ANTIBIOTIC RESISTANT ENTEROCOCCI IN LABORATORY REARED STORED-PRODUCT INSECT SPECIES AND THEIR DIETS

by

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Abstract

Stored-product insects and stored products from feed mills and swine farms contain antibiotic and potentially virulent *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus casseliflavus*, *Enterococcus gallinarum*, and *Enterococcus hirae*. Stored-product insects can serve as potential vectors of these enterococci which possess antibiotic resistance genes that can be spread by horizontal transfer to more serious human pathogens. In the present study, the species and concentration of enterococci from adults and larvae of key stored-product insects and insect diets and their antibiotic resistance profiles were characterized. Adults of five species out of the 15 stored-product insects were tested positive for enterococci, and these included *Callosobruchus maculatus* (F.), *Sitophilus granarius* (L.), *Stegobium paniceum* (L.), *Lasioderma serricorne* (F.), and *Sitophilus zeamais* Motschulsky. Three enterococcal species (*E. casseliflavus*, *E. faecalis*, and *E. faecium*) were found in 53 to 97% of the 30 adults screened for each insect species, and the enterococcal concentrations ranged from 1.4×10^3 to 3.1×10^6 CFU/adult. About 10 to 100% of the mature larvae of the respective five insect species had these three enterococcal species with concentrations ranging from 0.3×10^1 to 1.4×10^5 CFU/larvae. Only three of the eight insect diets screened had the same three enterococcal species in addition to *E. gallinarum* and *E. hirae* at concentrations of 0.2×10^1 to 5.9×10^3 CFU/g. The greatest enterococcal concentration was found in *C. maculatus* adults but not in their larvae or diet (cowpeas). In *C. maculatus* during a nine-day period after adult eclosion, the enterococcal concentrations increased exponentially from 0.6×10^1 to a maximum of 4.1×10^7 CFU/adult. Enterococci were detected in the fecal material of *C. maculatus* during a four-day period with a maximum concentration of 3.3×10^3 CFU/adult on the fourth day.

A total of 298 enterococcal isolates from adults, larvae, and diets were represented by *E. faecalis* (51.7% of the total), *E. faecium* (19.1%), *E. casseliflavus* (18.8%), *E. gallinarum* (5.7%), and *E. hirae* (4.7%). Enterococci were phenotypically resistant to quinupristin (51.3% of the total), erythromycin (38.9%), tetracycline (30.1%), enrofloxacin (29.2%), doxycycline (11.5%), and tigecycline (2.7%). All isolates were susceptible to ampicillin and vancomycin.

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Dedication

I dedicate this thesis to my parents Mori and Charlene Byington.

Chapter 1 - Literature review

1.1. Background data on enterococci in the postharvest system

The postharvest grain marketing system includes grain stored on farms, country and terminal elevators, processing facilities where the grain is processed into food for humans or animals, and retail stores where the end-use products for humans and animals are sold. The interconnectedness of this system is depicted in Figure 1-1.

Data from grain collected from farms and products from feed mills (Larson 2004, Channaiah 2009, Channaiah et al. 2010a,b) have shown that both the grain and grain products and the stored-product insect adults associated with these commodities were positive for several species of enterococci. The red flour beetle, *Tribolium castaneum* (Herbst), and confused flour beetle, *Tribolium confusum* (Jacquelin du Val), were the most frequently collected species from feed mills (Table 1-1), because of their abundance in these environments (Larson 2004). These species also are commonly found throughout the postharvest system (Hagstrum and Subramanyam 2006).

From each surface sterilized stored-product insect collected from mills, enterococci were cultured, isolated, and quantified on Enterococcus agar (Larson et al. 2008). The percentage of insects that were positive for enterococci ranged from 0 to 100%. Colony forming units (CFU) per insect ranged from 0 to 1.7×10^5 (Table 1-2). Compared to the insects, a greater percentage of products were positive for enterococci, and the enterococcal concentration from stored products ranged from 3.1×10^2 to 9.3×10^2 CFU/g (Table 1-3). It is unclear how the stored products became contaminated with enterococci, and whether stored-product insects are acquiring enterococci from the products or contributing to product contamination.

The frequency of antibiotic resistant enterococci from stored-product insects is shown in Figure 1-2 (Channaiah et al. 2010a) and from stored products is shown in Figure 1-3 (Channaiah 2009). *Enterococcus* species associated with stored-product insects and stored products are shown in Table 1-4. It is important to note that *E. faecalis* and *E. faecium* are nosocomial pathogens and medically important (de Perio et al. 2006, Ghosh and Zurek 2015).

Kansas State University researchers were the first group to report on the antibiotic resistance in enterococci isolated from grain, grain-based products, and from stored-product insects (Larson 2004, Channaiah et al. 2006, 2010). The prevalence, diversity, and antibiotic

resistance profiles of enterococci throughout the postharvest system is unknown. Additional studies are warranted to determine prevalence, concentrations, and antibiotic resistance profiles of enterococci in stored-product insets and stored products in grain stored on farms and elevators, feed mills, flour mills, retail stores, and laboratory-reared colonies. Ghosh and Zurek (2015) provide an excellent review on antibiotic resistance in enterococci from a food safety perspective.

1.2. Association of stored-product insects with bacteria

Stored-product insects have been reported to harbor many potentially pathogenic bacteria. For example, the lesser mealworm, *Alphitobius diaperinus* (Panzer), from poultry brooder houses was reported to carry *Salmonella* spp., *Escherichia coli* (Harein et al. 1970), *Micrococcus* spp., *Streptococcus* spp., and *Bacillus subtilis* (De Las Casas et al. 1972). The lesser mealworm sampled from turkey brooder houses were positive for *Streptococcus* spp. and *B. subtilis* (Harein et al. 1972). Skov et al. (2004) have shown that the lesser mealworm, hairy fungus beetle, *Typhaea stercorea* (L.); and foreign grain beetle, *Ahasverus advena* (Waltl), associated with litter in broiler chicken rearing facilities, served as reservoirs for *Salmonella indiana* and were responsible for transmission of this pathogen between two consecutive broiler flocks. The reservoir competence of *A. diaperinus* in broiler-rearing facilities for *Salmonella typhimurium* was also confirmed by McAllister et al. (1994). They isolated the pathogen from feces of *A. diaperinus* adults 28 days after feeding for 24 hours on 1 g of chicken feed inoculated with 3×10^8 bacteria/ml, and subsequently one-day old broiler chicks were positive for *Salmonella* 24 hours after eating an infected adult. Hald et al. (1998) have shown that *T. stercorea*, breeding in the poultry litter, was a carrier of *Salmonella enterica* var *infantis* in a Danish broiler house, and healthy 5-day-old chicks fed *S. enterica* positive beetles became infected in 4 days. The granary weevil, *Sitophilus granarius* (L.), from laboratory colonies and grain-storage facilities was identified as a potential reservoir for *Escherichia intermedia, Proteus rettgeri, Proteus vulgaris, B. subtilis, Serratia marcescens, Streptococcus* spp., *Micrococcus* spp., and members of the *Klebsiella-Aerobacter* group (Harein and de las Casas 1968). In addition, *S. granarius* has been shown to transfer *Salmonella montevideo* from contaminated to uncontaminated wheat under laboratory conditions (Husted et al. 1969). Most of the studies with stored-product insects and pathogenic bacteria involve poultry rearing facilities, with a few studies conducted in the laboratory. In a laboratory study, Yezerski et al. (2005) showed that two

stored-product insects, the cigarette beetle, *Lasioderma serricorne* (F.), and drug store beetle, *Stegobium paniceum* (L.), reared on sterile flour contaminated it with *Enterococcus* spp. However, sterile flour infested with two other stored-product insects, *T. castaneum* and *T. confusum*, were devoid of *Enterococcus* spp. They inferred that benzoquinones produced by the *Tribolium* spp. may have inhibited *Enterococcus* spp., but this finding is contrary to what we found in *Tribolium* spp., and in product samples (Larson 2004, Channaiah et al. 2006, 2010). Yezerski et al. (2005) did not indicate whether *Enterococcus* spp. was present on or inside the insects. Two studies (Larson 2004, Channaiah 2010a,b) are the only ones that address the association between stored-product insects in the postharvest system and enterococci, which are now becoming a major food safety issue (Franz et al. 1999, Ghosh and Zurek 2015).

1.3. Importance of enterococci

Enterococci are Gram-positive, catalase-negative cocci that are ubiquitous in the environment and occur in the digestive tract of animals, soil, contaminated water, animal feces, and food products derived from animals (Franz et al. 1999). Enterococci have been reported from several non-stored product insects collected from nonurban, wild, and cultivated fields, and woods (Martin and Mundt, 1972). Two species of enterococci, *E. faecalis* and *E. faecium*, are generally recognized as nosocomial (hospital-related infections) pathogens worldwide (Linden and Miller 1999), especially in immuno-compromised people (Awada et al. 1992). Moreover, enterococci possess antibiotic resistant genes (Huycke et al. 1998, Murray 1998, Hancock and Gilmore, 2000) that can be spread by horizontal transfer to more serious human pathogens (Devriese et al. 1992, Pembroke et al. 2002).

Enterococci are common symbionts in the gastro-intestinal tract of domestic animals including cattle, swine, and poultry (Kühn et al. 2003), and the use of antibiotics in feed for domestic animals has led to the selection of antibiotic resistant strains (Quednau 1998, Bauer-Garland et al. 2006). *Enterococcus faecalis, E. faecium,* and *E. hirae* have been isolated from feed samples in Sweden and Spain, *E. faecium* has been identified from feed samples in the United Kingdom (Kühn et al. 2003), and *E. faecium* highly resistant to vancomycin was isolated from chicken feed in the United States (Schwalbe et al. 1999). However, little emphasis has been placed on enterococci in raw stored grain and in grain processing or retail environments where stored-product insects are commonly found.

1.4. Insect species encountered in the postharvest system

Stored-product insects are adapted to infesting raw and processed cereal products, and present a constant threat to these commodities worldwide (Sinha and Watters 1985). These insect pests survive on dry, stored cereals and legumes in raw or processed form, and they are maintained year after year in storage systems by residual grain remaining in bins, poor sanitation in mills, food-processing facilities and warehouses, and retail stores, and immigration form natural (rodent caches, bird nests, wooded areas) and other infested sites. Losses due to stored grain insects are estimated at 5-10% for developed countries and more than 35% for developing countries. These pests cause significant quantitative and qualitative losses to the multi-billion dollar grain, food, and retail industries each year through their feeding, product adulteration, customer complaints, and product rejection at the time of sale.

A wealth of information is available on stored-product insect species and their occurrence, density, distribution, biology, damage, and management in stored grain (Sinha and Watters 1985, Subramanyam and Hagstrum 1995, 2000; Roesli et al. 2003 Larson 2004, Hagstrum and Subramanyam 2006) and processing environments (Heaps 2006). Many stored-product insect species are associated with raw grain (corn, wheat, sorghum, barley, rice, and oats) stored on farms (Barak and Harein 1981, Storey et al. 1983, Ingemansen et al. 1986, Meagher et al. 1986, Barker and Smith 1987, Reed and Pedersen 1987; Hagstrum 1989, 2001) and at elevators (Dowdy and McGaughey 1996, Reed et al. 2003, Arthur et al. 2006). Insect species commonly found in grain residues, stored grain and outside storage structures on farms include the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens); flat grain beetle, *Cryptolestes pusillus* (Schoenherr); lesser grain borer, *Rhyzopertha dominica* (F.); sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.); *T. castaneum*, grain weevils, *Sitophilus* spp.; *A. advena*; *T. stercorea*; larger black flour beetle, *Cynaeus angustus* (LeConte), warehouse beetle, *Trogoderma variabile* Ballion; and Indian meal moth, *Plodia interpunctella* (Hübner). The same insect species, along with a few additional ones not found in the grain environment, (e.g., *L. serricorne*) also are associated with flour mills, feed mills, and retail stores.

Flour mills are ideal habitats for economically damaging stored-product insect pests, because of year-round warm temperatures and constant availability of food resources (Wagner and Cotton 1935, Smallman and Loschiavo 1952). Stored-product insect species are found in moving mill stock (Wagner and Cotton 1935, Good 1937), static mill stock, and within and

around milling equipment (Dyte 1965, 1966; Rilett and Weigel 1956). In the United States, Good (1937) surveyed 19 flour mills in Kansas, Missouri, and Oklahoma during 1934 to 1935 by taking 227 g samples monthly from 24 elevator boots and mill streams. He reported 30 different species from 17 of the 19 mills, representing 15 families in five orders. Four of the eight most abundant stored product insect species made up nearly 96% of the 73,175 insects found in the samples, and the flour beetles made up 85% of the total insects. Adults and larvae of flour beetles were present throughput the milling system (Figure 1-4). Campbell and Arbogast (2004) monitored stored-product insects inside and outside a flour mill by using commercial pitfall and sticky traps and product samples during June 2001 to December 2003. They found 17 insect species, representing 12 families in 3 orders (Coleoptera, Hymenoptera, and Psocoptera). Captures of *P. interpunctella* adults in sticky traps and adults of *T. variable* were greater outside than inside the mill. However, captures of *T. confusum* adults in pitfall traps were greater inside than outside the mill. The presence of the same insect species inside and outside mills suggested movement between the two environments.

Commercial feed mills produce formulated feeds for a wide variety of livestock species such as cattle, horses, poultry, and pigs, and specialty feeds for animals such as geese, goats, moose, and buffalo. In the United States, there are about 3,000 feed mills which produce about 121 million tons of feed for various animal species (Feedstuffs 2003). A large portion of the feed formulation primarily includes cereal grains (usually ground) and by-products of cereals, legumes, and animals. Minor ingredients include vitamins, minerals, amino acids, fat, molasses, antibiotics, and flavor enhancers. 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, Larson 2004). Rilett and Weigel (1956) surveyed eight feed mills, two flour mills, and one flour and feed mill between October 1954 and March 1955 in Buffalo, New York, USA, by collecting 12.3-kg sample from each mill. They reported only the occurrence of insect species found in mills. A total of 2,632 insects, representing 23 species, were associated with the 11 mills. The black carpet beetle, *Attagenus piceus* (Olivier), and *O. surinamensis*, were found in 8 of the 11 mills. Triplehorn (1965) extracted insect adults from one 0.94-liter sample of grain, grain residues, and mill stock collected from each of the 118 grain elevators and feed mills in Ohio during May and September of 1961. He reported 44 species, representing 21 families in five orders. *A. piceus* was found in

104 and 110 facilities out of the 118 in May and September, respectively; all other species were found in ≤55 facilities. Loschiavo and Okumura (1979) surveyed four feed mills in Hawaii during 15 July to 31 December 1976 by examining product samples and captures in light traps and bait bags. Out of the seven insect species (all beetles) reported *A. diaperinus*, *R. dominica*, *S. oryzae*, and *T. casatenum* were found in all four mills. Twenty feed mills in southern Wisconsin were sampled between 24 June and 12 August of 1975 and 1976 by Pellitteri and Bousch (1983). They extracted insects from spilled grain and feed, as well as hand-collected visible insects during mill visits. They collected 18,410 insects, representing 100 species in 60 families and eight orders. Out of the 100 species, 19 stored-product insects constituted 83 and 92% of the total insects captured in 1975 and 1976, respectively. Out of the 19 species, *Cryptolestes* spp. made up 24 to 29% of the total insects found, followed by *S. granarius* (8 to 17%). *A. advena*, *A. piceus*, and larvae of mealworms, *Tenerbio* spp., were found in all 20 feed mills.

