POLYPHASIC CHARACTERIZATION OF ANTIBIOTIC RESISTANT AND VIRULENT ENTEROCOCCI ISOLATED FROM ANIMAL FEED AND STORED-PRODUCT INSECTS

by

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M.S., University of Agricultural Sciences, Dharwad, India, 2000

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
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Abstract

Feed samples and live stored-product insects from feed mills and swine farms were collected and cultured for *Enterococcus* spp. The mean concentration of enterococci in insect and feed were $2.7 \pm 0.5 \times 10^1$ cfu/insect and $6.3 \pm 0.7 \times 10^3$ cfu/g respectively. A total of 362 isolates of enterococci collected from 89 feed samples and 228 stored-product insects were identified to the species level using PCR. These isolates were represented by *Enterococcus casseliflavus* (53.0%), *E. gallinarum* (20.4%), *E. faecium* (16.2%), *E. hirae* (5.2%), and *E. faecalis* (5.0%). Enterococci were phenotypically resistant to tetracycline (48.0%), erythromycin (14.3%), streptomycin (16.8%), kanamycin (12.1%), ciprofloxacin (11.0%), ampicillin (3.3%), and chloramphenicol (1.1%). All isolates were susceptible to vancomycin and gentamicin. Tetracycline resistance was encoded by *tetM* (50.0%), *tetO* (15.1%), *tetK* (0.5%), *tetS* (0.2%) and other unknown tetracycline determinants. Enterococci carried virulence genes including gelatinase (*gelE*; 21.5%), an enterococcus surface protein (*esp*; 1.9%), and cytolysin (*cylA*; 2.2%). An aggregation substance (*asa1*) gene was detected in 61.0% of *E. faecalis* isolates. Fifty perncet of *E. faecalis* isolates were phenotipically tested positive for aggregation substances. Enterococci with *cylA* genes were hemolytic (52.0%) and with *gelE* genes were gelatinolytic (18.5%). The *ermB* gene, encoding erythromycin resistance was detected in 8.8% of the total isolates. The Tn*916/1545* family of conjugative transposons was detected in 10.7% of the isolates.

Laboratory experiments showed that adults of the red flour beetle, *Tribolium castaneum* (Herbst), fed on poultry and cattle feeds inoculated with *E. faecalis* OG1RF:pCF10, were able to successfully acquire enterococci and contaminate sterile poultry and cattle feeds. To assess the potential of horizontal gene transfer, conjugation assays were carried out with *E. faecalis* using a

donor (wild strains) and recipient (*E. faecalis* OG1SSP) in ratio of 1:10. Only one isolate (1 out of 18 *E. faecalis*) could transfer *tetM* to a recipient using broth mating. However, filter mating assay, followed by PCR confirmation revealed that 89.0% (16 out of 18 *E. faecalis)* of isolates could transfer *tetM* to *E. faecalis.* Transfer ratios of transconjugant per recipients ranged from 2.6×10^{-4} to 1×10^{-9}.

In summary, feed (52.0%) and stored-product insects (41.6%) collected from feed mills and swine farms carried antibiotic-resistant and potentially virulent enterococci. Our study showed that *T. castaneum*, a pest commonly associated with feed, served as a potential vector for enterococci in the feed environment. Conjugation assays followed by PCR confirmed presence of the *tetM* gene on a mobile genetic element(s) such as Tn*916* and may be horizontally transferred to other *Enterococcus* species and to other bacteria of clinical significance.

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Dedication

To my mother, Sri Jayamma and my father Sri Channaiah, H. C.

Chapter 1

Background and basis for studying antibiotic resistant and virulent enterococci in the feed environment

Introduction

The use of antibiotics in food animals is one of the major reasons for upsurge in cases of antibiotic resistant bacterial infections posing serious health risks to humans (Barton 2000; Saleha et al. 2009). Annually 90,000 people die from drug-resistant bacterial infections in the United States (Siddiqi et al. 2002; DeNap and Hergenrother 2005). Approximately 40-80% of the total antibiotics produced in the United States are used for food animals (Mellon et al. 2001; Sapkota et al. 2007; Orzech and Nichter 2008). There are concerns regarding transfer of pathogens such as *Salmonella* spp., *Campylobacter* spp., *Yersinia* spp., *Listeria* spp. and enterohaemorrhagic *Escherichia coli*, with various antimicrobial resistance genes from food animals to humans through the food chain (Dorn et al. 1975; Teuber 1999; Hawkes and Ruel 2006; Bailar and Travers 2002; Angulo et al. 2004; Lester et al. 2006; Maciorowski et al. 2006).

Although tracing contamination to its ultimate source is difficult, the source of several large food borne outbreaks is linked to consumption of contaminated feed (Crump et al. 2002; Maciorowski et al. 2006; Sapkota et al. 2007). Animal feed can potentially become contaminated with food borne pathogenic bacteria at several points throughout the feed production process including the primary production of feed ingredients, milling, mixing, extrusion, storage, and transportation (Cox et al. 1983; Hofacre et al. 2001; Kidd et al. 2002; Maciorowski et al. 2006; Myint et al. 2007; Sapkota et al. 2007). Additionally, the activity and byproducts (fecal material) of stored-product insect pests, birds, and rodents in the feed environment may increase the chance of pathogenic bacterial contamination. Stored-product insects are important as these insects have successfully adapted to survive on raw grains and processed food products (Sinha and Watters 1985; Larson et al. 2008a).

Stored-product insects have been reported to harbor many potentially pathogenic bacteria such as *Salmonella* spp., *Escherichia coli* (Husted et al. 1969; Harein et al. 1970; Harein et al. 1972), *Micrococcus* spp., *Streptococcus* spp., *Bacillus subtilis* (De Las Casas et al. 1972), and antibiotic-resistant enterococci (Larson et al 2008b). Of all these, enterococci which are ubiquitous in nature (Franz et al. 1999), have gained prominence in the last decade as an important reservoir of antibiotic resistance genes. These enterococci have the ability to transfer their resistance traits to other species of enterococci or to other serious human pathogens through horizontal gene transfer (HGT) (Devriese et al. 1992). Enterococci have been isolated from feed samples in Sweden, Spain, United Kingdom and United States (Schwalbe et al. 1999; Kidd et al. 2002; Kuhn et al. 2003) and from stored-product insects (Yezerski et al. 2005; Larson et al. 2008b). It is plausible that stored-product insects acquire bacteria from contaminated feed and serve as a potential vector in disseminating antibiotic resistant and virulent enterococci in the feed environment.

Antibiotics as feed additives

Antibiotics are used in food animals to treat sick animals, to prevent infections and to promote growth (Aarestrup et al. 1998). The United States animal feed industry is the largest producer of animal feed in the world (Sapkota et al. 2007). About 3,000 feed mills produce 121 million tones of feeds annually for various domestic animals (Feedstuffs 2003). In the United States, approximately 40-80% of the total antibiotics produced are used for food animals (Mellon et al. 2001; Sapkota et al. 2007; Orzech and Nichter 2008). The usage of antimicrobials in animal feed ranges from 2.5 to 125 mg/kg body weight, depending on the animal and on the antimicrobial (McEwen and Fedorka-Cray 2002). However, no concrete data are available on the amount of antibiotics that are used in the animal feed industry.

Antibiotics interfere with cell wall synthesis (e.g., penicillin, bacitracin) or protein synthesis (tetracyclines, streptomycin), or affect nucleic acid (DNA) synthesis (griseofulvin). These antibiotics are further divided based on mode of action as narrow-spectrum (penicillin) or broad spectrum (tetracycline). At least 17 classes of antibiotics are approved for animal feed and growth promotion in the United States (Mathew et al. 2007; Sapkota et al. 2007). Tetracyclines, macrolides, and pleuromutilins are frequently used in pigs for stabilization of the gut flora during the weaning phase. In cattle, antibiotics are used mainly to treat respiratory infections in calves and mastitis in cows (Phillips et al. 2003).

Many of these antibiotics are closely related to antibiotics that are used for treatment of human infections. For example, tetracyclines, macrolides, streptogramins and fluoroquinolones have analogue compounds that are also used in clinical settings (Turnidge 2004; FDA 2007; Sapkota et al. 2007). Furthermore, any development of resistance to one class of antibiotic, for instance avoparcin can promote resistance to other antibiotics in the same group such as vancomycin (Bates 1997).

Contamination of animal feed with pathogenic bacteria requires special attention to avoid contamination as these can cause significant impact on food animals. The use of animal by-products such as meat meal, bone meal, blood meal, feather meal, bone marrow, and dried poultry litter in animal feed are likely sources of potential pathogens such as *Salmonella* spp., *E. coli*, *Listeria* spp., *Campylobacter* spp., and opportunistic pathogens belonging to *Enterococcus* spp. (Orthoefer et al. 1968; Cox et al 1983; Schwalbe et al. 1999; Franco 2005; Mantovani et al 2006; Okoli et al. 2006; Okoli et al. 2007; Sapkota et al. 2007). Animal by-products are rich in protein, amino acids, and fats and together may act as enrichment media for bacterial growth and

multiplication.

Antibiotic resistance in bacteria associated with livestock production

There is a close correlation between the use of antibiotics and subsequent increase of antibiotic resistant bacteria isolated from various environments (Aarestrup et al. 1998; Aarestrup 1999; Martin et al. 2005). Starr and Reynolds (1951) were among the first to report streptomycin resistance in coliform bacteria (*E. coli*, and *Aerobacter* spp.) from turkeys that had been fed with streptomycin. Presence of antibiotic resistant bacteria in food animals, animal based food products, and the surrounding environment such as water, air, soil and also from farmers presents a passive link between antibiotics used in food animals and antibiotic-resistant human infections (McKeon et al. 1995; Roe and Pillai 2003; Sørum et al. 2006; Sapkota et al. 2007). The multidrug resistant *Salmonella* spp. is attributed to the contaminated feed made out of farmed fish fed with antibiotics (Angulo 1999). The widespread quinolone resistant *Campylobacter* spp. is attributed to the use of quinolones in chickens (Gupta et al. 2004). Ramchandani et al. (2004) reported that trimethoprim-sulfamethoxazole (TMP-SMZ) resistant *E. coli* bladder infections in humans were possibly from contaminated meat products. The DNA fingerprinting study in Italy showed 11 antibiotic resistance genes (*tetM*, *tetO*, *tetK*, *ermA*, *ermB*, *ermC*, *vanA*, *vanB*, *aac (6')-le aph (2")-la*, *mecA*, and *blaZ*) in raw pork, chicken meat and in fermented sausages as well as fecal samples from the these farm animals (Garofalo et al. 2007). It is estimated that 25-75% of the antibiotics administered to feedlot animals are excreted unaltered in feces (Roe and Pillai 2003). Antibiotic resistant bacteria in the animal feces can contaminate soil, ground water, and water streams. Furthermore, these bacteria with drug resistant genes can transfer the same to other bacteria, making the latter resistant for antibiotics (Roe and Pillai 2003).

Clones of vancomycin resistant enterococci have been isolated from humans (farm workers, butchers) and animals (pigs, and chickens) that received animal feed (analogue of vancomycin) containing avoparcin (Witte 1998; Stobberingh et al. 1999; Hammerum et al. 2000). Tetracycline resistant isolates of *Enterococcus hirae*, *Streptococcus cohinii*, *Lactobacillus reuteri*, *Bosea thiooxidans*, *Microbacterium oxydans*, *Afipia genospecies*, and *Pseudomonas peudoalcaligenes* were isolated from waste lagoons and ground water underlying two swine farms (Chee-Sanford et al. 2001). In a similar study, streptococci resistant to oxytetracycline, streptomycin, tetracycline, neomycin and erythromycin disseminated from beef cow (n =1,398), chickens (n =828), and dairy cows (n =728) have been isolated from surface and ground water in the mid-Atlantic region of the United States (Hagedorn et al. 1999). The DNA-DNA hybridization study revealed that the spread of resistant *Campylobacter coli* from swine farms to humans was due to swine byproducts ending up in Ground water. The DNA-DNA hybridization experiments show that all streptothricin resistant strains, which were isolated from one ecosystem or where an epidemiological link seems given, have sat4 positive signals at identical positions of the digested and blotted chromosomal DNA indicating the spread of *Campylobacter coli* from swine farms (Bischoff and Jacob 1996). Thirty isolates of *E. coli* resistant to ampicillin (33 μg), tetracycline (80 μg), streptomycin (100 μg), trimethoprim (5.2 μg), and sulfonamides (240μg) were isolated from six cattle and five humans working in the farm providing direct evidence for dissemination of antibiotic resistant bacteria from farm animals to humans (Oppegaard et al. 2001).

Multidrug resistant *Enterococcus* spp. and *Streptococcus* spp. have been recovered from the indoor air collected from large scale swine feeding operation which indicates the magnitude of contamination in confined animal feeding operations (Sapkota et al. 2006). Resistant

microorganisms are also known to travel from animals to humans via non-food sources. For example, throat and nare swab cultures of 10 pigs and family members of workers were characterized by pulsed-field gel electrophoresis (PFGE), staphylococcal protein A (*spa*) typing, multilocus sequence typing (MLST) analysis, subtyping of *staphylococcal cassette* chromosome *(*SSCmec), and accessory gene regulator (*agr*) typing, provides clear evidence for the spread and transmission of *Staphylococcus aureus* between pigs and humans (Huijsdens et al. 2006). In a similar study, upon oral infection of chickens with *E. coli* K12J5 (resistant to nalidixic acid), the *E. coli* strain was recovered from the workers who managed the experimental pen. Neither before nor after the experimental infection was any nalidixic acid-resistant *E. coli* isolated from workers who managed the poultry (Ojeniyi 1989). Several researchers documented the transfer of microorganisms through potential vector from animals in confined animal feeding operations. For example flies were identified as potential vector in disseminating *Campylobacter* spp. from animal production habitat (Nichols 2005). Rodents such as mice and rats were found carriers of *Salmonella enteritidis* in contaminated poultry farms (Henzler and Opitz 1992). In a similar study, geese that are in direct contact with liquid swine wastes had a significantly higher prevalence of *E. coli* resistant to antimicrobials (Ampicillin, amoxicillin/clavulanic acid, cefoxitin, cephalothin, tetracycline, sulfamethoxazole, gentamicin, kanamycin, streptomycin, and nalidixic acid) compared to *E. coli* isolates recovered from geese in regions with no known direct contact with liquid wastes. The investigation provides direct evidence for geese as potential vectors of pathogens carrying antimicrobial resistance genes between waste-handling facilities and other agricultural resources, such as crops and water (Cole et al. 2005).

Horizontal gene transfer

Horizontal gene transfer (HGT) refers to the acquisition of foreign genes by an organism. HGT occurs extensively among prokaryotes, especially in response to a changing environment and provides organisms access to genes in addition to those that are inherited (Jain et al. 2003). HGT is considered important in the divergence and adaptation of bacterial populations (Aoki and Syono 1999) to antibiotics or chemicals. For a DNA molecule to move between cells, it must cross the membrane of the donor and enter through the membrane of the recipient (Bushman 2004). The probability that a specific gene will be successfully transferred to a new host depends on the specific mechanistic details of transformation, transduction, and conjugation, and on the relationships of these mechanisms to the types of nucleic acids (single-stranded, double stranded, linear, and circular) that are being transferred (Jain et al. 1999). Usually short DNA fragments are exchanged during transformation. However, the process by which transformation and possible integration with bacterial chromosome occurs is still unclear (Molloy 2004). Conjugation is considered a major pathway for horizontal (usually single stranded) gene transfer among bacteria. Conjugation is mediated mainly by conjugative plasmids (Dionisio et al. 2002). Bacteriophage-mediated horizontal transduction has been known for nearly half a century (Jiang and Paul 1998). Although transformation, transduction, and conjugation can all result in DNA movement from one bacterium to another, conjugation is the only process that could transfer the whole chromosome (Thomas 2003).

Transposons play an important role in dissemination of antibiotic resistance genes among bacteria (Roe and Pillai 2003). Transposons, are transposable genetic elements conferring a selectable phenotype flanked by two insertion sequences, are involved in horizontal gene transfer events between bacteria. Transposons are unique in that they have the ability of excising

themselves from one genetic locus and migrating to another, whether it is within the same bacteria or bacteria in other taxa (Ochman et al. 2000). A mobile genetic element (MGE) provides strong evidences for DNA elements that have crossed species boundaries via horizontal transfer (Jordan et al. 1999). Transduction and conjugation depends on specialized MGEs which include most large plasmids and certain bacteriophages (phages). These MGEs are the main agents of HGT between organisms (Frost et al. 2005).

