PS2-4 – 6255

Association of *Enterococci* with stored products and stored-product insects: Medical importance and implications

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Abstract

Enterococci are ubiquitous, catalase negative, gram-positive cocci. *Enterococcus faecalis* and *E. faecium* have emerged worldwide in the last decade as one of the leading causes of nosocomial infectious diseases. Their intrinsic or acquired resistance to many antibiotics has become a major cause of concern. In the United States, the prevalence of antibiotic-resistant enterococci (ARE) is rising, in part, due to the use of antibiotics in feed as growth promoters for farm animals. There is a concern regarding the use of antibiotics in feed for livestock production and the development of resistance in strains of clinical importance. Several *Enterococcus* species obtained from feed and stored-product insects in livestock facilities and feed mills were examined for their antibiotic resistance profiles. Stored-products and stored-products insects were collected in sterilized polythene bags. Enterococci were isolated from stored products and stored-product insects by culturing approach using selective media. Isolates were identified as *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. casseliflavus* using species-specific primers. Phenotypic and genotypic analysis of these isolates for antibiotic resistance and virulence factors demonstrate that stored products and stored-product insects harbor antibiotic resistant and potentially virulent enterococci.

Key words: Stored-product insects, feed, enterococci, antibiotic resistance.

Introduction

Antibiotics are used as feed additives to promote the growth of animals, increase feed efficiency, and prevent diseases. In the United States, about 3,000 feed mills produce 121 million tons of feeds for various domestic animals annually (Feedstuffs, 2003). An estimated 70 % of the antibiotics used in the United States each year are used as feed additives for chickens, hogs, and beef cattle.

The development of bacterial resistance to antibiotics is now a major public health concern. For example, vancomycin-resistant enterococci have emerged in recent years as epidemiologically important pathogens. Enterococci are ubiquitous, catalase negative, gram-positive cocci. *Enterococcus faecalis* and *E. faecium* have emerged worldwide during the last decade as one of the leading causes of nosocomial infectious diseases. In the United States, the prevalence of antibiotic-resistant enterococci is rising, in part, due to the use of antibiotics as growth promoters for farm animals. *Enterococcus faecalis*, *E. faecium*, and *E. hirae* have been isolated from feed samples in Sweden and Spain, and *E. faecium* has been identified from feed samples in

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the United Kingdom (Kuhn et al., 2003). Any development of resistance in these bacteria constitutes a public health risk, primarily through the increased risk of treatment failure against human pathogens (Aarestrup, 1999). However, little emphasis has been placed on enterococci in the feed mill environment. The overuse of antimicrobials in feed for farm animals may cause problems during treatment for infectious disease as it results in the evolutionary selection for resistance among pathogenic bacteria (Quednau et al., 1998; Andrews et al., 2004). It is established that the widespread development of multi-drug resistance in many species of bacteria is due to horizontal gene transfer (HGT) (DeNap and Hergenrother, 2005). Horizontal gene transfer is a process, in which an organism, particularly bacteria, transfers genetic material to another cell that is not its offspring. There is increasing evidence that antibiotic-resistant bacteria is on the rise and is an increasing risk to human or animal health.

There are more than 300 identified species of bacteria that have been associated with insects and ticks. Although the gut bacteria of insects have been well studied, very few studies have focused on the antibiotic resistance gene or plasmid transfer from one bacterial species to another. To our knowledge, there are no studies examining the role of stored-product insects as vectors of antibiotic resistant genes in the environment, and on the horizontal transmission of these antibiotic resistant genes from enterococci bacteria in stored-product insects to pathogenic bacteria of medical importance. In this study enterococci associated with stored-product insects and stored products collected from feed mills and livestock facilities in the Midwestern United States were isolated, quantified, identified, and screened for antibiotic resistance.

Materials and methods

Sample collection and isolation of enterococci

Stored-product insects and stored products were collected from different feed mills, and livestock facilities located in five Midwestern Unites States (Kansas, Oklahoma, Nebraska, Indiana, and Missouri). A total of 392 stored-product insects were collected from March through November, 2003 (298 insects) and from April to June, 2006 (94 insects). A total of 28 stored product samples were collected from April to June, 2006, from the Kansas State University pilot feed mill, and three pig farms, one at Kansas State University and two at Salina, 120 miles from Kansas State University.

