J Korean Soc Appl Biol Chem (2013) 56, 295–299
DOI 10.1007/s13765-012-3212-0

ORIGINAL ARTICLE

Antibiotic Susceptibility and Molecular Typing of *Enterococcus faecalis* from Retail Pork Meat Products in Korea

Minseon Koo · Ae-Ri Cho · A-Ram Jeong · Hyun Jung Kim · Yong-Ho Park · Hyo-Sun Kwak · In-Gyun Hwang

Received: 19 September 2012 / Accepted: 3 March 2013 / Published Online: 30 June 2013

Abstract Enterococci have been used as starter cultures and probiotics. They also have been considered as indicator organisms for antibiotic resistance due to their ability to harbor and to easily acquire antibiotic resistance. This study aimed to show the antimicrobial resistance profiles and genotyping of *Enterococcus faecalis* in retail pork meat products in Korea. *Enterococcus* spp. were analyzed for 124 collected samples, which included minced pork meat, marinated pork meat with soy sauce or *kochujang* (fermented hot pepper-soybean paste), and frozen processed pork meat products. The isolates of *E. faecalis* (n =36) were resistant to tetracycline (58.3%), erythromycin (11.1%), and nitrofurantoin (2.8%). No vancomycin resistant enterococci were observed in the present study. Most of the *E. faecalis* isolates were sensitive to all antibiotics or resistant to single antibiotics. As a result of the automated repetitive-sequence-based PCR (rep-PCR), which was used as an approach for genotyping enterococci, 7 out of 36 isolates of *E. faecalis* were assigned to one cluster with a similarity >95%, and all isolates were found to have originated from minced pork meat, suggesting that this clone might circulate in minced pork meat products. Given the importance of antimicrobial resistance of enterococci in food safety as well as in public health, our results on the occurrence, antimicrobial resistance, and genotyping could provide useful information to derive risk management options.

Keywords antimicrobial resistance · *Enterococcus* · molecular typing · pork meat products · rep-PCR

Introduction

Enterococci are Gram-positive, facultative anaerobic bacteria that live as part of the natural gut flora of humans as well as animals. Selected strains of enterococci have been used not only as starter cultures in the production of traditional fermented food, but as probiotics for human and animal use (Weiss et al., 2010). Enterococci are not problematic for healthy people, but occasionally function as agents of urinary tract infections. However, the genus has been proven to cause opportunistic infections on hospital patients, including a wide variety of human infections, such as endocarditis, urinary and genital tract infections, meningitis, and septicemia (Woodford and Levermore, 2009).

The abilities to harbor and to easily acquire antibiotic resistance genes make enterococci of concern. They show increasing resistance to antimicrobial agents such as β-lactams, high-level resistance to aminoglycoside, and more recently, resistance to glycopeptides, particularly in *Enterococcus faecalis* strains (Murray, 1998; Woodford and Levermore, 2009). The genetic determinants conferring resistance to all classes of antimicrobials that are mediated by genes residing on plasmids or transposons could be transferred by pheromone-mediated, conjugative plasmids or transposons to other enterococci or even to more virulent pathogens (Barbosa et al., 2009). The heavy use of growth-promoting drugs in some food animals has caused an increase of antimicrobial resistance to

M. Koo and A. -R. Cho contributed equally.

M. Koo · A. -R. Cho · A. -R. Jeong · H. J. Kim (✉)
Food Safety Research Group, Korea Food Research Institute, Sungnam, Gyeonggi-do 463-746, Republic of Korea
E-mail: hjkim@kfri.re.kr

Y. -H. Park
Department of Microbiology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Republic of Korea

H. -S. Kwak · I. -G. Hwang
Food Microbiology Division, National Institute of Food and Drug Safety Evaluation, Cheongwon-gun 363-951, Republic of Korea

enterococci of animal origin, and this resistance also appears to have spread to enterococci in the human population (Bates, 1997).

Antibiotic-resistant enterococci have been found in meat products, dairy products, and ready-to-eat foods (Corpet, 1998; Quednau et al., 1998; Teuber et al., 1999; Baumgartner et al., 2001). However, little information is available on antibiotic resistance in enterococci isolated from retail meat products in Korea, in spite of the fact that antibiotic resistance has been reported in enterococci isolated from livestock and human (Hwang et al., 2009; Lee et al., 2011). In the present study, the occurrence of enterococci in retail pork meat products and the antibiotic resistance of *E. faecalis* isolates were examined. Automated repetitive-sequence-based PCR (rep-PCR) was used to investigate the genetic diversity of enterococci isolated from pork meat products.

