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Application of Multiplex PCR, Pulsed-Field Gel Electrophoresis (PFGE), and BOX-PCR for Molecular Analysis of Enterococci

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1. Introduction

Although it has been recovered from vegetation, soil, water, and food, *Enterococcus* is a ubiquitous Gram-positive bacterium found primarily in the intestine of nearly all animals (Giraffa 2002;Muller et al. 2001;Niemi et al. 1993;Svec and Sedlacek 1999). Different strains of enterococci populate the digestive tracts of humans and animals, making them a good indicator of water contamination (Svec and Sedlacek 1999). They are the second most studied group of bacteria in the field of microbial source tracking (following *Escherichia coli*) due to their connection to humans and animals as well as their recent significance as a clinical pathogen (Layton et al. 2010;Scott et al. 2005). The enterococci have been implicated in a number of clinical diseases including endocarditis, bacteremia, and urinary tract infections, most often in hospital settings (Huycke et al. 1998;Jett et al. 1994). They are a leading cause of nosocomial infections (hospital acquired infections), accounting for approximately 12% in the U.S. yearly; the majority of infections are caused by *Enterococcus faecalis* and *E. faecium* (Huycke et al. 1998). While their role as an opportunistic nosocomial pathogen has been well documented, their ability to cause food-borne illnesses still remains largely unknown. Their role in food processing can be desirable in some cases and unwanted in others. For example, they may be considered as beneficial because they harbor specific biochemical traits that are essential in manufacturing fermented milk products such as cheeses, but their presence can also indicate spoilage for fermented meats or unsanitary conditions in other food industries (Foulquie Moreno et al. 2006;Giraffa 2002). The production of biogenic amines in fermented foods by enterococci is also thought to result in food intoxication characterized by symptoms such as vomiting and headaches (Gardin et al. 2001;Giraffa 2002;Tham et al. 1990). In addition, because enterococci can potentially harbor antimicrobial resistance genes and genes which may

have a role in virulence, the presence of enterococci on foodstuffs is of concern especially since these enterococci may be passed to humans. Further complicating these issues is the ability of enterococci to transfer antimicrobial resistance genes and some virulence factors to other members of the intestinal microflora, as well as more pathogenic bacteria, increasing their threat as nosocomial pathogens (Chow et al. 1993; Hancock and Gilmore 2000; Murray 1990; Wirth 1994).

Enterococci are intrinsically more resistant than other bacteria to antimicrobial agents commonly used in hospitals (Facklam et al. 2002; Malani et al. 2002). The danger of enterococcal infections becomes more serious in light of increasing antimicrobial resistance, including resistance to multiple antibiotics and the possible transfer of resistance determinants to other bacterial genera (Huycke et al. 1998). Some enterococcal species, particularly *E. faecium*, are inherently resistant to some penicillins; and in the past few years, they have also shown increased resistance to vancomycin, cephalosporins, and aminoglycosides in nosocomial infections (Arias et al. 2010). Vancomycin and Synercid (quinupristin/dalfopristin) are often considered the last treatment available in serious, multi-drug resistant infections in humans (Boneca and Chiosis 2003;Marshall et al. 1998; Wilson et al. 1995). Because of their role in human infections and their potential for harboring antimicrobial resistance, it is important to identify genetic clones of enterococci. This has proven effective in clinical epidemiologic studies; however, genetic heterogeneity has been previously described for enterococci, particularly *E. faecium*, from both poultry and environmental sources (Jackson et al. 2004b;Jackson et al. 2006).

A number of DNA band-based molecular methods employing gel electrophoresis have been described for determining genetic relatedness between enterococci (Olive and Bean 1999). These methods vary in degree of difficulty of use, cost to perform, and level of discrimination. One of the first methods used was plasmid profiling (Hall et al. 1992;Lacoux et al. 1992). Although not technically challenging, restriction patterns of plasmids may be difficult to analyze due to varying amounts of plasmid DNA yields. Newer typing methods such as Multilocus Sequence Typing (MLST), Amplified Fragment Length Polymorphism (AFLP), and Multiple-locus Variable-Number Tandem Repeat Analysis (MLVA) may be more suitable for investigations of epidemiologically-related strains than source tracking (Bruinsma et al. 2002;Homan et al. 2002;Top et al. 2004). The same concern can also be applied to Pulsed-Field Gel Electrophoresis (PFGE), considered the gold standard for typing enterococci and, in particular, epidemiologically-related strains even though it may misrepresent isolates which are unrelated epidemiologically (Olive and Bean 1999). Other limitations of use of PFGE also include the time needed to complete the procedure and the costs associated with equipment and consumables necessary to perform PFGE. Another disadvantage of using PFGE analysis is its inability to separate very large DNA molecules. In comparison, PCR-based molecular typing methods are usually less complicated, cost less to perform, and have a shorter time from initiation to analysis. First described in *Streptococcus pneumoniae*, one commonly used PCR-based method, BOX-PCR, produces amplicons based upon repetitive sequences in the bacterial genome (Malathum et al. 1998;Olive and Bean 1999). Although based upon sequences in the *S. pneumoniae* genome, BOX primers have been used to discriminate many bacterial species including enterococcal isolates (Malathum et al. 1998;Olive and Bean 1999).

In our previous study, prevalence and antimicrobial resistance of enterococci isolated from retail fruits, vegetables, and meats collected from grocery store chains in the North Georgia USA area were evaluated (McGowan et al. 2006). *E. faecalis* isolates from that study were further characterized to determine if any association between antimicrobial resistance and virulence genes existed (McGowan-Spicer et al. 2008). Genetic analysis of *E. faecalis* from retail food items revealed that the isolates did not cluster according to retail store or year of isolation. The objective of the present study was to use band-based methods including BOX-PCR and PFGE to determine if genetically related enterococci were found among different stores, food types, or years.

2. Materials and methods

2.1 Sample collection, isolation, and identification of enterococci

Enterococci used in this study were collected and described in previous studies (McGowan et al. 2006;McGowan-Spicer et al. 2008). During 2000-2001, fresh fruits and vegetables and pre-packaged ground and whole meat were purchased from six retail food stores in the Athens, GA area. Approximately two pounds of each food product were purchased and each food was collected to ensure that cross-contamination from lab personnel to the product did not occur. Foods were bagged separately and kept refrigerated until processed. Fruits and vegetables were placed in a sterile bag to which 50 ml of phosphate buffered saline (PBS, 1X) was added. One hundred mls of PBS was added to each ground or whole meat sample bag. Bags were vigorously shaken for two minutes to remove bacteria from the surface. One ml of each rinsate was then transferred to nine mls of Enterococcosel Broth (Becton Dickinson, Sparks, MD) and incubated for 24 h at 37°C. A swab was used to transfer broth from positive cultures to Enterococcosel Agar (Becton Dickinson, Sparks, MD) for isolation of enterococci. Plates were incubated overnight at 37oC. Ten food samples were randomly chosen and direct plated onto CHROMagar™ Orientation Rodac plates (Hardy Diagnostics, Santa Maria, CA). The agar plates were "stamped" directly onto meat, packaging, or fruit/vegetable surfaces and incubated at 37°C for 24 h. Positive isolates on CHROMagar™ were blue to teal blue in color. One presumptive positive colony from Enterococcosel Agar and presumptive positive colonies of each color from CHROMagar™ were plated to blood agar, and the resulting clones were identified to enterococcal genus and species using multiplex PCR as previously described (Jackson et al. 2004a) with the following modification. PCR multiplex Group 3 consisted of *E. dispar*, *E. pseudoavium*, *E. saccharolyticus*, and *E. raffinosus* while multiplex Group 6 consisted of *E. cecorum* and *E. hirae* only. *E. raffinosus* multiplexing primers were subsequently moved to multiplex Group 6 in the final enterococcal multiplexing groups (Table 1). Typical results of the PCR multiplex are shown in Figure 1.