Individual insects of each species were surface sterilized with sodium hypochlorite and ethanol (Zurek et al., 2000), homogenized in 1 ml of potassium buffer saline (pH 7.2; ICN Biomedicals, Ohio, USA), serially diluted in potassium buffer saline, and drop-plated on Trypticase soy broth agar (TSBA; BBL, Sparks, Maryland, USA), mEnterococcus agar (mENT; Difco, Sparks, Maryland, USA) with cycloheximide (36 mg per 1L to inhibit fungi), and MacConkey agar (MAC; Difco, Sparks, Maryland, USA). TSBA was used to determine the diversity of bacteria within the insect. Enterococci (red and pink colonies) were enumerated on mENT, which is a selective medium for this genus. MAC was used as a selective medium for Enterobacteriaceae. Plates were allowed to dry and then placed into an incubator at 37 °C (mENT, MAC) or 28 °C (TSBA). The colony forming units (CFU) was recorded at 24, 48 and 72 h after plating. Up to five presumptive enterococcal colonies with different colony morphologies from each sample were streaked on TSBA (Becton Dickinson, Massachusetts), incubated at 37 °C for 24 h, and stored at 4 °C until further analysis.

Isolation of enterococci from stored products

Feed samples of approximately 100 g were collected from different feed mills and livestock facilities located in Kansas. A total of 28 feed samples starting from raw materials to the

finished products were collected in a sterilized zipper-sealed plastic bag, immediately brought to the laboratory for further microbial analysis.

All samples were mixed thoroughly before 1 g of sample was added to 1 ml of potassium buffer saline under sterile conditions, shaken and allowed to settle (Yezerski et al., 2005). One-hundred microliters of each sample was serially diluted in potassium buffer saline, drop-plated on TSBA, mENT, and MAC media. Plates were allowed to dry and then placed into an incubator at 37 °C (mENT, MAC) or at 28 °C (TSBA). The CFU were recorded at 24, 48 and 72 h after plating.

Bacterial identification and screening for antibiotic resistance

The presumptive identities of enterococcal colonies were confirmed at the genus level by the esculin hydrolysis test using Enterococcossel broth (Becton Dickinson, Massachusetts, USA). Multiplex PCR (polymerase chain reaction) was used to identify four common species, *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum* (Kariyama et al., 2000; Dutka-Malen et al., 1995). The strains used as positive controls and the primer sequences are listed in Table 1. Antibiotic resistance was determined by the diffusion disk test technique using Muller-Hinton agar (Difco, Sparks, Maryland, USA). The six antibiotics and disc concentrations tested were tetracycline (30 µg), ampicillin (10 µg), erythromycin (15 µg), vancomycin (30 µg), chloramphenicol (30 µg), and ciprofloxacin (5 µg) (all BBL, Sparks, Maryland, USA). All plates were incubated at 37 °C. After 24 h of incubation, the zone of inhibition was measured to differentiate resistant, intermediate, or susceptible isolates.

Results

Isolation and quantification of enterococci from stored-product insects

A total of 392 individual stored-product insects were screened in this study. The insect species screened included the red flour beetle, *Tribolium castaneum* (Herbst) [number of insect, n = 156; 39.7 % of the total insects screened]; confused flour beetle, *Tribolium confusum* (Jacquelin du Val) [145; 36.9 %]; warehouse beetle, *Trogoderma variabile* (Ballion) [36; 9.1 %]; *Cryptolestes* spp. [17; 4.3 %]; lesser grain borer, *Rhyzopertha dominica* (F.) [15; 3.8 %] ; drugstore beetle, *Stegobium paniceum* (L.) [12; 3.0 %]; foreign grain beetle, *Ahasverus advena* (Waltl) [4; 1 %]; lesser mealworm, *Alphitobius diaperinus* (Panzer) [4; 1 %]; maize weevil, *Sitophilus zeamais* (Motschulsky) [2; 0.5 %]; and smalleyed flour beetle, *Palorus ratzeburgi* (Wissmann) [1; 0.2 %].

On mEnt media, enterococci were detected in 33.7 % of the 392 stored-product insects screened. The mean concentration of enterococci per insect among the samples ranged from 3.7×10^1 to 6.5×10^3 CFU (Table 2). On TSBA, 65 % of the insects from mill 3 and 81% of insects from mill 5 were positive for bacterial growth. Insects from mills, 1, 5, and 6 had the greatest CFU per insect (>1.6×10^4) (Table 2). About 16.6 % of stored-product insects were found positive on MAC plates. The mean CFU per insect ranged from 4×10^1 (mill 8) to 6.0×10^3 (mill 3). Lesser grain borer yielded the highest percentage of positive samples of all the species, followed by the red flour beetle and confused flour beetle (data not shown).