Materials and Methods

Sample collection and preparation. Pork samples were purchased from retail markets in Gyeonggi Province in South Korea over a period of three months in 2010. The one hundred twenty four samples included minced pork meat (n =40), marinated pork meat with soy sauce (n =34) or *kochujang* (n =34), and frozen processed meat products (n =16). Samples were placed on ice in a cooling box after purchase and were transported to the laboratory within 2 h. Samples that included minced pork meat and marinated pork meat were immediately stored at 4°C. Frozen processed meats were kept at −20°C.

Isolation and enumeration of *Enterococcus* spp. The enumeration of enterococci was carried out according to ISO-7899-1 (IOS, 1998) and Weiss et al. (2005) with some modifications. In brief, 25 g of the sample was cut with sterilized scissors and homogenized with 225 mL sterile peptone water in a Stomacher ®400 Circulator (Seward, England) for 2 min at 230 rpm. The homogenates were then subjected to serial 10-fold dilution in peptone water, and 100 μL of each dilution was spread on Bile Aesculin Azide (BAAA) agar (MERCK, Germany) plates. After 24 h incubation at 37°C, the plates with typical numbers of colonies between 25 and 250 were selected for presumptive enumeration of *Enterococcus*. Typical colonies on BAAA agar were transferred to Tryptic soy agar (TSA, Merck, Germany) and were subjected to biochemical testing in a VITEK® 2 compact system (Biomerieuk, France) and to a genetic test by conventional PCR to identify *E. faecalis*. The template DNA of each strain was obtained with a DNeasy tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. PCR amplifications were routinely carried out at a 20 μL reaction volume, which consisted of 1 μL DNA templates: the primer for *E. faecalis* (5'-TCAAGTA CAGTTAGTCTTTATTAG', 5'-ACGATTCAAAGCTAACTGAA TCAGT, 941 bp) (Dutka-Malen et al., 1995) at 5 pmole/μL, *rrs* primer (R-5'-GGATTAGATACCCTGGTAGTCC, R-5'-TCGTTG CGGGACTTAACCCAAC, 320 bp) (Kariyama et al., 2000) at 1 pmole/μL; 1 unit of Taq-polymerase, 2.5 mM of dNTP mixture, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, and 1.5 mM of $MgCl_2$ (AccuPower™ PCR PreMix, Bioneer, Korea). All PCR reactions were carried out in a Mastercycler pro (Eppendorf, Germany) using the following parameters: initial denaturation at 94°C for 5 min; 30 cycles at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min; with the final extension at 72°C for 10 min. The PCR products were electrophoresed on 1.8% agarose gel and stained with ethidium bromide. The DNA bands were visualized and documented with a GelDoc™ XR+ imaging system (Bio-Rad, USA). The confirmed isolates were kept in 50% glycerol at −80°C for further analysis.

Antibiotic susceptibility. Antibiotic susceptibility of the identified enterococci isolates was examined by the broth dilution test and disc diffusion test. The broth dilution test was performed with an AST-P601 test card in a VITEK® 2 compact system according to the manufacturer's instructions, and the disc diffusion test was carried out using the antibiotic susceptibility testing disc (Oxoid Ltd, UK) based on the Clinical and Laboratory Standards Institute (CLSI, 2010) standards. The tested antimicrobials were as follows: ciprofloxacin, erythromycin, linezolid, teicoplanin, vancomycin, tetracycline, tigecycline, nitrofurantoin, ampicillin, amoxycillin/clavulanic acid, and chloramphenicol. The qualitative interpretation (resistant or sensitive) is based on the breakpoints for enterococci proposed by the CLSI standards (CLSI, 2010). Quality control for this antibiotic susceptibility was performed using *E. faecalis* ATCC 29212, and all values were within accepted limits.

Molecular typing by DiversiLab system. For rep-PCR, DNAs of enterococci isolates were extracted using the UltraClean microbial DNA isolation Kit (Mo Bio Laboratories, USA). All DNA solutions were standardized to a concentration of ca. 25 ng/μL. The DiversiLab *Enterococcus* Kit (Bacterial Barcodes, Inc, USA) was used for rep-PCR amplification of non-coding intergenic repetitive elements in the genomic DNA, according to the manufacturer's instructions. PCR was performed on a Mastercycler pro using the following parameters: initial denaturation at 94°C for 5 min; 30 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 1 min; with the final extension at 72°C for 10 min. Amplicons were analyzed using the DiversiLab system, which includes fragment separation using microfluidic chips and Agilent B2100 Bioanalyzer (Agilent Technologies Inc., USA). The DNA standard markers, which were applied for normalization of the sample runs, and Chip kit molecular weight ladders were used. Results were analyzed using the DiversiLab software (version 3.3), which uses the Pearson correlation coefficient to determine distance matrices and the unweighted pair group method with arithmetic averages (UPGMA) to create dendrograms.