2.2 Antimicrobial susceptibility

Minimum inhibitory concentrations (MICS, µg/ml) for enterococci were determined by broth microdilution using the Sensititre semi-automated antimicrobial susceptibility system (Trek Diagnostic Systems, Inc., Cleveland, OH) and the Sensititre Gram-Positive Custom Plate CMV1AGPF according to the manufacturer's directions. Results were

interpreted according to CLSI (Clinical and Laboratory Standards Institute) guidelines when defined (Clinical and Laboratory Standards Institute (CLSI) 2006;Clinical and Laboratory Standards Institute (CLSI) 2007). Breakpoints for bacitracin, flavomycin, gentamicin, kanamycin, lincomycin, salinomycin, streptomycin, and tylosin were those defined by National Antimicrobial Resistance Monitoring System (NARMS) (http://www.ars.usda.gov/Main/docs.htm?docid=6750&page=3). Antimicrobials and breakpoints were: bacitracin ($\geq$128 µg/ml), chloramphenicol ($\geq$32 µg/ml), ciprofloxacin ($\geq$4 µg/ml), erythromycin ($\geq$8 µg/ml), flavomycin ($\geq$16 µg/ml), gentamicin ($\geq$500 µg/ml), kanamycin ($\geq$500 µg/ml), lincomycin ($\geq$4 µg/ml), linezolid ($\geq$8 µg/ml), nitrofurantoin ($\geq$128 µg/ml), penicillin ($\geq$16 µg/ml), salinomycin ($\geq$16 µg/ml), streptomycin ($\geq$1000 µg/ml), quinupristin/dalfopristin ($\geq$4 µg/ml), tetracycline ($\geq$16 µg/ml), tylosin ($\geq$20 µg/ml), and vancomycin ($\geq$32 µg/ml). *E. faecalis* ATCC 29212, *E. faecalis* ATCC 51299, *Staphylococcus aureus* ATCC 29213, and *Escherichia coli* ATCC 25922 were quality controls for determination of MIC.

Group 1	Sequence (5'-3')	Size (bp)	Group 2	Sequence (5'-3')	Size (bp)
E. faecalis ATCC 19433	ACTTATGTGACTAACTTAACC TAATGGTGAATCTTGGTTTGG	360	*E. solitarius* ATCC 49428	AAACACCATAACACTTATGTGACG AATGGAGAATCTTGGTTTGGCGTC	371
E. durans ATCC 19432	CCTACTGATATTAAGACAGCG TAATCCTAAGATAGGTGTTTG	295	*E. casseliflavus* ATCC 25788	TCCTGAATTAGGTGAAAAAAC GCTAGTTTACCGTCTTTAACG	288
E. faecium ATCC 19434	GAAAAAACAATAGAAGAATTAT TGCTTTTTTGAATTCTTCTTTA	215	*E. gallinarum* ATCC 49673	TTACTTGCTGATTTTTGATTCG TGAATTCTTCTTTGAAATCAG	173
E. malodoratus ATCC 43197	GTAACGAACTTGAATGAAGTG TTGATCGCACCTGTTGGTTTT	134			

Group 3		Size (bp)	Group 4		Size (bp)
E. saccharolyticus ATCC 43076	AAACACCATAACACTTATGTG GTAGAAGTCACTTCTAATAAC	371	*E. flavescens* ATCC 49996	GAATTAGGTGAAAAAAAAGTT GCTAGTTTACCGTCTTTAACG	284
E. dispar ATCC 51266	GAACTAGCAGAAAAAAGTGTG GATAATTTACCGTTATTTACC	284	*E. sulfureus* ATCC 49903	TCAGTGGAAGACTTAATCGCA CCAAATGTATCTTCGATCGCT	173
E. pseudoavium ATCC 49372	TCTGTTGAGGATTTAGTTGCA CCGAAAGCTTCGTCAATGGCG	173	*E. mundtii* ATCC 43186	CAGACATGGATGCTATTCCATCT GCCATGATTTTCCAGAAGAAT	98
E. raffinosus ATCC 49427	GTCACGAACTTGAATGAAGTT AATGGGCTATCTTGATTCGCG	287[a]			

Group 5		Size (bp)	Group 6		Size (bp)
E. avium ATCC 14025	GCTGCGATTGAAAAATATCCG AAGCCAATGATCGGTGTTTTT	368	*E. cecorum* ATCC 43198	AAACATCATAAAACCTATTTA AATGGTGAATCTTGGTTCGCA	371
E. columbae ATCC 51263	GAATTTGGTACCAAGACAGTT GCTAATTTACCGTTATCGACT	284	*E. hirae* ATCC 8043	CTTTCTGATATGGATGCTGTC TAAATTCTTCCTTAAATGTTG	187
E. seriolicida ATCC 49156	ACACAATGTTCTGGGAATGGC AAGTCGTCAAATGAACCAAAA	100			

Table 1. PCR multiplex groups and band sizes for each enterococcal species (Jackson et al. 2004a). [a]Revised primers. Original primers produced a PCR product of 98 bp (this study). Original primers were redesigned to produce a product of 287 bp for *E. raffinosus*. *E. raffinosus* was moved from multiplex group 3 to multiplex group 6. For each species, the top primer listed is the forward primer and the bottom primer listed is the reverse primer.