Bacteria evolve rapidly not only by mutation and rapid multiplication, but also by transfer of DNA, which can result in strains with beneficial mutations from more than one parent. Mutation and HGT continually give rise to new bacterial genotypes, therefore, bacterial genomes are in a constant state of flux, and any segment of DNA in a large bacterial population might have the opportunity to be horizontally transferred (Thomas and Nielsen 2005). Prokaryotes can make evolutionary leaps by sharing genes with another, thereby opening a back door (HGT) to an adaptation (Jain et al. 1999; Raymond and Blankenship 2003). There are evidences showing horizontal exchange of genetic material between prokaryotes and between prokaryotes and eukaryotes (Huynen et al. 1999; Kurland et al. 2003; Raymond and Blankenship 2003). The transfer of genes from one species to another through HGT plays a significant role in evolution of microorganisms (Syvanen 1985). However, the extent and mechanisms of horizontal gene transfer between prokaryotes are not well understood (Won and Renner 2003).

Horizontal gene transfer plays an important role in dissemination of antibiotic resistance genes in the environment. Kasuya (Kasuya 1964) demonstrated transfer of drug resistance genes (tetracycline, chloramphenicol, Streptomycin and sulfathiazole) from *Shigella flexneri* to *Klebsiella pneumoniae*, *S. flexneri* to *E. coli*, *E.coli* to *K. pneumoniae*, and *K. pnemoniae* to *S. flexneri* in the intestine of mouse. Transfer of vancomycin resistance gene *vanA,* from

vancomycin reistant *E. faecalis* to vancomycin resistant *Staphylococcus aureus* strain has been well established by many researchers (Flannayan et al. 2003; Weigel et al. 2003; Sung and Lindsay 2007). Antibiotic resistance observed in clinical settings or in animal products or food animals may be due to dissemination of antibiotic resistance genes among bacteria of different genera and origin through HGT. Moubarek et al. (2003) showed the transfer of drug resistance genes *vanA, ermB, tetL, ant6* and *tetM* from *E. faecium* isolates of porcine origin to *E. faecium* of human faecal isolate. Recent *ex vivo* tooth model show the horizontal transfer of the conjugative plasmid pAM81 carrying erythromycin resistance between *Streptococcus gordonii* and *Enterococcus faecalis* (Sedgley et al. 2008). These findings clearly support the role of HGT in disseminating antibiotic resistance genes from livestock origin to human origin and reverse.

Enterococci

Enterococci are catalase negative, Gram positive cocci that occur singly, in pairs, and in short chains (Franz et al. 1999). Enterococci are inhabitants of the intestines of humans, and nearly all animals (Devriese et al. 1992). Enterococci have emerged worldwide in the last decade as one of the leading causes of nosocomial agent responsible for nearly 110,000 urinary tract, 25,000 systemic blood, 40,000 wound, and 1,100 heart valve infections annually in the United States (Huycke et al. 1998). Enterococci readily develops resistance to various antibiotics upon exposure inside the intestines of livestock animals and it become more dangerous when they pass the drug-resistance genes to pathogens of greater clinical significance. Added to this, enterococci have been reported to be a reservoir for antibiotic resistance genes (Devriese et al. 1992). Enterococcus species are intrinsically resistant to several antibiotics, such as cephalosporins, β-lactams, sulphonamides and low levels of clindamycin and aminoglycosides (Larson et al.

2008b). *Enterococcus* has the ability to develop or acquire resistance to chloramphenicol, erythromycin, clindamycin, aminoglycosides, tetracycline, β-lactams, fluoroquinon, and glycopeptides such as vancomycin (Franz et al. 2003).

Avoparcin, a glycopeptide showing cross-resistance to medically important glycopeptides, in particular vancomycin, has been used in the European community as a growth promoter in animal feeds is believed to be the cause of vancomycin-resistant enterococci (VRE) outbreak in clinical settings. Since VRE were clinically isolated in Europe in 1986 and the United States in 1987, VRE infections have been reported throughout the world (Schouten et al. 2000; Thapa et al. 2007). Vancomycin resistant enterococci are found in the agricultural setting despite the banning of glycopeptides. Infection resulting from VRE is a dangerous and expensive broad-spectrum antibiotic therapy (Brandl et al. 2008). Six types of vancomycin resistance have been characterized on both phenotypic and genotypic basis in enterococci; *vanA*, *B, D, E*, and *G* correspond to acquired resistance: *vanC* is an intrinsic property found in *E. gallinarum,* and *E. casseliflavus* (Courvalin 2006). Concern is on transfer of vancomycin resistance traits from enterococci to pathogens such as *Staphylococcus aureus.* Recent study shows that *Staphylococcus aureus* strains are hypersusceptible to vancomycin resistance genes, such as *vanA*, from VRE (Sung and Lindsay 2007). Vancomycin resistance encoded by *vanA* is the most frequently encountered type of glycopeptide resistance in enterococci and to date is the only one detected in *Staphylococcus aureus* (Courvalin 2006).

Insect-bacterial interactions

Insects represent three-quarters of all extant animal species occupying nearly all the ecological niches on earth and hence are confronted with innumerable potential pathogenic bacteria, viruses, fungi, protozoan and helminthes parasites (Hoffmann 1997; Steinhaus 1946).

The association between insects and bacteria can be of two kinds, symbiotic, or to the detriment of the insect. The symbionts (bacteria) reside in specialized 'host' (insect) cells called bacteriocytes (Zientz et al. 2001). Generally these bacteria may occupy intracellular and extracellular localization in the midgut, muscle, salivary glands, fat body and haemolymph (Cheng and Aksoy 1999; Rio et al. 2003), where bacteria get shelter and food from the host, and in turn these bacteria synthesize essential amino acids required for the insects. Bacteria within the invertebrate gut are known to be important in the nutrient breakdown, methanogenesis, mineralization of organic compounds and nitrogen fixation (Zientz et al. 2001; Reeson et al. 2003). Additionally, gut microflora in insects are implicated in pheromone production, vitamin synthesis, pesticide degradation, and also there is evidence that gut bacteria can prevent the growth of some insect pathogens (Ohkuma and Kudo 1996; Reeson et al. 2003). Few insects maintain a sophisticated mutualistic symbiosis involving consortia of co-inherited organisms. Although symbiotic association exists between insects and bacteria, the density of particular symbiotic bacteria varies with type of insect, habitat and food availability. Bacteria associated with insects can either cause disease and death in insects or insects can serve as vectors transmitting bacterial diseases to crops, domestic animals, and humans (Durvasula et al. 1997). First written record about insect borne disease dates back to 1577, then the cause of Bubonic plague was believed to be carried by flies (Steinhaus 1946).

There are two probable ways by which insects come in contact with microorganisms. Few insects come in association with bacteria by birth. Here, eggs have the bacteria (obligate symbiont) descended from previous generation and this mechanism is a passive process. As soon as egg hatches the bacteria start multiplying in the insect body. However, few insects (both

larvae and insects) acquire bacteria due to their movements from the surrounding environment, and this mechanism is an active process.

A variety of insects have been reported to occur in context with veterinary animal production and found to carry bacterial pathogens (Hald et al. 1998; Harein et al. 1972). There are many ways by which an insect causes infection in farm animals. In recent years, stored-product insects gained prominence as a source of potential vector in disseminating potentially pathogenic bacteria affecting animal production (Husted et al. 1969; Harein et al. 1970; De Las Casas et al. 1972; Harein et al 1972; Lambkin 2001; Larson et al. 2008b).

Stored-product insects

Stored-product insects cause significant quantitative and qualitative losses to the multi-billion dollar grain, food, feed, and retail industries each year through their feeding, and product adulteration (Hagstrum and Subramanyam 2006). Incidences of stored-product insects in feed mills, farm house, stored house, brooder house, and flour mill are well documented (Rilett and Weigel 1956; Loschiavo and Okumura 1979; Pellitteri and Boush 1983; Skov et al. 2004; Larson et al. 2008a). Stored-product insects have been reported to harbor many bacteria. The darkling beetle, *Alphitobius diapernius* (Panzer), from poultry brooder houses was reported to contain *Salmonella* spp. *Escherichia coli, Campylobacter* spp. (Harein et al. 1970; Bates et al. 2004; Templeton et al. 2006), *Micrococcus* spp. *Streptococcus* spp. and *Bacilus subtilis* (De Las Casas et al. 1972). In a similar study, *Alphitobius diapernius* sampled from turkey brooder houses had *Streptococcus* spp. and *B. subtilis* (Harein et al. 1972). The granary weevil, *Sitophilus granarius* (L.), from laboratory colonies and grain storage facilities was identified as a potential reservoir for *Escherichia intermedia*, *Proteus rettgeri*, *P. vulgaris*, *B. subtilis*, *Serratia marcescens*,

Streptococcus spp. *Micrococcus* spp. and members of the *Klebsiella-Aerobacter* group (Harein and De Las Casas 1968). In addition, *Sitophilus granarius* have been shown to transfer *Salmonella* Montevideo from contaminated wheat to fresh sterile wheat (Husted et al. 1969). The mealworm beetle, *Tenebrio molitor* (L.), a pest of poultry sheds and egg barns, is capable of transmitting poultry diseases (Lambkin 2001).

Although incidences of stored-product insects in feed mills, farm houses, stored houses, brooder houses, and flour mills are well documented (Rilett and Weigel 1956; Loschiavo and Okumura 1979; Pellitteri and Boush 1983; Skov et al. 2004), very few studies (Yezerski et al. 2006; Larson et al. 2008b) have addressed the importance of stored-product insects carrying enterococci. Stored-product insects are often associated with feed mills because of warm temperatures in production areas and the availability of cereal ingredients in raw and processed form (Mills 1992; Mills and White 1993). Additionally, insect infestations in mills and retail environments are not completely eliminated due to inadequate inbound inspection of materials, poor sanitation and exclusion practices and improper timing of pest management interventions (Toews et al. 2006). Therefore, stored-product insects tend to survive and continue to cause damage to animal feed. Furthermore, the alimentary tract of insects offers a congenial microclimate for the survival of symbiont's (bacteria), and could be a "hot spot" for gene transfer (Dillon and Dillon 2004). Larson et al. (2008b) isolated antibiotic resistant enterococci from stored-product insects collected from eight feed mills located in the Mid-Western region of United States. Antibiotic resistant profiles showed that, enterococci isolated from stored-product insects had varying levels of resistance to tetracycline, erythromycin, ampicillin and streptomycin (Larson et al. 2008b). Since enterococci have been isolated from feed samples in Sweden, Spain, UK and USA (Schwalbe et al. 1999; Kidd et al. 2002; Kuhn et al. 2003), it is

plausible that stored-product insects may acquire antibiotic resistant and potentially virulent enterococci from contaminated feed and serve as a potential vector in the environment. Many stored product insects (*T. castaneum, Rhyzopertha dominica and T. variable*) are highly prolific and mobile (Hagstrum and Subramanyam 2006) in the postharvest environment causing increased dissemination of antibiotic resistant and virulent enterococci. Therefore, management of stored-product insects in feed environment should be given utmost priority to avoid contamination and dissemination of resistant bacteria.

Research objectives

Specific objectives of my dissertation research include: (1) Polyphasic characterization of antibiotic resistant and potentially virulent enterococci associated with stored-product insects. (2) Polyphasic characterization of antibiotic resistant and potentially virulent enterococci isolated from animal feed. (3) Survival of *Enterococcus faecalis* (OG1RF:pCF10) in poultry and cattle feed and vector competence of the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae).

Justification for research

One of the sources of antibiotic resistant bacteria is possibly animal feed. Enterococci have been isolated from feed samples in Sweden, Spain, UK and USA (Schwalbe et al. 1999; Kidd et al. 2002; Kuhn et al. 2003). Previous study in our laboratory (Larson et al. 2008b) revealed that *Enterococcus* spp. isolated from stored-product insects collected from eight feed mills located in the Midwestern United States showed varying levels of resistance to tetracycline, erythromycin, ampicillin and streptomycin. However, it was unclear how these insects acquired antibiotic resistant enteroccci. It is plausible that enterococcal contamination was present in the raw ingredients or the finished feed on which the insects were feeding. Larson et al. (2008b) did

not sample feed ingredients from mills to determine if they were the source of contamination. Since stored-product insects are cosmopolitan in distribution and occur frequently in feed mills, poultry brooder house, storage facilities, and flour mills in the United States and around the world, it is likely that these insects are of medical and veterinary importance, as they could serve as reservoirs and potential vectors for antibiotic resistant enterococci. Therefore, my present study focuses on isolation, identification and polyphasic characterization of antibiotic resistant and virulent enterococci associated with animal feed and stored-product insects collected from feed mills, grain storage silo, retail store and swine farms located in the Midwestern United States. Conjugation assays to study the potential for horizontal gene transfer among isolated enterococci, and incubation studies to assess the survival of *E. faecalis* in poultry and cattle feeds are also part of my investigation. Furthermore, my study involves elucidating the role of the red flour beetle, *Tribolium castaneum*, pest commonly associated with feed mills as a potential vector in acquisition and transmission of antibiotic resistant enterococci from contaminated feeds to sterile feeds.

Larson (2008b) reported the presence of antibiotic resistant enterococci in the midgut of stored-product insects. This present work complements and extends Larson's (2008b) findings by determining the species of enterococci present both in feed and stored-product insects collected from feed mills, retail store, and swine farms using single and multiplex PCR. In addition, the research also characterizes the antibiotic resistance and virulence profiles of enterococci isolated in this study. My research also involves evaluating the vector competence of red flour beetle, *T. castaneum* in transmitting antibiotic resistant enteroccci from contaminated to sterile feeds. I hope my research will help in gaining better understanding on the ecology of antibiotic resistant enterococci associated with feed and stored-product insects, and will lead to

additional studies addressing the importance of antibiotic resistant enterococcal contamination in the food chain.

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Chapter 2

Polyphasic characterization of antibiotic resistant and potentially virulent enterococci associated with stored-product insects

Abstract

A total of 154 enterococcal isolates from 228 stored-product insects collected from a feed mill, a stored grain silo, and a retail store were isolated and identified to the species level using single and multiplex PCR. Insects from the feed mill yielded $3.7 \pm 0.8 \times 10^1$ colony forming units (cfu)/insect, while insects from a stored grain silo and retail store yielded $2.3 \pm 0.1 \times 10^1$ and $2.1 \pm 0.1 \times 10^1$ cfu/insect, respectively. *Enterococcus casseliflavus* represented 51.0% of the total isolates, followed by *E. gallinarum* (24.0%), *E. faecium* (14.3%), *E. faecalis* (7.0%), and *E. hirae* (5.0%). About 47.3% were phenotypically resistant to tetracycline, followed by streptomycin (21.0%), erythromycin (14.0%), kanamycin (13.0%), ciprofloxacin (12.0%), ampicillin (4.0%), and chloramphenicol (<1.0%). All isolates were susceptible to vancomycin and gentamicin. The tetracycline resistance was encoded by *tetM* (45.4%), *tetO* (16.0%) and other unknown tetracycline determinants. Enterococci carried genes coding virulence factors including gelatinase (*gelE*; 26.0%), an enterococcal surface protein (*esp*; 1.2%), and cytolysin (*cylA*; 2.0%). An aggregation substance (*asa1)* gene was detected in 60.0% of *E. faecalis* isolates. Fifty perncet of *E. faecalis* isolates were phenotipically tested positive for aggregation substances. The erythromycin resistance encoded by *ermB* gene was detected in 6.0% of the total isolates. Enterococci were phenotypically positive for hemolytic (56.0%) and gelatinolytic (22.0%) activities. The Tn*916*/*1545* family of conjugative transposons was detected in 9.7% of the isolates. The filter-mating assay showed that the tetracycline resistance gene, *tetM*, was successfully transferable by conjugation to a recipient. The rates of gene transfer were in the range of 5.7×10^{-5} to 6.0×10^{-9} (transconjugant per recipient). Phenotypic and genotypic assay of these isolates for antibiotic resistance and virulence factors demonstrated that stored-product insects (41.6%) carried antibiotic-resistant and potentially virulent enterococcal isolates. These

isolates may potentiallyspread the antibiotic resistance and virulence genes to other bacteria of clinical importance by horizontal gene transfer. Therefore, management of stored-product insect vectors through effective integrated pest management programs is important and essential in feed mills and in grain and retail store environments.