Isolation and quantification of enterococci from stored products

Table 3 provides details on the number of stored product samples, which ranged from 4 to 12 per facility. The percentage of samples found positive for enterococci on mENT plates ranged from 60 (site 2) to 100 (site 1). The mean concentration of enterococci on mENT agar ranged from 2.7×10^2 to 9.3×10^2 CFU per sample. All stored product samples were found positive on TSBA plates and the mean CFU was in range of 2.7×10^3 (site 2) to 6.4×10^3 (site 1) per sample. The mean CFU per sample ranged from 8.3×10^2 (site 2) to 2.8×10^3 CFU (site 4) on the MAC media.

Table 1. Primers and multiplex PCR conditions used for identification of *Enterococcus* species.

Primer type	Positive control	Direction[a]	Sequence (5'–3')	Primer concn (pmol)	Annealing temp. (°C)	Product size (bp)	Reference
E. gallinarum	ATCC 49579	F	GGTATCAAGGAAACCTC	2.5	54	822	Kariyama et al., 2000
		R	CTTCCGCCATCATAGCT				
E. casseliflavus	ATCC 25788	F	CGGGGAAGATGGCAGTAT	2.5	54	484	Kariyama et al., 2000
		R	CGCAGGGACGGTGATTTT				
E. faecalis	ATCC 19433	F	TCAAGTACAGTTAGTCTTTATTAG	5.0	54	941	Dutka-Malen et al., 1995
		R	ACGATTCAAAGCTAACTGAATCAGT				
E. faecium	ATCC 19434	F	TTGAGGCAGACCAGATTGACG	1.25	54	658	Dutka-Malen et al., 1995
		R	TATGACAGCGACTCCGATTCC				
16S rRNA gene		F	GGATTAGATACCCTGGTAGTCC	2.5	54	320	Kariyama et al., 2000
		R	TCGTTGCGGGACTTAACCCAAC				

[a] F, forward; R, reverse.

Table 2. Number of stored product insects sampled from each mill and number of colony forming units (CFU) per insect on three media.

Mill[1]	N[2]	mEnterococcus agar		Trypticase soy broth agar		MacConkey agar	
		n (%)[3]	mean CFU ± SE	*n* (%)[3]	mean CFU ± SE	*n* (%)[3]	mean CFU ± SE
1	57	5 (8.7)	$1.7 \times 10^5 \pm 9.0 \times 10^4$	12 (21.1)	$3.0 \times 10^4 \pm 3.0 \times 10^4$	1 (1.8)	3.5×10^3
2	22	0	0	11 (50.0)	$5.9 \times 10^2 \pm 4.8 \times 10^2$	1 (4.5)	2.4×10^3
3	65	1 (1.5)	4.8×10^3	42 (64.6)	$3.1 \times 10^3 \pm 1.7 \times 10^3$	4 (6.2)	$6.0 \times 10^3 \pm 2.7 \times 10^3$
4	45	1 (2.2)	1.2×10^1	9 (20.0)	$4.1 \times 10^2 \pm 4.0 \times 10^2$	1 (2.2)	3.6×10^2
5	53	45 (84.9)	$6.5 \times 10^3 \pm 2.0 \times 10^3$	43 (81.1)	$1.6 \times 10^4 \pm 2.6 \times 10^3$	10 (18.9)	$2.4 \times 10^2 \pm 8.4 \times 10^1$
6	56	1 (1.8)	8.4×10^3	23 (41.1)	$2.4 \times 10^4 \pm 1.6 \times 10^4$	2 (3.6)	$3.5 \times 10^3 \pm 3.4 \times 10^3$
7	10	3 (30)	$4 \times 10^1 \pm 1 \times 10^1$	7 (70)	$4.2 \times 10^2 \pm 1.5 \times 10^2$	6 (60)	$2.5 \times 10^2 \pm 7.6 \times 10^1$
8	08	6 (75)	$4 \times 10^1 \pm 6.3 \times 10^1$	7 (87.5)	$4.3 \times 10^2 \pm 1.5 \times 10^2$	6 (75)	$4 \times 10^1 \pm 1.2 \times 10^1$
9	09	7 (77.7)	$3.8 \times 10^1 \pm 5.5 \times 10^1$	8 (88.8)	$2.4 \times 10^3 \pm 1.4 \times 10^3$	7 (77.7)	$4.3 \times 10^2 \pm 6.1 \times 10^1$
10	47	47 (100)	$5 \times 10^2 \pm 1.1 \times 10^2$	47 (100)	$6.2 \times 10^3 \pm 1 \times 10^3$	10 (100)	$2.6 \times 10^3 \pm 5 \times 10^2$
11	12	8 (66.6)	$3.7 \times 10^1 \pm 4 \times 10^1$	11 (91.6)	$3.7 \times 10^2 \pm 6.3 \times 10^1$	9 (75)	$7.5 \times 10^1 \pm 1.1 \times 10^1$
12	08	8 (100)	$4.1 \times 10^1 \pm 0.79 \times 10^1$	8 (100)	$1.4 \times 10^3 \pm 7.7 \times 10^2$	8 (100)	$3.4 \times 10^2 \pm 5.3 \times 10^1$