2.3 Plasmid extraction

Plasmids were extracted using alkaline lysis as previously described (Uttley et al. 1989;Woodford et al. 1993) with minor modifications. A 5 ml culture was grown overnight in BHI broth (Becton Dickinson, Sparks, MD) at 37oC. Cells from the culture were pelleted, resuspended in TE buffer containing sucrose (10 mM Tris, 1 mM EDTA, 25% sucrose, pH 8) and 1 mg/ml lysozyme and incubated for 1 h at 37oC. Cells were lysed by adding 0.2M NaOH, 1% SDS and incubating for 30 min at 37oC followed by the addition of 3M potassium acetate (pH 4.8) and incubation on ice for 15 min. Cell debris was removed by successive phenol chloroform extractions; DNA was precipitated using 100% ethanol. Plasmid DNA was resuspended in TE buffer and stored at -20oC until use.
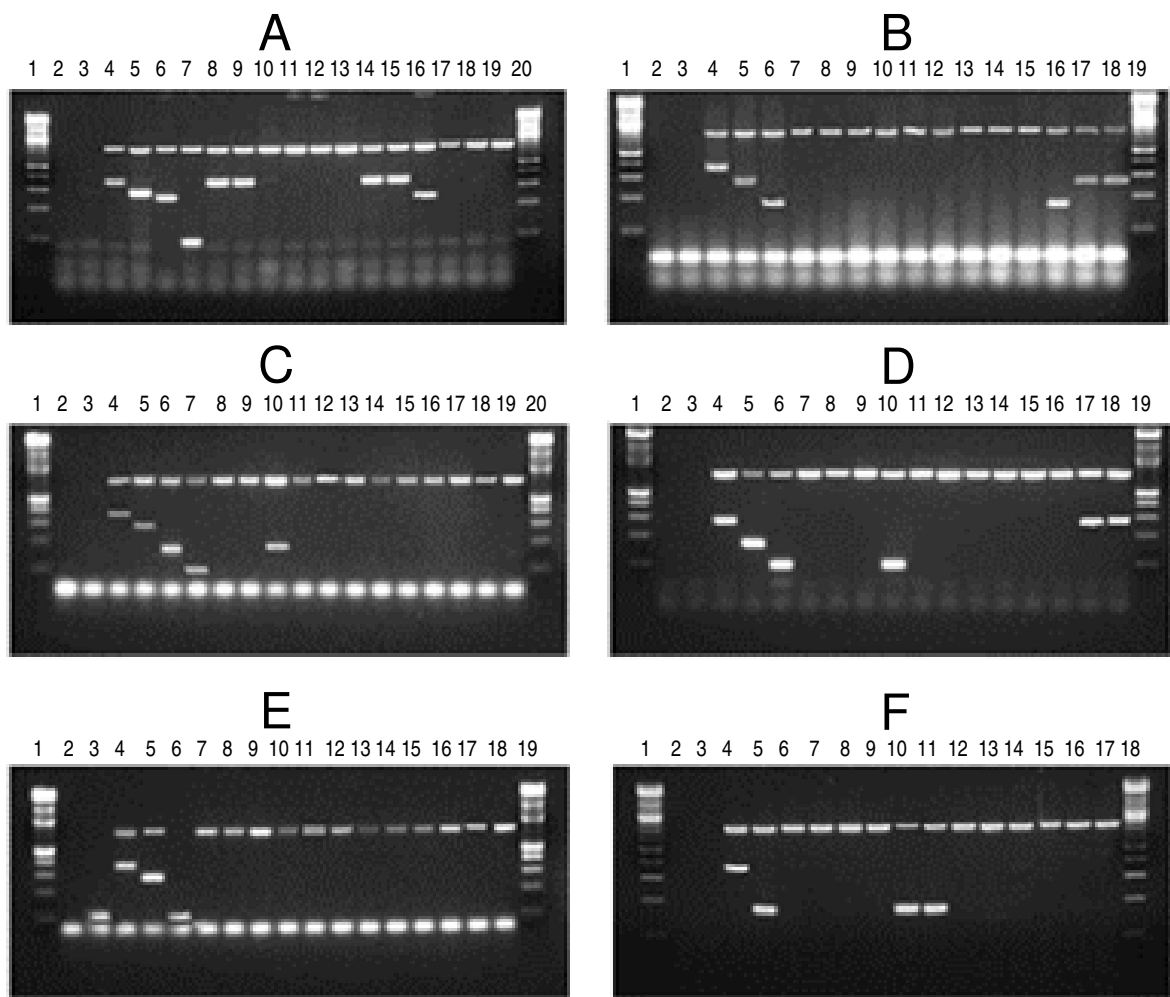
Fig. 1. *Enterococcus* genus and species multiplex PCR. First and last lanes on each panel are 100 bp molecular weight markers; Lane 2, sterile ddH₂O (no DNA control); Lane 3, *Lactococcus garvieae* ATCC 43921. Panel A: Lane 4, *Enterococcus faecalis* ATCC 19433; Lane 5, *E. durans* ATCC 19432; Lane 6, *E. faecium* ATCC 19434; Lane 7, *E. malodoratus* ATCC 43197; Panel B: Lane 4, *E. solitarius* ATCC 49428; Lane 5, *E. casseliflavus* ATCC 25788; Lane 6, *E. gallinarum* ATCC 49673; Panel C: Lane 4, *E. saccharolyticus* ATCC 43076; Lane 5, *E. dispar* ATCC 51266; Lane 6, *E. pseudoavium* ATCC 49372; Lane 7, *E. raffinosus* ATCC 49427; Panel D: Lane 4, *E. flavescens* ATCC 49996; Lane 5, *E. sulfureus* ATCC 49903; Lane 6, *E. mundtii* ATCC 43186. Panel E: Lane 4, *E. avium* ATCC 14025; Lane 5, *E. columbae* ATCC 51263; Lane 6, *E. seriolicida* ATCC 49156; and Panel F: Lane 4, *E. cecorum* ATCC 43198; Lane 5, *E. hirae* ATCC 8043. Remaining lanes are unidentified enterococcal isolates in the following order on each gel: A17, A19, B1, B2, B17, B21, D16, D17, C-O30, E16, E-O12, and E-O12a. The top band in control and unknown enterococcal lanes is the *Enterococcus* genus band; other bands represent specific enterococcal species.

Plasmid DNA was also extracted using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) according to manufacturer's directions with the following modifications. A 5 ml overnight culture was pelleted, resuspended in 250 µl of Buffer P1 containing lysozyme (5 mg/ml), and incubated at 37°C for 10 min. Proteinase K (100 µl, 20 mg/ml) was added and incubation continued for 10 min at 50°C. Cells were lysed and plasmid DNA was purified according to manufacturer's protocols. Plasmid DNA was also purified using phenol