Introduction

Numerous stored-product insect species are associated with stored grain, animal feed and retail stores (Triplehorn 1965; Loschiavo and Okumura 1979; Pellitteri and Boush 1983; Roesli et al. 2003; Larson et al. 2008). These pests cause significant quantitative and qualitative losses to the multi-billion dollar grain, food, feed, and retail industries each year through their feeding, and product adulteration. In addition, several stored-product insects have been reported to harbor potentially pathogenic bacteria. For example, the darkling beetle, *Alphitobius diaperinus* (Panzer), a cosmopolitan general stored-product pest from poultry brooder houses was reported to contain *Salmonella* spp., *Escherichia coli*, *Campylobacter* spp. (Harein et al. 1970; Bates et al. 2004; Templeton et al. 2006), *Micrococcus* spp., *Streptococcus* spp., and *B. subtilis* (De Las Casas et al. 1972). In a similar study, *Alphitobius diapernius* sampled from turkey brooder houses had *Streptococcus* spp. and *B. subtilis* (Harein et al. 1972). The mealworm beetle, *Tenebrio molitor* (L.), a pest of poultry sheds and egg barns, is capable of transmitting poultry diseases (Lambkin 2001). *Escherichia intermedia*, *Proteus rettgeri*, *P. vulgaris*, *B. subtilis*, *Serratia marcescens*, *Streptococcus* spp., *Micrococcus* spp., and members of the *Klebsiella-Aerobacter* group were isolated from laboratory colonies of the granary weevil, *Sitophilus granarius* (L.) (Harein and De Las Casas 1968). Husted et al. (1969) have shown that the granary weevils are capable of transferring *Salmonella* Montevideo from contaminated wheat to fresh wheat. Although, incidences of stored-product insects in feed mills, farm houses, stored houses, brooder houses, and flour mills is well documented (Rilett and Weigel 1956; Loschiavo and Okumura 1979; Pellitteri and Boush 1983; Skov et al. 2004), very few studies (Yezerski et al. 2005; Larson et al. 2008) have addressed the importance of stored-product insects carrying enterococci. Enterococci are one of the leading causes of nosocomial infections and are an

important reservoir for antibiotic resistance genes (Huycke et al. 1998). Enterococci have been isolated from feed samples in Sweden, Spain, United Kingdom, and Unites States (Schwalbe et al. 1999; Kidd et al. 2002; Kuhn et al. 2003). It is plausible that stored-product insects associated with storage environments may be able to acquire and serve as a potential vector in disseminating antibiotic resistant and virulent enterococci. The primary objective of this study was to determine the prevalence, concentration, and diversity of antibiotic resistant and potentially virulent enterococci associated with stored-product insects collected from feed mill, stored grain silo, and retail store.

Materials and methods

Insect collection

Stored-product insects were collected from a feed mill, stored grain silo, and retail store in Kansas. A total of 228 stored-product insects were collected from March through August 2006, using pheromone-baited traps in the feed mill (Trécé Inc., Adair, OK) (Roesli et al. 2003), perforated plastic pitfall traps in the silo (Trécé, Inc., Adair, OK) (Subramanyam and Hagstrum 1995) and by sifting the infested wheat flour obtained from the retail store. Insects were collected using sterile forceps and individually placed into sterile plastic vials, labeled, and transported to the laboratory for microbial analysis.

Isolation, enumeration, and identification of enterococci using PCR

Individual insects of each species were identified to species and surface sterilized with 10.0% sodium hypochlorite and 70.0% ethanol (Zurek et al. 2000), homogenized in potassium buffer saline (pH 7.2; MP Biomedicals, Solon, OH), and drop-plated on mEnterococcus agar (mENT; Difco Laboratories, Detroit, MI). Plates were allowed to dry and then placed in an incubator at 37°C. After incubation, the colony forming units (cfu) were recorded to determine

the concentration of enterococci per insect. Up to three presumptive enterococcal colonies with different colony morphology from each insect sample were isolated on Trypticase Soy Broth Agar (TSBA; Difco Laboratories, Detroit, MI), incubated at 37°C for 24 h, and stored at 4°C until further analysis.

The presumptive identities of enterococcal colonies were confirmed to the genus level by the esculin hydrolysis test using Enterococcossel broth (Difco Laboratories, Detroit, MI). All enterococcal isolates were grown in esculin broth using 96 well plates (Fisher Scientific, Pittsburg, PA), incubated at 44.5°C for 6 h. All positive enterococcal isolates were stabbed in TSB (0.3% agar) in 2.0 ml vials and stored at room temperature.

Multiplex PCR was used to identify four common species of enterococci including *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum* (Dutka-Malen et al. 1995; Kariyama et al. 2000). *Enterococcus mundtii* ATCC 43186 was used as a negative control. A single PCR was run to identify *E. hirae* using a specific primer (Arias et al. 2006). *Enterococcus hirae* ATCC 8043 was used as a positive control. The unidentified isolates (41.6%) were identified by amplifying the *sodA* (superoxide dismutase) gene using PCR and then sequencing (Genomics center, University of California, Davis, CA) and confirmed by Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) gene bank database (Poyart et al. 2000). All identified isolates were characterized further by screening for antibiotic resistance and virulence determinants by PCR, as well as by phenotypic tests.

Phenotypic screening of enterococci for antibiotic resistance

The protocols used for screening antibiotic resistance were based on the established guidelines of the National Committee for Clinical Laboratory Standards (NCCLS 2000). The antibiotics selected for this screening were based on their usage in animal agriculture.

Enterococcal isolates were screened for antibiotic sensitivity by the disk diffusion method (Soussy et al. 2000; Dargere et al. 2002) on Muller-Hinton Agar (Difco Laboratories, Detroit, MI) using seven antibiotics: ampicillin (10 µg), ciprofloxacin (15 µg), tetracycline (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), vancomycin (30 µg), and gentamicin (120µg). Susceptibility of isolates to kanamycin (2000 µg/ml) and streptomycin (2000 µg/ml) were assessed by agar dilution method using TSBA plates.

Genotypic screening of enterococci for antibiotic resistance and virulence determinants

Identified enterococcal isolates were screened for four important virulence determinants namely *asa1* (aggregation substances), *cylA* (cytolysin), *esp* (Enterococcus surface protein), and *gelE* (gelatinase) genes using multiplex PCR (Vankerckhoven et al. 2004). Similarly, the identified enterococcal isolates were screened for tetracycline and erythromycin resistance genes using multiplex and single PCR (Macovei and Zurek 2006). Tetracycline resistance genes were divided into three groups. The group I multiplex PCR reaction consisted of *tetA*, *tetC*, and *tetQ* genes, while group II consisted of *tetM*, *tetS*, tet*K*, and *tetO* genes (Ng et al. 2001; Turnidge 2004; Wilcks et al. 2005). A single PCR reaction was used to detect the *tetW* gene (Aminov et al. 2001). The *ermB* gene was used to screen all isolates for erythromycin resistance using single PCR (Sutcliffe et al. 1996).

*Screening enterococci for mobile genetic elements (*Tn*916/1545 family transposons)*

All identified enterococcal isolates were screened for integrase gene (*int*) for detection of Tn*916/1545* family conjugative transposons (Doherty et al. 2000; Gevers et al. 2003) that frequently carry tetracycline (*tetM)* resistance gene. *Enterococcus faecalis* OG1RF:pCF10 was used as a positive control.

Phenotypic screening of enterococci for gelatinase, hemolysin, and aggregation substance

Todd Hewitt Broth (THB; Difco Laboratories, Detroit, MI) with 2.0% milk powder was used for the detection of gelatinase enzyme activity. All the enterococcal isolates were streaked on THB plates and incubated at 37°C for 24 h. After incubation, the plates were examined for a zone of clearance surrounding the colonies (Gevers et al. 2003). Columbia Blood Agar (Difco Laboratories, Detroit, MI) with 5.0% human blood was used to phenotypically assess cytolysin (β-hemolysis) activity. All the enterococcal isolates were streaked and incubated at 37°C for 48 to 72 h. Clear zones around the colonies (beta-hemolysis) were considered positive for cytolysin activity. The *E. faecalis* strain MMH-594 was used as a positive control for both gelatinase and cytolysin activity tests.

The clumping assay for enterococcal aggregation substances was carried out exclusively using *E. faecalis* isolates (Dunny et al. 1978). *Enterococcus faecalis* JH2-2 was used for cCF10 peptide formation. *E. faecalis* JH2-2 was grown in THB for 18 h at 37°C. The supernatant containing pheromone (peptide) was collected by centrifuging at 10,000 rpm for 10 min followed by autoclaving for 15 min. The test isolates were grown in THB (5 ml) for 18 h at 37°C, then 1 ml *E. faecalis* JH2-2 supernatant was added to each culture and incubated at 37°C overnight in a incubator with a shaker (Excel E24 incubator shaker series, Edison, NJ). After the incubation the isolates were considered positive if clumping or aggregation of cells was observed with the naked eye or under a microscope (Dunny et al. 1978). *Enterococcus faecalis* OG1RF:pCF10 was used as a positive control.

Conjugation assay

Broth mating and filter matings were carried out as described by Ike et al. (1998) and Tendulkar et al. (2006) respectively, to study the mobility of the *tetM* gene. Both assays were performed with a donor:recipient ratio of 1:10. *Enterococcus faecalis* (10 isolates) isolated in this

study, resistant to tetracycline and sensitive to streptomycin, were used as the donor, whereas *E. faecalis* OG1SSp resistant to streptomycin and sensitive to tetracycline was used as the recipient.

For broth mating, overnight cultures of donor (0.5 ml) and recipient (5.0 ml) grown in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI) were mixed in 5ml fresh BHI broth and incubated at 37°C for 4 h, with gentle agitation in a incubator with shaker. After incubation, 100 µl of mixed culture was plated on BHI agar plates supplemented with tetracycline (40mg/L), streptomycin (2g/L), and plates containing both tetracycline (40mg/L) and streptomycin (2g/L) antibiotics. Plates were incubated overnight at 37°C.

For filter mating, overnight cultures of the donor (0.5 ml) and recipient (5 ml) were mixed, and cells were collected on 0.2 µ cellulose nitrate membrane filter (Whatman International Ltd., Dassel, Germany). The filter was placed on BHI agar plates with bacteria side up and incubated at 37°C for 16 h. After incubation, the cells from the filter were suspended in 1ml BHI broth and appropriate dilutions were plated on BHI agar plates containing tetracycline (40mg/L), streptomycin (2g/L), and plates containing both tetracycline (40mg/L) and streptomycin (2g/L) antibiotics. Plates were incubated overnight at 37°C. After the incubation the colony forming units for broth and filter matings were recorded to determine the transfer frequency, which was expressed as the number of transconjugants per recipient (T/R) and transconjugants per donor (T/D). The presence of the *tetM* gene in the transconjugants was confirmed by PCR.

Results

Isolation, enumeration, and identification of enterococci

Adults of eleven insect species were identified from feed mill, grain storage silo, and retail store (Table 1). A total of 154 enterococcal isolates were obtained from 228 stored-product

insects representing nine species. A majority of the insect species was from the feed mill (85.0% of the total insects), followed by the retail store (10.5% of the total insects) and the grain storage silo (4.4% of the total insects). The percentage of stored-product insects positive for enterococci ranged from zero for the small eyed flour beetle, *Palorus ratzeburgii* (Wissmann) and maize weevil, *Sitophilus zeamais* Motschulsky, to 50.0% for the lesser meal worm, *Alphitobius diaperinus* (Panzer), lesser grain borer, *Rhyzopertha dominica* F., and foreign grain beetle, *Ahasverus advena* (Waltl.), followed by the red flour beetle, *Tribolium castaneum* Herbst (45.0%) and warehouse beetle, *Trogoderma variabile* Ballion (44.4%). Of all the insect species, *T. castaneum* yielded 31.0% of the total enterococcal isolates followed by the confused flour beetle, *Tribolium confusum* (Jacquelin du Val) (23.0%). Enterococcal loads in insects that tested positive were low and ranged from 2.0 to 4.1 x 10^1 cfu/insect. The mean enterococcal load for insects collected from a feed mill was $3.7 \pm 0.8 \times 10^1$ cfu/insect and those from a grain storage silo was $2.3 \pm 0.1 \times 10^1$ cfu/insect, and from retail storage was $2.1 \pm 0.1 \times 10^1$.

The enterococcal isolates, screened by multiplex and single PCR using specific primers, resulted in the identification of 90 (58.0%) isolates. The rest of the isolates (42.0%) were successfully identified by amplifying conserved *sod*A (superoxide dismutase) gene using PCR and then sequenced and confirmed by BLAST search in the National Center for Biotechnology Information gene bank database. A majority of the isolates (51.0%) belonged to *E. casseliflavus* followed by *E. gallinarum* (24.0%), *E. faecium* (14.0%), *E. faecalis* (7.0%) and *E. hirae* (5.0%) (Table 2). All *E. faecium* and *E. faecalis* isolates came from stored-product insects associated with a feed mill.

Antibiotic resistant profiles of enterococci

Enterococcal isolates were phenotypically resistant to tetracycline (Tet^r, 47.0%) followed by streptomycin (Str^r, 21.0%), erythromycin (Erm^r, 14.0%), kanamycin (Kan^r, 13.0%), ciprofloxacin (Cip^r, 12.0%), ampicillin (Amp^r, 4.0%), and chloramphenicol (Chl^r, <1%) (Fig. 1). All enterococcal isolates were susceptible to vancomycin and gentamicin. Some of the enterococcal isolates showed resistance to more than one antibiotic; *E. casseliflavus* (16.0%), *E. gallinarum* (49.0%), *E. faecium* (36.0%), *E. faecalis* (50.0%) and *E. hirae* (43.0%). The combinations of Tet^r-Str^r (18.0%) were more common, followed by the combination of Tet^r-Erm^r (8.0%), and Tet^r-Cip^r (6.0%).

Screening enterococci for virulence determinants

The genotypic analysis of virulence determinants revealed that enterococcal isolates contained *gelE* (26.0%), *cylA* (2.0%), and *esp* (1.0%) genes (Fig. 2). The aggregation substance gene (*asa1*) was detected only in *E. faecalis* isolates (6 out of 10 isolates) (Fig. 3). The phenotypic assay to study gelatinase, cytolysin (β-hemolysis), and aggregation substances (exclusively for *E. faecalis*) were carried out to see how many isolates expressed these virulence factors. A total of 22.0% isolates were positive for gelatinase activity. Among enterococcal species, 80.0% of *E. faecalis*, 23.0% of *E. faecium*, 46.0% of *E. gallinarum*, and 6.0% of *E. casseliflavus* were found positive for gelatinase activity. None of the *E. hirae* isolates was found positive for gelatinase activity. About 56.0% of the total enterococcal isolates were found positive for cytolysin activity. Among enterococcal species, 23.0% of *E. faecium,* 29.0% of *E. hirae*, 60.0% of *E. faecalis*, 62.0% of *E. casseliflavus* and 73.0% of *E. gallinarum* were found positive for cytolysin activity. The phenotypic test showed that 50.0% of *E. faecalis* isolates were positive aggregation substance (Fig. 3).

Prevalence of tet^r, erm^r, and Tn*916/1545 family of conjugative transposons*

The most common resistance genes detected were *tetM* (45.0%), and *tetO* (16.0%) (Fig. 4). No other tetracycline genes (*tetA*, *tetC*, *tetQ*, *tetK*, *tetS*, and *tetW*) were detected. The ribosomal protection mechanism encoded by *tetM* was most frequently detected in the order of *E. casseliflavus*, *E. gallinarum*, *E. faecium*, and *E. faecalis*. The *ermB* gene encoding mechanism for macrolide resistance was detected in 6.0% of the isolates. The Tn*916/1545* family conjugative transposon was detected in 15 (10.0%) isolates. Most of these transposons (92.0%) carried either *tetM* or *ermB* genes. All isolates that carried Tn*916/1545* family conjugative transposons were from stored-product insects collected from feed mill.

Broth mating assay did not yield any transconjugants. However, filter mating assay, followed by PCR confirmation revealed that *E. faecalis* isolated from stored-product insects could transfer tetracycline resistance gene encoded by *tetM* to the recipient strain. The rates of gene transfer were in the range of 5.7×10^{-5} to 6.0×10^{-9} (transconjugant per recipient) (Table 3).