[1] Samples in mills 1-6 were collected during March to November, 2003 (Larson, 2004); samples in mills 7-12 were collected by Lakshmikantha during April to June, 2006.

[2] *N* = Total number of insects sampled from each mill.

[3] *n* = Number of positive samples (% of positive samples).

Table 3. Number of stored products sampled from each mill/facility and number of colony forming units (CFU) per sample on three media.

		mEnterococcus agar		Trypticase soy broth agar		MacConkey agar	
Site[1]	N[2]	n (%)[3]	mean CFU ± SE	n (%)[3]	mean CFU ± SE	n (%)[3]	mean CFU ± SE
1	4	4 (100)	$9.3 \times 10^2 \pm 1.3 \times 10^2$	4 (100)	$6.4 \times 10^3 \pm 1.9 \times 10^3$	4 (100)	$2.2 \times 10^3 \pm 2.7 \times 10^2$
2	5	3 (60)	$2.7 \times 10^2 \pm 3.4 \times 10^1$	5 (100)	$2.7 \times 10^3 \pm 8 \times 10^2$	3 (60)	$8.3 \times 10^2 \pm 1.8 \times 10^2$
3	12	8 (66.6)	$3.1 \times 10^2 \pm 8 \times 10^1$	12 (100)	$5 \times 10^3 \pm 7.7 \times 10^2$	10 (83.3)	$1.4 \times 10^3 \pm 2.4 \times 10^2$
4	7	5 (71.4)	$3.12 \times 10^2 \pm 4.5 \times 10^1$	7 (100)	$5.5 \times 10^3 \pm 1.6 \times 10^3$	6 (85.7)	$2.8 \times 10^3 \pm 1 \times 10^3$

[1] 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.

[2] N = Total number of stored products sampled from each site.

[3] n = Number of positive samples (% of positive samples).

Identification of enterococcus isolates using multiplex PCR

A total of 67 enterococci colonies from stored-product insects on mENT plates were selected and confirmed presumptively using Enterococcossel broth (esculin hydrolysis test). These isolates were further screened by multiplex PCR for species identification. Forty three of the isolates (64.1 %) belonged to the *Enterococcus* species. These were *E. faecium* 10 (23.2 %), *E. gallinarum* 7 (16.2 %) and *E. casseliflavus* 26 (60.4 %) (Table 4). Similarly, a total of 125 colonies from stored products on mENT plates were selected and confirmed presumptively using Enterococcossel broth. Further screening by multiplex PCR for species identification revealed that 45 isolates (36 %) were of *Enterococcus* species. These were identified as *E. faecalis* 2 (4.4%), *E. faecium* 8 (17.7 %), *E. gallinarum* 2 (4.4 %) and *E. casseliflavus* 33 (73.3 %) (Table 4).

Enterococci and antibiotic resistance profiles

Majority of the identified isolates (genera Enterococcus) were phenotypically resistant to tetracycline, followed by erythromycin, ciprofloxacin, vancomycin, ampicillin and chloramphenicol (Figure 1). Of the four species of *Enterococcus*, *E. faecium* and *E. casseliflavus* were most frequently resistant to tetracycline and ciprofloxacin (data not shown). Majority of the enterococcus genera were sensitive to ampicillin, followed by chloramphenicol, vancomycin, ciprofloxacin, tetracycline, and erythromycin. Most of the enterococcus isolates were resistant to at least one antibiotic. For multidrug resistance, majority of the isolates exhibited resistance to tetracycline, ciprofloxacin, vancomycine and erythromycin.

Discussion

Resistance to antibiotics has been well studied in bacterial pathogens associated with human health. However, there is limited information on the impacts of antibiotics used in animal feed. Species of *Enterococcus* are found in the intestine of nearly all animals. Although *Enterococcus* species are generally innocuous, they have been shown to readily develop resistance to various antibiotics upon exposure inside the intestines of animals. These species also have the capability to pass the drug-resistant genes to other human bacterial pathogens of clinical importance.