A limited number of surveys were conducted in the United States to determine insect species associated with retail grocery and pet stores. Loschiavo and Okumura (1979) sampled stored-product insects with food-baited traps in 33 supermarkets and six feed and pet food stores in Hawaii. They captured stored-product insects in traps at all locations; species most commonly found were *L. serricorne*, merchant grain beetle, *Oryzaephilus mercator* (Fauvel), and *T. castaneum*. Platt et al. (1998) reported stored-product insects to be more common and abundant near pet food aisles compared with other areas of the store. The most common insect species captured in commercial food-baited and pheromone traps were adults of *P. interpunctella*, *S. paniceum*, and *O. mercator*. Arbogast et al. (2000) also used food- and pheromone-baited traps and captured stored-product insects in all five stores. *C. ferrugineus*, *L. serricorne*, *O. mercator*, *T. castaneum*, and *P. interpunctella* were the most common insects. In the surveyed department stores, infestations generally were associated with food products in or near the pet department. In the pet stores, infestations were generally associated with cat food, dog food, and horse feed.

Stored-product insects have been reported from wild hosts in the nature (Hagstrum and Subramanyam 2006), grain harvesting equipment (Sinclair and White 1980), grain transportation vehicles such as rail cars (Cogburn 1973, Perez-Mendoza et al. 2004), warehouses where products intended for retail distribution are stored (Vick et al. 1986, Soderstrom et al. 1987), and in residences as pantry pests (Loschiavo and Okumura 1979, Turner and Maude-Roxby 1989, Baz and Monserrat 1999, Subramanyam and Nelson 1999). Information on stored-product

insects in these habitats, omitted in Figure 1-1, has not been well studied, and source populations of these insects are generally associated with stored grain, grain processing, and retail stores where the environmental conditions are conducive for survival and reproduction (Hagstrum and Subramanyam 2006).

Insect infestations in stored raw commodities on farms and at elevators generally arise from resident populations in and around the storage environments (Reed et al. 1991, Dowdy and McGaughey 1994, 1996; Throne and Cline 1994, Vela-Coiffier et al. 1997, Subramanyam and Nelson 1999, Hagstrum 2001, Arthur et al. 2006).

In the United States, common preventive practices to protect bulk stored raw grain from insect infestations include application of insecticides, some with long residual life, to empty bins/silos and to the grain or grain surface. Surveys have shown that many pest management practices are not properly followed resulting in survival of insects both on farms and at elevators (Reed and Pedersen 1987, Kenkel et al. 1992, Martin et al. 1997). Furthermore, grain storage structures and elevator buildings are not designed to exclude insects. Fumigation with phosphine gas is commonly and routinely used to eliminate active stored-product infestations in the grain marketing system, but this treatment is rarely effective (Perez-Mendoza et al. 2004), and only less than 16% of the total stored grain (e.g., corn and wheat) in the postharvest system is treated with phosphine (NASS 1999). In flour and feed mills, sanitation, fumigation with sulfuryl fluoride, and/or heat treatment are commonly used. In retail stores, sanitation is commonly used, with occasional application of residual insecticides. Sanitation and chemical treatments have little impact on insect populations in retail stores (Roesli et al. 2003, Nansen et al. 2004), because potential sources of insects are often not eliminated by these practices. Additionally, insect infestations in mills and retail environments are not completely eliminated due to inadequate inbound inspection of materials, poor sanitation and exclusion practices (Heaps 2006, Subramanyam et al. 2005), and improper timing of pest management interventions (Toews et al. 2006). Therefore, stored-product insects tend to survive and continue to cause damage to grain and grain products in the postharvest system.

1.5. Rationale and significance of research

Antibiotics have saved countless human and animal lives and treated many infectious diseases since they were first widely used during World War II. However, after more than 50 years of widespread use, many antibiotics have lost their usefulness because of resistance

development in bacteria. According to the Centers for Disease Control and Prevention (CDC), one in ten patients, or 2 million patients a year, in the United States admitted to hospitals acquire a nosocomial infection resulting in 88,000 deaths (Mead et al. 1999). The Institute of Medicine of the National Academy of Sciences, Washington, D.C., estimates the annual cost of infections caused by antibiotic-resistant bacteria to be in the range of US $ 4.5 to 11 billion.

Enterococci are ubiquitous in human and animal digestive tracts, and in the environment (Martin and Mundt 1972, Noble 1978, Huycke et al. 1998). Some species of enterococci are used as probiotics (Franz et al. 1999), while other species are important as opportunistic and nosocomial human pathogens (Kayser 2003). There are 26 species in the genus *Enterococcus*, but two species, *E. fecalis* and *E. faecium*, are implicated in nosocomial infections. About 13% of all nosocomial infections are attributed to enterococci (NNIS 1997), and 80% of these infections are due to *E. faecalis.* One of the reason of the over representation of *E. faecalis* may due to its enhanced virulence (Huycke et al. 1998) or its natural abundance, because this species can tolerate high temperatures, dryness, and acidic environments (Hancock and Gilmore 2000).

Resistance of enterococci to several antibiotics have been reported from hospital infections (Huycke et al. 1998), and from food and gastrointestinal tract (Klein 2003). Several excellent reviews are available on the antibiotic resistance of enterococci in terms of prevalence, virulence, and virulence mechanisms (Jett et al. 1994, Johnson 1994, Murray 1998, Hancock and Gilmore 2000, Johnston and Jaykus 2004). Patterns of multidrug resistance to antibiotics vary among enterococcal species. The resistance to ampicillin and vancomycin is relatively uncommon among *E. faecalis* isolates (<2%), while isolates of *E. faecium* showed a general trend towards increasing resistance to both ampicillin (83%) and vancomycin (52%) (Huycke et al. 1998). Majority of enterococci are resistant to at least one of the common antibiotics (Huycke et al. 1998, Larson 2004). Vancomycin drugs are frequently reserved as the last available therapeutic agent to treat the most serious infections (Franz et al. 1999). According to CDC, there were no vancomycin-resistant enterococci (VRE) prior to 1989, but subsequently, such resistance has become more common in the United States (2006 National Institute of Allergy and Infectious Disease; http://www.niaid.nih.gov/factsheets/antimicro.htm), and the number of resistance reports are increasing yearly. The rapid increase in vancomycin resistance (Huycke et al. 1998) indicates that enterococcal infections will pose an increasing challenge. A study found that presence of vancomycin-resistant enterococci in the bloodstream was associated with

increased mortality of humans. Patients with vancomycin-resistant enterococci were observed to be twice as likely to die from infections compared to patients with susceptible enterococci (37% versus 16%) (Edmond et al. 1996).

Channaiah (2009) isolated *E. faecalis* (identified using multiplex PCR) from samples of stored wheat collected from a farm bin in Abilene, Kansas. Twenty-three of the 30 isolates were intermediately resistant to vancomycin based on the disk diffusion assay Figure 1-5. The minimum inhibitory concentration (MIC) test (reference dose, 2 µg/ml; Andrews 2001) was used to further characterize resistance of the 23 isolates, and all isolates were intermediately resistant at 6 µg/ml. In contrast, Schwalbe et al. (1999) isolated a strain of *E. faecium* collected from chicken feed in the United States that possessed high levels of resistance (MIC >256 µg/ml) to vancomycin. These findings are significant because vancomycin is not registered for use in animal feed in the United States (Schwalbe et al. 1999, van den Boogard et al. 1999).

Enterococci have been reported from milk, cheese, and meat (Klein et al. 1998, Franz et al. 1999, Hayes et al. 2003), raw produce (Johnston and Jaykus 2004), animal feed (Schwalbe et al. 1999, Channaiah et al. 2006), and stored-product insects (Larson 2004). The work of Channaiah (2009) and Larson (2004) and that of others have implicated enterococci as reservoirs of antibiotic resistant genes (Gilmore 2002). There may be a connection between the antibiotic resistance of isolates collected from food/feed, antibiotic resistance to clinical isolates, and community health (Eaton and Gasson 2001, Salyers 2002, Smith et al. 2002), but this issue is controversial (Phillips et al. 2004a,b,c), because very little is known about the ecology of antibiotic resistance in the environment, especially in postharvest environments. The data on the prevalence of enterococci in grain and feed samples and their resistance to antibiotics, including vancomycin, raises serious food safety concerns. As Schwalbe et al. (1999) stated, "…..the identification of a highly resistant enterococcal strain in feed raises disturbing questions about the potential for penetration of VRE strains into farms and food animal populations in the USA and the subsequent risk of transfer into human populations." Additional studies are needed to establish this connection.

The role of stored-product insects in the transmission of enterococci and transfer of antibiotic resistance has not been studied in detail. Most of the studies on the prevalence of enterococci are from nosocomial infections (Noble 1978, Huycke et al. 1998, Hancock and Gilmore 2000). These studies did not point out the source of enterococcal contamination at

hospitals. Martin and Mundt (1972) found that non-stored product insects were vectors of enterococci. Enterococci were found from 50% of the 403 insects collected from nonurban, wild, plant, and wooded habitats. Most of the enterococci were isolated from insects representing the orders of Coleoptera and Lepidoptera. Nearly 32% of *E. faecalis* and 22% of *E. faecium* were recovered from 37 insect taxa (Martin and Mundt 1972). These authors also found enterococci in overwintering insects suggesting survival of these bacteria in insects during winter months.

Martin and Mundt's (1972) data showing association of enterococci with non-stored product insects and our data on antibiotic resistant enterococci in stored-product insects suggest that insects may serve as potential reservoirs and vectors. Because stored-product insects, especially beetles, are long-lived (3 months to a year), highly mobile, and intimately associated with grain and grain products throughout the postharvest system (Hagstrum and Subramanyam 2006), they may be able to transfer antibiotic resistant enterococci to food materials during their course of colonization, feeding, and breeding. In order to understand the role of stored-product insects in the transmission of antibiotic resistance enterococci to food, it is important to first determine the competency of stored-product insects as reservoirs and vectors of antibiotic resistant enterococci. Information in this area is currently lacking, except for one study (Channaiah et al. 2010b) which showed *T. castaneum* adults to acquire enterococci from spiked diet and transfer them to sterile cattle and poultry feed.

It is established that the widespread multidrug resistance in many bacterial species is mainly due to horizontal gene transfer (HGT) (DeNap and Hergenrother 2005). HGT and selection pressure drive the evolution of virulence in enterococci that acquire resistance to antibiotics. There are two types of gene transformations. Acquired resistance is usually transposon or plasmid encoded. Intrinsic resistance is based in chromosomal genes and typically not transferable. Conjugative transfer of bacterial plasmids is the most efficient way of HGT (Grohmann et al. 2003). The transfer frequency of the investigated conjugative transposons is between 10^{-4} and 10^{-9} (Grohmann et al. 2003). Pembroke et al. (2002) provide an excellent review of conjugative transposons in *Enterobacteriaceae.* During transfer, conjugative transposons are integrated into DNA elements that excise themselves to form a covalently closed circular intermediate. This circular intermediate can either reintegrate in the same cell (intracellular transposition) or transfer by conjugation to a recipient and integrate into the recipient's genome (intercellular transposition) (Salyers et al. 1995). Many enterococci are

considered to be intrinsically resistant to several antibiotics. On the other hand, *E. faecalis* commonly acquires resistance through exchange of resistance genes carried on conjugative transposons, pheromone-responsive plasmids and other broad host-rage plasmids (Franz et al. 1999). Genes such as *vanA, vanB* and *vanC* more frequently confer resistance to vancomycin and glycopeptide analogues in enterococci (Kariyama et al. 2000) by altering cell wall synthesis. Another major gene, *vanD,* was reported by Perichon et al. in 1997. Laboratory transformation of these resistance genes from enterococci to other bacteria have been confirmed (Arthur and Courvalin 1993). Transposon *Tn1546,* carrying vancomycin resistance genes in *E. faecalis,* has transferred to a clinical isolate of *S. aureus* (Weigel et al. 2003). This work emphasized the importance of the horizontal gene transfer in the transformation of resistance genes in the environment.

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**).** It was reported that more than 30% of *E. faecalis* isolates collected from house flies contained a broad-host conjugative transposon *Tn916* (Macovei and Zurek 2006). This finding highlights the importance of resistant gene transformation by insects. Therefore, there is a need to investigate the antibiotic resistance prevalence, transfer, and role of economically important stored-product insects in terms of food safety, antibiotic resistance management, and integrated pest management.

Stored-product insects and stored products collected from a grain silo, feed mills, and swine farms are positive for enterococci, and the isolates collected from insects and products show varying levels of resistance to different antibiotics (Larson et al. 2008, Channaiah 2009, Channaiah et al. 2010a). Several isolates were also resistant to more than one antibiotic. Antibiotic resistant enterococci are potentially virulent and are able to transfer resistant genes to recipient enterococci (Channaiah 2009, Channaiah 2010a). Adults of a stored-product insect, *T. castaneum,* was able to successfully acquire and transfer antibiotic resistant enterococci to sterile poultry and cattle feed (Channaiah et al. 2010b). However, it is unclear whether insects are acquiring enterococci from contaminated stored products or whether insects are contributing to enterococcal contamination of products. Stored-product insects and stored products were collected from feed mills or swine farms where there is use of antibiotics and hence greater chance of enterococci to acquire antibiotic resistance. It is unclear whether enterococci,

especially antibiotic resistant enterococci can be found in stored-product insects or stored products collected from areas where antibiotics are not typically used. Therefore, studies were designed to collect stored-product insects from laboratory colonies where the insects have been in rearing at controlled conditions on standard diets for at least 17 years in the Department of Grain Science and Industry, Kansas State University, and assay the insects to determine prevalence, concentration, and antibiotic resistant profiles of enterococci. Specific objectives of the study were to first determine enterococci in adults and larvae of various species of stored-product insects and their rearing diets, and determine species of enterococci associated with the insect life stages, and characterize antibiotic resistant profiles of enterococcal isolates.