Discussion

Stored-product insects collected from feed mill (43.3% prevalence), grain storage silo (30.0% prevalence), and retail store (33.3% prevalence), tested positive for enterococci. Adults of *T. castaneum* and *T. confusum* were the most frequently collected insects in this study as these two species are commonly found throughout the post-harvest system (Hagstrum and Subramanyam 2006). Of the 228 insects studied, 85.0% of insects were from the feed mill environment and the remaining 15.0% were from grain storage silo and retail store. Stored-product insects are often associated with feed mills because of warm temperatures in production areas and the availability of cereal ingredients in raw and processed form throughout the year (Triplehorn 1965; Mills 1992; Mills and White 1993; Larson et al. 2008). Fewer insects and

species were recovered from the grain storage silo and retail store compared to feed mill, because we took did not samples these habitats as intensively as the feed mill.

We identified five species of enterococci from stored-prodduct insects. Of these,namely *E. casseliflavus*, *E. gallinarum*, and *E. hirae* are primarily associated with plants, soil, and domestic waste (Thal et al. 1995; Coque et al. 1996; Muller et al. 2001; McGowan et al. 2006). Although, *E. gallinarum* and *E. casseliflavus* are infrequently isolated from clinical settings, they tend to cause a wide variety of invasive infections in humans, especially in immuno-compromised or chronically-ill patients (Facklam and Collins 1989; Ruoff et al. 1990; Pompei et al. 1991; Gordon et al. 1992; McNamara et al. 1995; Toye et al. 1997; Reid et al. 2001; Dargere et al. 2002). A small minority (<3.0%) of clinical enterococcal isolates belong to *E. hirae* (Prakash et al. 2005). To our knowledge, three reported cases of human infection by *E. hirae* were reported in the medical literature worldwide (Gilad et al. 1998; Poyart et al. 2002; Canalejo et al. 2008).

Stored-product insects primarily feed on plant based products including raw grains stored on farms and at feed mills, and flour mills, and on processed food products manufactured in feed, and flour mills (Sinha and Watters 1985; Mills 1992; Subramanyam and Hagstrum 1995, 2000; Roesli et al. 2003; Hagstrum and Subramanyam 2006), and this may explain the presence of *E. casseliflavus*, *E. gallinarum*, and *E. hirae* in stored-product insects.

We found *E. faecalis* and *E. faecium* in stored-product insects collected only from the feed mill. Animal by-products such as meat meal, blood meal, bone meal, feather meal, egg-shell meal, and fish meal are common ingredients used in animal feed, and may be the source of *E. faecalis* and *E. faecium* in feed samples (Orthoefer et al. 1968; Cox et al. 1983; Franco 2005; Mantovani et al. 2006; Okoli et al. 2006; Okoli et al. 2007; Sapkota et al. 2007). One study

reported the presence of vancomycin resistant *E. faecalis* in animal (chicken) feed in the United States (Schwalbe et al. 1999). It is plausible that stored-product insects feeding on contaminated feed may have acquired *E. faecalis* and *E. faecium* in their digestive tract.

The diffusion disk assay revealed relatively high resistance to tetracycline and streptomycin as these two classes of antibiotics are widely used for growth promotion and therapeutic purposes in animal feed (Schroeder et al. 2002; Sapkota et al. 2007). Resistance to erythromycin, ciprofloxacin and kanamycin were found in enterococci isolated in this study. The aminoglycosides streptomycin and kanamycin are commonly used in animal feed to manage intestinal infections. The resistance to ciprofloxacin may be due to the use of the closely related drug (enrofloxacin) in animal feed (Delsol et al. 2004; Lovine and Blaser 2004). We found very low percentage of isolates resistant to ampicillin and chloramphenicol, which is expected as these antibiotics are banned in the United States for use in animal agriculture (Hofacre et al. 2001; Dawson 2005). Chloramphenicol has been banned in several countries, including the European Union (EU) for treatment of livestock animals (Shakila et al. 2007). The reason for antibiotic resistance in enterococci isolated from grain storage silo and retail store are unclear and warrant further investigation.

Genotypic analysis revealed that most of the tetracycline resistance was due to *tetM* and *tetO* genes. Similar results were also found by other researchers (Fairchild et al. 2005; Macovei and Zurek 2006), who showed that *tetM* and *tetO* are widespread among antibiotic resistant enterococci.

Phenotypically resistant erythromycin enterococcal isolates carried the *ermB* gene. This could be due to wide spread use of erythromycin in animal agriculture for the treatment of infections caused by Gram-positive bacteria (Skeeles 1991; Corpet 1996). The *ermB* gene

confers cross resistance to other macrolides, lincosamide, and streptogramin type B antimicrobials (Roberts et al. 1999; Jackson et al. 2004).

Less than 10.0% of the enteroccal isolates carried Tn*916*/*1545* family of conjugative transposons that encode erythromycin via *ermB* gene and tetracycline resistance via *tetM* gene (Courvalin and Carlier 1987; Kresken et al. 2004). Similar results were also found by other researchers (Huys et al. 2004; Kresken et al. 2004; Macovei and Zurek 2006). Conjugative transposons (Tn*916*/*1545*) are the most efficient way of transferring resistance genes to other bacteria due to their high integration ability into the host chromosome or plasmids (Clewell 1986; Murray 1998).

Our study showed that virulence determinants (*gelE*, *asa1*, *cylA*) are relatively common in enterococcal isolates and can be comparable to the virulence determinants in some clinical isolates (Gilmore et al. 2002; Creti et al. 2004). The results obtained by genotypic assay for cytolysin factor (*cylA*) was less compared to phenotypic test, indicating the involvement of other unknown cytolysin determinants. We found high prevalence of *gelE* gene among enterococcal isolates than gelatinase activity. This may be due to the presence of silent genes that are expressed only under *in vivo* conditions (Creti et al. 2004). Isolates of *E. faecalis* that contained *cylA* gene also possessed the aggregation substance gene *asa1*. Generally, these *cylA* and *asa1* genes are carried on plasmids (such as pAD1) and can be transferred to other enterococcal species (Gilmore et al. 2002; Creti et al. 2004).

The filter mating assay showed higher conjugation frequency in transferring *tetM* gene from *E. faecalis* isolates to *E. faecalis* OG1SSp. Mobilization of gene/transposons such as Tn*916* present on chromosome needs close contact of donor and recipient cells on solid surface provided by the filter mating technique as compared to the broth mating technique (Ike et al.

1998; Clewell and Dunny 2002). Filter mating assays followed by PCR confirmation for tetracycline resistance encoded by *tetM* gene in transconjugants suggest that *tetM* locus is present on mobile genetic element such as Tn*916/1545* (Courvalin and Carlier 1987; Salyers et al. 1995; Gilmore et al. 2002).

Stored-product insects are cosmopolitan in distribution. These insects are adapted to infesting raw cereal grains and processed cereal products posing a constant threat to stored food commodities worldwide (Sinha and Watters 1985). Several researchers reported the presence of stored-product insects in the near vicinity of feed mills, flour mills, and grain storage facilities (Rilett and Weight 1956; Triplehorn 1965; Loschiavo and Okumara 1979; Pelliteri and Bousch 1983). Many stored product insects are highly prolific and mobile (Hagstrum and Subramanyam 2006) and may cause increased dissemination of antibiotic resistant and virulent enterococci throughout the postharvest marketing system.

The animal feeds produced in the United States are often contaminated with clinically important human food borne bacteria such as *Salmonella* spp. (Krytenburg et al. 1998; Hofacre et al. 2001; Crump et al. 2002; Davies et al. 2004; Dargatz et al. 2005; Maciorowski et al. 2006), *Enterococcus* spp. (Schwalbe et al. 1999), *Campylobacter* spp. (Myint et al. 2007), *Listeria* spp. (Maciorowski et al. 2006) and *Escherichia coli*, including *E. coli* O157:H7 (Lynn et al. 1998; Davies et al. 2004; Sargeant et al. 2004; Dargatz et al. 2005; Myint et al. 2007). The digestive tract of insects offers congenial microclimate for the survival and multiplication of bacteria (Dillon and Dillon 2004). It is likely that stored-product insects are of medical and veterinary importance, because they could serve as reservoirs for antibiotic-resistant bacteria (Larson et al. 2008). Despite low concentrations of enterococci in stored-product insects in this study, we hypothesize that stored-product insects feeding on contaminated feed or grain based products are

capable of acquiring antibiotic resistant and virulent enterococci in their digestive tract and may act as a potential vector in postharvest environments. It is unclear as to how these stored-product insects were contaminated with antibiotic resistant and potentially virulent enterococci, and whether stored-product insects are acquiring enterococci from the feed or contributing to product contamination. These aspects require further study. A detailed study of stored-product insects and feed samples collected from same source (feed mill) is needed to elucidate the role of stored-product insects (i.e., reservoir and vector competence) in the acquisition, retention, and transmission of antibiotic resistant enterococci within the feed mill environments. Nevertheless, our study reinforces the need for good sanitation, exclusion, and pest management practices to minimize stored-product insect incidence and abundance in grain and retail stores and in feed mills.

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Table 1. Incidence of enterococci from stored-product insect species collected from different storage environments.

Insect species	No. insects	No. insects positive for enterococci (%)	No. enterococcal isolates (%)	Mean ± SEM cfu/insect
Feed mill				
Red flour beetle	75	34 (45.3)	44 (28.5)	$4.1 \pm 0.1 \times 10^1$
Confused flour beetle	70	30 (42.8)	39 (25.3)	$3.8 \pm 0.1 \times 10^1$
The warehouse beetle	18	8 (44.4)	16 (10.4)	$4.0 \pm 0.2 \times 10^1$
Rusty grain beetle	10	3 (30.0)	8 (5.2)	$3.7 \pm 0.3 \times 10^1$
Lesser grain borer	8	4 (50.0)	8 (5.2)	$3.6 \pm 0.2 \times 10^1$
Drugstore beetle	7	3 (42.8)	9 (5.8)	$3.5 \pm 0.2 \times 10^1$
Lesser mealworm	2	1 (50.0)	2 (1.3)	$3.5 \pm 0.5 \times 10^1$
Foreign grain beetle	2	1 (50.0)	2 (1.3)	$3.5 \pm 0.5 \times 10^1$
Small-eyed flour beetle	1	0 (0)	0 (0)	0.0
Maize weevil	1	0 (0)	0 (0)	0.0
Total	194	84 (43.3)	129 (83.7)	$3.7 \pm 0.8 \times 10^1$
Grain storage silo				
Red flour beetle	6	2 (33.3)	3 (1.9)	$2.3 \pm 0.3 \times 10^1$
Confused flour beetle	4	1 (25.0)	2 (1.3)	$2.0 \pm 0.0 \times 10^1$
Total	10	3 (30.0)	5 (3.3)	$2.3 \pm 0.1 \times 10^1$
Retail store				
Sawtoothed grain beetle	24	8 (33.3)	20 (13.0)	$2.1 \pm 0.1 \times 10^1$
Grand Total	228	95 (41.6)	154 (100.0)	$2.7 \pm 0.5 \times 10^1$

Table 2. Enterococcal species diversity in stored-product insects.

Species	No. isolates (% of total)
E. casseliflavus	78 (50.6)
E. gallinarum	37 (24.0)
E. faecium	22 (14.3)
E. faecalis	10 (6.5)
E. hirae	7 (4.5)
Total	154 (100.0)

Table 3. Transfer frequency of *tetM* gene by filter mating in *E. faecalis* isolated from stored-product insects to recipient, *E. faecalis* OG1SSp.

Donor	Transconjugants/Recipient	Transconjugants/Donor
E. faecalis IF1	2.2×10^{-8}	1.4×10^{-4}
E. faecalis IF2	5.7×10^{-5}	2.0×10^{-4}
E. faecalis IF3	2.8×10^{-9}	4.0×10^{-5}
E. faecalis IF4	1.7×10^{-6}	1.0×10^{-4}
E. faecalis IF5	3.1×10^{-8}	6.3×10^{-5}
E. faecalis IF6	4.8×10^{-8}	2.0×10^{-5}
E. faecalis IF7	4.0×10^{-8}	1.6×10^{-5}
E. faecalis IF8	8.1×10^{-7}	1.4×10^{-4}
E. faecalis IF9	6.0×10^{-9}	2.0×10^{-4}
E. faecalis IF10	1.0×10^{-8}	2.2×10^{-5}

Fig. 1. Antibiotic resistance profiles of enterococci (n = 154) isolated from stored-product insects. TET, tetracycline; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; ERM, erythromycin; KAN, kanamycin; and STR, streptomycin

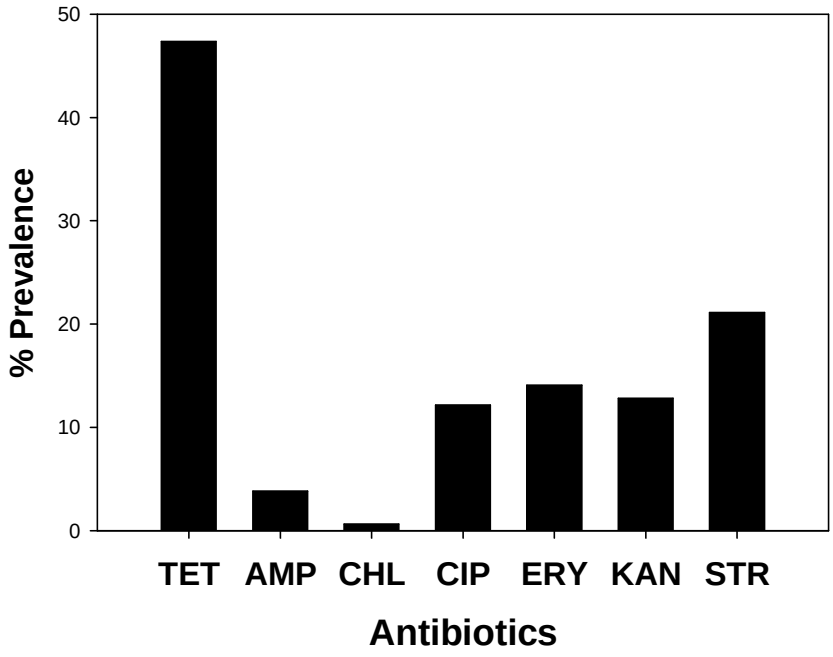


Fig. 2. Genotypic virulence profiles of enterococci (*n* = 154) isolated from stored-product insects

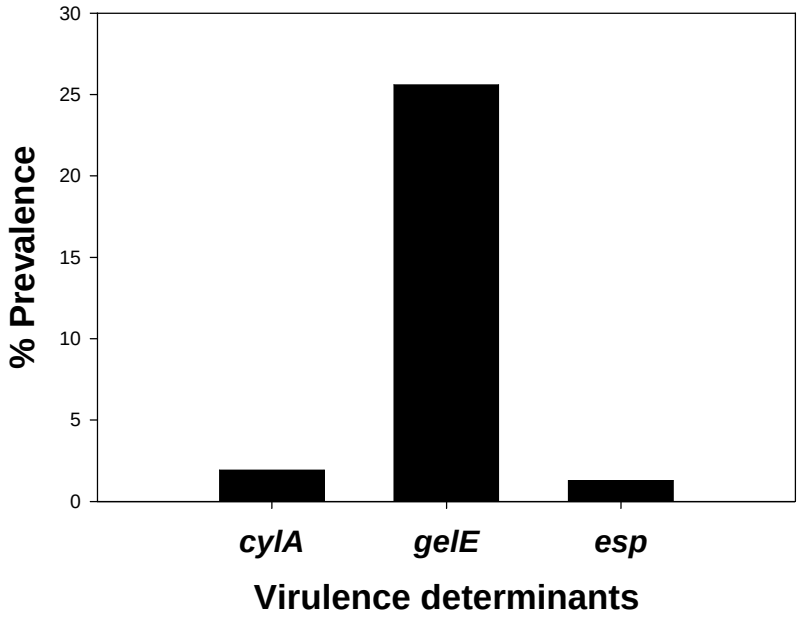


Fig 3. Prevalence of aggregation substance (*asa1*) in *E. faecalis* (*n* = 10) isolated from stored-product insects

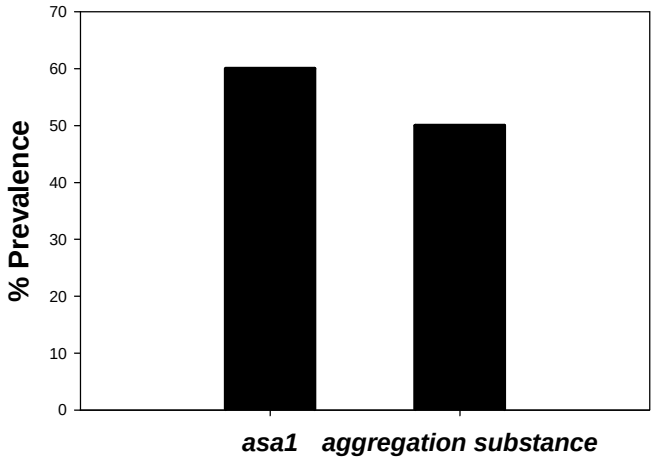
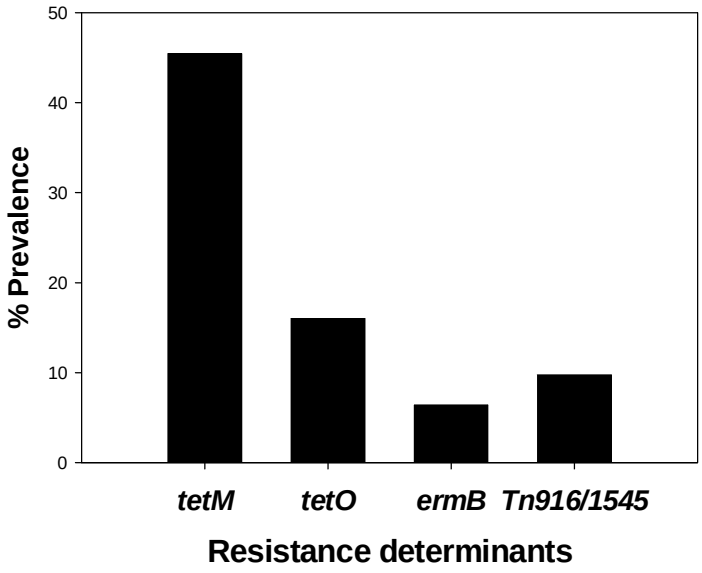


Fig. 4. Prevalence of tetracycline, erythromycin genes and Tn*916/1545* family of transposons in enterococci (*n* = 154) isolated from stored-product insects.