Enterococcus have been reported to be reservoirs for antibiotic resistance genes (Devriese et al., 1992). *Enterococcus* species are intrinsically resistant to several antibiotics, such as cephalosporins, beta-lactans, sulphanamides and

Table 4. Prevalence and identification of enterococci isolated from stored products and stored-product insects collected during April to June, 2006.

Source	No of samples	Total no. enterococci isolates	No. isolates identified (%)	No. species identified (%)			
				E. faecalis	*E. faecium*	*E. gallinarum*	*E. casseliflavus*
Stored-product insect	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)

low levels of clindamycin and aminoglycosides (Larson, 2004). *Enterococcus* species have the ability to develop or acquire resistant to chloramphenicol, erythromycin, clindamycin, aminoglycosides, tetracycline, beta-lactans, fluoroquinoles, and glycopeptides such as vancomycin (Franz et al., 2003).

Nearly 35 % of isolates from stored-product insects and 64 % of isolates from stored products were not identified by multiplex PCR protocol. These isolates may represent other enterococcal species and the use of additional primers is necessary for identification of species not reported in this paper. The majority of these isolates were phenotypically resistant to at least one antibiotic and many isolates were found to be multidrug resistant, raising the concerns for safety of the animal feed. The exact origin and subsequent dispersal is unknown, but our data suggest that stored-product insects may serve as a potential vector in acquisition, multiplication, and subsequent dispersal of drug resistant bacteria in feed mill and livestock environment.

Conclusions

Our findings provide evidence that stored products and stored-product insects harbor antibiotic resistant and potentially virulent enterococci, with antibiotic resistant and virulent genes. Our data reinforces the need for pest management to reduce the availability of insect vectors, particularly stored-product insects, in the feed mill and livestock environments. However, a better understanding of the insect biology coupled with factors for successful bacterial symbiotic associations and acquisition of genes resistant to antibiotics are very important to overcome transfer of vector (insect) mediated dissemination of drug resistant genes in nature.