Table 1-1. Insect species collected from feed mills in 2003 and 2006 for screening of enterococci.

Insect species	Number of insects screened (% of total insects positive for enterococci)
Alphitobius diaperinus	4 (100.0)
Palorus ratzeburgi	1 (0.0)
Rhyzopertha dominica	11 (81.8)
Sitophilus zeamais	2 (50.0)
Stegobium paniceum	12 (0.0)
Tribolium castaneum	115 (24.3)
Tribolium confusum	116 (0.9)
Trogoderma variabile	20 (5.0)

Source: Larson et al. (2008).

Table 1-2. Number of stored-product insects collected from each mill site and the mean number of colony forming units (CFU) per insect on mEnterococcus agar.

Site[a]	*N*[b]	mEnterococcus agar	
		n (%)[c]	Mean CFU
1	57	5 (8.7)	1.7×10^{5}
2	22	0	0
3	65	1 (1.5)	4.8×10^{3}
4	45	1 (2.2)	1.2×10^{1}
5	53	45 (84.9)	6.5×10^{3}
6	56	1 (1.8)	8.4×10^{3}
7	10	3 (30)	4×10^{1}
8	8	6 (75)	4×10^{1}
9	9	7 (77.7)	3.8×10^{1}
10	47	35 (74.4)	3.7×10^{1}
11	12	8 (66.6)	3.7×10^{1}
12	8	8 (100)	4.1×10^{1}

[a]Samples in mills 1-6 were collected during March to November, 2003 (Larson et al. 2008); samples in mills 7-12 were collected during April to June, 2006 (Channaiah 2009).
[b]*N* = Total number of insects sampled from each mill.
[c]*n* = Number of positive samples (% of positive samples).

Table 1-3. Number of stored products collected from four mills sites and mean colony forming units (CFU) per gram of the product on mEnterococcus agar.

Site[a]	*N*[b]	mEnterococcus agar	
		n (%)[c]	mean CFU
1	4	4 (100)	9.3×10^2
2	15	3 (60)	2.7×10^2
3	12	8 (66.6)	3.1×10^2
4	7	5 (71.4)	3.1×10^2

[a]Sites 1 and 3 are swine farms in Salina, Kansas; site 2 is the Kansas State University pilot feed mill and site 4 is the swine farm at Kansas State University (Channaiah 2009).
[b]*N* = Total number of stored products sampled from each site.
[c]*n* = Number of positive samples (% of positive samples).

Table 1-4. Enterococcal isolates and species associated with stored-product insects and stored products collected from mills.

Source	No. samples	Total no. enterococci isolates	No. isolates identified (%)	No. species identified (%)			
				E. faecalis	*E. faecium*	*E. gallinarum*	*E. casseliflavus*
Stored-product insects	94	67	43 (64.1)	0	10 (23.2)	7 (16.2)	26 (60.4)
Stored products	28	125	45 (36)	2 (4.4)	8 (17.7)	2 (4.4)	33 (73.3)

Source: Channaiah (2009).

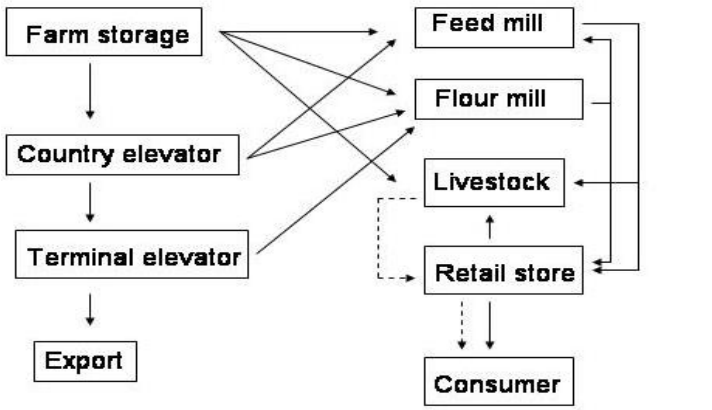

Figure 1-1. Grain and grain product flow patterns in the postharvest market system.

Source: Sinha and Wallace (1985).

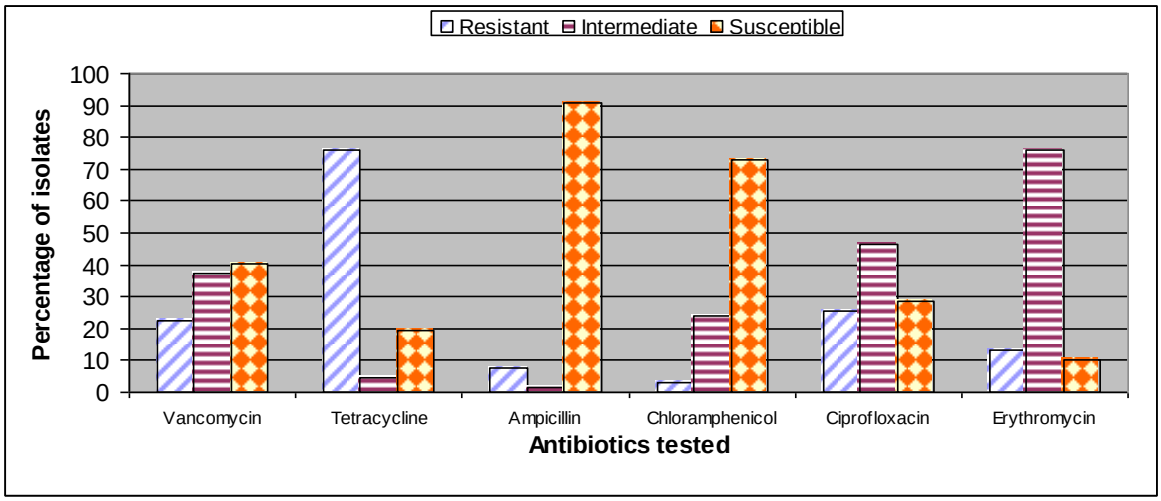

Figure 1-2. Resistance of enterococci isolated from stored-product insects to six antibiotics (*n*=67).

Source: Channaiah (2009).

The resistance, intermediate, and susceptible isolates were classified based on inhibition zone using diffusion disk assays; see Figure 1-5.

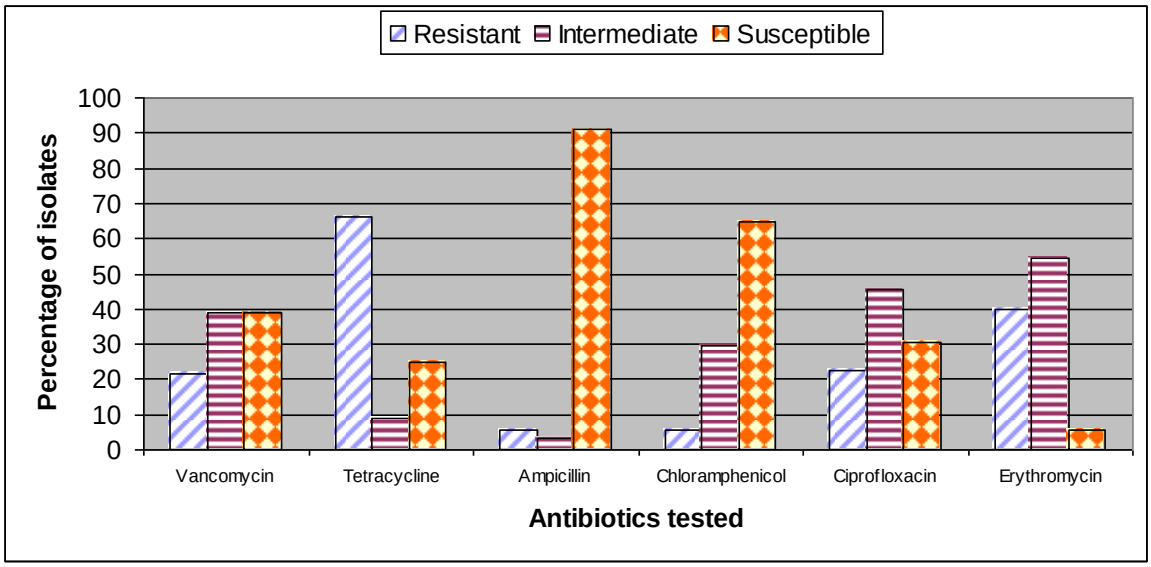

Figure 1-3. Resistance of enterococci isolated from stored products to six antibiotic (*n*=125).

Source: Channaiah (2009).

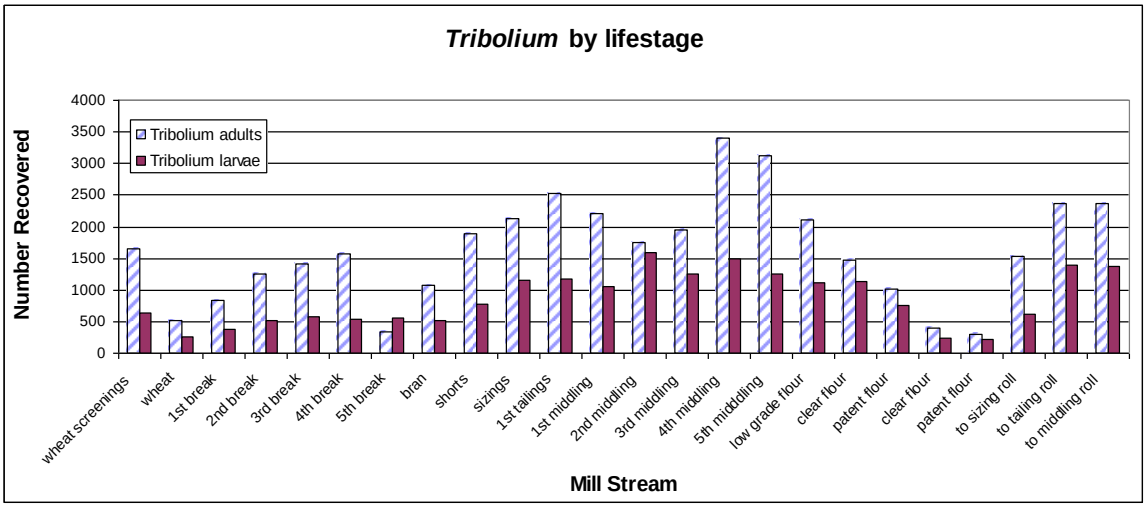

Figure 1-4. Number of Tribolium spp. found throughout the milling system.

Source: Good (1937)

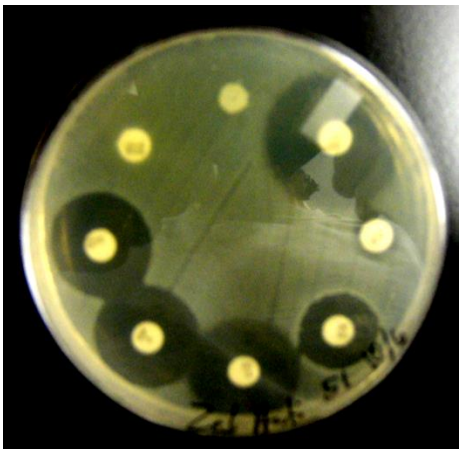

Figure 1-5. The zone of clearance of bacteria around each antibiotic-laced disk.

A zone of clearance (used to classify an isolate's resistance status) indicates the isolates susceptibility to that particular antibiotic.

Chapter 2 - Antibiotic Resistant Enterococci in Laboratory Stored-Product Insect Colonies

Abstract

Stored-product insects and stored products from feed mills and swine farms contain antibiotic and potentially virulent *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus casseliflavus*, *Enterococcus gallinarum*, and *Enterococcus hirae*. Stored-product insects can serve as potential vectors of these enterococci which possess antibiotic resistance genes that can be spread by horizontal transfer to more serious human pathogens. In the present study, the species and concentration of enterococci from adults and larvae of key stored-product insects and insect diets and their antibiotic resistance profile were characterized. Adults of five species out of the 15 stored-product insects tested positive for enterococci. The species testing positive were *Callosobruchus maculatus* (F.), *Sitophilus granarius* (L.), *Stegobium paniceum* (L.), *Lasioderma serricorne* (F.), and *Sitophilus zeamais* Motschulsky. Three enterococcal species (*E. casseliflavus*, *E. faecalis*, and *E. faecium*) were found in 53 to 97% of the 30 adults screened for each insect species, and the enterococcal concentrations ranged from 1.4×10^3 to 3.1×10^6 CFU/adult. About 10 to 100% of the mature larvae of the respective five insect species had the above-mentioned three enterococcal species with concentrations ranging from 0.3×10^1 to 1.4×10^5 CFU/larvae. Only three of the eight insect diets screened had the same three species of enterococci in addition to *E. gallinarum* and *E. hirae* at concentrations of 0.2×10^1 to 5.9×10^3 CFU/g. The greatest enterococcal concentration was found in *C. maculatus* adults but not in larvae or diet (cowpeas). In *C. maculatus* during a nine-day period after adult eclosion, the enterococcal concentrations increased exponentially from 0.6×10^1 to a maximum of 4.1×10^7 CFU/adult. Enterococci were detected in the fecal material of *C. maculatus* during a four-day period with a maximum concentration of 3.3×10^3 CFU/adult on the fourth day. A total of 298 enterococcal isolates from adults, larvae, and diets were represented by *E. faecalis* (51.7% of the total), *E. faecium* (19.1%), *E. casseliflavus* (18.8%), *E. gallinarum* (5.7%), and *E. hirae* (4.7%). Enterococci were phenotypically resistant to quinupristin (51.3% of the total), erythromycin (38.9%), tetracycline (30.1%), enrofloxacin (29.2%), doxycycline (11.5%), and tigecycline (2.7%). All isolates were susceptible to ampicillin and vancomycin.