Chapter 3

Polyphasic characterization of antibiotic resistant and potentially virulent enterococci isolated from animal feed

Abstract

In order to assess the level of contamination of animal feed with antibiotic resistant enterococci, feed samples from feed mills and swine farms were collected and cultured for enterococci. A total of 89 feed samples were collected, and 52% of these samples tested positive for enterococci. The mean concentration of enterococci in feed samples collected from feed mills and swine farms was 1.2×10^2 and 8.1×10^3 cfu/g, respectively. A total of 208 isolates of enterococci from the 89 feed samples were identified to the species level using single and multiplex PCR. These isolates were represented by *Enterococcus casseliflavus* (54.8% of the total isolates), *E. gallinarum* (17.7%), *E. faecium* (17.7%), *E. hirae* (5.7%), and *E. faecalis* (3.8%). Enterococcal isolates were phenotypically resistant to tetracycline (48.0% of the total isolates), erythromycin (14.4 %), streptomycin (13.4%), kanamycin (11.5%), ciprofloxacin (10.0%), ampicillin (3.0%), and chloramphenicol (1.4%). All enterococcal isolates were susceptible to vancomycin and gentamicin. Tetracycline resistance in enterococci was encoded by *tetM* (52.8% of the total isolates), *tet*O (14.4%), *tetK* (1.0 %), and *tetS* (0.5 %). Some enterococci carried genes coding for virulence factors, including gelatinase (*gelE*; 18.2%), an enterococcal surface protein (*esp*; 2.4%), and cytolysine (*cylA*; 2.4%). An aggregation substance (*asa1)* gene was detected in 5 out of 8 (62.5%) of *E. faecalis* isolates. Four out of 8 *E. faecalis* isolates were phenotypically positive for aggregation substance. Enterococci were hemolytic (49.0% of total isolates) and gelatinolytic (15.8%). The erythromycin resistance encoded by *ermB* was detected in 10.5% of the total isolates. The conjugative transposon Tn*916/*Tn*1545* family was detected in 11.5% of the total isolates. Only one isolate (1 out of 8 *E. faecalis*) could transfer *tetM* to a recipient using broth mating. Filter mating assay, followed by PCR confirmation revealed that 6 out of 8 of *E. faecalis* isolates (75.0%) could transfer *tetM* to *E.*

faecalis. The rate of gene transfer (transconjugant per donor and recipient) was in the range of 2.6×10^{-4} to 1.0×10^{-9}. Our data revealed that feed samples collected from feed mills and swine farms carried antibiotic resistant and potentially virulent enterococci. We have shown that these isolates have the potential to spread the antibiotic resistance and virulence genes to other bacteria of clinical importance by horizontal gene transfer.

Introduction

There are numerous reports regarding animal feed contaminated with potential food-borne bacterial pathogens such as *Salmonella* spp. (Isa et al. 1963; Cox et al. 1983; Krytenburg et al. 1998; Maciorowski et al. 2006), *Enterococcus* spp. (Schwalbe et al. 1999), *Campylobacter* spp. (Myint et al. 2007), *Listeria* spp. (Maciorowski et al. 2006), and *Escherichia coli*, including *E. coli* O157:H7 (Lynn et al. 1998; Davies et al. 2004; Sargeant et al. 2004; Dargatz et al. 2005; Myint et al. 2007). Of all these pathogens, enterococci which are ubiquitous in nature (Franz et al. 1999), have gained prominence in the last decade because they serve as important reservoirs for antibiotic resistance genes and can transfer the resistance traits to serious human pathogens by horizontal gene transfer (HGT) (Devriese et al. 1992). Enterococci have been implicated as one of the leading causes of nosocomial infections (Huycke et al. 1998) and several virulence factors including aggregation substances, cytolysin activity, enterococcal surface protein, and gelatinase activity have been reported. There are concerns regarding transfer of pathogens with various antimicrobial resistance genes from food animals to humans through the food chain (Angulo et al. 2004; Hawkes and Ruel 2006; Lester et al. 2006; Maciorowski et al. 2006; Macovei and Zurek 2006). Hence, there is a need to better understand the ecology of enterococci associated with animal feed.

Enterococcal contamination of poultry feed has been reported by Schwalbe et al. (1999), and in their paper they reported *Enterococcus faecalis* to be resistant to the antibiotic vancomycin. Larson et al. (2008) reported that stored-product insects collected from eight feed mills in the United States tested positive for two species of enterococci (*E. faecium* and *E. gallinarum*). They reported resistance profiles of enterococci isolated from stored-product insects. However, it was unclear how these insects acquired enteroccci. It is plausible that

enterococcal contamination may have been present in the raw ingredients or the finished feed on which the insects were feeding. Larson et al. (2008) did not sample feed ingredients from mills to determine if they were the source of contamination. Therefore, in the present investigation, feed samples collected from mills and swine farms were analyzed for enterococcal contamination. The objectives of the present investigation were to determine the prevalence, concentration, and diversity of antibiotic resistant and potentially virulent enterococci associated with animal feed.

Materials and methods

Collection of feed samples

Feed samples were collected from six feed mills and two confined swine facilities located in four Midwestern United States (KS, IN, IA and WI). A total of 89 feed samples (41 from swine farms and 48 from feed mills) were collected from April through August, 2006. Feed samples (250g) from mills included raw materials as well as various processed fractions and finished feed. Samples of feed (250 g) from storage facilities were collected from swine farms. All samples were collected in a sterilized zipper-sealed plastic bag, labeled, and transported in a cooler to the laboratory for microbial analyses.

Isolation, enumeration, and identification of enterococci using PCR

One gram of feed sample collected from swine facilities was added to 9 ml of potassium buffer saline (PBS) (pH 7.2; MP Biomedicals, USA), shaken and allowed to settle (Yezerski et al. 2005). One millileter of the sample was then serially diluted in 9 ml of PBS and a 100µl sample from serial dilutions was drop-plated on mEnterococcus agar (mENT; Difco Laboratories, Detroit, MI), and incubated at 37°C for 48 h. After the incubation the colony forming units (cfu) were recorded to determine the concentration of enterococci. Enterococcal colonies with different colony morphologies from each sample were isolated on Trypticase Soy

Broth Agar (TSBA; Difco Laboratories, Detroit, MI), incubated at 37°C for 24 h and later stored at 4°C.

To confirm the genus level, all isolates were grown in Enterococcosal broth (Difco Laboratories, Detroit, MI) in 96-well plates (Fisher Scientific, Pittsburgh, PA) and incubated at 44.5°C for 6 h. All positive enterococcal isolates were stabbed in TSBA (0.3% agar) in 2 ml vials and stored at room temperature until further analysis.

The enterococcal species were identified using multiplex PCR using species-specific primers for *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. casseliflavus* (Dutka-Malen et al. 1995; Kariyama et al. 2000). *E. mundtii* ATCC 43186 was used as a negative control. A single PCR was run to identify *E. hirae* using a specific primer (Arias et al. 2006), and *E. hirae* ATCC 8043 was used as a positive control. The unidentified isolates were identified by amplifying conserved *sodA* (superoxide dismutase) gene using PCR (Poyart et al. 2000), then sequenced (Genomics center, University of California, Davis, CA), and confirmed by Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) genebank database (Poyart et al. 2000). All identified isolates were characterized further by screening for antibiotic resistance and virulence determinants.

Phenotypic screening of enterococci for antibiotic resistance

The identified enterococcal isolates were screened for antibiotic resistance using established guidelines of the National Committee for Clinical Laboratory Standards (NCCLS 2000). The antibiotics selected in this study were based on their usage in animal agriculture as well as their clinical significance. Enterococcal isolates were screened for antibiotic sensitivity by the disc diffusion method (Soussy et al. 2001; Dargere et al. 2002) on Muller-Hinton Agar (MHA; Difco Laboratories, Detroit, MI) with seven antibiotics: ampicillin (10 µg), ciprofloxacin

(15 µg), tetracycline (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), vancomycin (30 µg), and gentamicin (120µg). Sensitivity of enterococcal isolates to kanamycin (2000 µg/ml) and streptomycin (2000 µg/ml) were assessed by agar dilution method using TSBA plates.

Genotypic screening of enterococci for antibiotic resistance and virulence determinants

All identified enterococcal isolates were screened for four important virulence determinants: *asa1* (aggregation substance), *cylA* (cytolysin), *esp* (enterococcal surface protein), and *gelE* (gelatinase) using multiplex PCR (Vankerckhoven et al. 2004). Similarly, all isolates were screened for tetracycline and erythromycin resistance genes using multiplex and single PCR (Macovei and Zurek 2006). Tetracycline resistance genes were divided into three groups. The group I multiplex PCR reaction included *tetA*, *tetC*, and *tetQ*. The group II multiplex PCR reaction consisted *tetM*, *tetS*, tet*K*, and *tetO* (Ng et al. 2001; Wilcks et al. 2005), and group III single PCR reaction consisted *tetW* gene (Aminov et al. 2001). The *ermB* gene was used to screen for erythromycin resistance (Sutcliffe et al. 1996).

Screening enterococci for mobile genetic elements, Tn*916/1545 family transposons*

All identified enterococcal isolates were screened by PCR for integrase gene (*int*) for detection of Tn*916/1545* conjugative family transposons (Doherty et al. 2000; Gevers et al. 2003) that frequently carry tetracycline (*tetM)* resistance gene. *E. faecalis* OG1RF:pCF10 was used as a positive control.

Phenotypic screening of enterococci for gelatinase, hemolysis, and aggregation substance

Todd Hewitt Broth (THB; Difco Laboratories, Detroit, MI) with 2.0% milk powder was used to detect gelatinase enzyme activity. All identified enterococcal isolates were streaked on THB plates and incubated at 37°C for 24 h. After incubation, the plates were examined for zone of clearance of casein surrounding the colonies (Gevers et al. 2003).

Columbia Blood Agar (Difco Laboratories, Detroit, MI) with 5.0% human blood was used to assess cytolysin (β-hemolysis) activity. All enterococcal isolates were streaked and incubated at 37°C for 48-72 h. Clearance zones around the colonies were considered positive for cytolysin activity. The strain, *E. faecalis* MMH-594 was used as a positive control for both, gelatinase and cytolysin activities. The clumping assay (for enterococcal aggregation substance) was carried out for *E. faecalis* isolates (Dunny et al. 1978). *E. faecalis* strain JH2-2 was used for peptide formation. *E. faecalis* JH2-2 was grown in THB for 18 h at 37°C. The supernatant containing pheromone was collected by centrifugation (10,000 rpm for 10 min) and autoclaved for 15 min. The test isolates were grown in THB (5ml) for 18 h at 37°C, then 1 ml of *E. faecalis* JH2-2 supernatant was added to each culture and incubated at 37°C overnight in a shaker incubator (Excell E24 incubator shaker Series, New Brunswick Scientific, Rutgers, NJ). After incubation, the isolates were considered positive if clumping or aggregation of cells was observed by naked eye or under a microscope (Dunny et al. 1978). For clumping assay, *E. faecalis* OG1RF:pCF10 was used as a positive control.

Conjugation assay

Both broth mating and filter mating assays were performed as described by Ike et al. (1998) and Tendulkar et al. (2006) respectively, to study the mobility of transposon Tn*916/1545* containing *tetM* gene. Both assays were performed with donor/recipient ratio of 1:10. *E. faecalis* isolated in this study, resistant to tetracycline and sensitive to streptomycin, was used as a donor, whereas *E. faecalis* OG1SSP resistant to streptomycin and sensitive to tetracycline was used as a recipient.

For broth mating, overnight cultures of donor (0.5 ml) and recipient (5.0 ml) grown in BHI broth were mixed in 5ml fresh BHI broth and incubated at 37°C for 4 h, with gentle

agitation in a shaker incubator. After the incubation, 100 µl of mixed culture was plated on BHI agar plates supplemented with tetracycline (40mg/l), streptomycin (2g/l), and plates containing both tetracycline (40mg/l) and streptomycin (2g/l) antibiotics. Plates were incubated overnight at 37°C. For filter mating, overnight cultures of donor (0.5 ml) and recipient (5 ml) were mixed, and the cells were collected on 0.2 µm cellulose nitrate membrane filter (Whatman International Ltd., Dassel, Germany). The filter was placed on Brain Heart Infusion (BHI) agar plates with bacteria side up and incubated at 37°C for 16 h. After the incubation, the cells on the filter were suspended in 1 ml BHI broth and appropriate dilutions were plated on BHI agar plates containing tetracycline (40mg/l), streptomycin (2 g/l), and plates containing both tetracycline (40 mg/l) and streptomycin (2 g/l) antibiotics. Plates were incubated overnight at 37°C. After the incubation, colony forming units (cfu) for broth mating and filter matings were recorded to determine the transfer frequency, which is expressed as the number of transconjugants per donor and recipient. The presence of *tetM* gene in transconjugant was confirmed by PCR.

Results

Isolation, enumeration, and identification of enterococci

The 89 feed samples (41 from swine farms and 48 from feed mills) yielded 208 enterococcal isolates. A majority of the isolates (160) were from the two swine farms located in Kansas. About 58.0% of the 41 feed samples from swine farms and 46.0% of the 48 samples from feed mills were positive for enterococci. The mean concentration of enterococci in feed samples ranged from $1.1\pm 0.2 \times 10^1$ to $8.1\pm 0.8 \times 10^3$ cfu/g of feed (Table 1). Single and multiplex PCR using species specific primers resulted in identification of 125 (60.0% of total) isolates. The remaining isolates were identified by sequencing the *sod*A gene. A majority of the isolates belonged to *E. casseliflavus* (54.8%), followed by *E. gallinarum* (17.7%), and *E. faecium*

(17.7%), *E. hirae* (5.7%), and *E. faecalis* (3.8%) (Table 2). Among the samples collected from feed mills, a majority of the enterococci were isolated from unprocessed feed samples than processed or finished feed samples. For example, 69.0% of the enterococcal isolates were recovered from unprocessed feed (raw ingredients) while the remaining 31.0% were recovered from processed or finished feed samples (Table 2).

Phenotypic screening for antibiotic resistance

Nearly 58.0% of the isolates were resistant to at least one antibiotic. The isolates were phenotypically resistant to tetracycline (Tet, 48.0%), followed by erythromycin (Ery, 14.4 %), ciprofloxacin (Cip, 10%), streptomycin (Str, 13.4%), kanamycin (Kan, 11.5%), ampicillin (Amp, 3%), and chloramphenicol (Chl, 1.4%) (Fig. 1). None of the isolates was resistant to vancomycin or gentamicin. Some enterococcal isolates showed resistance to two or more antibiotics: *E. casseliflavus* (13.1%), *E. gallinarum* (43.0%), *E. faecium* (24.3%), *E. hirae* (66.6%), and *E. faecalis* (50.0%). The combinations of Tet^r-Str^r (10.5%) were more common, followed by the combination of Tet^r-Cip^r (8.6%), and Tet^r-Erm^r (7.7%).