chloroform extractions described in the alkaline lysis protocol above. After cell lysis using the QIAprep Spin Miniprep kit, phenol chloroform (equal volume) was added to the supernatant. Following a second phenol chloroform extraction, DNA was precipitated using 100% ethanol. Plasmid DNA was resuspended in TE buffer and stored at -20°C until use as described above. Ten microliters of product was electrophoresed on a 0.8% 1 X TAE agarose gel at 90 V. Supercoiled DNA ladder (Invitrogen, Carlsbad, CA) was used as the standard.
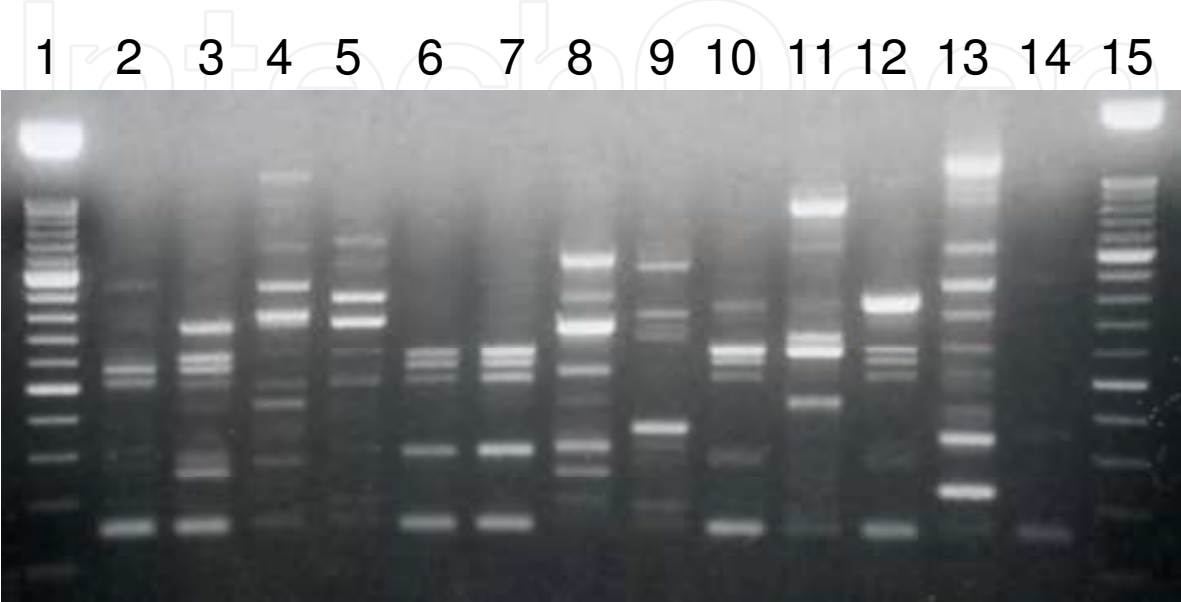

Fig. 2. BOX-PCR of enterococci. First and last lanes are 100 kb ladder. Lane 13, *Enterococcus durans*; Lane 14, sterile ddH$_2$O (no DNA control). Lanes 2-12 are various enterococcal species tested using the BOX A2R primer.
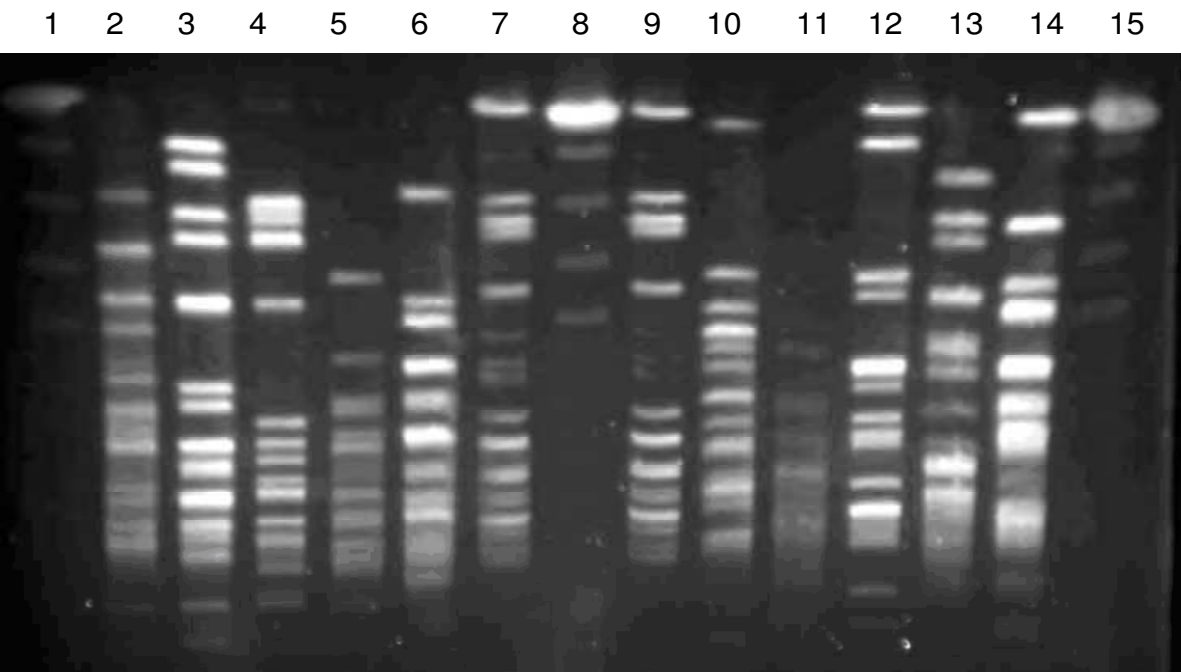

Fig. 3. PFGE of enterococci. Lanes 1, 8, and 15 are *Saccharomyces cerevisiae* standards. All other lanes are various enterococcal isolates. Enterococcal DNA was digested using *Sma*I.

2.4 BOX-PCR

The protocol for BOX-PCR by van Belkum and Hermans (van Belkum and Hermans 2001) was performed as described with the following modifications. A master mix was prepared in sterile microcentrifuge tubes containing 46 μl sterile ddH$_2$O, 20 μl 20 mM MgCl$_2$ containing ficol and tartrazine (Idaho Technologies, Salt Lake City, UT), 10 μl 1:10 DMSO (Sigma, St. Louis, MO), 10 μl BOX A2R primer (1.25 mM), 2 μl of a 10 mM dNTP, and 2 μl *Taq* DNA polymerase (Roche, Indianapolis, IN). Using a 96-well microtiter plate, 9 μl of the master mix and 1 μl of each sample (samples: 16 isolates, 1 *E. durans* ATCC 19432 positive control, and 1 sterile ddH$_2$O negative control) were added to the corresponding wells of a 96-well microtiter plate. Each master mix sample was drawn up into a 10 μl thin-walled capillary tube (Idaho Technologies) and each end of the tube was sealed using a butane torch. Tubes were placed in a Rapid Cycler (Idaho Technologies) and fragments amplified using the following parameters: (1) hold at 95^{0}C for 7 minutes; (2) cycle at 90^{0}C for 1 second; (3) 40^{0}C for 7 seconds; (4) 65^{0}C for 59 seconds; (5) repeat for 35 cycles with slope of 6.0; (6) hold at 65^{0}C for 16 minutes. Ten microliters of product was electrophoresed on a 1.5% 1 X TAE agarose gel containing ethidium bromide. Electrophoretic separation was at 100 V for 85 min. DNA molecular weight marker XVII (500 bp, Roche) was used as the standard. An example of typical BOX-PCR results using enterococcal DNA is shown in Figure 2.