2.1. Introduction

Enterococci are frequently found in the intestinal tracts and fecal matter of humans and animals. For many years it was used as the marker for fecal contamination in water systems (Aarestrup et al. 2001). It is the most abundant Gram-positive cocci found in human feces and can be present in numbers as high as 10^8 CFU/g (Jett et al. 1994). There are hundreds of different species of Enterococcus, but the 5 most commonly found and important species are *Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae, Enterococcus casseliflavus, Enterococcus gallinarum* (Debnam et al. 2005). They are generally considered to be harmless, but can cause nosocomial and opportunistic infections in immune-compromised individuals, especially from *E. fecalis* and *E. faecium*. In humans, enterococcal infection can cause endocarditis, urinary tract infections, and other symptoms (Murray 1990). The secondary infections can be potentially life threatening if the bacteria are resistant to antibiotics.

Enterococci represent a small portion of intestinal microflora typically around 1% of the total microflora, but they have significant impact on human health. A survey of infections in hospitals revealed that a portion of them are caused by enterococcal species. Approximately 12% of total nosocomial infections in the United States were caused by enterococci (Edmund et al. 1999). In blood stream infections 11% were from enterococci, and in surgical-site infections enterococci were responsible for 17% of the infections and were the single most common pathogen (Richards et al. 2000). Historically, more infections in humans caused from enterococci were by *E. faecalis* making up 80-90% of the infections. *E. faecium* accounts for 5-15% of infections (Bhat et al. 2011).

Enterococci are rugged bacteria and are capable of surviving under a wide range of harsh conditions that would normally destroy other bacteria. They can survive a wide range of temperatures, pH levels, and high salinity. They are also capable of resisting detergents that would otherwise remove or kill bacteria such as bile salts and sodium dodecyl sulfate. *E. faecalis* is capable of surviving exposure to 60°C for 30 minutes. It has a high salt tolerance (up to 6.5% NaCl) and a pH range of 3.5 to 11 (Flahaut et al. 1996). This high level of tolerance to stress and detergents is one of the reasons they are known for being a nosocomial pathogen. The cleaners used in hospitals are not always effective against enterococci and they are transmitted to a patient during surgery or in recovery.

In addition to potentially causing disease directly they are also a vector for antibiotic resistance. They have the capability to contain and transmit antibiotic resistance to other bacteria including pathogens such as *Salmonella* or *Staphylococcus*. Enterococci have about 25% of the genome that is mobile as transpons, which are insertion sequence elements that make up the genome and plasmids (Lam et al. 2012, Manson et al. 2010). These make up a high amount of the genome and are able to be moved and combined with other genetic elements making new genes possible. There are integrative conjugative elements (ICEs) that give enterococci the ability to transmit gene elements to other enterococci or species of bacteria. ICEs have been linked to resistance to tetracyclines, kanamycin, streptogramin, and glycopeptide antibiotics. The most important factor of enterococcal ICEs is the ability to transfer other plasmids and transposons and facilitate the transfer of large chromosomal DNA fragments (Ghosh and Zurek 2015). This transfer of genes is referred to as horizontal gene transfer and occurs readily between enterococci and other species.

Antibiotic resistance is characterized and tested by many different methods with various levels of effectiveness. The common methods include agar dilution screening, broth micro-dilution, and disk diffusion (Facklam et al. 2002). However, the most commonly and routinely used method is disk diffusion. There are different methods used to confirm when the occurrence of resistance to an antibiotic like vancomycin or ampicillin occurs. These antibiotics are of concern when infections occur in the hospital. The screening processes commonly used are best at detecting relatively high levels of resistance. The recommendation for screening for vancomycin resistance is to perform the agar screening test as it has higher accuracy than disk diffusion method. The problems with traditional methods for detecting antibiotic resistance is that they can be prone to user error in interpreting results and sometimes the sensitivity level is not high enough when resistance levels are moderate or intermediate.

Genetic methods are commonly used to test for antibiotic resistance and the known factors. It is faster than the conventional methods to detect antibiotic resistance and not prone to as many errors. However, it can miss resistance if the bacteria have developed a different mechanism than previously known, or if the type of resistance present is not included in the screening. Genetic probes and polymerase chain reaction (PCR) are used to screen for specific genes present (Facklam et al. 2002). These methods are capable of detecting low levels of

resistance and can be done in just a few hours instead of the days needed for conventional methods.

Enterococci have acquired antibiotic resistance to many of the major classes of antibiotics including β-lactam, aminoglycoside, glycopeptide, macrolide, lincosamide, streptogramin, chloramphenicol, tetracycline, oxazolidinone, evernimicin, and quinolones (Aminov et al. 2001, Bager et al. 2002, Kak and Chow 2002). These represent most of the pathways antibiotics use to inhibit bacterial growth. β-lactam antibiotics are the most common class of antibiotic and include penicillin and its derivatives, and ampicillin. This class of antibiotic inhibits cell wall synthesis in the bacteria which causes cell death. Enterococci have developed resistance to most of the β-lactams. When isolated from a hospital environment isolates were found to resist this class of antibiotic by producing an alternative penicillin-binding protein or specific amino acid substitutions that result in penicillin being ineffective (Fontana et al. 1994, Zorzi et al. 1996). Some *E. faecium* have been found to produce ß-lactamase, which they acquired from *Staphylococcus aureus* (Murray 1992). β-lactamase is an enzyme that can break the bond holding the antibiotic together and render it dysfunctional. The minimum inhibitory concentration (MIC) of ampicillin is very high in the enterococci when compared to other bacteria such as streptococci which ranges from 0.006- 0.25 µg/ml. In enterococci the MIC is 1.0-16 µg/ml.

Another important class of antibiotics enterococci have shown high resistance to are the aminoglycosides. This class includes streptomycin. They are normally used against Gram-negative bacteria and primarily act by interfering with protein synthesis by binding to the 16s rRNA of the 30S ribosomal subunit. Since these are normally for Gram-negative bacteria enterococci already have intrinsically present resistance. The mechanism that enterococci typically use to resist the aminoglycosides involves a single mutation of a protein within that ribosome which is the target of the activity (Chow 2000). As many different species of bacteria are now resistant to the very commonly used streptomycin there was a switch to using gentamicin, which uses a slightly different mechanism, but widespread resistance to it is now reported. The mechanism of resistance is different in that the enterococci involved develop a bifunctional enzyme 6′-aminoglycoside acetyltransferase 2″-aminoglycoside phosphotransferase [AAC(6′)-Ie-APH(2″)-Ia] (Ferretti et al. 1986). Widespread resistance is now found in enterococci to this antibiotic class though most strongly observed in *E. faecium*.

Glycopeptide antibiotics include vancomycin and are used as the last effective antibiotics against some strongly resistance bacterial infections such as methicillin-resistant *Staphlyococcus aureus* (MRSA). It is usually only prescribed as a last resort to prevent widespread vancomycin resistant bacteria. Glycopeptides function similarly to the β-lactams in that they inhibit cell wall biosynthesis, but instead of reacting on the cell wall synthesis enzymes they react on the precursors to them, peptidoglycan pentapeptide precursors, preventing the precursors from cross-linking leading to a loss in cell wall integrity and rupture (Arthur and Courvalin 1993). Resistance to the glycopeptides involves modifying the target switch in the bacteria replacing it with a different amino acid thereby removing the susceptible target (Bugg et al. 1991). Multiple different genetic mechanisms can be expressed by the enterococci either all of the time or when a glycopeptide is added and the trait expresses itself (Billot-Klein et al. 1994). There are several known phenotypes of vancomycin resistance in enterococcus, *VanA*, *VanB*, *VanD* and many others which produce different amino acid combinations. Some of the phenotypes are more effective than others and are more common.

The prevalence of enterococci resistant to the different classes of antibiotics has increased dramatically over the years and is attributed to increased and widespread use of antibiotics. Vancomycin resistant enterococci (VRE) were first found in the United States in 1986. In 1989 the percentage of vancomycin resistant nosocomial infections reported in hospitals was 0.3% of total infections. By 1999, just a decade later, the rate was up to 24.7% of reported infections, despite efforts to prevent the spread of vancomycin resistance in hospitals (NNIS 1997, 2000). *E. faecium* is commonly exhibits resistance to vancomycin. Ampicillin resistance has been reported in hospital settings. There has been in increasing incidence of isolates identified that are resistant to both vancomycin and ampicillin (Huycke 1998).

In Europe agricultural reservoirs are primary sources of antibiotic resistant enterococci, especially in sewage treatment plants and food sources such as poultry and pork. It has been suggested that agricultural settings and food products may be the source for community wide infections of vancomycin resistance (Bates et al. 1994, McDonald et al. 1997). One of the most notable sources of VRE is farm animals exposed to antimicrobial drugs and human and animal foods (Thal et al. 1996). VRE has been isolated from dog food sold in the United States (Dunne et al. 1996). There has been evidence from Europe which showed VRE in household pets such as dogs and cats, and this resistance can be transmitted to humans (van Belkum et al. 1996). This

could be a means of community transmission of VRE. It is even possible for VRE to be transferred from person to person through household contact, which includes food preparation.

Enterococci are frequently isolated in poultry environments with high levels of antibiotic resistance (Hayes et al. 2004, Asadpour 2012). Poultry farms are susceptible to high levels of antibiotic resistance due to the use of therapeutic levels of antibiotics in the feed. In a survey of 82 poultry farms in the east coast of the United States, isolates were recovered from the poultry litter used for the chickens. Among the 541 isolates recovered 53% were *E. faecalis* and 31% were *E. faecium.* There were high levels of resistance including multidrug resistance observed in many different isolates of multiple enterococci species. In *E. faecalis* isolates there was high levels of resistance to lincosamide, macrolide, and tetracycline. Isolates of *E. faecium* were resistant to fluoroquinolone and penicillins. Nearly 63% of the *E. faecium* isolates were resistant to streptogramin quinupristin-dalfopristin.

Stored-product insects are cosmopolitan in distribution and are capable of infesting a wide variety of cereal products from raw grains to processed flour and feed (Hagstrum and Subramanyam 2006). Numerous species are associated with stored grain, grain stores, warehouses, grain-processing facilities, and retail environments. When uncontrolled the insects can cause qualitative and quantitative losses to the multi-billion dollar grain and food industries.

Stored-product insects have been reported to carry potentially pathogenic bacteria. The granary weevil (*Sitophilus granarius* (L.)) was found to harbor *Escherichia intermedia, Proteus rettgeri, Bacillus subtilis, Serratia marascens, Streptococcus spp., and Micrococcus* spp (Harein and De Las Casas 1968). The lesser mealworm, *Alphitobius diaperinus* (Panzer), found in poultry brooder houses carried *Salmonella, E. coli, Micrococcus, Streptococcus and B. subtilis (*Harein et al. 1970, De Las Casas et al. 1972, Asaniyan and Agbede 2007). The hairy fungus beetle, *Typhaea stercorea* (L.), and foreign grain beetle, *Ahasverus advena* (Waltl.) found in chicken litter in broiler houses were a reservoir for *Salmonella indiana* and the pathogen was transmitted between two different broiler flocks. *A. diaperinus* also carried and transmitted the same pathogen (McAllister et al. 1994). The newly hatched chicks tested positive for *Salmonella typhimurium* after eating infected adults of *A. diaperinus.* Adults of *S. granarius* from both laboratory raised colonies and grain storage facilities were identified as potential reservoirs for many pathogens including *E. intermedia, P. rettgeri, P. vulgaris, B. subtilis, S. marcescens,*

Streptococcus spp., *Micrococcus* spp., and members of the *Klebsiella-Aerobacter* group (Harein and De las Casas 1968).

In addition to harboring the pathogens, the insects can pass these bacteria onto the grain. *S. granarius* adults were shown to transfer *Salmonela montevideo* to uncontaminated wheat in a laboratory study (Husted et al. 1969). Yezerski et al. (2005) demonstrated how contaminated diet can infect insects with pathogens. Four different species of stored product insects, the cigarette beetle, *Lasioderma serricorne* (L.); drug store beetle, *Stegobium paniceum* (L.); red flour beetle, *Tribolium castaneum* (Herbst), and confused flour beetle, *Tribolium confusum* Jacquelin du Val, were reared on flour contaminated with *Enterococcus* spp. Only *L. serricorne* and *S. paniceum* later tested positive for *Enterococcus* spp.

Stored-product insects and stored products collected from a grain silo, feed mills, and swine farms were positive for enterococci, and the isolates collected from insects and products show varying levels of resistance to different antiniotics (Larson et al. 2008, Channaiah 2009, Channaiah et al. 2010a). Several isolates were also resistant to more than one antibiotic. Antibiotic resistant enterococci are potentially virulent and are able to transfer resistant genes to recipient enterococci (Channaiah 2009, Channaiah 2010a). Adults of a stored-product insect, *T. castaneum*, was able to successfully acquire and transfer antibiotic resistant enterococci to sterile poultry and cattle feed (Channaiah et al. 2010b). However, it is unclear whether insects are acquiring enterococci from contaminated stored products or whether insects are contributing to enterococcal contamination of products. In Larson et al. (2008) and Channaiah et al. (2010a,b) studies, stored-product insects and stored products were collected from feed mills or swine farms where antibiotics are regularly used, and hence there was a greater chance of enterococci to acquire antibiotic resistance. It is unclear whether enterococci, especially antibiotic resistant enterococci can be found in stored-product insects or stored products collected from areas where antibiotics are not typically used. Therefore, studies were designed to collect stored-product insects from laboratory colonies where the insects have been in rearing at controlled conditions on standard diets for at least 17 years in the Department of Grain Science and Industry, Kansas State University, and assay the insects to determine prevalence, concentration, and antibiotic resistant profiles of enterococci. Specific objectives of the study were to first determine enterococci in adults and larvae of various species of stored-product insects and their rearing

diets, and determine species of enterococci associated with the insect life stages, and characterize antibiotic resistant profiles of enterococcal isolates.

2.2. Materials and Methods

2.2.1. Enterococci from insect diets

A total of 30 samples of each the following standard insect rearing diets were taken from frozen stocks used in culturing insects. Organic whole wheat flour (Heartland Mills, Marienthal, KS), organic corn (Heartland Mills), organic hard red winter wheat (Heartland Mills), organic rolled oats (Heartland Mills), poultry pellets (Kansas State Feed Mill), Meow- Mix cat food (Del Monte Foods, San Francisco, CA), poultry mash diet, and cowpeas (from a local grocery store) were used for rearing insects. Poultry mash diet was mixed in the laboratory consisting of 68% Chick Starter Grower (Nutrena®, Nature wise no antibiotic or hormones, Minneapolis, MN), 13% glycerin, 14% honey, and 5% water. One gram samples were collected aseptically using a sterile scoop and placed in a 15 ml sterile plastic centrifuge tubes. A 9 ml Phosphate Buffered Saline (PBS) was added to each sample and homogenized in a blender for 60 seconds. The 100 µl grain solution was spread plated onto a mEnterococcus agar plate (mENT; Difco laboratories, Detroit, MI), incubated at 37°C for 48 hours then colonies were counted. If necessary, dilutions were performed by removing 100 µl homogenate and adding to 900 µl PBS until a readable sample was possible.