Screening for virulence determinants

The genotypic assay for virulence determinants showed that identified isolates had *gelE* (18.2%), *cylA* (2.4%), and e*sp* (2.4%) genes (Fig 2). An aggregation substance gene (*asa1*) was found in 62.5% of *E. faecalis* isolates (Fig 3). Out of 208 isolates, 24.0% possessed at least one virulence factor and very few isolates (4.8%) possessed two or more virulence factors.

About 16.0% (33) of the 208 isolates were positive for gelatinase activity. Out of the 33 isolates that were positive 7 belonged to *E. faecalis*, 15 to *E. gallinarum*, 3 to *E. faecium*, and 8 to *E. casseliflavus*.. One *E. faecalis* isolate was negative for gelatinolytic activity.All *E. hirae* isolates were negative for gelatinolytic activity. About 49.0% (102) of the 208 isolates were

positive for hemolytic. Out of the 102 isolates that were positive for hemolytic activity, 70 belonged to *E. casseliflavus*, 20 to *E. gallinarum*, 8 to *E. faecium*, 2 to *E. hirae*, and 5 to *E. faecalis*. The phenotypic test for aggregation substance showed that 4 of the 8 *E. faecalis* isolates (50.0%) were positive for aggregation substance (Fig 3).

Prevalence of tet, erm, and Tn*916/1545 determinants*

The most common resistant genes were *tetM* (53.0% of 208 isolates), followed by *tetO* (14.4%) *tetK* (1.0%), and *tetS* (0.5%) (Fig. 4). The *tetA, tetC, tetQ,* and *tetW* genes were not detected. The ribosomal protection mechanism encoded by *tetM* was detected most frequently in descending order in*E. faecalis, E. faecium*, *E. casseliflavus*, and *E. gallinarum*. The *tetO* gene was found mainly in *E. casseliflavus*. The *ermB* gene was detected in 10.5% of 208 isolates and was restricted to *E. casseliflavus* and *E. faecium*.

The Tn*916/1545* conjugative transposon family was detected in 24 (11.5%) of the 208 isolates (Fig 4). The Tn*916/1545* family of transposons was detected mostly in *E. gallinarum*, *E. faecium*, and *E. faecalis*. Out of the 24 isolates, most of these transposons (91.6%) carried either *tetM* or *ermB* genes. All isolates that carried Tn*916/1545* family of transposons were from feed samples collected from swine farms.

Only one isolate (1 out of the 8 *E. faecalis* isolates could transfer *tetM* to a recipient using broth mating. However, filter mating assay, followed by PCR confirmation revealed that 6 out of 8 *E. faecalis* isolated from animal feed transferred *tetM* to *E. faecalis*. The rate of gene transfer (transconjugant per recipient) was in the range of 2.6×10^{-4} to 1.0×10^{-9} (Table 3).

Discussion

Species of *E. casseliflavus*, *E. gallinarum,* and *E. hirae* are uncommon in clinical settings. To our knowledge, there are only three reported cases of human infection by *E. hirae*

(Gilad et al. 1998; Prakash et al. 2005; Canalejo et al. 2008). The use of plant by-products such as forage, grains, plant protein products, and by-products of fruits in feed manufacturing process may be the likely source of *E. casseliflavus*, *E. gallinarum, and E. hirae* in the feed samples (Muller et al. 2001; Sapkota et al. 2007). The use of animal by-products such as meat meal, blood meal, feather meal, bone meal, fish meal, egg shell meal, bone marrow, and dried poultry litter, as feed ingredients are the likely source of *E. faecalis* and *E. faecium* encountered in our study and association of enterocci with these ingredients have been reported by several authors (Orthoefer et al. 1968; Cox et al. 1983; Franco 2005; Mantovani et al. 2006; Okoli et al. 2006; Okoli et al. 2007; Sapkota et al. 2007). Furthermore, activities of stored-product insects, birds and rodents are common in feed mills (Pelliteri and Bousch 1983) and contamination of the feed through the feces of these animals may also be a source of *E. faecalis* and *E. faecium.*

The high frequency of resistance to tetracycline and erythromycin in enterococci could be due to the widespread use of these antibiotics in animal feed for growth promotion and therapeutic purposes (Schroeder et al. 2002; Sapkota et al. 2007). Resistance to kanamycin and streptomycin can be attributed to use of aminoglycosides in animal feeds to manage intestinal infections. The use of closely related drug (enrofloxacin) in poultry feed (Zervos 2001; Delsol et al. 2004; Lovine and Blaser 2004) may be the likely cause for ciprofloxacin resistance among the enterococcal isolates recovered from feed. The low prevalence of ampicillin and chloramphenicol resistance in entercoccal isolates was expected because in the United States ampicillin and chloramphenicol are no longer allowed in animal feed (Hofacre et al. 2001; Dawson 2005). Chloramphenicol has been banned in several countries, including the European Union for treatment of livestock animals (Shakila et al. 2007). None of the isolates was resistant to vancomycin and gentamicin. Avoparcin, an analogue of vancomycin has never been used in

animal feed in the United States (Chapin et al. 2005), and only one study so far reported the presence of vancomycin resistant enterococci in a chicken feed sample in the United States (Schwalbe et al. 1999).

Genotypic analysis for tetracycline resistance revealed that most of the tetracycline resistance was due to *tetM* and *tetO* genes. The *tetM* and *tetO* genes are widespread among antibiotic resistant enterococci (Fairchild et al. 2005; Macovei and Zurek 2006). The majority of the phenotypically resistant erythromycin enterococcal isolates carried the *ermB* gene. Erythromycin is widely used in animal feed for the treatment of infections caused by Gram-positive bacteria (Skeeles 1991; Corpet 1996).

The conjugative transposons Tn*916/1545* generally encodes erythromycin through *ermB* and tetracycline resistance through *tetM* genes, respectively (Courvalin and Carlier 1987; Huys et al. 2004; Kresken et al. 2004), and these transposons are efficient in transferring resistance genes to other bacteria due to their high integration ability into the host chromosome or plasmids (Clewell 1986; Murray 1998; Rice 1998).

We found higher percentage of *gelE* gene than what was observed phenotypically, and this may be due to the presence of silent genes that are expressed only under *in vivo* conditions (Creti et al. 2004). The cytolysin factor (*cylA*) was less prevalent compared to the phenotypic test, indicating the involvement of other unknown cytolysin determinants in hemolysis. *E. faecalis* that contained *cylA* also possessed the *asa1*. Generally *cylA* and *asa1* are carried on plasmids (such as pAD1) and transferred to other enterococcal species as reported by Gilmore et al. (2002) and Creti et al. (2004).

The filter mating assay was more efficient in transferring *tetM* from the donor to the recipients when compared to broth mating assay. This could be due to the presence of the *tetM*

gene on the chromosome instead of the free plasmid. Mobilization of gene/transposons such as Tn*916* present in chromosome needs close contact of the donor and recipient on solid surface, such as filter paper (Ike et al. 1998; Clewell and Dunny 2002). Filter mating assay followed by PCR confirmation for tetracycline resistance encoded by *tetM* gene in transconjugants suggest that *tetM* locus is present on mobile genetic element such as Tn*916/1545* (Courvalin and Carlier 1987; Salyers et al. 1995; Gilmore et al. 2002).

The presence of antibiotic resistance and virulence genes in *E. casseliflavus, E. gallinarum*, and *E. hirae* cannot be ignored. These isolates can potentially transfer resistance and virulence genes to clinically important species such as *E. faecalis* and *E. faecium*. These latter two species have been implicated in nosocomial infections.

Several authors have raised the concern on safety of animal feed because it could be a source of foodborne pathogens and multidrug resistant bacteria, especially bacteria that are pathogenic to humans (Kidd et al. 2002; Lu et al. 2004; Dargatz et al. 2005). The need for preventing contamination particularly with antibiotic resistant bacteria such as enterococci is very important to minimize food safety hazards. The cleanliness, construction, and ecology of feed storage warehouses, feeding operations, and the farms may have a direct impact on the presence and diversity of antibiotic resistant bacteria (Singer et al. 2006). It has been well documented that the air within concentrated animal feeding operations (CAFOs) is highly contaminated with antibiotic resistant bacteria (Gibbs et al. 2004; Chapin et al. 2005; Gibbs et al. 2006; Sapkota et al. 2006). The potential sources of antibiotic resistant bacteria besides the animal digestive tract may be the feed itself. Rodents and stored-product insects are often associated with feed environments. In addition, the feed ingredients some of which include

animal proteins or by products may carry pathogenic bacteria (Maciorowski et al. 2006; Sapkota et al. 2007; Larson et al. 2008).

In our study more enterococi were isolated from unprocessed feed samples than processed or finished feed samples, indicating the need for inspecting and testing raw ingredients for enterococcal contamination before using them as feed ingredients. The presence of enterococci in processed animal feed samples suggests that commercial pelleting, where temperatures exceed 82^{o}C, may not totally eliminate bacterial contamination, and the feed itself could be a source of antibiotic resistant and potentially virulent enterococci.. Therefore, our data reinforce the need for improved quality standards in feed mills and animal farms to prevent further contamination of feed before it is consumed by farm animals.

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Table 1. Prevalence and distribution of enterococci in animal feed collected from swine farms and feed mills

Source	No. feed samples	Prevalence No. (%)	No. enterococcal isolates	Mean ± SE (cfu/g)
Swine farms[a]	41	24 (58.0)	160	$8.1 \pm 0.8 \times 10^3$
Feed mills[b]				
Unprocessed feed	32	16 (50.0)	39	$1.2 \pm 0.2 \times 10^2$
Processed feed	16	6 (37.2)	9	$1.1 \pm 0.3 \times 10^2$
Total	89	46 (51.6)	208	$6.3 \pm 0.7 \times 10^3$

[a]Data based on 2 swine farms.

[b]Data based on 6 feed mills.

Table 2. Enterococcal species diversity in animal feed

Species	No. isolates (% of isolates)		
	Feed on farm	Unprocessed feed	Processed feed
E. faecalis	6 (3.7)	2 (6.0)	0
E. faecium	29 (18.1)	4 (12.1)	4 (26.6)
E. gallinarum	29 (18.1)	6 (18.1)	2 (13.3)
E. casseliflavus	85 (53.1)	20 (60.6)	9 (60.0)
E. hirae	11 (6.8)	1 (3.0)	0
Total	160	33	15

Table 3. Transfer frequency of *tetM* gene by filter mating in *E. faecalis* isolated from animal feeds to recipient, *E. faecalis* OG1SSp

Donor	Transconjugants/Recipient	Transconjugants/Donor
E. faecalis SF1	4.5×10^{-6}	1.0×10^{-4}
E. faecalis SF2	1.0×10^{-9}	1.5×10^{-4}
E. faecalis SF3	4.8×10^{-4}	2.0×10^{-3}
E. faecalis SF4	1.2×10^{-5}	1.2×10^{-4}
E. faecalis SF5*	1.2×10^{-6}	2.0×10^{-4}
E. faecalis SF5	2.6×10^{-4}	2.0×10^{-3}
E. faecalis SF6	7.4×10^{-8}	1.6×10^{-5}

* Broth mating

Fig. 1. Antibiotic resistance profiles of enterococci (*n* = 208) isolated from animal feed. TET, tetracycline; AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; ERM, erythromycin; KAN, kanamycin; STR, streptomycin

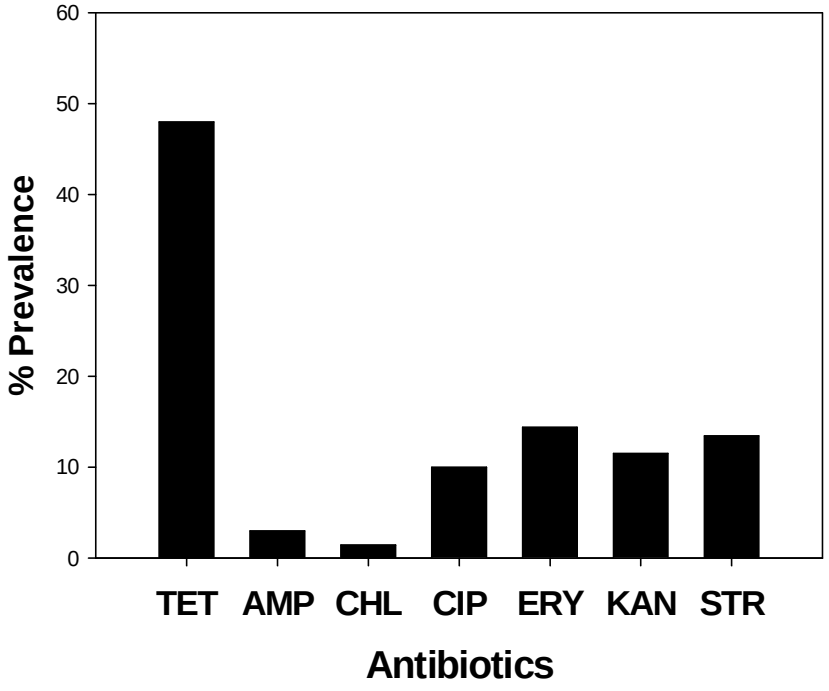


Fig. 2. Genotypic virulence profiles of enterococci (*n* = 208) isolated from animal feed

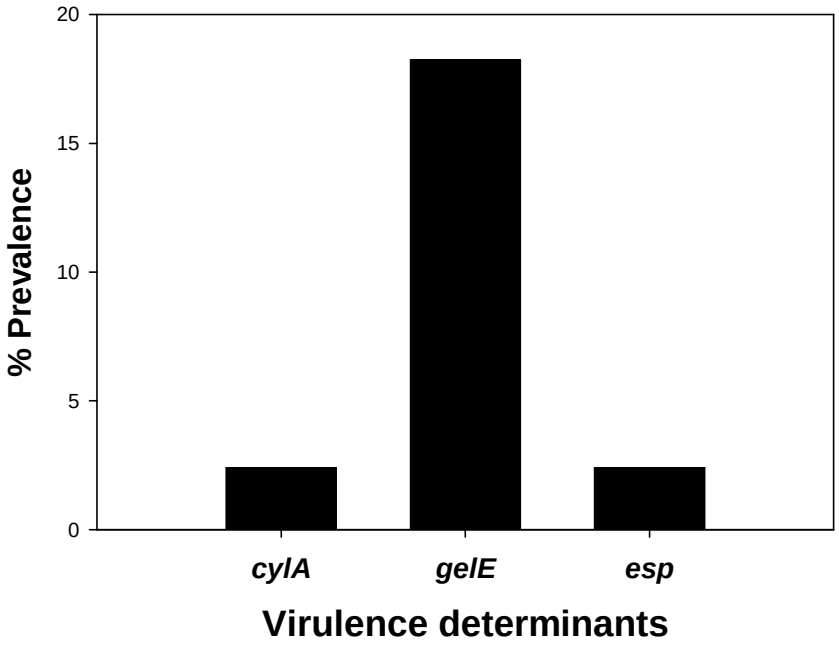


Fig 3. Prevalence of aggregation substance (*asa1*) in *E. faecalis* (*n* = 8) isolated from animal feed