2.5 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis was performed as previously described (Turabelidze et al. 2000). Briefly, cells from a 5 ml overnight culture were pelleted, embedded in agarose plugs and lysed. Plugs were digested overnight with 20 U of *Sma*I (Roche, Indianapolis, IN) and digested DNA separated on a 1.2% SeaKem agarose gel using a CHEF-DRII pulsed-field electrophoresis system (Bio-Rad, Hercules, CA). Electrophoresis was carried out at 6V for 21 h with a ramped pulse time of 5 to 30 s in 0.5X Tris-borate-EDTA (TBE) buffer (14°C). *Saccharomyces cerevisiae* (BioWhittaker Molecular Applications (BMA), Rockland, ME) was used as the standard on each end and center as a marker, and *E. faecalis* JH2-2 as a positive control. An example of some PFGE patterns obtained using enterococcal DNA is shown in Figure 3.

2.6 Data analysis

Cluster analysis of BOX-PCR and PFGE results was determined using BioNumerics software program (Applied Maths, Sint-Martens-Latem, Belgium) using Dice coefficient and the unweighted pair group method (UPGMA). Optimization settings for both BOX-PCR and PFGE dendrograms were 1.06% and a band tolerance of 1%.

Using the Statistical Analysis Software (SAS) System (SAS Institute, Inc., Cary, NC), data from the antimicrobial susceptibility testing and isolate characterization were compared to see if any trends were apparent. Comparisons were made between stores, years, food types (meats versus vegetables and fruits), and species (*E. faecalis* and *E. casseliflavus*, the two most predominant species identified). Probability values of statistical significance were generated using Chi-square analysis. Statistical significance was defined as a probability value of less than or equal to 0.05 (P $\leq$0.05). Chi-square p-values between 0.05 – 0.1 indicated a possible significant difference.

To relate the data gathered from antimicrobial susceptibility testing to the dendrograms created using BioNumerics, Sigma Plot (Version 8.02, Sigma Plot Scientific Software, Chicago, IL) was used. Isolates in certain clusters in each dendrogram may also share common antimicrobial susceptibility patterns. Antimicrobial susceptibility data for isolates in clusters showing ≥ 75% homology were grouped together. Only data for those antimicrobials showing significant differences and only clusters containing five or more isolates were analyzed. Another table showing actual MIC values for each antimicrobial for each isolate was created and loaded into the program. The newly organized data was analyzed using Sigma Plot to create scatter plots, which were then compared to each corresponding cluster.

3. Results

3.1 PCR analysis of *Enterococcus* isolates

A total of 111 isolates for year 2000 were tested in this study. Seventy-five isolates were collected from fruits and vegetables and 36 isolates were collected from meats. Of the vegetable/fruit isolates, 40 (53%) were identified as *E. casseliflavus*, 17 (23%) were identified as *E. faecalis*, 10 (13%) were identified as *E. mundtii*, 2 (3%) isolates each of *E. flavescens* and *E. hirae* were identified along with 1 isolate (1%) each of *E. gallinarum*, *E. durans*, and *E. pseudoavium*; 1 (1%) remained unidentified by PCR (Figure 4). From meat samples, *E. faecalis* represented the largest number of isolates (n=28; 78%); 3 (8%) *E. faecium* isolates were identified, 2 isolates (6%) were identified as *E. hirae* and 1 isolate (3%) each of *E. gallinarum* and *E. durans* were identified. One (3%) isolate remained unidentified (Figure 4).
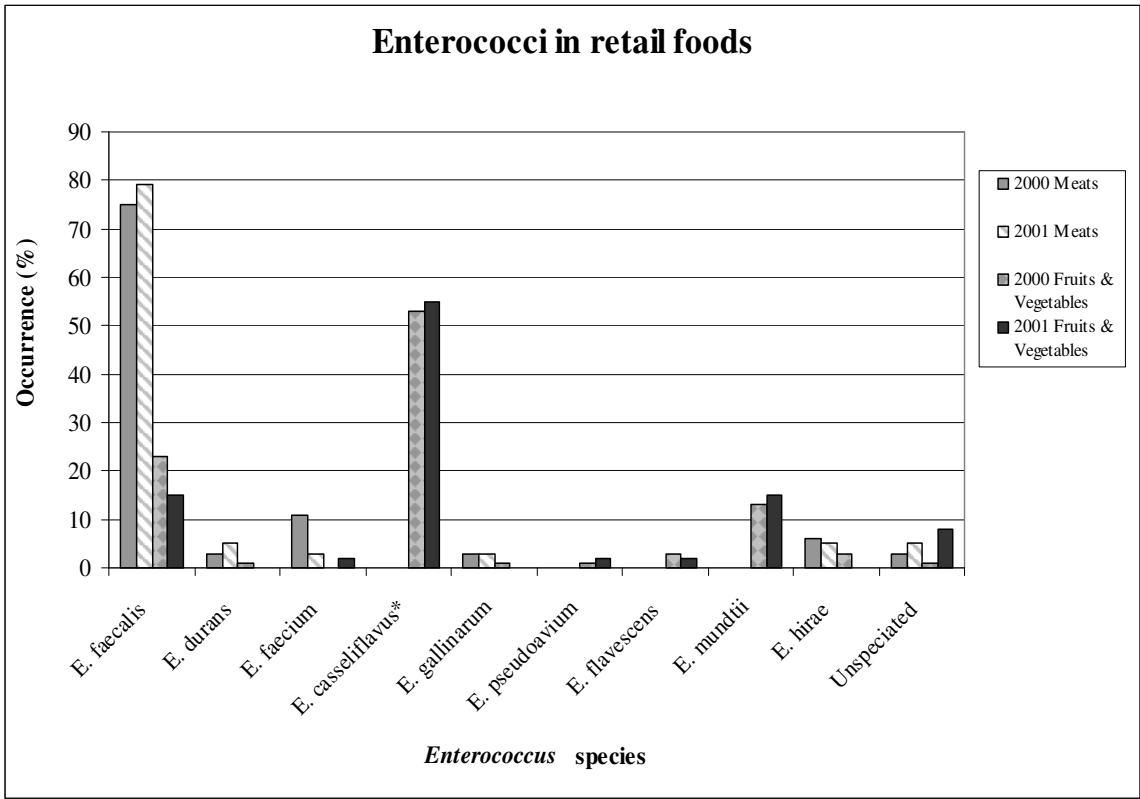

Fig. 4. Distribution of *Enterococcus* species separated by food type.

In 2001, a total of 78 isolates were tested, 40 from fruits and vegetables and 38 from meats. Of the vegetable/fruit isolates 22 (55%) speciated as *E. casseliflavus*, 6 (15%) speciated as *E. faecalis*, 6 (15%) were *E. mundtii*; 3 (8%) remained unidentified by PCR; 1 each (2%) were identified as *E. flavescens*, *E. pseudoavium*, and *E. faecium*. A total of 38 isolates were collected from a variety of meat samples in the different stores in 2001. *E. faecalis* represented 30 (79%) of these isolates; 2 (5%) isolates each of *E. durans* and *E. hirae* were identified; 2 (5%) remained unidentified by PCR; and 1 (3%) isolate each of *E. faecium* and *E. gallinarum* were identified. The distribution of species by food type is shown in Figure 4.