2.2.2. Insect Culturing

A total of 250 stored-product insects were collected from colonies of stored-product insects in rearing in the department of Grain Science and Industry, Kansas State University, Manhattan, KS. These cultures have been in rearing since 1999. A total of 30 adults each of the following species were tested for prevalence, concentration, and antibiotic resistance of enterococcal species: *L. serricorne, S. paniceum*, the lesser grain borer, *Rhyzopertha dominica* (F.), cowpea weevil, *Callosobruchus maculatus* (L.), *S. granarius;* rice weevil, *Sitophilus oryzae* (L.), maize weevil, *Sitophilus zeamais* (Motschulsky)*;* warehouse beetle, *Trogoderma variable* Ballion*;* rusty grain beetle, *Cryptolestes ferrugineus* (Stephens), larger black flour beetle, *Cynaeus angustus* (LeConte), *T. castaneum, T. confusum*, Angoumois grain moth, *Sitotroga cerealella* (Olivier), and Indian meal moth, *Plodia interpunctella* (Hübner). All insects were reared on standard diets (see below) in 0.95-L glass jars at 28°C and 65% RH. Insects (unsexed adults of mixed ages) were collected from different locations of each jar using forceps and

placed in a glass vial. Ethyl alcohol (95% pure) was added to cover the insects in the vial before transporting for further analysis. Insects were immediately processed for microbial analysis.

2.2.3. Isolation, enumeration, and identification of enterococci from adults

Individual insects from each species were surface sterilized in 10% sodium hypochlorite and then 70% ethanol and finally rinsed in sterile distilled water for one minute in each of the solutions (Zurek et al. 2000). Sterile insects were homogenized in 200 µl phosphate buffered saline (PBS) (ph 7.2; MP Biomedicals, Solon, OH), with a pellet pestle in a 1.5-ml centrifuge tube and spread plated on mEnterococcus agar (mENT; Difco laboratories, Detroit, MI). Plates were placed in an incubator at 37°C for 48 hours. After incubation the CFU's per insect were recorded to determine the number of enterococci per insect. Well isolated presumptive enterococcal colonies from each mENT plate were streaked for isolation onto tripticase soy broth agar (TSBA; Difco Laboratories) and incubated at 37°C for 48 hours. TSBA plates were stored at 4°C until further analysis.

Species identification of isolates was conducted using multiplex PCR (Ng et al. 2001). Five species of enterococci primers were used, including *E. faecalis, E. faecium, E. casseliflavus, E. gallinarium,* and *E. hirae.* A total of 91% of isolates were identified using multiplex PCR (Table 2-1). The remaining 9% were identified by amplifying the soda (superoxide dismutase) gene by sequencing then comparing to the National Center for Biotechnology Information Genbank database. The following was used as confirmed controls: *E. faecium* ATCC19634, *E. faecalis* ATCC 19434, *E. gallinorium* ATCC 49573, *E. hirae* ATCC 8043, *E. casseliflavus* ATCC 49604. Multiplex PCR was conducted in the following manner: 50µl of 8% Chelix resin solution was mixed in a sterile 1.5-ml centrifuge tube, with 3 or 4 enterococcal colonies using a sterile toothpick. The mixture was heated in a heating block at 100°C for 10 minutes. Tubes were submerged in ice for 1 minute to cool. Tubes were centrifuged at 7200 rpm for 1 minute. The supernatant was combined in a PCR reaction tube with 10 µl PCR master mix, 8 µl DNA-free water, 0.5 µl primer and 1 µl extracted DNA. Tubes were placed in a thermocycler and allowed to go through the preprogramed cycle for Enterococcal DNA amplification. Gel electrophoresis was performed using an Agilent Tape station machine to identify bands against the control strains. All isolates were subjected to further testing for antibiotic resistance and virulence determinants.

2.2.4. Enterococcal growth in adults of *Callosbruchus maculatus*

Due to the relatively large numbers of enterococci associated with *C. maculatus* sampled when compared to other insectd testing positive for enterococci, adults over a 9-day period after eclosion. Newly emerged (less than 24 hours old) *C. maculatus* adults were collected and 20 adults were placed on 10 g of cowpeas in a small plastic sample cup with a mesh screen lid for ventilation. A total of 20 sample cups were prepared. A total of 15 insects were collected each day. Adults collected were tested for enterococci in the same manner as for the other adult stored-product insects on mENT agar. Colonies were isolated and analyzed using Multiplex PCR (Table 2-1).

2.2.5. Enterococci from *C. maculatus* fecal material

Pint sized mason jars and lids were autoclaved for 15 minutes at 121°C. A total of 10 cowpeas and 10 newly emerged *C. maculatus* were added to the jars. Every day 4 jars were randomly selected and any feces present was collected from the jar with a sterile spatula. Feces was placed in a 1.5-ml centrifuge tube and weighed, 100 µl PBS was added and homogenized with a pellet pestle. On a mENT plate, 100 µl was spread plated and incubated at 36°C for 48 hours. Colonies were counted and unique colonies from each plate were isolated on TSBA for further DNA analysis. DNA analysis was done with multiplex PCR as previously described. Calculations were conducted to convert the experimental results into CFU/g to match how diet samples were collected and read.

2.2.6. Enterococci from larvae

About 30 g of cowpeas were placed in a 0.45-L mason jar. In each jar, 200 unsexed adults of various ages were added. Jars were fitted with wire-mesh screened lids. Adults were sifted from cowpeas 3 days later. For *S. granarius and S. zaemais*, 30 grams clean organic whole wheat kernels were added to separate 0.45-L mason jars into which 200 unsexed adults of a species were added. After 3 days adults were sifted from wheat. All jars were placed in a growth chamber at 28°C and 65% RH for 21 days to allow the larva developing with seeds to mature to 4^{th} instars. Larvae were extracted from the grain by using a sharp scalpel to cut apart either the wheat kernel or to remove the skin of the cowpeas under a stereomicroscope. Head capsule width was measured under a stereomicroscope to identify the instar.

For the external feeders such as *L. serricorne* and *S. paniceum* larvae were collected by placing 200 adults in 30 g of flour pre-sifted with a US Standard No. 60 sieve (250 µm

openings). After 3 days adults were removed and flour was sifted through a No. 60 sieve to separate the eggs. Eggs were added to 2 g of flour in a plastic vial and allowed to mature for 21 days until large larva were visible. Exactly 30 larvae were sifted out of the flour with a No. 40 sieve (420 µm openings) and placed in glass vial and taken to laboratory for further analysis. Insect sterilization, enterococcal extraction, enumeration, and isolation were conducted in the same manner as for adults.

2.2.7. Enterococci from eggs

About 20 g of flour was sifted through a No. 60 sieve and autoclaved in 0.45-L mason jar. Clumped flour was resifted through an autoclaved No.60 sieve and returned to the jar, and 200 adults of *L. serricorne* or *S. paniceum* were added to the jar. An autoclaved wire-screen and lid were used to close the jars. After 3 days the adult insects were separated from the eggs by sifting through a No. 25 sieve (707 µm openings), and eggs were collected on the No. 60 sieve that had been autoclaved (sterilized). A total of 50 eggs were added to a 1.5-ml centrifuge tube. To the tube 100 µl PBS was added and a pellet pestle was used to homogenize the mixture. On mENT agar 100 µl egg PBS solution was spread-plated. The plate was incubated at 36°C for 48 hours. Colonies were counted and unique colonies from each plate were isolated on TSBA for further DNA analysis. DNA analysis was conducted as described above with multiplex PCR.

2.2.8. Phenotypic screening of enterococci for antibiotic resistance

Enterococci isolates were screened for antibiotic sensitivity using the disk diffusion method on Muller-Hinton Agar (Difco Laboratories, Franklin Lakes, NJ) for nine antibiotics. Antibiotics were chosen based on prevalence of use and past studies conducted. The following antibiotics were used in screening tigemycin (TGC, 15 µg), vancomycin (VA, 30 µg), tetracycline (TE, 30 µg), erythromycin (E, 15 µg), doxycycline (D, 30 µg), ampicillin (AM, 10 µg), gentamicin (GM, 10µg), enrofloxacin (ENO, 5 µg), and quinupristin (SYN, 15 µg). A total of 119 positively identified enterococcal colonies isolated from the stored-product insects were tested. The class these antibiotics belong to is given in Table 2-2. The test was performed using the National Committee for Clinical Laboratory Standards (NCCLS) method for the Kirby-Bauer disk diffusion test on Muller-HintonII Agar (Difco Laboratories).

2.2.9. Minimum inhibitory concentration screening to estimate resistance levels

Colonies identified as resistant to either vancomycin or ampicillin through disk diffusion were further tested for the minimum inhibitory concentration (MIC) of each antibiotic to

estimate the level of resistance. The colonies were taken out of -80°C storage and grown fresh on TSBA for 24 hours. Multiple (1-3) colonies from each plate were suspended in 200 µl PBS in a 1.5-ml centrifuge tube to a McFarland standard of 0.5. Eight different concentrations of antibiotic broth were prepared to the following 2-fold dilution levels: 1 µl/ml, 2 µl/ml, 4 µl/ml, 8 µl/ml, 16 µl/ml, 32 µl/ml, 64 µl/ml, and 128 µl/ml. The proportional level of antibiotic was added to Mueller-Hinton broth to make 1100 µl broth antibiotic solution in a 1.5-ml centrifuge tube. About 200 µl broth antibiotic solution was added to wells in a 96 well plate and 1% PBS enterococci suspension. Both antibiotics were tested with all isolates. The plate was incubated at 36°C for 24 and 48 hours. A 96 well-plate reader set at 600 nm was used to determine growth. Readings were compared to inoculated broth with culture and no antibiotic and broth with no inoculum. The minimum dose to be resistant for ampicillin was 16 µl and for vancomycin it was 32 µl.

2.2.10. Statistical analysis

Data were analyzed using the Statistical Analysis System (SAS Institute 1988). The enterococcal prevalence was expressed as a percentage based on total insects that were positive for enterococci. The enterococcal load was expressed based on a gram of diet, an adult or larva. Differences among mean enterococcal loads were determine by subjecting data to one-way analysis of variance (ANOVA) after transforming counts to log (x) or log (x + 1) scale to normalize herteroscedastic treatment variances. Mean separations were done using the Ryan-Einot-Gabriel-Welsch multiple comparison test (REGWQ) at $\alpha = 0.05$. SigmaPlot®12.5 (Systat Software, San Jose, CA) was used for graphing. The exponential growth of enterococci in *C. maculatus* gut over a 9-day period was fitted to an exponential curve using SigmaPlot®12.5.

2.3. Results

2.3.1. Enterococci from insect diets

Only poultry pellets, flour, and cowpeas were positive for enterococci among all the diets screened. All 30 samples of poultry pellets were positive. DNA analysis revealed that poultry pellets carried four species of enterococcus, *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. hirae.* Only 3 of the 30 flour samples had *E. casseliflavus* and 2 of the 30 cowpea samples had *E. casseliflavus* (Table 2-3). There were large differences in CFU/g of poultry pellets. For example, 6 of the 30 samples had a mean ± SE CFU/g that ranged from 7×10^3 to 56×10^3, with a mean ± SE of $5.9\times10^3 \pm 2.7\times10^3$. The remaining 24 samples had concentrations that ranged from 2 to 69

CFU/g. In flour and cowpeas, enterococcal concentrations ranged from 1-7 CFU/g, with mean ± SE concentrations of $0.2\times10^1 \pm 0.3\times10^1$ and $0.4\times10^1 \pm 0.3\times10^1$, respectively. A total of 71 isolates were obtained from poultry pellets, of which 41% were *E. faecium*, 39% were *E. faecalis*, 24% were *E. gallinarum*, and 20% were *E. hirae*. Some isolates from poultry pellets had anywhere from 2 to 4 different enterococcal species. The 3 isolates from flour and the 2 from cowpeas were all *E. casseliflavus*. The remaining 6 diets were negative for enterococci.

Enterococci from flour and cowpea samples were susceptible to all nine antibiotics. Only enterococci associated with poultry pellets had any antibiotic resistance (Figure 2-1). Out of the 71 isolates from poultry pellets 9 were resistant to antibiotics tested. About 56% of the 9 isolates were each resistant to doxycycline, enrofloxacin, and tetracycline. Gentamicin resistance was found in 18% of the isolates, followed byquinupristin (18%) and erythromycin (11%). No resistance was seen to ampicillin, tigecycline, and vancomycin. Two isolates of *E. faecalis* from poultry pellets were resistant to two antibiotics, whereas one isolate was resistant to three antibiotics (Table 2-4). Two isolates of *E. faecium* were resistant to three antibiotics, and an isolate each of *E. gallinarum* was resistant to one and two antibiotics, respectively.

2.3.2. Enterococci from adults of insect species

Five out of the 16 species tested were positive for enteroccci on mENT plates (Table 2-5). The five species were *L. serricorne, C. maculatus, S. paniceum, S. granarius, S. zeamais.* The five species feed on four different types of diets. *L. serricorne* and *S. paniceum* were cultured on poultry pellets, while *C. maculatus*, *S. granarius*, and *S. zeamais* were reared on cowpeas, whole maize kernels, whole wheat kernels, respectively. Enterococci were detected in poultry pellets and cowpeas, and not in whole corn and wheat. *L. serricorne* and *S. paniceum* had *E. faecalis*, although the diet had three additional enterococci. The cowpeas had *E. casseliflavus* exclusively, but the gut of *C. mac*ulatus had *E. faecalis* and *E. faecium*, with the former being more prevalent. *S. granarius* had about equal prevalence of *E. casseliflavus* and *E. faecalis*, whereas in *S. zeamais E. casseliflavus* was more prevalent, followed by *E. faecalis* and *E. faecium*. The greatest concentration of enterococci was found in *C. maculatus* followed by *L. serricorne, S. granarius, S. paniceum*, and *S. zeamais* (Table 2-5). Flour was positive with 2 isolates of *E. casseliflavus* but both *Tribolium* spp. were negative for any enterococci. All other insect species tested from different diets were all negative for enterococci.