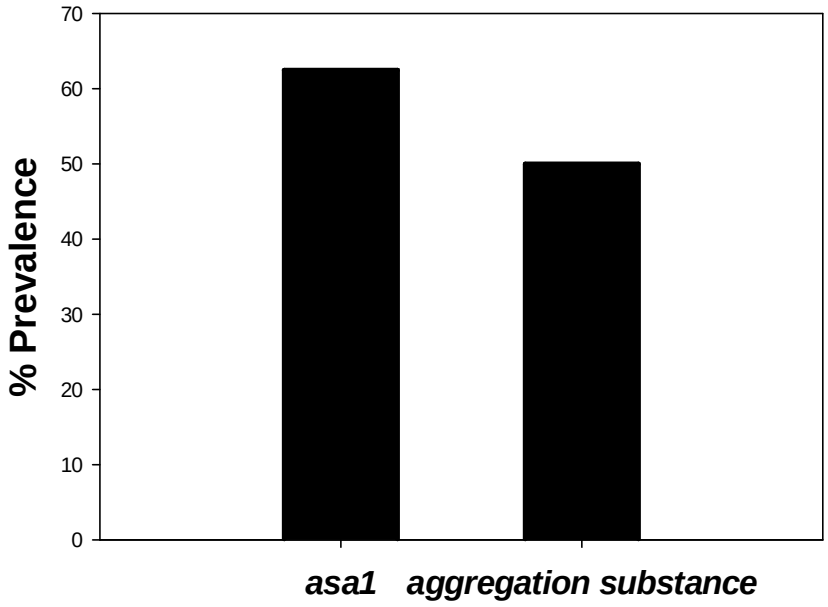
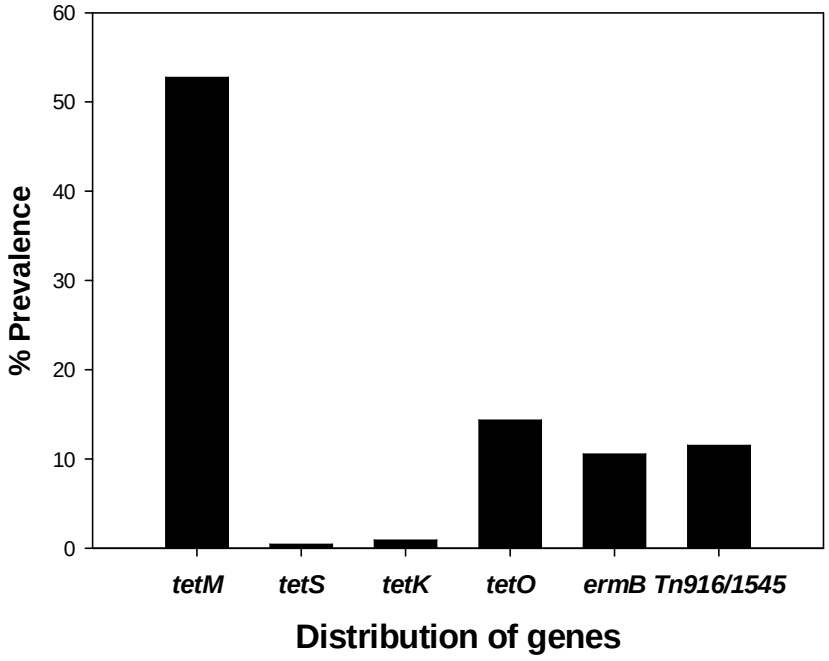


Fig. 4. Prevalence of tetracycline, erythromycin resistance genes and Tn*916/1545* family of transposons in enterococci (*n* = 208) isolated from animal feed



Chapter 4

Survival of *Enterococcus faecalis* (OG1RF:pCF10) in poultry and cattle feed: Vector competence of the red flour beetle, *Tribolium castaneum* (Herbst)

Abstract

Laboratory experiments were designed to determine the survival of *Enterococcus faecalis* OG1RF:pCF10 on inoculated poultry and cattle feed, and subsequent acquisition and transmission of this *Enterococcus* strain by adults of the red flour beetle, *Tribolium castaneum* (Herbst), to sterile feed. Adults of *T. castaneum* were introduced into poultry and cattle feed inoculated with *E. faecalis* and incubated at 28°C and 65% RH for 7-d in a growth chamber. Inoculated *E. faecalis* survived in both poultry and cattle feed during the 7-d test period. The *E. faecalis* strain persisted on the surface and within *T. castaneum* adults for 7-d when adults were on *E. faecalis*-contaminated poultry feed and for 5 d on *E. faecalis*-contaminated cattle feed. There was a logarithmic decrease in *E. faecalis* concentration in poultry and cattle feed, and in or on insects. The concentration of *E. faecalis* decreased more slowly on poultry feed than on cattle feed, and this may explain why adults of *T. castaneum* were able to successfully acquire and transfer *E. faecalis* from contaminated poultry feed to sterile poultry feed during the 7-d test period. In contrast, *T. castaneum* adults on contaminated cattle feed were unable to contaminate sterile cattle feed on day 7. To our knowledge, this is the first report documenting *T. castaneum* adults to successfully acquire antibiotic resistant enterococci from contaminated animal feed and transfer it to sterile feed. Management of *T. castaneum* vectors through effective integrated pest management programs is therefore important to prevent spread of antibiotic resistant and virulent enterococci in animal feed and feed mill environments.

Introduction

There is substantial evidence that the animal feeds produced in the United States are often contaminated with bacterial pathogens such as *Salmonella* spp. (Krytenburg et al. 1998; Hofacre et al. 2001; Crump et al. 2002; Davies et al. 2004; Dargatz et al. 2005; Maciorowski et al. 2006; Myint et al. 2007), *Enterococcus* spp. (Schwalbe et al. 1999; Kidd et al. 2002; Da Costa et al. 2007; Myint et al. 2007), *Campylobacter* spp. (Myint et al. 2007), *Listeria* spp. (Maciorowski et al. 2006), and *Escherichia coli*, including *E. coli* O157:H7 (Lynn et al. 1998; Davies et al. 2004; Sargeant et al. 2004; Dargatz et al. 2005; Da Costa et al. 2007; Myint et al. 2007). In addition to microbial contamination, animal feed is infested by several stored-product insects (Mills 1992; Mills and White 1993; Larson et al. 2008a,b). These stored-product insects have been reported to harbor many pathogenic bacteria. For example, the granary weevil, *Sitophilus granarius* (L.), a common pest of stored grains has been shown to transfer *Salmonella enterica* serotype Montevideo from contaminated wheat to fresh wheat (Husted et al. 1969). In a similar study, *S. granarius* adults collected from laboratory colonies and grain storage facilities were identified as potential reservoirs for *Salmonella intermedia*, *Proteus rettgeri*, *P. vulgaris*, *Bacillus subtilis*, *Serratia marcescens*, *Streptococcus* spp., *Micrococcus* spp. and members of the *Klebsiella-Aerobacter* group (Harein and De Las Casas 1968). The darkling beetles, *Alphitobius diaperinus* (Panzer), a stored-product pest generally associated with poultry brooder houses, was reported to harbor *Salmonella* spp., *E. coli*, *Campylobacter* spp. (Harein et al. 1970; Bates et al. 2004), *Micrococcus* spp., *Streptococcus* spp., and *B. subtilis* (De Las Casas et al. 1972). The same insect species sampled from turkey brooder house also had *Streptococcus* spp. and *B. subtilis* (Harein et al. 1972). The mealworm, *Tenebrio molitor* (L.), a pest of poultry sheds and egg barns, is capable of transmitting poultry diseases (Lambkin 2001). Recently, Larson et al. (2008b) isolated

antibiotic resistant *Enterococcus faecium* from the red flour beetle, *Tribolium castaneum* (Herbst), confused flour beetle, *Tribolium confusum* (Jacquelin du Val), warehouse beetle, *Trogoderma variabile* (Ballion), and maize weevil, *Sitophilus zeamais* (Motschulsky), collected from six feed mills located in the Mid-Western United States. This is not surprising considering the fact that enterococci have been previously isolated from feed samples (Schwalbe et al. 1999; Kidd et al. 2002; Kuhn et al. 2003).

Enterococci, which are ubiquitous in nature (Franz et al. 1999), have gained prominence in the last decade as an important reservoir of antibiotic resistance genes. Furthermore, enterococci can transfer their resistance traits to bacteria of clinical significance through horizontal gene transfer (Devriese et al. 1992). While antibiotic resistant enteroccci are associated with stored-product insects (Larson et al. 2008b), it is not clear how these insects are contaminated with enterococci. We hypothesize that stored-product insects are capable of acquiring enterococci from the feed and contribute to feed contamination in the feed environments. In order to answer these questions, laboratory experiments were designed to evaluate the survival of *Enterococcus faecalis* in poultry and cattle feed, and to elucidate the role of adult *T. castaneum*, a pest commonly associated with feed mills (Larson et al. 2008a), in the acquisition, retention, and transmission of enterococci from inoculated poultry and cattle feed to sterile feed.

Materials and methods

Poultry and cattle feed substrates

Poultry and cattle feed, freshly prepared in the Department of Grain Science and Industry's pilot feed mill, were used in this study. These feed materials are similar to poultry and cattle feed produced commercially. The ingredients in poultry feed include ground corn (70.0%

by wt), soybean meal (20.4%), fishmeal (5.5%), soybean oil (3.0%), monocalcium phosphate (0.5%), limestone (0.6%), salt (0.3%), D-methionine (0.3%), L-lysine (0.1%), and poultry vitamin mix (0.25%). The poultry feed was batched using a Wisconsin Electrical Manufacturing automated batching system (WEM Automation Inc., New Berlin, WI). Batched feed was then mixed in a 500 kg Forberg double shaft paddle mixer (Forberg International AS, Larvik, Norway) after which it was steam-conditioned in a single pass steam conditioner to a temperature of ~82°C followed by pelleting in a California Pellet Mill (Master Model 1000; CPM Crawfordsville, IN). A pellet die with 0.4 cm pellet diameter and 3.2 cm pellet thickness was used to make the pelleted poultry feed. The pelleted feed was cooled on a double pass, perforated bed, horizontal cooler before bagging pellets into a 22.7 kg (50 lb) paper bags.

The ingredients in cattle feed include ground corn (77.0% by wt), cottonseed meal (5.2%), liquid molasses (4.0%), soybean oil (3.0%), ruminant vitamin mix (2.6%), dehydrated alfalfa (5.5%), and cotton seed hulls (3.0%). Cattle feed was batched, mixed, and bagged following procedures described for poultry feed, but the feed did not go through the pelleting process.

E. faecalis cultures and feed inoculation

An overnight culture of *E. faecalis* OG1RF:pCF10 strain, resistant to tetracycline, grown in Brain Heart Infusion broth (BHI; Difco Laboratories, Detroit, MI) at 37°C in an incubator with a shaker (Excel E24 incubator shaker series, Edison, NJ), was selected for this study. The bacterial cells were centrifuged and the supernatant was discarded. The pellets were re-suspended in potassium buffer saline (pH 7.2; MP Biomedicals, Solon, OH), diluted, and used to inoculate poultry and cattle feed.

Poultry and cattle feed, prior to inoculation, were ground in a mixer (Braun, KSM2 grinder, Kronberg, Germany) and then sifted through a 180 µm opening sieve (Seedburo Equipment Company, Chicago. IL). The sifted material was dry sterilized at 121°C for 20 min. Poultry or cattle feed (100 g) was placed in separate 500 g capacity sterilized glass jars. The sample was inoculated with 5 ml of *E. faecalis* cell suspension. After inoculation, the feed material in each jar was shaken manually for 15 min to ensure thorough mixing of *E. faecalis* cells with the feed. Feed samples that received aliquots of double-distilled water served as the control treatment.

Enumeration of *E. faecalis* in poultry and cattle feed in triplicate samples was done following procedures outlined by Yezerski et al. (2005). Poultry or cattle feed (1 g) was added to 9 ml of potassium buffer saline (pH 7.2; MP Biomedicals, Solon, OH) under sterile conditions, shaken, and allowed to settle. A milliliter of the sample was serially diluted in 9 ml of potassium buffer saline. A 100 µl sample from serial dilutions was drop plated on mEnterococcus agar (Difco Laboratories, Detroit, MI) and incubated at 37°C for 48 h. The number of *E. faecalis* colonies were counted to determine the concentration of enterococci in poultry and cattle feed. The mean (n = 3) initial enterococcal concentration in poultry feed and cattle feed was $4.6 \pm 0.3 \times 10^6$ and $4.7 \pm 0.3 \times 10^6$ cfu/g, respectively. The control feed samples tested negative for *E. faecalis* contamination.

Insect exposure to E. faecalis inoculated feed

Inoculated feed samples were placed in sterilized glass Petri dishes. In each Petri dish 2 g of feed was introduced. Cultures of *T. castaneum* were reared in the Department of Grain Science and Industry, Kansas State University, Manhattan, KS, in environmental chambers (Model I-36 VL, Percival Scientific, Perry, IA) on sterile whole wheat flour plus 5%% (by wt)

brewer's yeast diet at 28°C and 65% RH. Adults (2-wk-old) were separated from the diet using 840 µm opening sieve. Adults separated from the diet were starved for 24 h before use in tests. Before commencing the actual experiments, 10 randomly selected adults from the laboratory colony, were individually tested for the presence of *E. faecalis* using serial dilutions and drop plate techniques described by Zurek et al. (2000). Individual insects were surface sterilized with 10% sodium hypochlorite and 70% ethanol, homogenized in potassium buffer saline, and drop-plated on mEnterococcus agar (mENT; Difco Laboratories, Detroit, MI) containing tetracycline. Plates were allowed to dry and then placed in an incubator at 37°C for 24 to 48 h. After the incubation, the colony forming units were counted to determine the concentration of enterococci within the insect. The tested insects were negative for *E. faecalis*.

Four separate tests were conducted with poultry and cattle feed as food substrates to determine the vector competence of *T. castaneum* adults. In the first test, 24 four sterile glass Petri dishes, each containing five *T. castaneum* adults with 2 g of poultry feed inoculated with *E. faecalis*, were incubated at 28°C, 65% RH for 7 d. On 1, 3, 5, and 7 d postinfestation, three Petri dishes (replicates) at random were sampled to assess for the presence of *E. faecalis* in feed as well as in insects as described above. For enumerating *E. faecalis* in feed, 1 g of the feed was used following procedures described above. To determine concentration of *E. faecalis* on the surface and within *T. castaneum* (non-surface sterilized insects), 2, 2, and 3, adults from replicates 1, 2, and 3, respectively, were selected and *E. faecalis* was isolated from individual insects. The remaining 3, 3, and 2 adults from replicates 1, 2, and 3, respectively, were surface sterilized with 10% sodium hypochlorite and 70% ethanol before isolating and enumerating *E. faecalis* . In the second test, a set up similar to that used for poultry feed was used to determine the concentration of *E. faecalis* in cattle feed on days 1, 3, 5, and 7, and on surface and non-

surface sterilized *T. castaneum* adults. In the third and fourth tests, the vector competence of starved (24 h) *T. castaneum* adults in transferring *E. faecalis* from inoculated poultry and cattle feeds to fresh sterilized poultry and cattle feeds, respectively, was assessed. This test for each feed involved two separate sets of 24 glass Petri dishes. Each dish with either poultry or cattle feed was infested with five adults. On alternate days (1, 3, 5, and 7 d postinfestation) three dishes at random were selected and the five beetles from each dish were transferred to three new dishes with sterile poultry or cattle feed. After 48 h the sterile poultry feed samples (1 g) were tested for the presence of *E. faecalis* following procedures described above.

Experimental design and statistical analysis

A completely random design was used for all experiments. Data on *E. faecalis* colony forming units (x) in poultry feed over time, in surface sterilized and non-surface sterilized *T. castaneum* adults in poultry feed, and transfer of *E. faecalis* by *T. castaneum* adults from inoculated to sterile poultry feed were transformed to $\log_{10}(x)$ scale before statistical analysis to normalize heteroscedastic treatment variances. Similar data for the cattle feed were transformed to $\log_{10}(x + 1)$ scale because of zero counts on day 7 for three of the four tests. Significant differences over time of transformed count data for *E. faecalis* in poultry or cattle feed, in surface or non-surface sterilized insects, and in sterile poultry or cattle feed were determined by subjecting data to one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (lsd) test at $\alpha = 0.05$ level (SAS Institute 1999).

The logarithmic decrease of *E. faecalis* counts in poultry or cattle feed, in surface or non-surface sterilized insects in poultry and cattle feed, and in sterile poultry or cattle feed over time (days 1 through 7) was characterized by linear regression (SAS Institute 1999). The inverse of

the slope gave a '*D*-value', which is the number of days required for one log reduction of enterococcal concentration (Stumbo 1973).

Results

Survival of E. faecalis in feed substrates and insects

The presence of *E. faecalis* in poultry and cattle feeds indicated that the inoculations were successful. The adults of *T. castaneum* allowed to infest the feed for 7 d also tested positive for *E. faecalis*. Table 1 showed that the survival of *E. faecalis* decreased significantly in poultry ($F = 1174.85$; df = 3, 8; $P < 0.0001$) and cattle feed ($F = 319.35$; df = 3, 8; $P < 0.0001$). Adults of *T. castaneum* in poultry feed acquired *E. faecalis* within 24 h of infestation and retained it during the 7-d test period (Table 2). However, surface and non-surface sterilized *T. castaneum* adults in cattle feed tested negative for *E. faecalis* on day 7.