Results and Discussion

Occurrence of *Enterococcus* spp. *Enterococcus* spp. was detected in 111 of the samples of pork meat products collected in

Table 1 Occurrence of *Enterococcus* spp. in retail pork meats products

Food	No. of samples, Positive/ Total (%)	Range of enterococci in positive samples (log CFU/g)		
		<3	3~5	5<
Minced pork meat	39/40 (97.5)	14	24	1
Marinated pork meat				
with *kochujang* (fermented hot pepper-soybean sauce)	29/34 (85.3)	22	7	0
with soy sauce	29/34 (85.3)	23	6	0
Frozen processed meat	14/16 (87.5)	11	3	0
Total	111/124 (89.5)	70	40	1

this study. The contamination of *Enterococcus* spp. was observed in 89.5% of the samples in the present study, whereas in a study undertaken by Kuhn et al. (2003), enterococci were found as contaminants in 77% of the samples, including human, food, animal, and water samples in an examination of 2,868 samples. In another study, 27.2% of 101 samples (chicken products, cooked pork, and cold turkey meat) were shown to be contaminated with enterococci (Robredo et al., 2000). In the present study, the samples most frequently shown to have contamination were those of minced pork meat products (97.5%) followed by frozen processed pork meat products (87.5%), marinated pork meat with soy sauce (85.3%), and marinated pork meat with *kochujang* (85.3%) (Table 1). The detection rate of enterococci in minced pork meat was higher than that in samples marinated with two types of sauce and higher than that in frozen pork meat products. Thirty six percent of the enterococci-positive minced pork meat was contaminated with *Enterococcus* spp. at less than 3 log CFU/g. On the contrary, most of the enterococci-positive samples of marinated pork meat with *kochujang*, marinated pork meat with soy sauce, and frozen processed meat were contaminated with *Enterococcus* spp. at below 3 log CFU/g. The differences in detection rate might be explained by several factors, including additives from *kochujang* and soy bean sauce, storage temperature and period before selling, and cross contamination from processing machines. The majority of *Enterococcus* spp. are able to grow from 10 to 45°C, in 6.5% sodium chloride, and at pH 9.6 (Hardie and Whiley, 1997). Further study is needed to elucidate the possible contamination route of enterococci and the influence of other factors on the survival of the organisms. Among enterococci isolated from the processed pork meat products *E. faecalis* was the most predominant isolate, and was identified in all four types of pork meat products.

Antibiotic resistance. The antibiotic resistances of 36 isolates of *E. faecalis* were analyzed (Table 2). The number and origin of *E. faecalis* isolates were as follows. Minced pork meat (n=19), marinated pork meat with soy sauce (n=4) and *kochujang* (n=5), and frozen processed meat products (n=8). The isolates of *E. faecalis* were resistant to tetracycline (58.3%), erythromycin (11.1%) and nitrofurantoin (2.8%), lower than the level of resistance to tetracycline of *E. faecalis* isolated from pigs in Korea (Jung, 2003). The main reason for the high resistance to tetracycline may be that this antimicrobial is the most commonly used in Korea as both a therapeutic and non-therapeutic antimicrobials in veterinary medicine. Tetracycline accounted for over 50% of the sales of antimicrobials for animals in Korea (Jung, 2003). In the present study, none of the strains were resistant to vancomycin. The use of the glycopeptide antibiotic avoparcin in animal husbandry as an antimicrobial growth promoter is reportedly associated with the appearance of vancomycin-resistant enterococci (VRE); the use of avoparcin was forbidden in Korea in 1997 (Seo et al., 2005). Banning avoparcin might be associated with the observed lack of VRE in the present study. Thirteen enterococci isolates were susceptible to all antimicrobials tested in this study. Most of the *E. faecalis* (n=20) isolated from processed pork meat products were resistant to single antibiotics (Table 3). The multiple antibiotic resistances were only observed in *E. faecalis* isolated from marinated pork meat products, which showed resistance against two antibiotics, i.e. tetracycline and erythromycin. The principal concern about enterococci in the food supply is the pathogenic potential of bacteria based on the horizontal transfer of genes associated with virulence and antibiotic resistance.