In the adult insects not every isolate collected from the plates was identifiable through DNA analysis. It was assumed that these isolates are either uncommon enterococci or another species that was able to grow on the selective media. The majority of these unidentifiable colonies were collected from *L. serricorne* with 5 unidentified colonies out of the 16 collected.

Antibiotic resistance is enterococcal isolates to the 9 antibiotics is shown in Table 2-6. About 93% of the isolates from *C. maculatus* were resistsnt to quinupristin, followed by gentamycin, enroflaxacin, and erythromycin. About 39% of isolates from *S. granarius* were each resistant to erythromycin and enroflaxacin followed by quinupristin. About 80 and 70% of isolates from *S. paniceum* were resistant to tertracycline and erythromycin, respectively. Isolates from both *L. serricorne* and *S. zeamais* were each resistant to 6 antibiotics. A majority of *L. serricorne* isolates (88%) were resistant to tetracycline with 12% being resistant to quinupristin. In the case of *S. zeamais*, 44% of isolates were each resistant to quinupristin and enroflaxacin with 16% being resistant to gentamycin.

The antibitoc resistancde profiles of individual species of enterococci by species is shown in Table 2-7. Across the species of insects and enterococcal species, isolates were resistant to at least 4 antibiotics, but many isolates were resistant to 1 to 2 antibitoics.

2.3.3. *C. maculatus* enterococci growth curve development

Enterococcal concentration soon after eclosion and over a nine day period is shown in Table 2-8. The life expectancy of this insect is 10-14 days. By day nine the majority of insects were dead and only three were found alive. All insects were dead on day 10. Due to insect death sample size changed over time. However, the exponential model satisfactorily described enterococcal growth in the midgut of *C. maculatus* adults (Figure 2-2). .

2.3.4. *C. maculatus* fecal isolation

The samples taken from the 10 adult *C. maculatus* were miniscule in weight. The weight did not change much over the 4-day sample period. The culture count was converted to CFU/gram to better fit the way the rest of the data was presented (Table 2-9). When compared on a gram scale the CFU's are on track with what was seen from mature *C. maculatus.* In adults the average CFU/insect was 3×10^6 and in the feces the concentration was 3×10^3.

2.3.5. Enterococci from larvae

Larvae of the five species were positive for enterococci, and percent of 30 larvae of each of the five species that were positive for enterococci ranged from 10% (*L. serricorne,* C. maculatus) to 100% (*S. zeamais*) (Table 2-10). The concentration of enterococci was lower than it was in the adults only for *C. maculatus* and *S. zeamais*. The same species found in adults were found in larvae, with one exception. Larvae of *C. maculatus* had *E. faecalis* but no *E. faecium*, which was found in the adults. Enterococcal loads were significantly greater in *S. paniceum* and lowest in *C. maculatus*.

2.3.6. Enterococcus from eggs of the five insect species

This test yielded conflicting results. There were enterococci found in the eggs tested but it was difficult to separate all of the flour from eggs as the sterilization process used on the flour caused it to clump. The eggs could not be readily sterilized after separating from the adults with sodium hypochlorite or ethanol because the integrity of the eggs was destroyed. Due to the inconsistency and the possible contamination from the adults the results were discarded.

2.3.7. Antibiotic resistance profiles of enterococcal isolates from insect larvae

Isolates from larvae were resistant to 2 to 5 antibiotics tested (Figure 2-3). Isolates from *C. maculatus* and *L. serricorne* were 100% resistant to doxycycline and tetracycline. All isolates from *S. paniceum* were resistant to erythromycin and tetracycline. *S. granarius* was resistant to erythromycin and quinupristin, whereas less than 40% of isolates from *S. zeamais* were resistant to enrofoxacin, followed by erythromycin. Less than 10% of isolates were also resistant to doxycycline, quinupristin, and tetracycline.

Antibiotic resistance in enterococcal species for each insect species is shown in Table 2-11. About 67% of the isolates of *E. faecalis* from *C. maculatus* were resistant to two antibiotics whereas 33% were resistant to one antibiotic. About 33% of *E. faecalis* isolates from *S. granarius* each were resistant to 1 and 2 antibiotics, respectively. All 3 *E. casseliflavus* isolates were resistant to 1 antibiotic. About 10, 60, and 30% of *S. panecium* isolates of *E. faecalis* were resistant to 2, 3, and 4 antibiotics. About 80% of *E. faecalis* isolates from *L. serricorne* were resistant to 4 antibiotics whereas 20% of isolates were resistant to 2 antibiotics. About 67% of isolates of *E. faecalis* from *S. zeamais* were resistant to one antibiotic while 33% were resistant to 4 antibiotics. All isolates of *E. casseliflavus* from *S. zeamais* were resistant to one antibiotic.

Only 67% of E. faecium isolates from *S. zeamais* were resistant to one antibiotic whereas 33% were not resiatant to any of the antibiotics tested.

2.3.8. Minimum inhibitory concentration

This test was done to confirm true antibiotic resistance to ampicillin and vancomycin. In the disk diffusion assay test 5 colonies from *S. zeamais* were recorded as being resistant to either vancomycin or ampicillin. All 5 colonies were tested for the minimum dose that inhibited growth of the specific antibiotic (Andrews 2001). For all of the colonies the only growth observed was seen at the lowest concentration of antibiotic below the threshold for resistance. These were classified as susceptible to vancomycin and ampicillin. Due to negative data these data are not presented.

2.4. Discussion

In this study the research methods endeavored to answer the question if the enterococci present in the insects came from the insects or if the enterococci present in diet infected the insects. The study was designed to determine if the enterococci were present in the diet, the insects themselves in both the adult and larval stages, the species of enterococci present in the insects, and finally characterize antibiotic resistance of the isolates from insects.

All diet samples tested had very low enterococci levels or no enterococci with the exception of poultry pellets which had the highest enterococcal loads. The poultry pellets are obtained from the Kansas State Feed Technology Innovation Center. They are composed of many different ingredients including bone meal and various grains. The product is mixed in a ribbon blender with steam and moved to a pressing forming to shape it into pellets. During pressing the temperature is elevated in the product. The data in this study indicates that the resulting pellets were positive for enterococci. Despite the fact that poultry pellets contained enterococci they were utilized to culture the *S. paniceum* and *L. serricone* because these insects are secondary feeders and prefer to feed on cracked grains and they prefer higher protein found in the pellets (Mason et al. 2010). They thrive on a diet of poultry pellets so it is what is continuously utilized to culture them, and could explain enterococci in these insect species.

The grains used in the insect diets are whole and did not undergo any processing steps, except for rolled oats. The poultry mash diet's base is poultry starter crumbles. Three samples of the poultry starter crumbles were analyzed and all three contained enterococci. No insects are cultured on the crumbles alone. When mixed with honey, glycerin, and water for use in culturing

P. interpunctella diet was negative for enterococci. Honey is a natural preservative and has antimicrobial properties stemming from many factors including the hydrogen peroxide content, osmolarity and acidity (Morre et al. 2001). It is likely that the honey killed the enterococci that were present. *P. interpunctella* reared on poultry mash diet had no enterococci in their gut.

Poultry pellets had four different species of enterococcus present including *E. faecium* and *E. faecalis,* the two species of enterococcus that are nosocomial pathogens. While the sample size was small, many colonies isolated were resistant to up to three different types of antibiotics.

In other studies, *E. hirae* isolates were found in feed samples, but when isolated in flies that fed on the diet they are negative for *E. hirae* (Ali et al. 2014). The same was found in this study. *E. hirae* was found in the poultry pellet diet but when isolated in the insects that are cultured on that diet, *S. paniceum* and *L. serricorne,* the only isolate found was *E. faecalis.* The reason for this is unknown, but it is thought that something in the insect's gastro intestinal tract eliminates the generally harmless *E. hirae* but allows *E. faecalis* to survive. In the case of *C. maculatus* adults, the *E. faecalis* were capable of multiplying exponentially suggesting that the gut environment is conducive for the growth.

Enterococcal species were present in five out of the total fifteen stored-product insect species sampled. Some interesting results were observed when compared to the previous data. In Channaiah et al. (2010a) study the insects had a maximum enterococcal concentration of up to 2×10^2 CFU/insect. In their study, insects were collected in the field from grain silos. In our study there were as many as 1.0×10^6 CFU/insect observed in *C. maculatus* adults. The reason for this difference was that the insects used in this study lived in the cramped laboratory colony environment with a population density higher than what would normally be seen in a grain silo environment. In the overcrowded jar environment bacteria would spread between insects easier and the longer life spans of laboratory reared insects would mean a longer incubation period for the long lived species like *S. zeamais.* In a study conducted by Harein and de las Casas (1968) enterococcal species were not observed in *S. granarius* adults. In the current research conducted there was *E. faecalis* and *E. casseliflavus* found in 66% of adults.

The Enterococci Species *E. faecalis* and *E. faecium* are the most commonly found isolates in human Enterococci infections, although other species are found to cause infection including *E. casseliflavus* (Ghosh 2015). All three of these isolates were found in high numbers in the insects.

The *S. paniceum* and *L. serricorne* both had larger CFU concentrations in the larval stage compared to that in the adult stage. The larval stages of five species were also examined and the larval stages were positive for enterococcus. All *S. zeamais* larvae were positive for enterococcus and the adults had a lower percent positive samples. The pupal stage of these species was not sampled. Generally, in the pupae stages the bacterial load decreases because the old digestive tract is shed before emerging as adults. This is what likely occurred in the beetles. The enterococcus could grow in the actively feeding stages of the larvae then when they matured into adults the enterococcus load decreased and could not recover in the non-feeding short lived adult stage.

The larvae of *S. zeamais, C. maculatus* and *S. granarius* are internal feeders, and the diets were devoid of any enterococci. The larva develops directly inside grain kernels which should be a fairly sterile environment. In all probability, the larva may be obtaining the enterococci from their Parental adults tranovarially.

Tests to isolate enterococci from eggs was not possible or this could have shed some light on whether or not these pathogens are infecting the eggs from adults.

The *C. maculatus* bacterial growth curve is unique. None of the other insects tested in the diet had such high numbers of enterococci present. The average load per insect was 3.1×10^6 CFU/insect. The load started out relatively small and as the insects aged to their natural life expectancy of approximately 10 days the bacterial load increased exponentially, indicating that the insect gut is a suitable environment to support enterococcal growth. Enterococci were also found in *C. maculatus* feces, suggesting that the adults are capable of contaminating healthy cowpeas with enterococci. The next highest CFU/insect was *S. granarius* with $1.8\times1\ 0^4$ CFU/insect. What makes this species able to incubate enterococci in the gut is unknown.

It has been shown with flies in poultry production operations that they are capable of transferring their antibiotic resistant enterococci into their litter and the surrounding diet (Graham et al. 2009). Adults of *T. castaneum* were able to obtain enterococci when fed on infected diet and transfer enterococci to sterile diet (Channaiah et al. 2010b). These findings mean that insects could infect the grain they grow in with a potential pathogen. The findings of this research suggest that antibiotic resistance in enterococci is naturally occurring from an isolated source. The significance is that if the enterococci remained in the grain through

processing they could possibly infect susceptible people making them ill with antibiotic resistant bacteria. How frequently this occurs in nature is unknown, but would make for an interesting study.

In *S. zeamais* the first 20 adults were sampled at a different times than the last 10 adults. It is unknown as to why the second sampling yielded *E. faecalis* and *E. faecium* while the first sampling only found *E. casseliflavus*. The reasons for this are unknown. In *S. zeamais* the larvae had only 24% *E. casseliflavus* present and in the adults it was present in 84% of the adults. In the larvae 60% *E. faecium* was present and only 4% of adults had *E. faecium.* It is unknown where the significant discrepancy between adults and larvae originated. It is possible that the mature gut favors *E. casseliflavus* over the other species or if differences exist could be a result of pupae shedding the midgut prior to adult eclosion.

The antibiotics screened in this study were from different classes: penicillin, tetracyclines, macrolides, aminoglycosides and streptogramins, and are all used in poultry production. All of the drugs used are categorized by the Food and Drug Administration (FDA) as critically or highly important to human medicine (FDA, 2003). In terms of importance, streptogramins have been used in animal husbandry for 30 years, have been approved for use in patients with vancomycin resistant *E. faecium* or methicillin-resistant *Staphylococcus aureus* (Jensen et al. 2002 and McDermott et al. 2005). Enterococcal infections are commonly treated with ampicillin, vancomycin. *E. faecium* is successfully treated with quinupristin-dalfopristin (Kristich et al. 2014).

No colonies were resistant to the antibiotics vancomycin and ampicillin. These two antibiotics are generally used as a last resort to try and prevent widespread resistance. Enterococci resistant to these antibiotics are generally found in hospital environments where these antibiotics are used. It is not surprising to not find vancomycin and ampicillin resistance in the stored-product insects tested. The antibiotics are not approved to use in United States animal production operations (Aarestrup et al. 2001). The laboratory colonies were collected from grain silos, an environment where antibiotics are not used and no environmental pressures would develop resistance through antibiotic use, as is seen in the feed mill and feed lot environments (Smith et al. 2003). The enterococci used in this study have been isolated in a laboratory environment that have not been exposed to antibiotics to develop resistance from environmental pressures. Quinupristin was identified as having 51% of the isolates tested be resistant to it. *E.*

faecalis is naturally resistant to Quinupristin (Kristich et al. 2014). High resistance to this antibiotic was expected. It was, however, effective against the *E. faecium* isolates which is where quinupristin is used clinically.

The enterococci are resistant to multiple antibiotics. So the question arises, is this a natural characteristic of enterococci or was it contaminated at one point in time and the resistance remained? There is no way to determine precisely where the antibiotic resistance originated.

Two insects that feed on poultry pellets had a high level of enterococci to begin with, *L. serricorne* and *S. paniceum.* The enterococci in these two species can be directly related to the diet they feed upon. The poultry feed had enterococci that were resistant to up to three different antibiotics. The poultry feed had four species of enterococci, the insects that feed upon that diet only displayed one species of enterococci, *E. faecalis.* Having *E. faecalis* in the gut may be beneficial and that is why it was the one to colonize the insect versus the other three species the insects were consuming. When comparing the antibiotic resistance of these two insects to the diet there are some similarities. Part of the resistance is likely to be coming directly from the diet. However, isolates were only resistant to three different antibiotics found in the diets an isolate in *L. serricorne* was resistant to up to five different antibiotics.