Seven total isolates remained unidentified by PCR. Bands appearing in the appropriate size range for the *Enterococcus* genus band were evident among all seven isolates, indicating they were *Enterococcus* sp. New species are being incorporated into the PCR procedure and these may be among them.

3.2 Antimicrobial susceptibility

All isolates were subjected to antimicrobial susceptibility testing to varying concentrations of 17 antimicrobials. Two isolates from each year did not grow in the Sensititre plates and were excluded from the results. One isolate from the 2000 set that did not grow in the Sensititre plates was identified as *E. casseliflavus*, while the other isolate from 2000 and both isolates from the 2001 set that did not grow in the Sensititre plates remained unidentified. Figure 5 summarizes the overall antimicrobial resistance results for each year by store.

High levels of resistance were seen among isolates from both years for lincomycin (90-98%) and bacitracin (69-95%). Low levels of resistance were found with chloramphenicol (3-15%), nitrofurantoin (2.5-8%), penicillin (0-3%), and salinomycin (0-3%). Varying ranges of resistance were observed with the other antimicrobials. Vegetable and fruit isolates typically showed higher rates of resistance to ciprofloxacin and flavomycin. Alternatively, meat isolates had higher resistance rates for erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, and tylosin. When comparing levels for quinupristin/dalfopristin resistance, higher levels of resistance were seen among meat isolates for both years. Resistance ranged from 45-58% for vegetable isolates, whereas resistance for meat isolates ranged from 83-91%. Although no isolates were resistant to linezolid or vancomycin, intermediate resistance was higher among vegetable and fruit isolates and were more often *E. casseliflavus* (data not shown). Intermediate resistance levels for fruits and vegetables ranged from 27.5-31% and 35-49% for linezolid and vancomycin, respectively, compared to 11-14% and 3-9% for meat isolates, respectively.

3.3 Plasmid analysis

Plasmids were extracted from enterococci isolated from retail foods using two methods. The alkaline lysis procedure was based on the classic plasmid extraction protocols which used alkaline solutions to segregate plasmid DNA from chromosomal DNA (Uttley et al. 1989;Woodford et al. 1993). Although it has a number of steps, alkaline lysis produced plasmid bands with reduced smearing with nearly every isolate tested (Figure 6, Panel A).
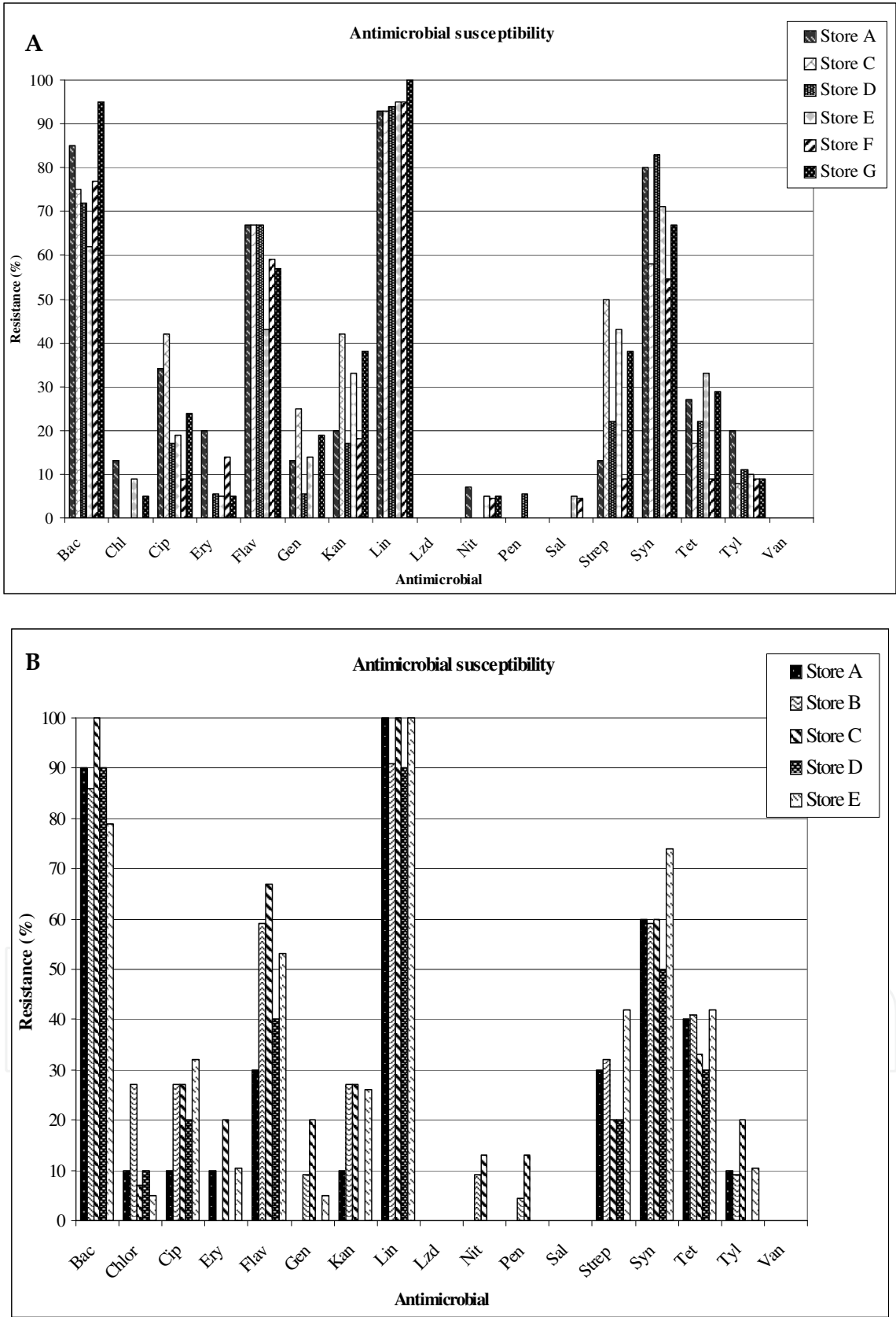
Fig. 5. Antimicrobial resistance data for all isolates tested during 2000 (Panel A) and 2001 (Panel B) grouped by store.
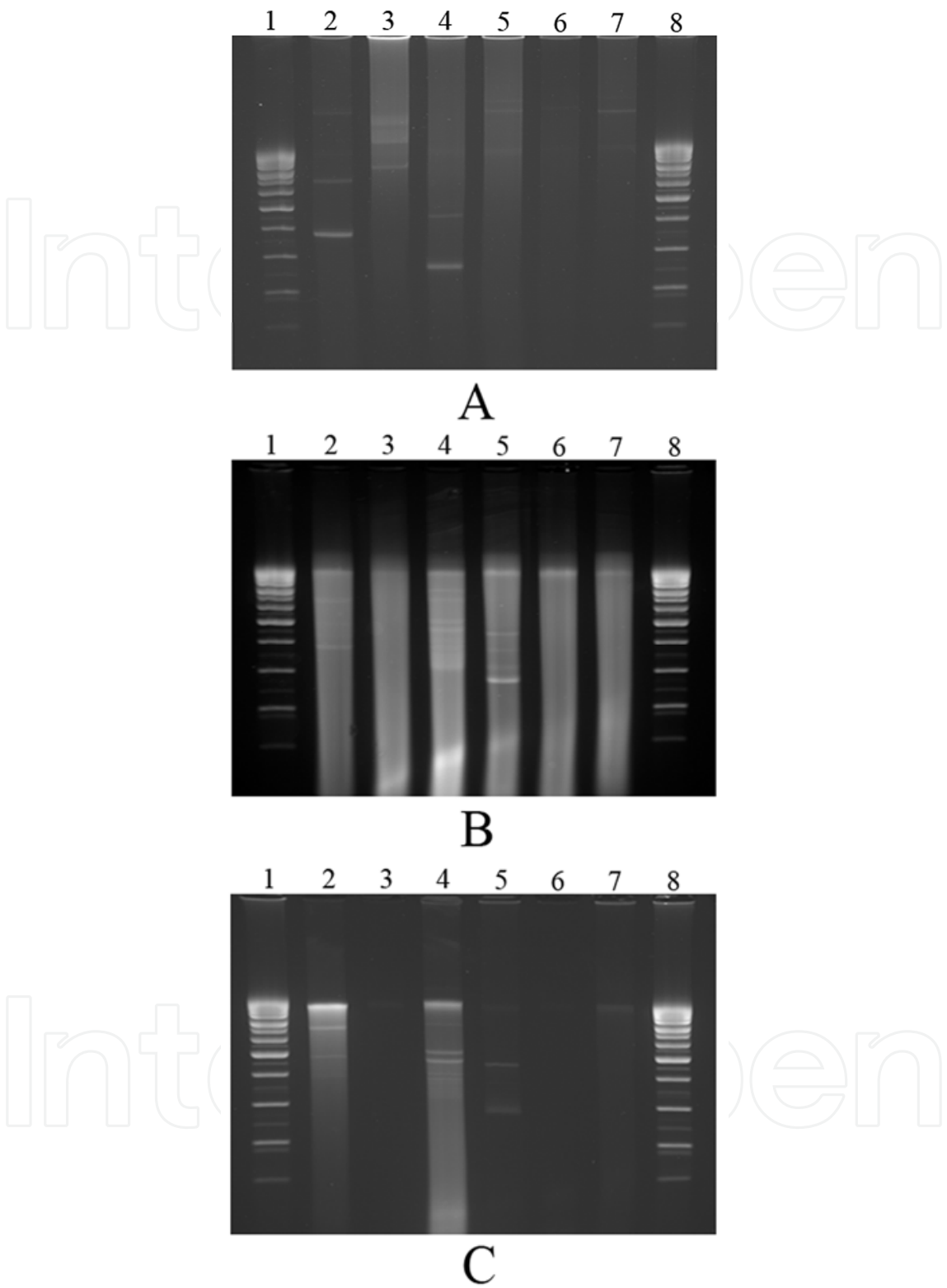
Fig. 6. Plasmids from enterococci from retail food using different extraction methods. Panel A, alkaline lysis; Panel B, Qiagen miniprep; Panel C, Qiagen miniprep with phenol chloroform extraction. First and last lanes on each panel are supercoiled DNA ladder; Lane 2, *E. gallinarum* ARS 9402; Lane 3, *E. casseliflavus* (radish); Lane 4, *E. faecalis* (beef); Lane 5, *E. faecium* (pork); Lane 6, *E. hirae* (beef); and Lane 7, *E. mundtii* (salad).