Table 2 Antibiotic resistance of *Enterococcus faecalis* against 11 antibiotics

	No. of isolates (%)		
	[a]R	I	S
Ciprofloxacin (CIP)	0 (0)	4 (11.1)	32 (88.9)
Erythromycin (E)	4 (11.1)	14 (38.9)	18 (50.0)
Linezolid (LNZ)	0 (0)	1 (2.8)	35 (97.2)
Nitrofurantoin (FT)	1 (2.8)	8 (22.2)	27 (75.0)
Teicoplanin (TEC)	0 (0)	0 (0)	36 (100)
Tetracycline (TE)	21 (58.3)	0 (0)	15 (41.7)
Tigecycline (TGC)	0 (0)	0 (0)	36 (100)
Vancomycin (VA)	0 (0)	0 (0)	36 (100)
Amoxycillin/clavulanic acid (AMC)	0 (0)	0 (0)	36 (100)
Ampicillin (AMP)	0 (0)	0 (0)	36 (100)
Chloramphenicol (CHA)	0 (0)	0 (0)	36 (100)

[a]R: resistant to antibiotics, I: intermediate resistant to antibiotics, and S: sensitive to antibiotics.
Total number of enterococci isolates subjected to antibiotics resistance test was 36.

Table 3 Multidrug resistance of *Enterococcus faecalis* isolated from processed pork meat products

Species (n)	Resistant antibiotics	Number of strains (%) for different types of pork meat products				
		Minced	Marinated with *kochujang* (fermented hot pepper-soybean paste)	Marinated with soy sauce	Frozen	Total
E. faecalis (36)	None	10 (25.0)	1 (2.8)	0 (0)	2 (5.6)	13 (33.3)
	[a]E	0 (0)	1 (2.8)	0 (0)	0 (0)	1 (2.8)
	FT	1 (2.8)	0 (0)	0 (0)	0 (0)	1 (2.8)
	TE	7 (19.4)	3 (8.3)	2 (5.6)	6 (16.7)	18 (50.0)
	TE, E	0 (0)	1 (2.8)	2 (5.6)	0 (0)	3 (8.3)

[a]E, Erythromycin; FT, Nitrofurantoin; TE, Tetracycline.

Enterococci acquire multiple antibiotic resistances from their efficient gene transfer mechanisms, which may also encode factors that are associated with virulence (Corpet, 1998). Whether or not these bacteria should be considered pathogens in food, the concern is that they can rapidly acquire plasmid-encoded genes for antibiotic resistance and virulence traits, and can become pathogenic (Franz et al., 1999). Therefore, enterococci isolates from food should be investigated for the presence of other potential virulence factors as well as for the source of horizontal spread of antimicrobial determinants to various pathogens. In addition, further studies should focus on determining possible differences among antibiotic resistance patterns and other potential virulence factors of food isolates as well as enterococci isolated from community and clinical sources.

Molecular typing by DiversiLab system. The rep-PCR technique, adapted to an automated format on the DiversiLab system, was used to analyze the molecular characteristics of *E. faecalis* at the strain level. The antimicrobial resistance and the source of enterococcal isolates analyzed for genotyping are shown in Table 3. Rep-PCR on the DiversiLab system assigned the 36 isolates of