The insects used in this study have been isolated in the K-State stored product insect entomology lab for over 17 years. This study was unable to determine an exact source of enterococcal infection in the insects but it was able to make some inferences. In the two species studied that fed on the poultry pellets, *L. serricorne* and *S. paniceum,* the diet had enterococci and the insects had one of the same strains in their gut microflora, *E. faecalis.* It can be inferred that these two insect species obtained enterococci from the diet they were feeding on. The presence of enterococci in insects in some insect species when the diets tested negative is difficult to explain. The diets either did not have enterococci or very low levels in the case of cowpeas. The insects could have at one time been infected by the diet and passed the bacteria down to their larvae.

2.5. Conclusions

Out of the diets sampled, three were positive for enterococci. Of the three, cowpeas and flour both had small enterococcal loads. Poultry pellets had teh greatest enterococcal loads. Four

species of enterococci were present in the poultry pellets, *E. faecalis, E. faecium, E. heria, E. gallanerium.* In both flour and cowpeas only *E. casseliflavus* was present.

The results of this study demonstrate that laboratory colonies of stored-product insects carry antibiotic resistant enterococci. The larval and adult stages of the insect are both carriers of the pathogen, although differences in species found between the internal and external feeders. The insect can pass the potential pathogen to its offspring as demonstrated by the larva having enterococci. The eggs were sampled but no reliable method for obtaining an uncontaminated sample was devised. In insects three species of enterococci were found, *E. faecalis, E. faecium,* and *E. casseliflavus. E. faecalis* was the most prevalently found strain in every adult species of stored-product insect. The larval stages of the five species had similar results as adults. The exception was *S. granarius* which did not have *E. faecalis* in the larval stage but did in the adult stage.

For antibiotic resistance none of the samples tested were resistant to ampicillin or vancomycin. There was high resistance to doxycycline, erythromycin, quinupristin, and tetracyclin seen in adult insects. There was little difference between the antibiotic resistance of adults and larvae. The frequency of resistance was similar. In the diet samples only the poultry pellets had any antibiotic resistance. The flour and cowpea samples were susceptible to every antibiotic.

In this study, storedproduct insects had antibiotic resistance even after continuous rearing for 17 years. In two of the insect species, *L. serricorne* and *S. paniceum* the diet had enterococci and so did the insects, the diet is the probable source of the infection. In the other three species tested, *C. maculatus, S. granarius* and *S. zeamais*, still had enterococci while the diet was devoid of it.

Table 2-1. Polymerase chain reaction (PCR) primer sequences.

Enterococcal species	Direction	Primer sequence	Product size (bp)	Reference
E. gallinarum	F R	5′-GGTATCAAGGAAACCTC-3′ 5′-CTTCCGCCATCATAGCT-3′	822	Arias et al. (2006)
E. casseliflavus	F R	5′-CGGGGAAGATGGCAGTAT-3′ 5′-CGCAGGGACGGTGATTTT-3′	484	Kariyama et al. (2000)
E. faecalis	F R	5′-ATCAAGTACAGTTAGTCTTTATTAG-3′ 5′-ACGATTCAAAGCTAACTGAATCAGT-3′	941	Kariyama et al. (2000)
E. faecium	F R	5′-TTGAGGCAGACCAGATTGACG-3′ 5′-TATGACAGCGACTCCGATTCC-3′	656	Kariyama et al. (2000)
E. hirae	F R	5’-CGTCAGTACCCTTCTTTTGCAGAGTC-3’ 5’-GCATTATTACCAGTGTTAGTGGTTG-3’	521	Arias et al. (2006)
Soda	F R	5′-CCITAYICITAYGAYGCIYTIGARCC-3’ 5′-ARRTARTAIGCRTGYTCCCAIACRTC-3’	480	Poyart et al. (2000)

Table 2-2. Classification of antibiotics used.

Antibiotic	Classification	Reference
Ampicillin	Penicillins	Ciranowicz (2010)
Quinupristin	Streptogramins	Facklam et al. (2002)
Vancomycin	Glycopeptides	Ciranowicz (2010)
Gentamicin	Aminoglycosides	Facklam et al. (2002)
Erythromycin	Macrolides	Facklam et al. (2002)
Tetracycline	Tetracyclines	Facklam et al. (2002)
Doxycycline	Tetracyclines	FDA (2015)
Tigecycline	Tetracyclines	FDA (2014)
Enrofloxacin	Quinolones	Facklam et al. (2002)

Table 2-3. Prevalence, concentration, and enterococcal species isolated from insect diets[a].

Diet	No. positive per 30 samples (%)	Mean ± SE CFU/g	Total isolates	*E. faecalis* No. (%)	*E. faecium* No. (%)	*E. casseliflavus* No. (%)	*E. gallinarum* No. (%)	*E. hirae* No. (%)
Poultry Pellets	30 (100.0)	$5.90\times10^{3} \pm 2.70\times10^{3}$	71	28 (39.4)	29 (40.8)	0	17 (23.9)	14 (19.7)
Cowpeas	2 (6.7)	$0.40\times10^{1} \pm 0.30\times10^{1}$	2	0	0	3 (100.0)	0	0
Flour	3 (10.0)	$0.20\times10^{1} \pm 0.03\times10^{1}$	3	0	0	2 (100.0)	0	0
Cat Food	0	0	0	0	0	0	0	0
Corn	0	0	0	0	0	0	0	0
Oats	0	0	0	0	0	0	0	0
Poultry mash	0	0	0	0	0	0	0	0
Whole Wheat	0	0	0	0	0	0	0	0

[a]Out of 30, 1 g samples of poultry pellets, *E. faecalis*+*E. faecium*+*E. gallinarum* were found 13 times, *E. faecalis*+*E. faecium*+*E. hirae* were found 11 times, *E. faecalis*+*E. faecium*+*E. gallinarum*+*E. hirae* were found 2 times, *E. faecium*+*E. gallinarum* were found 1 time, *E. faecalis*+*E. faecium* were found 1 time, and *E. faecalis*+*E. hirae* were found 1 time.

Table 2-4. Enterococcal species from insect diets that are not antibiotic resistant and resistant to single or multiple antibiotics tested.

No. of antibiotics	Poultry Pellets			
	E. faecalis *n=3*	*E. faecium* *n=2*	*E. gallinarum* *n=2*	*E. hirae* *n=2*
0	0	0	0	0
1	0	0	1 (50.0)[d]	0
2	2 (66.6)[a]	0	1 (50.0)[e]	2 (100.0)[a]
3	1 (33.3)[b]	2 (100.0)[c]	0	0
4	0	0	0	0

[a]Doxycycline and tetracycline.

[b]Enrofloxacin, doxycycline, and erythromycin.

[c]Quinupristin, enrofloxacin, and gentamycin.

[d]Enrofloxacin.

[a]Enrofloxacin and tetracycline.

Table 2-5. Prevalence and concentration, enterococcal species isolated from individual adults of insect species.

Insect species	No. positive per 30 insects (%)	Number of isolates	Mean ± SE CFU/insect[a]	*E. faecalis* No. (%)	*E. faecium* No. (%)	*E. casseliflavus* No. (%)
Callosobruchus maculatus	29 (96.7)	29	$3.1\times10^{6} \pm 7.1\times10^{5}$a	24 (82.8)	5 (17.2)	0
Sitophilus granarius	20 (66.7)	39	$1.8\times10^{4} \pm 8.0\times10^{3}$b	18 (46.2)	0	19 (48.7)
Stegobium paniceum	20 (66.7)	20	$1.3\times10^{4} \pm 7.8\times10^{3}$b	19 (95.0)	0	0
Lasioderma serricorne	16 (53.3)	16	$9.8\times10^{3} \pm 6.2\times10^{3}$ b	11 (68.8)	0	0
Sitophilus zeamais	23 (76.7)	19	$1.3\times10^{3} \pm 7.0\times10^{2}$ b	3 (12.0)	1 (4.0)	21 (84.0)

All other species tested were negative for enterococci.

[a]Means followed by different letters are significantly different ($P < 0.05$; by REGWQ test).

Table 2-6. Antibiotic resistance in enterococcal isolates from adults of five insect species.

Antibiotic	*C. maculatus* *n=29*	*S. granarius* *n=39*	*S. paniceum* *n=20*	*L. serricorne* *n=16*	*S. zeamais* *n=25*
Ampicillin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Doxycycline	0 (0)	0 (0)	0 (0)	11 (64.7)	5 (20.0)
Erythromycin	1 (3.4)	15 (38.5)	14 (70.0)	9 (52.9)	9 (36.0)
Enrofloxacin	4 (13.8)	15 (38.5)	0 (0)	4 (23.5)	11 (44.0)
Gentamicin	5 (17.2)	0 (0)	0 (0)	3 (17.6)	4 (16.0)
Quinupristin	27 (93.1)	14 (35.9)	0 (0)	2 (11.8)	11 (44.0)
Tetracycline	0 (0)	0 (0)	16 (80.0)	15 (88.2)	6 (24.0)
Tigecycline	0 (0)	2 (5.1)	0 (0)	0 (0)	1 (4.0)
Vancomycin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 2-6. Enterococcal species from adults of five stored-product insect species that are not antibiotic resistant and resistant to single or multiple antibiotics.

No. of anti-biotics	*C. maculatus*		*S. granarius*		*S. paniceum*	*L. serricorne*	*S. zeamais*		
	E. faecalis n=24	*E. faecium n=5*	*E. casseliflavus n=19*	*E. faecalis n=18*	*E. faecalis n=20*	*E. faecalis n=16*	*E. faecalis n=5*	*E. casseliflavus n=21*	*E. faecium n=1*
0	1 (4.2)	0 (0)	4 (21.1)	9 (50.0)	2 (10.0)	1 (6.3)	0 (0)	9 (42.9)	1 (100)
1	17 (70.8)[a]	3 (60)[d]	3 (15.8)[f]	6 (33.3)[j]	6 (30.0)[m]	3 (18.8)[o]	1 (20.0)[t]	8 (38.1)[x]	0 (0)
2	5 (20.8)[b]	2 (40)[e]	6 (31.6)[g]	2 (11.1)[k]	12 (60.0)[n]	4 (25.0)[p]	1 (20.0)[u]	1 (4.8)[y]	0 (0)
3	1 (4.2)[c]	0 (0)	5 (26.3)[h]	1 (5.6)[l]	0 (0)	4 (25.0)[q]	0 (0)	1 (4.8)[z]	0 (0)
4	0 (0)	0 (0)	4 (21.1)[i]	0 (0)	0 (0)	4 (25.0)[r]	1 (20.0)[v]	0 (0)	0 (0)
5	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (6.3)[s]	0 (0)	1 (4.8)[aa]	0 (0)
6	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (40.0)[w]	1 (4.8)[ab]	0 (0)

[a]Quinupristin; [b]Quinupristin and enrofloxacin/quinupristin and gentamicin; [c]Quinupristin, erythromycin, and gentamicin; [d]Quinupristin; [e]quinupristin and gentamicin; [f]Erythromycin/enrofloxacin; [g]Enrofloxacin and erythromycin/enrofloxacin and doxycycline; [h]Quinupristin, enrofloxacin, and erythromycin/tetracycline, enrofloxacin, and erythromycin; [i]Tigecycline, quinupristin, enrofloxacin, and erythromycin; [j]Enrofloxacin/quinupristin/erythromycin; [k]Quinupristin and enrofloxacin; [l]Quinupristin, enrofloxacin, and erythromycin; [m]Tetracycline/erythromycin; [n]Erythromycin and tetracycline; [o]Erythromycin/tetracycline; [p]Doxycycline and tetracycline/erythromycin and tetracycline; [q]Erythromycin, doxycycline, and tetracycline/doxycycline, gentamicin, and tetracycline; [r]Enrofloxacin, doxycycline, gentamicin, and tetracycline/quinupristin, enrofloxacin, doxycycline, and tetracycline/erythromycin, doxycycline, gentamicin, and tetracycline; [s]Quinupristin, enrofloxacin, doxycycline, and tetracycline; [t]Enrofloxacin; [u]enrofloxacin and erythromycin; [v]Quinupristin, enrofloxacin, and erythromycin; [w]Enrofloxacin, erythromycin, doxycycline, gentamicin, and tetracycline; [x]Enrofloxacin, erythromycin, and quinupristin; [y]Quinupristin and enrofloxacin; [z]Erythromycin, doxycycline, and tetracycline; [aa]Quinupristin, enrofloxacin, doxycycline, gentamicin, and tetracycline.

Table 2-8. Enterococcal prevalence and load in adults of *C. maculatus* over a 9-day period after adult eclosion.

Day	No. of beetles	% positive for enterococci		Mean ± SE CFU/beetle
	0	15	40	$0.61\times10^{1} \pm 0.42\times10^{1}$
	1	15	60	$3.0\times10^{3} \pm 2.0\times10^{3}$
	2	15	86	$3.2\times10^{4} \pm 2.1\times10^{4}$
	3	15	86	$2.6\times10^{3} \pm 1.3\times10^{3}$
	4	15	100	$3.6\times10^{4} \pm 3.3\times10^{4}$
	5	15	100	$2.6\times10^{6} \pm 3.2\times10^{5}$
	6	15	93	$5.1\times10^{6} \pm 1.6\times10^{6}$
	7	13	100	$1.3\times10^{7} \pm 1.9\times10^{6}$
	8	5	100	$2.3\times10^{7} \pm 1.7\times10^{6}$
	9	3	66	$4.1\times10^{7} \pm 3.3\times10^{7}$

Table 2-9. Enterococcal prevalence and concentration from fecal material produced by adult *C. maculatus* over a 4-day period after adult eclosion.

Adults age (days)	% positive for enterococci	Weight of fecal material (g)	Mean ±SE CFU/g feces
1	0	0.00190 ± 0.00062	0
2	50	0.00073 ± 0.00041	$2.7\times10^3 \pm 1.8\times10^3$
3	25	0.00520 ± 0.00200	$1.9\times10^2 \pm 1.9\times10^2$
4	25	0.00290 ± 0.00210	$3.3\times10^3 \pm 3.3\times10^3$

Table 2-10. Prevalence, concentration, and enterococcal species isolated from individual larvae of five insect species.