Alternatively, while the modified Qiagen miniprep procedure was rapid, the quality of the resulting plasmids was inferior to that of the alkaline lysis procedure. Smearing and difficulty in distinguishing plasmid bands from background was difficult using the Qiagen miniprep (Figure 6, Panel B). Background and smearing for some enterococcal isolates was reduced when the cells were lysed using the Qiagen miniprep kit followed by successive phenol chloroform extractions although no clear bands were observed for isolates in lanes 3 and 6 (Figure 6, Panel C). Interestingly, even though the samples were processed the same day from enterococcal cultures, the plasmid profiles were not identical (Figure 6).

3.4 BOX-PCR analysis

Overall, there were two main groups or clusters identified by BOX-PCR dendrograms for each year (Figure 7 and 8). One cluster contained mostly *E. faecalis* and one was mostly comprised of *E. casseliflavus*. Each of the dendrograms showed, in a few cases, species outside their respective clusters, i.e. a few *E. casseliflavus* isolates were identified as more closely related to *E. faecalis* isolates and vice versa. This was seen in both years. For example, isolate F35 was identified as *E. faecalis*, but according to BOX-PCR results, it is more related genetically to F31, which was identified as *E. casseliflavus*. Also, in the same dendrogram, isolates A37, A33, and E40 all were identified as *E. casseliflavus*, but BOX-PCR results classified them as more closely related to other *E. faecalis* isolates.

For the year 2000 isolates (Figure 7), 17 sets of identical clones were found, meaning BioNumerics identified 100% homology in banding patterns between two or more isolates given the parameters. In a few cases, three or even four isolates were genetically identical to each other. Nine of the 17 sets included isolates from different stores. Interestingly, two of those nine sets also included isolates from different food types (F40 was isolated from a potato while G29 was isolated from ground beef, and E34 was from a red potato while A28, A29, and G26 were from various meats). One set of identical banding patterns among isolates from different stores consisted of an *E. faecalis* isolated from a white potato and an *E. casseliflavus* isolated from a red potato (A33 and G41). Ten other sets of the 17 with identical banding patterns also consisted of isolates from different food types. For example, G10 came from a tomato while F30 came from a red potato. Fourteen additional sets of isolates were ~95% homologous in banding pattern, with only one of those clusters containing isolates of different species from the same food source (C37 and E36, identified as *E. casseliflavus* and *E. mundtii*, respectively, both from red potatoes). Of note is one of those 14 clusters which contained a set of four isolates (A20, A34, A36, and A35) with identical banding patterns exhibiting 95% homology in banding patterns with a separate set of two identical isolates (G10, F30). The set of four were isolated from the same store from two different food sources (alfalfa sprouts and red potatoes). The set of two identical isolates were from different stores and food types (tomato and red potato).