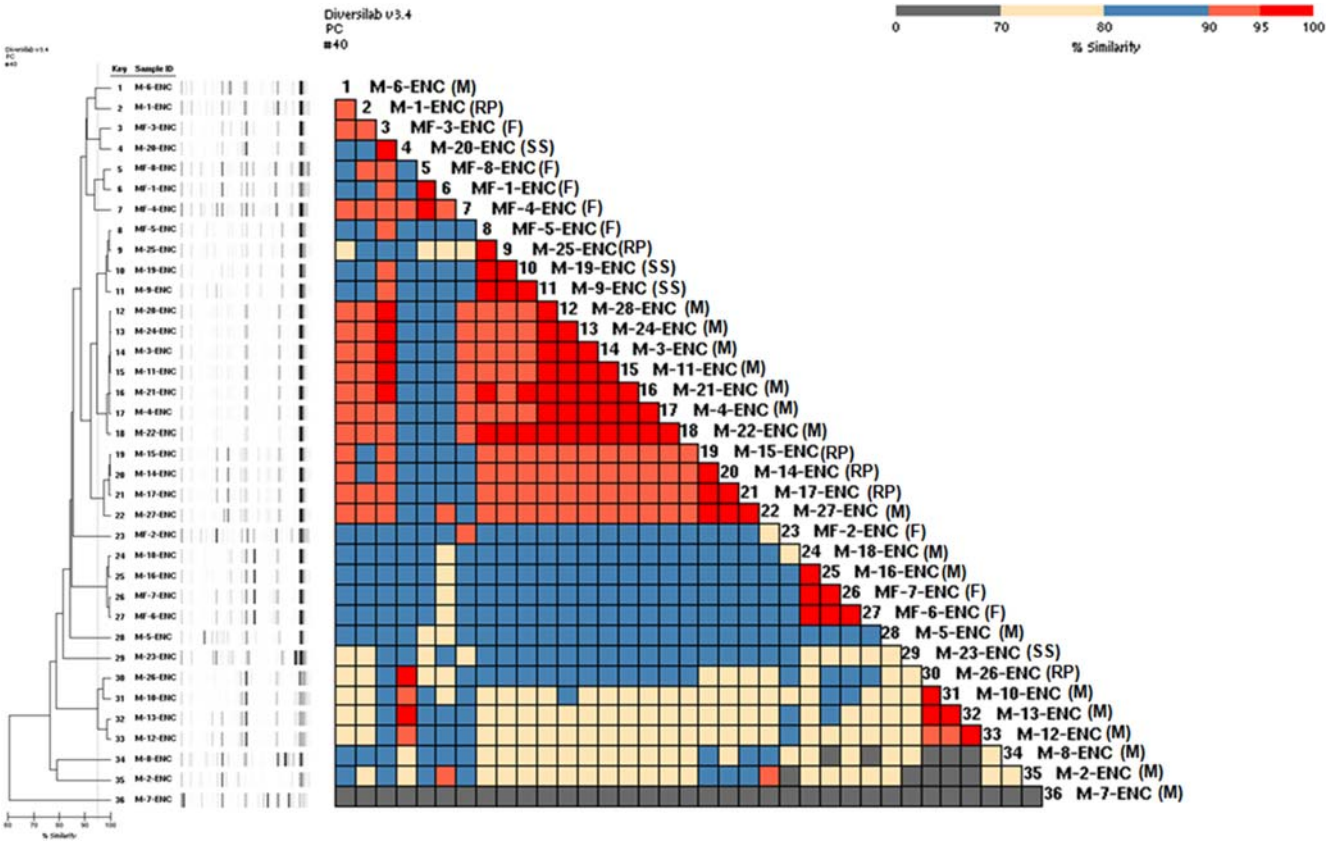

Fig. 1 PCR generated dendrogram, virtual gel image, and the similarity matrix of the 36 *E. faecalis* isolates typed with DiversiLab. Letters in parenthesis of similarity matrix indicate source of the isolates. M; minced pork meat, SS; marinated pork meat with soy sauce, RP; marinated pork meat with *kochujang* (fermented hot pepper-soybean paste), F; frozen processed pork meat products.

E. faecalis to seven clusters consisting of two or more isolates with a similarity >95%. In addition, nine isolates (No. 1, 2, 7, 23, 28, 29, 34, 35, and 36) remained ungrouped and appeared to represent unique genotypes (Fig. 1). The biggest cluster had seven isolates (No. 12–18) that originated from minced pork meat, suggesting that this clone might be circulated in minced pork meat products. Other clusters of *E. faecalis* were found to consist of isolates from various sample sources, such as marinated pork meat, frozen pork meat products, and minced pork meat.

In conclusion, we investigated the occurrence, antibiotic resistance profiles against 11 antimicrobials, and genotyping of *E. faecalis* in 124 retail pork meat samples including minced pork meat, marinated pork meat with soy sauce or *kochujang*, and frozen pork meat products in Korea. The isolates of *E. faecalis* (n =36) were resistant to tetracycline (58.3%), erythromycin (11.1%), and nitrofurantoin (2.8%). The isolates were susceptible to other antimicrobials. No VRE were observed in the present study. Most of the strains isolated from processed pork meat products were sensitive or single drug resistant. Multiple resistant strains against two antibiotics were observed for *E. faecalis* isolated from marinated pork meat products. As a result of the automated rep-PCR, which was used as an approach for genotyping enterococci, 7 out of 36 isolates of *E. faecalis* were assigned to one cluster with a similarity >95% and all the isolates were found to have originated from minced pork meat, suggesting that this clone might be circulated in minced pork meat products. Given the importance of the antimicrobial resistance of enterococci in food safety as well as in public health, our results on the level of contamination, antimicrobial resistance and genotyping could provide useful information to derive risk management options.

