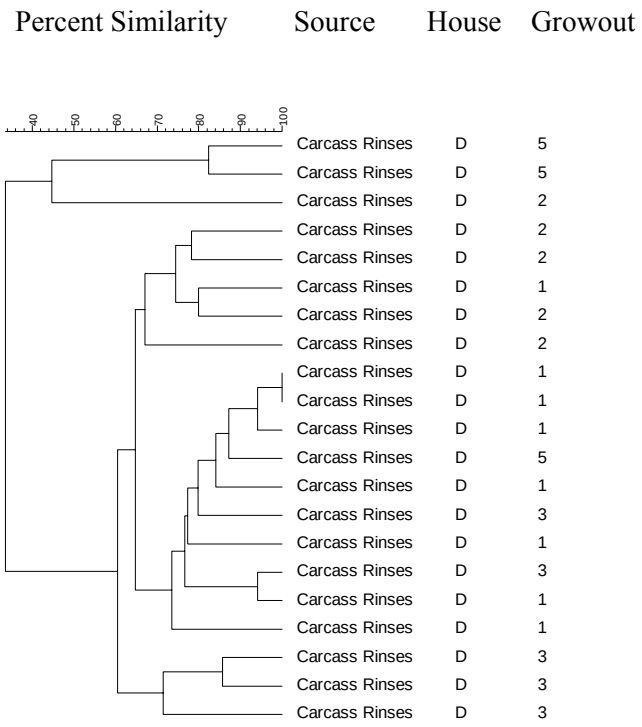

FIG. 26 Cluster analysis of *E. faecium* from poultry carcass rinses using BOX-PCR. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

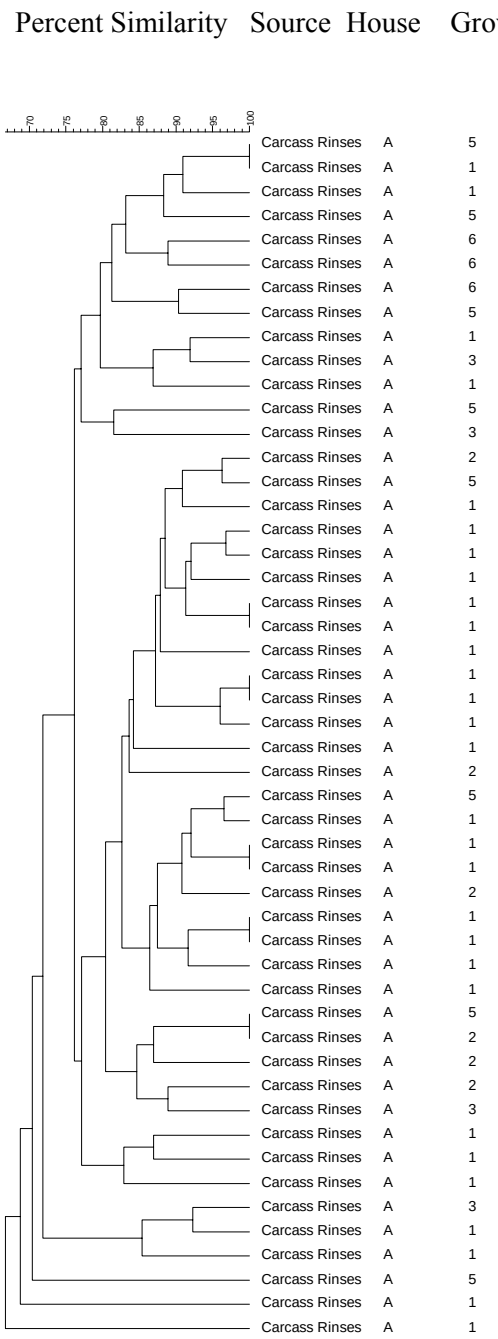

FIG. 27. Cluster analysis of *E. faecium* from poultry carcass rinses using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