The *E. faecalis* survival in *T. castaneum* adults fed inoculated poultry feed also decreased significantly for non-surface sterilized ($F = 393.71$; df = 3, 8; $P < 0.0001$) and surface sterilized insects ($F = 27.11$; df = 3, 8; $P = 0.0002$). Similarly, *E. faecalis* survival decreased significantly only in non-surface sterilized *T. castaneum* adults fed inoculated cattle ($F = 469.26$; df = 3, 8; $P < 0.0001$). However, this trend was not apparent in surface sterilized *T. castaneum* adults ($F = 3.15$; df = 3, 8; $P = 0.0865$).

Vector competence of T. castaneum adults

Adults of *T. castaneum* were able to acquire *E. faecalis* and transfer it to sterile poultry feed (Table 1) and cattle feed (Table 2) during the 7-d test period. The adults were unsuccessful in transferring *E. faecalis* to sterile cattle feed on day 7 because no *E. faecalis* was present in cattle feed by day 7. The number of *E. faecalis* transferred by *T. castaneum* adults to sterile poultry feed showed a significant decrease during the 7-d test period ($F = 4.69$; df = 3, 8; $P =$

0.0358). A similar trend was evident in tests with the sterile cattle feed (F = 12.16; df = 3, 8; P = 0.0024).

D values for E. faecalis survival in feed substrates and insects

The relationship between logarithmic reduction of *E. faecalis* concentration in the feed substrates and in non-surface sterilized and sterilized insects, and in sterile feeds and time was satisfactorily described by the linear regression (Table 3; Fig. 1-3). The r^2 values ranged from 0.79 – 0.99. The logarithmic reduction of *E. faecalis* concentration decreased more rapidly in cattle feed than in poultry feed as indicated by the steeper slope values in cattle than poultry feed (Fig. 1). (Table 1). Similarly, *E. faecalis* concentration from insects fed cattle feed when compared with poultry feed also showed a similar trend (Fig. 2). On sterile cattle feed, *E. faecalis* concentration also decreased rapidly than in sterile poultry feed (Fig. 3). The *D*-values revealed that for one log reduction of *E. faecalis* counts in cattle feed took 2.43 days whereas in poultry feed it took 2.56 d. The non-surface and surface sterilized insects in poultry feed had *D*-values that ranged from 4.76 – 8.30 d, whereas corresponding values for insects in cattle feed ranged from 1.85 – 2.63 d. The *D*-value in sterile poultry feed was 3.14 d while comparable value for cattle feed was 2.21 d.

Discussion

In feed samples collected from feed mills, enteroccal concentration typically is in the range of 1.2×10^2 to 8.2×10^3 cfu/g (Chapter 3). Similarly, enteroccal concentration in surface sterilized *T. castaneum* adults collected from feed mills ranged from 2.0×10^1 to 5.0×10^1 cfu/insect (Chapter 2), which is much lower than what we observed in this study. A high concentration of *E. faecalis* was used in the present study to ensure survival in the feed substrates

and increase the probability of acquisition of *E. faecalis* by *T. castaneum* and its transmission to sterile feed.

In this investigation *E. faecalis* survived longer in poultry feed compared to cattle feed. This is supported by the fact that the type of animal and plant origin by-products used in feed play an important role in bacterial contamination and survival in animal feed (Maciorowski et al. 2007; Sapkota et al. 2007). For example, poultry feed used in this study contained fish meal and added amino acids which are absent in cattle feed. However, the exact role of these ingredients in survival of *E. faecalis* in poultry feed requires further study.

Tribolium castaneum are commonly associated with feed mills (Loschiavo and Okumura 1979; Roesli et al. 2003; Trematerra and Sciarretta 2004; Hagstrum and Subramanyam 2006; Larson et al. 2008a). Adults of *T. castaneum* successfully acquired, retained, and transmitted *E. faecalis* from inoculated feed to sterile feed. This suggested survival of *E. faecalis* for a short time in the digestive tract of *T. castaneum* adults. The digestive tract of adult insects provides an ideal microclimate for bacterial growth and development (Martin and Mundt 1972; Dillon and Dillon 2004), and several authors have also reported acquisition, retention, and transmission of bacterial pathogens in adult stored-product insects (Husted et al. 1969; Crumrine et al. 1971; Harein et al. 1970; Harein et al. 1972; De Las Casas et al. 1972; Pelliteri and Boush 1983; Skov et al. 2004; Yezerski et al. 2005; Larson et al. 2008b).

The enteroccal concentration in non-surface sterilized *T. castaneum* adults was generally higher than in surface sterilized adults, irrespective of the feed, because the former insects had *E. faecalis* contamination on their body surface. Interestingly, the *D*-values were higher for *E. faecalis* from surface sterilized insects on both feeds compared to non-surface sterilized insects. For example, in tests with sterile poultry feed, the *D*-value was 8.30 d for surface sterile insects

whereas it was 4.76 d for non-surface sterilized insects. The plausible explanation is the rapid loss of *E. faecalis* on the surface of the insects in non-surface sterilized insects.

The United States animal feed industry is the largest producer of animal feed in the world (Sapkota et al. 2007). About 3,000 feed mills produce over 121 million tons of formulated feeds for cattle, horses, poultry, and swine, as well as specialty feeds such as geese, goats, moose, and buffalo (Larson et al. 2008b). Animal feed requires special attention to avoid contamination as contaminated feed can adversely impact food animals. In recent years, public concern regarding feed safety has increased due to outbreaks of illness associated with clinically important pathogens such as *Salmonella* spp., *E. coli*, *Campylobacter* and *L. monocytogenes*. The source of contamination may be due to consumption of contaminated feed itself.

Our results show that poultry and cattle feed can support *E. faecalis* infection, but the inoculum tends to disappear at a logarithmic rate over time. It is during these short time periods that any *E. faecalis* inoculum can be potentially acquired and transmitted to fresh feed by *T. castaneum* adults. The perception that stored-product insects are just aesthetic contaminants is no longer tenable, because these adults can serve as potential vectors of antibiotic resistant enterococci within the feed environment. Therefore, it is important to follow proper pest management practices to reduce potential insect vectors in feeds and in the feed environments.

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Table 1. Counts of *E. faecalis* (mean ± SE) in poultry feed, in non-surface sterilized and surface sterilized *T. castaneum* adults, and transmission of *E. faecalis* by *T. castaneum* to sterile feed[a,b]

Time (d)	Poultry feed (cfu/g)	*T. castaneum* adults (cfu/insect)		Transmission by *T. castaneum* (cfu/g)
		Non-surface sterilized	Surface sterilized	
1	$3.3 \pm 0.3 \times 10^6$ a	$3.8 \pm 0.6 \times 10^4$ a	$2.1 \pm 0.1 \times 10^3$ a	$1.8 \pm 0.1 \times 10^3$ a
3	$6.7 \pm 0.5 \times 10^4$ b	$5.1 \pm 0.2 \times 10^3$ b	$2.8 \pm 0.4 \times 10^3$ a	$7.6 \pm 0.8 \times 10^2$ a
5	$2.0 \pm 0.1 \times 10^4$ c	$2.5 \pm 0.3 \times 10^3$ c	$1.0 \pm 0.2 \times 10^3$ b	$2.6 \pm 0.3 \times 10^2$ a
7	$1.1 \pm 0.1 \times 10^4$ d	$1.8 \pm 0.1 \times 10^3$ c	$4.6 \pm 0.6 \times 10^2$ b	$1.0 \pm 0.5 \times 10^2$ b

The mean initial enterococcal concentration in poultry feed ($n = 3$) was $4.6 \pm 0.3 \times 10^6$ cfu/g.

[a]Each mean is based on $n = 3$ replicates.

[b]Means within a column followed by different letters are significantly different ($P < 0.05$, by Fisher's protected lsd test).

Table 2. Counts of *E. faecalis* (mean ± SE) in cattle feed, in non-surface sterilized and surface sterilized *T. castaneum* adults, and transmission of *E. faecalis* by *T. castaneum* to sterile feed[a,b]

Time (d)	Poultry feed (cfu/g)	*T. castaneum* adults (cfu/insect)		Transmission by *T. castaneum* (cfu/g)
		Non-surface sterilized	Surface sterilized[c]	
1	$1.3 \pm 0.3 \times 10^6$ a	$2.9 \pm 0.1 \times 10^3$ a	$1.1 \pm 0.1 \times 10^3$	$3.3 \pm 0.3 \times 10^2$ a
3	$2.5 \pm 0.2 \times 10^4$ b	$1.1 \pm 0.6 \times 10^3$ b	$3.3 \pm 0.1 \times 10^2$	$1.6 \pm 0.6 \times 10^2$ a
5	$1.2 \pm 0.1 \times 10^4$ b	$4.0 \pm 0.1 \times 10^2$ c	$2.0 \pm 0.1 \times 10^2$	$3.3 \pm 0.3 \times 10^1$ b
7	$2.0 \pm 0.3 \times 10^3$ c	0 d	0	0 c

The mean initial enterococcal concentration in cattle feed ($n = 3$) was $4.7 \pm 0.3 \times 10^6$ cfu/g.

[a]Each mean is based on $n = 3$ replicates.

[b]Means within a column followed by different letters are significantly different ($P < 0.05$, by Fisher's protected lsd test).

[c]Means among days are not significant ($F = 3.15$; df = 3, 8; $P = 0.0865$; one-way ANOVA)

Table 3. Regression estimates showing logarithmic reduction in *E. faecalis* counts in feed substrates and in non-surface sterilized and sterilized *T. castaneum* adults

Parameter	Inoculated feed		*T. castaneum* adults in:				Sterile feed	
	Poultry	Cattle	Poultry feed		Cattle feed		Poultry	Cattle
			Non-surface sterilized	Surface sterilized	Non-surface sterilized	Surface sterilized		
Intercept	6.50 ± 0.54	6.10 ± 0.46	4.60 ± 0.30	3.60 ± 0.20	4.40 ± 0.84	3.50 ± 0.12	3.60 ± 0.22	3.10 ± 0.33
Slope	-0.40 ± 0.12	-0.41 ± 0.20	-0.20 ± 0.06	-0.10 ± 0.04	-0.54 ± 0.20	-0.40 ± 0.02	-0.30 ± 0.05	-0.45 ± 0.10
r^2	0.84	0.90	0.85	0.80	0.80	0.99	0.94	0.94
D-value (d)[a]	2.60	2.43	4.80	8.30	1.85	2.63	3.44	2.20

[a]The *D*-value shows one log reduction in *E. faecalis* counts as a function of time.

Fig. 1. Logarithmic decrease in *E. faecalis* counts in inoculated poultry and cattle feed

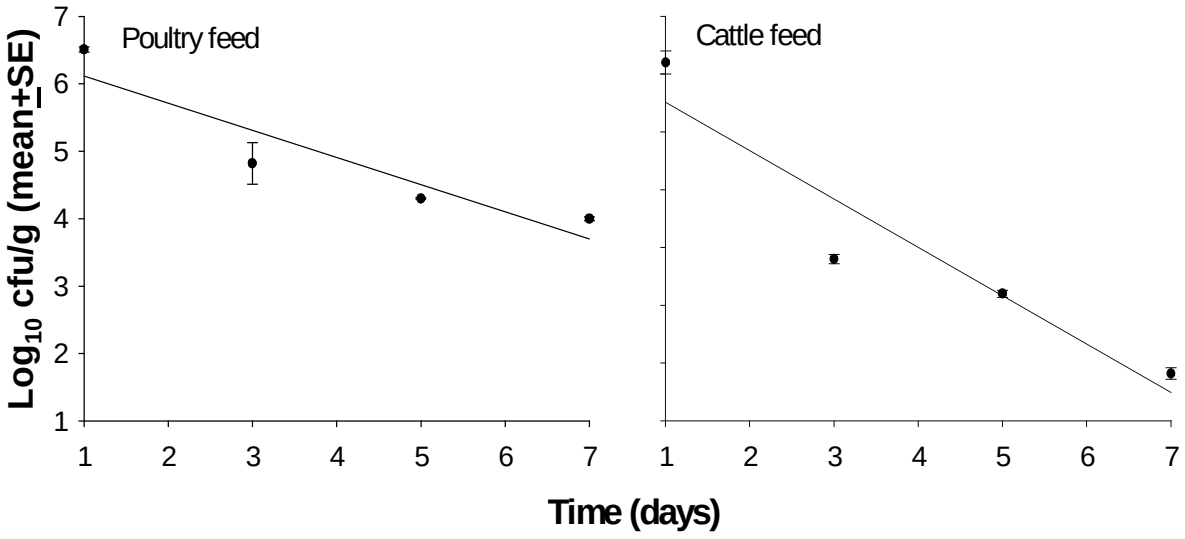

Fig. 2. Logarithmic decrease in *E. faecalis* counts in non-surface sterilized and sterilized *T. castaneum* fed inoculated poultry and cattle feed

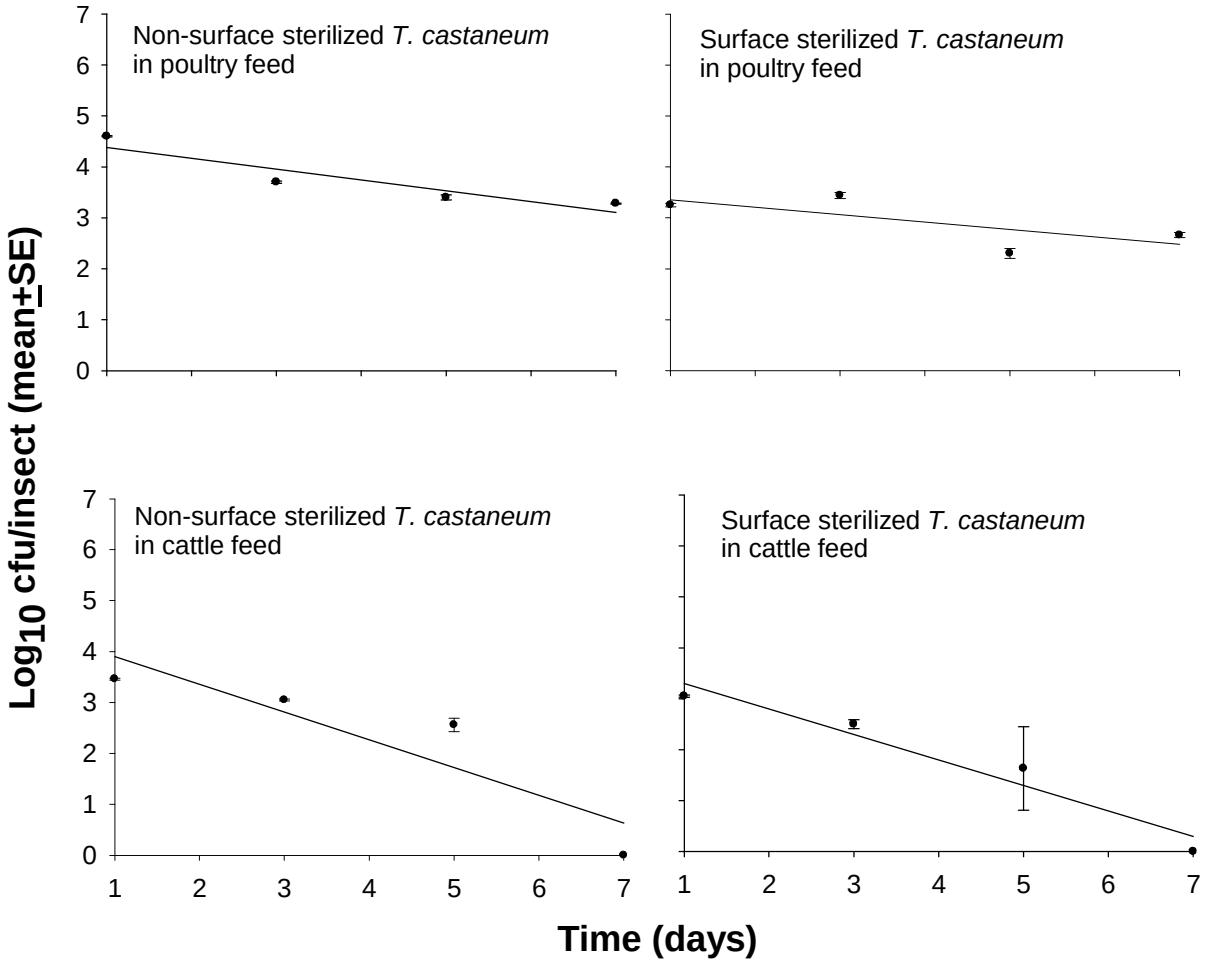

Fig. 3. Logarithmic decrease in *E. faecalis* counts transmitted by *T. castaneum* adults in sterile poultry and cattle feed