Acknowledgments This research was supported by a grant from Korea Food and Drug Administration in 2010 and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A1A2008740).

References

Barbosa J, Ferreira V, and Teixeira P(2009) Antibiotic susceptibility of enterococci isolated from traditional fermented meat products. *Food Microbiol* **26**, 527–32.

Bates J (1997) Epidemiology of vancomycin-resistant enterococci in the community and the relevance of farm animals to human infection. *J Hosp Infec* **37**, 89–101.

Baumgartner A, Kueffer M, and Rohner P (2001) Occurrence and antibiotic resistance of enterococci in various ready-to-eat foods. *Arch Lebens* **52**, 1–24.

Clinical and Laboratory Standards Institute (CLSI) (2010) Performance standards for antimicrobial susceptibility testing; Twentieth informational supplement. CLSI document M100-S20. Wayne, PA: Clinical and Laboratory Standards Institute, USA.

Corpet DE (1998) Antibiotic resistant bacteria in human food. *Rev Med Vet* **149**, 819–22.

Dutka-Malen SD, Evers S, and courvalin P (1995) Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J Clin Microbiol* **33**, 24–7.

Franz CMAP, Holzapfel WH, and Stiles ME (1999) Enterococci at the crossroads of food safety? *Int J Food Microbiol* **47**, 1–24.

Hardie JM and Whiley RA (1997) Classification and overview of the genera *Streptococcus* and *Enterococcus*. *J Appl Microbiol* **83**, 1–11S.

Hwang IY, Ku HO, Lim SK, Park CK, Jung GS, Jung SC et al. (2009) Species distribution and resistance patterns to growth-promoting antimicrobials of enterococci isolated from pigs and chickens in Korea. *J Vet Diagn Invest* **21**, 858–62.

International Organization for Standardization (IOS) (1998) ISO 7899-1: Water quality-Detection and enumeration of intestinal enterococci in surface and waste water. Part I: Miniaturized method (Most Probable Number) by inoculation in liquid medium. International Organization for Standardization, Switzerland.

Jung SC (2003) Establishment of control system of antibiotics for livestock. Report of Korea Food and Drug Administration Project. National Veterinary Research and Quarantine Service, Anyang, Korea.

Kariyama R, Mitsuhata R, Chow JW, Clewell DB, and Kumon H (2000) Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. *J Clin Microbiol* **38**, 3092–5.

Kühn I, Iversen A, Burman LG, Olsson-Liljequist B, Franklin A, Finn M et al. (2003) Comparison of enterococcal populations in animals, humans and the environment: An European study. *Int J Food Microbiol* **88**, 133–45.

Lee SJ, Lee DS, Choe HS, Shim BS, Kim CS, Kim ME et al. (2011) Antimicrobial resistance in community-acquired urinary tract infections: results from the Korean antimicrobial resistance monitoring system. *J Infect Chemother* **17**, 440–6.

Murray BE (1998) Diversity among multidrug-resistant enterococci. *Emerg Infect Dis* **4**, 37–46.

Quednau M, Ahrné S, Petersson AC, and Molin G (1998) Antibiotic-resistant strains of *Enterococcus* isolated from Swedish and Danish retailed chicken and pork. *J Appl Microbiol* **84**, 1163–70.

Robredo B, Singh KV, Baquero F, Murray BE, and Torres C (2000) Vancomycin resistant enterococci isolated from animals and food. *Int J Food Microbiol* **54**, 197–204.

Seo KS, Kim JY, Yoo HS, Bae WK, and Park YH (2005) Comparison of vancomycin-resistant enterococci isolates from human, poultry and pigs in Korea. *Vet Microbiol* **106**, 225–33.

Teuber M, Meile L, and Schwarz F (1999) Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie van Leeuwenhoek* **76**, 115–37.

Weiss A, Konrad JD, Kneifel W, and Mayer HK (2010) Evaluation of PCR-based typing methods for the identification of probiotic *Enterococcus faecium* strains from animal feeds. *Animal Feed Sci Technol* **158**, 187-196.

Weiss A, Domig KJ, and Kneifel W (2005) Comparison of selective media for the enumeration of probiotic enterococci from animal feed. *Food Technol Biotechnol* **43**, 147–55.

Woodford N and Levermore DM (2009) Infections caused by Gram-positive bacteria: a review of the global challenge. *J Infect* **59**, S4–16.