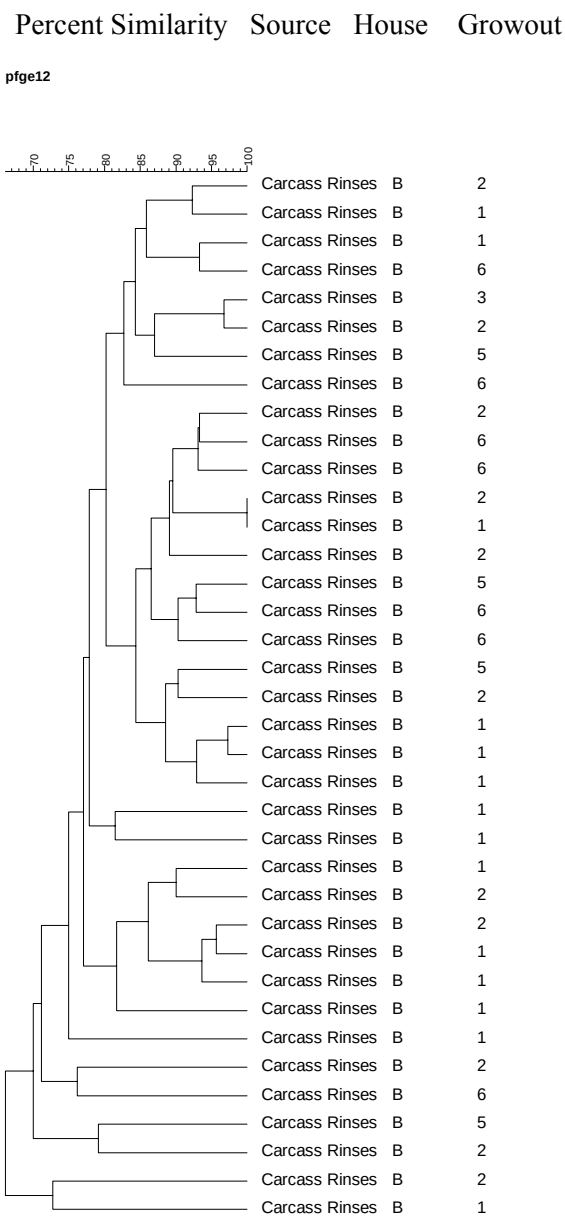

FIG. 28. Cluster analysis of *E. faecium* from poultry carcass rinses using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

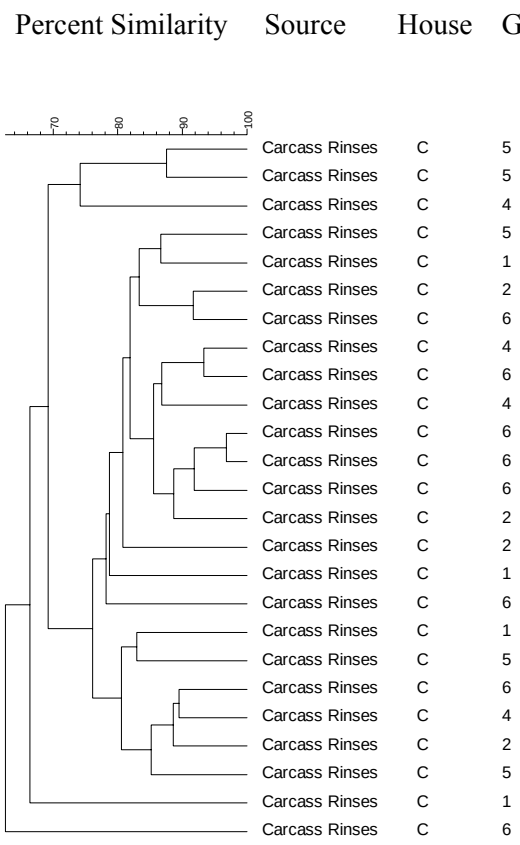

FIG. 29. Cluster analysis of *E. faecium* from poultry carcass rinses using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