Insect species	No. positive per 30 larvae (%)	Mean + SE CFU/larva[a]	Total no. of isolates	*E. faecalis* No. (%)	*E. faecium* No. (%)	*E. casseliflavus* No. (%)
S. paniceum	28 (93.3)	1.4×105 ± 2.2×104 a	28	28 (100.0)	0	0
L. serricorne	13 (43.3)	1.1×105 ± 2.2×104 ab	13	13 (100.0)	0	0
S. granarius	3 (10.0)	2.5×104 ± 2.5×104 bc	6	3 (50.0)	0	3 (50.0)
S. zeamais	30 (100.0)	1.3×102 ± 3.9×101 c	37	7 (18.9)	22 (59.5)	9 (24.3)
C. maculatus	3 (10.0)	0.27×101 ± 0.067×101 d	3	3 (100.0)	0	0

[a]Means followed by different letters are significantly different ($P < 0.05$; by REGWQ).

Table 2-7. Enterococcal species from larvae of five insect species that are not antibiotic resistant and resistant to single or multiple antibiotics tested.

No. of A*	*C. maculatus*	*S. granarius*		*S. paniceum*	*L. serricorne*	*S. zeamais*		
	E. faecalis n=3	*E. faecalis* n=3	*E. casseliflavus* n=3	*E. faecalis* n=10	*E. faecalis* n=10	*E. faecalis* n=3	*E. casseliflavus* n=2	*E. faecium* n=9
0	0 (0)	1 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (33.3)
1	0 (0)	1 (33.3)[c]	3 (100)[e]	0 (0)	0 (0)	2 (66.7)[k]	2 (100)[m]	6 (66.7)[n]
2	1 (33.3)[a]	1 (33.3)[d]	0 (0)	1 (10.0)[f]	2 (20.0)[i]	0 (0)	0 (0)	0 (0)
3	2 (66.7)[b]	0 (0)	0 (0)	6 (60.0)[g]	0 (0)	0 (0)	0 (0)	0 (0)
4	0 (0)	0 (0)	0 (0)	3 (30.0)[h]	8 (80.0)[j]	1 (33.3)[l]	0 (0)	0 (0)
5	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

*A = antibiotics
[a]Doxycycline and tetracycline; [b]Erythromycin, doxycycline, and tetracycline; [c]Erythromycin; [d]Quinupristin and erythromycin;
[e]Quinupristin/erythromycin; [f]Erythromycin/tetracycline; [g]Erythromycin, doxycycline, and tetracycline/Quinupristin, erythromycin, and tetracycline;
[h]Quinupristin, erythromycin, doxycycline, and tetracycline; [i]Doxycycline and tetracycline; [j]Quinupristin, erythromycin, doxycycline, and tetracycline;
[k]Enrofloxacin and erythromycin; [l]Quinupristin, enrofloxacin, doxycycline, and tetracycline; [m]Enrofloxacin and erythromycin; [n]Enrofloxacin.

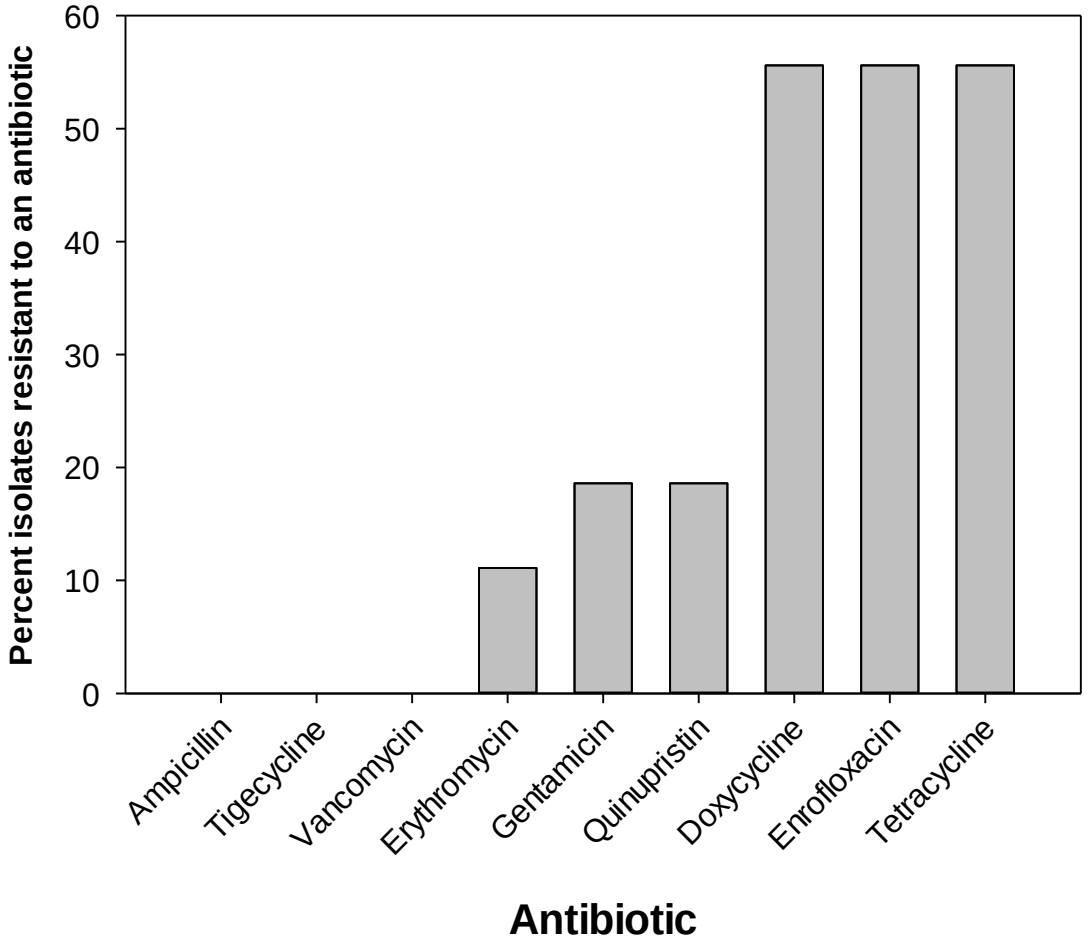

Figure 2-1. Enterococcal colonies resistant to antibiotics from poultry pellet diet.

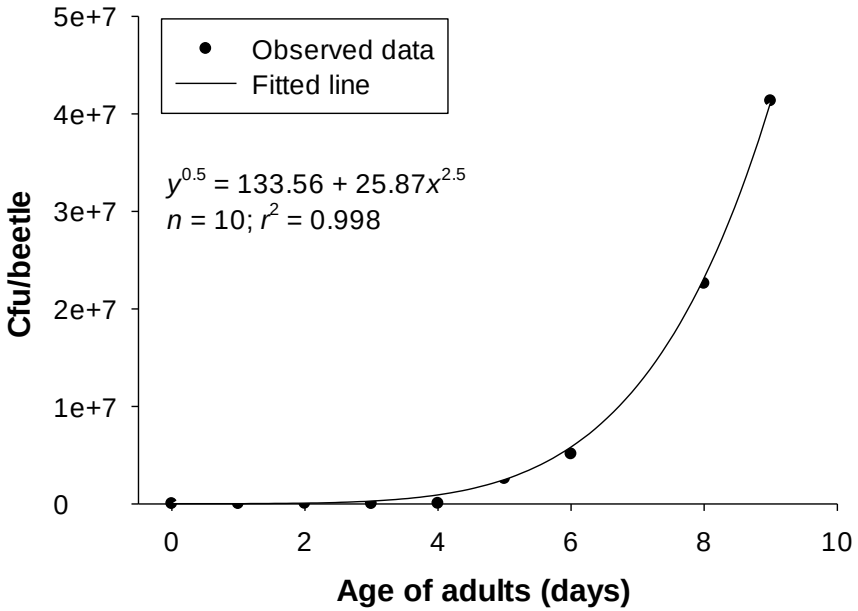

Figure 2-2. Observed and fitted line showing exponential growth of enterococci in C. maculatus adults (beetles) over a 9-day period after eclosion.

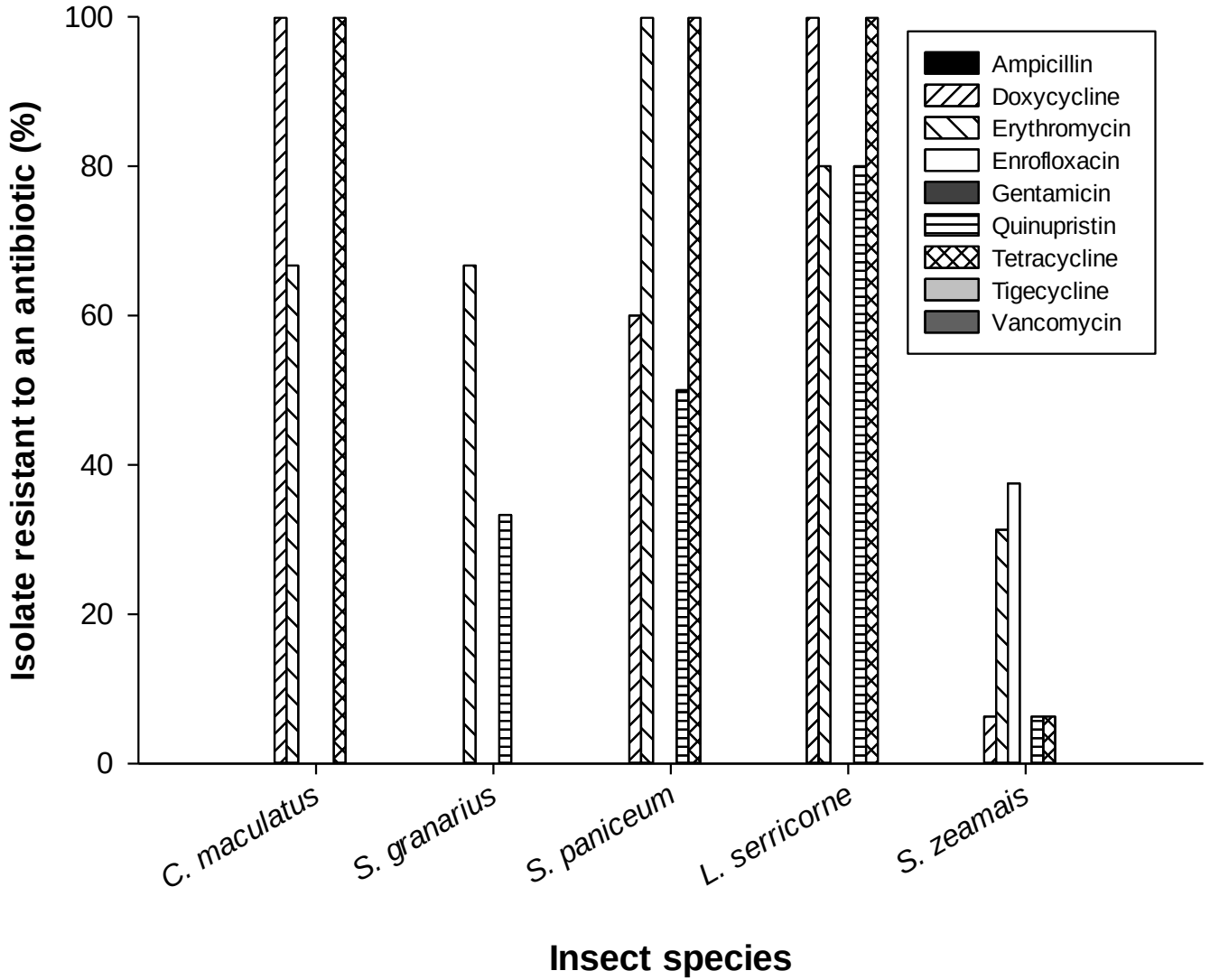

Figure 2-3. Antibiotic resistance in enterococcal isolates from larvae of five insect species.

Chapter 3 - Research Summary and Future Steps

3.1. Summary of the findings

Five out of a total of 15 tested species of stored-product insect adults taken from laboratory reared colonies were tested and found positive for enterococci. These insect species were *Callosobruchus maculatus, Sitophilus granarius, Stegobium paniceum, Lasioderma serricorne*, and *Sitophilus zeamais.* These species were positive for enterococci in the larvae stages as well.

Among the insect diets tested, poultry pellets had high levels of enterococci and four enteroccoal species were isolated. These included *Enterococcus faecalis, E. hirae, E. faecium,* and *E. gallinarium*. Two other diets were identified to have enterococci present, flour and cowpeas. They both had *E. casseliflavus.* They both had two samples out of a total of 30 that were positive for enterococci. Poultry pellets had the highest enterococcal load close to 4800 CFU/g. Cowpeas and flour had a CFU/g count of less than 10.

Nine different antibiotics were tested for resistance with every colony isolated from the adult insects, larvae, and diet samples. For antibiotic resistance none of the samples tested were resistant to ampicillin or vancomycin. There was high resistance to doxycycline, erythromycin, quinupristin, and tetracycline in isolates from adult insects. There was little difference between the frequency of antibiotic resistance of adults and larvae. In the diet samples only the poultry pellets had any antibiotic resistance. The flour and cowpea samples were susceptible to every antibiotic.

C. maculatus had a larger mean enterococcal load compared to the other adult insects. The adult insects were isolated upon emergence and allowed to mature in a jar until ready to sample in one day intervals until all of the adults were dead, nine days. At the end of the nine days an exponential growth curve was developed that showed the enterococci were growing inside the insect midgut.

3.2. Future work

There are many questions this work brought up that need to be answered by further research. One of the studies that needs to be conducted is why *C. maculatus* had such high levels of enterococci compared to the other species of insects studied. This work developed a growth curve for the enterococci, but no weights were taken from the insect to determine if weight loss

could be a factor in the numbers. Further studies should be conducted with antibiotics to determine if this growth in the midgut can be prevented.

A reliable method for sterilizing adult insects without killing them needs to be derived so eggs of external feeders can be obtained without contamination of the outside of the egg. Possibly a way to surface sterilize or remove all of the flour from the egg samples needs to be developed. Further research into egg collection and isolation is warranted.

In Channaiah et al. (2010b) it was shown that *T. castaneum* loads in acquired *E. faecalis* decreased over time. No larvae from other insects that were negative as adults were examined. Testing the larvae and pupal stages of the other species is needed to determine if these stages have enterococci that die out in the adult stages.

Another worthwhile study would be to expand upon the current research by collecting insects from multiple locations including flour mills, feed mills, processing plants that use flour and grains, retail stores, and private residences. The patterns of enterococci species identified in different ecological niches and their antibiotic resistance could be tracked to determine if development of antibiotic resistance has a bearing on prior exposure to antibiotics or is already prevalent in insects and perpetuates even under controlled laboratory conditions transovarially.

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