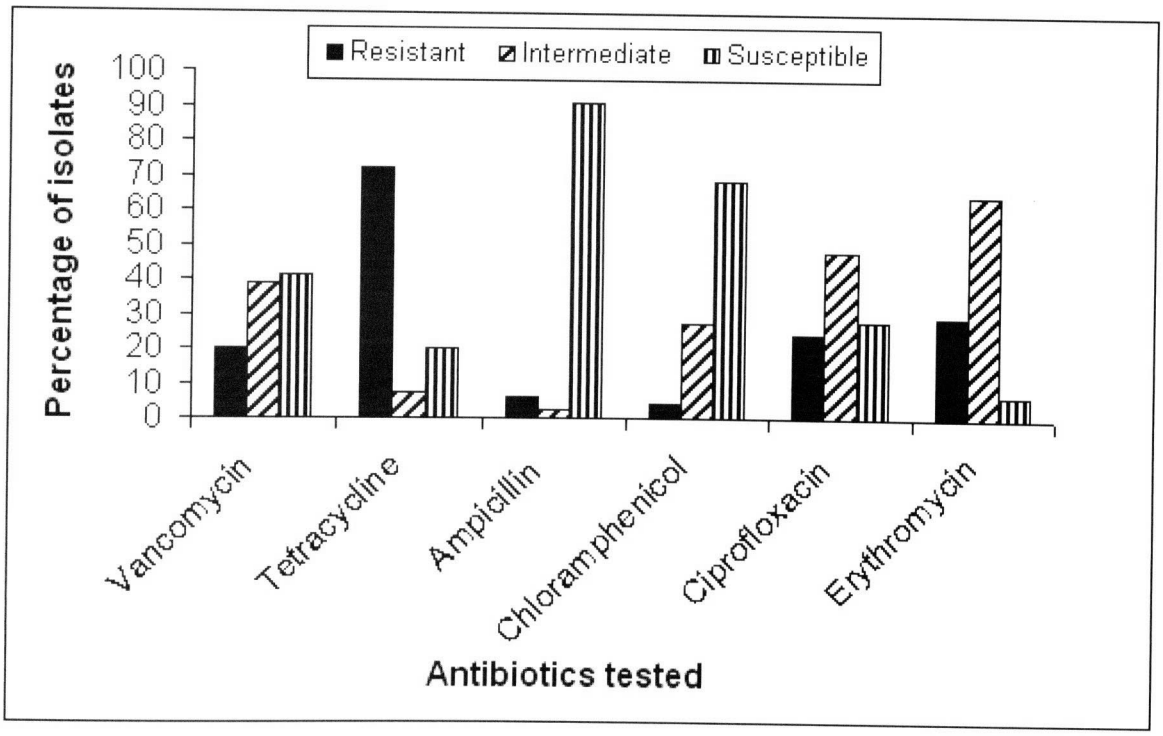

Figure 1. Percentage of enterococcal isolates (n = 192) with antibiotic resistance associated with stored products and stored product insects collected during April to June, 2006.

Acknowledgments

We wish to thank Dr. Aqeel Ahmad for providing us accessibility to the hog farms where product samples were collected, and to Dr. Keith Behnke for allowing us to sample the Kansas State University feed mill. This research is part of a large-scale, long-term effort by the Consortium for Integrated Management of Stored Product Insect Pests (http://www.oznet.ksu.edu/spiramp) to investigate and develop alternative prevention, monitoring, sampling, and suppression measures for organophosphate insecticides used directly on post-harvest grains and under scrutiny as a result of the 1996 U.S. Food Quality Protection Act (FQPA) requirements and for the loss of methyl bromide in food-processing facilities as a result of the Montreal Protocol. Research reported here was funded by the United States Department of Agriculture, under the Cooperative State Research and Education, and Extension Service's Risk Assessment and Mitigation Program under Agreement Number 2002-3438-112187.

References

Aarestrup, F. M., 1999. Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. Journal of Applied Microbiology 12, 279-285.

Andrews, R.E. J., Johnson , S.W., Guard , A., Marvin, J.D., 2004. Survival of *Enterococci* and Tn*916*-like conjugative transposons in soil. Canadian Journal of Microbiology 50, 957-966.

DeNap, J.C.B., Hergenrother, P.J., 2005. Bacterial death comes full circle: targeting plasmid replication in drug-resistant bacteria. Organic and Bimolecular Chemistry 3, 959-966.

Devriese, L.A., Collins , M.D., Wirth, R., 1992. The genus Enterococcus, pp. 1465-1481. In Balows, A., H.G. Traper, M. Dworkin, W. Harder, and K,-H. Schleifer [eds]. The Prokaryotes: A Handbook on the Biology of Bacteria: Vol I-IV, Springer-Verlag, New York.

Dutka-Malen, S., Evers , S., Courvalin, P., 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. Journal of Clinical Microbiology 33, 24-27.

Feedstuffs, 2003. Feed marketing and distribution, 2003-04, Feedstuffs reference issue and guide 38: 17.

Franz, C.M., Stiles , M.E., Schleifer , K.H., Holzapfel, W.H., 2003. *Enterococci* in foods-a conundrum for food safety. International Journal of Food Microbiology 88, 105-122.

Kariyama, R., Mitsuhata , R., Chow , J.W., Clewell , D.B., Kumon, H., 2000. Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. Journal of Clinical Microbiology 38, 3092-3095.

Kuhn, I., Iversen , A., Burman , L.G., Olsson-Liljequist , B., Franklin , A., Finn , M., Aarestrup , F., Seyfarth , A.M., Blanch , A.R., Vilanova , X., Taylor , H., Caplin , J., Moreno , M.A., Dominguez , L., Herrero , I.A., Mollby, R., 2003. Comparison of enterococcal populations in animals, humans, and the environment—a European study. International Journal of Food Microbiology 88, 133-145.

Larson, Z.A., 2004. Diversity and medical importance of stored-product insects in feed mills, M.S. thesis, Kansas State University, Manhattan, Kansas.

Martin, J.D., Mundt, J.O., 1972. *Enterococci* in insects. Applied Microbiology 21, 572-580.

Quednau, M., Ahrne , S., Petersson , A.C., Molin, G., 1998. Identification of clinically important species of *Enterococcus* within 1 day with randomly amplified polymorphic DNA (RAPD). Current Microbiology 36, 332-336.

Yezerski, A., Cussatt, G., Glick, D., Evancho, M., 2005. The effects of the presence of stored-product pests on the microfauna of a flour community. Journal of Applied Microbiology 9, 1365-2672.

Zurek, L., Schal, C., Watson, D.W., 2000. Diversity and contribution of the intestinal bacterial community to the development of *Musca domestica* (Diptera: Muscidae) larvae. Journal of Medical Entomology 37, 924-928.