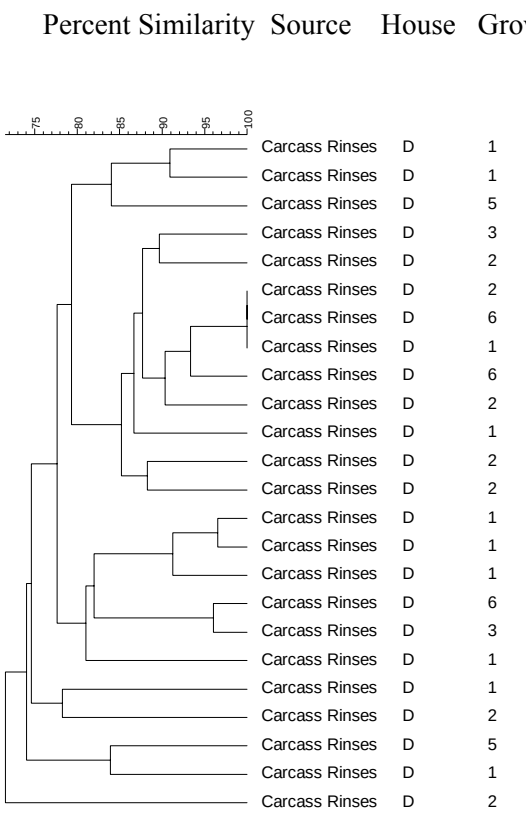

FIG. 30. Cluster analysis of *E. faecium* from poultry carcass rinses using PFGE. The percent similarities between clusters are shown. The corresponding house [A,B (control); C,D (treated)] and growout treatment (1;flavomycin, 2;virginiamycin, 3;virginiamycin, 4;virginiamycin, 5;flavomycin, 6;bacitracin) of each isolate are also included.

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Chapter 4

Conclusions

Enterococci has become of significant interest in the past few years. These organisms, originally thought to be harmless commensals, have emerged as a leading cause of nosocomial infections (4). Most enterococci infections are caused by *Enterococcus faecalis* and *Enterococcus faecium*. Furthermore, enterococci multiple drug resistance creates a therapeutic challenge to the medical community. The approval of new drugs to fight these infections is often followed by new resistant enterococci including vancomycin resistant enterococci (VRE) and linezolid resistant enterococci (1). Therefore, the medical community is searching for understanding of the development and dissemination of enterococci antimicrobial resistance.

There is speculation that cross- resistance to therapeutic, antimicrobial agents to combat human pathogens can be transferred through the food chain via the normal mircoflora of animals (3). Recently, the FDA has approved Synercid, a streptogramin a and b antibiotic, for the treatment of vancomycin resistant *Enterococcus faecium* (VREF) as *E. faecalis* is naturally resistant to Synercid. Virginiamycin is an analog of Synercid and has been used in animal production for over two decades (3). Concerns about virginiamycin use are based on the theory that its use in animals has established a reservoir of streptogramin resistant bacteria in poultry and other food animals (5).

The purpose of this study was to examine the effect, if any, of growth promotants in the prevalence of *Enterococcus* species among different sampling areas on a commercially integrated poultry farm from 2002 to 2003. Sampling areas included boxliners, litter, feed, and

carcass rinses. This study examined the *Enterococcus* population throughout the total poultry processing up to slaughter. Growth promotants tested included, virginiamycin, flavomycin and bacitracin. Furthermore, phylogenetic analysis was conducted to examine the genetic relatedness of the isolates according to sampling area and growth promotant administered, if any. Enterococcal species were identified using a new multiplex PCR method (2) in conjunction with Vitek 32 (bioMerieux Vitek, Hazelwood, MO, USA) for confirmation. Phylogenetic analysis was conducted by BOX-PCR and PFGE to create genetic profiles. Finally, the two molecular typing methods were evaluated to examine similarities and differences among clustering and efficiency of each method.

The summary of results provide a good foundation of knowledge of *Enterococcus spp*. found on a poultry farm. Nine species were isolated from the farm including *E. faecalis*, *E. faecium*, *E. casseliflavus*, *E. gallinarum*, *E. durans*, *E. hirae, E. cecorum, E. malodoratus,* and *E. avium. E. faecalis* and *E. faecium* were the predominant species. The sampling area where *Enterococcus* was isolated had an effect on which species was more numerous. Boxliners and carcass rinses were dominated by *E. faecalis*, whereas litter and feed were dominated by *E. faecium*. Additionally, the usage of virginiamycin in three out of six growouts did not cause a shift in the species. Furthermore, enterococci from poultry samples can be successfully genetically fingerprinted with BOX-PCR and PFGE. The two methods were comparable in their results, which indicated that *E. faecium* had a high degree of genetic diversity. Additionally, the usage of virginiamycin or any of the other growth promotants did not appear to have an impact on a genetic population of the *E. faecium* isolates.

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