Fewer sets of isolates with identical banding patterns were found with the BOX-PCR for the year 2001 isolates; only five compared to 17 from 2000 (Figure 7 and 8). Three of these sets consisted of isolates from different stores and different food sources. Isolates from one of these sets, both identified as *E. faecalis*, came from completely different stores and food types (A22 from ground beef and B16 from a tomato). Isolates A4 and E-O12, both classified as

identical *E. casseliflavus* isolates, were isolated from different food types (white potato and alfalfa sprout, respectively). Similar results were noted for A23 and E21, both characterized as identical *E. faecalis* samples, but isolated from whole pork and ground beef, respectively. Four additional sets of isolates exhibited ≥ 95% genetic homology. Three of these consisted of isolates from different stores. Only one of the sets also had isolates of different species from different food types (B9 and E16), while two others (A2 and E1, as well as B22 and E21 rodac) were isolated from different food sources.

3.5 PFGE analysis

In dendrograms from the PFGE results, more clusters were evident, indicating more genetic variability. As with the BOX-PCR dendrograms, two large clusters were present, for *E. faecalis* and *E. casseliflavus*. For the year 2000 isolates (Figure 9), eight sets of isolates exhibited identical banding patterns. Five of the sets were isolated from different food sources. Of these, four sets contained isolates from different stores and different food sources: A28 and G26, both identified as *E. faecalis,* were isolated from turkey and chicken, respectively; E18 and G34, both *E. casseliflavus*, were isolated from a radish and a red potato, respectively; G20 and F37, both *E. casseliflavus*, were isolated from alfalfa sprouts and a red potato, respectively; and A20 and C1, identified as *E. casseliflavus*, were isolated from alfalfa sprouts and an apple. None of the sets contained isolates of different species. Twenty sets of isolates were ≥ 95% genetically homologous. Fourteen of these sets contained isolates from different stores, most of these occurring in the *E. casseliflavus* cluster. Of the 14 sets, only two contained isolates of different species. Two of the 14 sets also contained isolates of the same species, from different stores and/or food types (D27 and G32; E32, E31, and E34).

For the year 2001 isolates (Figure 10), only two sets of identical banding patterns were produced. Both sets were isolated from the same stores and were identified as the same species. Eleven more sets of isolates showed ≥ 95% genetic homology in the PFGE dendrogram. Two of these sets were isolated from different stores and are among the unidentified enterococci. Five of the sets were isolated from different stores, but were identified as being the same species. Two of the sets, isolated from different stores, were identified as different species (B11 and E11; B17 and E-O4). One set, isolated from the same store, but from different foods, was identified as different species (C-O12 and C-10). Of note were three isolates from the 2000 set (C42, D26, and D31) that were unable to be added to the PFGE dendrogram. They were identified as *E. mundtii*, *E. faecalis*, and *E. faecalis*, respectively, but produced unclear bands from PFGE analysis.

3.6 Comparison between BOX-PCR and PFGE

One aspect of this study was to determine if isolates grouped by BOX-PCR would remain in the same groupings using PFGE analysis. For year 2000 isolates, 17 sets of identical clones were identified using BOX-PCR, whereas only eight sets were observed using PFGE. For year 2001 isolates, five sets of isolates exhibiting identical banding patterns were seen with BOX-PCR, compared with only two by PFGE. These results were expected, as PFGE is more discriminatory. More bands were produced with *Sma*I digestion of isolates in PFGE, so more variations among banding patterns were possible.

When comparing the two procedures, differences in the percent similarity was observed. For example, one cluster in the 2000 BOX-PCR dendrogram, A16, E20, E19, E21, and F33, exhibited ~90% similarity between the isolates (Figure 7). For these same isolates in the PFGE dendrogram, there was only ~70% similarity (Figure 9). Isolates G32, G19, and G17 exhibited 100% similarity in the BOX-PCR dendrogram, but only ~86% similarity using PFGE. Alternatively, when comparing isolates such as A33 (*E. casseliflavus*) and G41 (*E. faecalis*), which showed 100% similarity with BOX-PCR, with PFGE, only 73% similarity was found between the isolates. Similar results can be seen when comparing dendrograms from 2001 isolates. In the BOX-PCR dendrogram, isolates A23, E21, E17 (rodac), and E18 exhibited ~90% homology (Figure 8). In the PFGE dendrogram, these same isolates were only ~76% homologous (Figure 10).

Of the 17 sets of identical isolates identified by BOX-PCR for 2000, five were also identified as identical using PFGE analysis. These were A28 (*E. faecalis* from turkey) and G26 (*E. faecalis* from chicken), A35 and A36 (both *E. casseliflavus* from red potatoes), A25 small and A25 large (*E. faecalis* from chicken), F29 small and F29 large (*E. faecalis* from pork), and G17 and G19 (both *E. faecalis* from a cucumber and carrot, respectively). Fewer sets of isolates in 2001 showed 100% homology using BOX-PCR; only five compared to 17 in 2000. None of the sets in 2001 showed 100% homology using PFGE analysis.

3.7 Statistical analysis

Some association between antimicrobial resistance and year of isolation, food item, and enterococcal species was observed using Chi-square analysis (Table 2). Antimicrobials for which a significant difference existed (bacitracin, ciprofloxacin, flavomycin, kanamycin, linezolid, streptomycin, Synercid (quinupristin/dalfopristin), tetracycline, and vancomycin) were further analyzed using the clustering generated by BOX-PCR and PFGE analysis. BOX-PCR and PFGE clusters used for Sigma Plot analysis are shown in Table 3 and Table 4, respectively.

Table 5 shows the results from the Sigma Plot analysis. Values for the most common MIC values are listed as percent of isolates having these MICs. Only MIC values containing more than one-half of the isolates, or MIC values where equal numbers of isolates were represented in the same cluster were included in the table.

From the analysis, BOX-PCR 2000 clusters 3 and 4 had the highest percent of isolates resistant to ciprofloxacin (Table 5). Isolates in cluster 4 also had the highest level of resistance to flavomycin, with 100% of isolates with an MIC of 32 µg/ml. Clusters 1 and 11 had the highest percent of isolates resistant to kanamycin and streptomycin. Isolates in cluster 11 also had higher levels of resistance to Synercid (quinupristin/dalfopristin) (57% with MIC=32 µg/ml) and tetracycline (40% with MIC=32 µg/ml). Notably, clusters 3, 4, and 9 all had a majority of isolates with intermediate resistance to vancomycin.

For isolates in 2000 analyzed by PFGE, clusters 1 and 3 clearly showed higher resistance levels to flavomycin (62 and 80% having MIC=32 µg/ml, respectively). Clusters 1 and 2 contained more isolates with higher resistance levels to Synercid (38 and 52% of isolates with MIC=32 µg/ml, whereas cluster 3 only had 45% of isolates with MIC=2 µg/ml). Only in cluster 2 did a majority of isolates exhibit resistance to tetracycline. Furthermore, only in cluster 3 did a majority of isolates exhibit intermediate resistance to vancomycin.
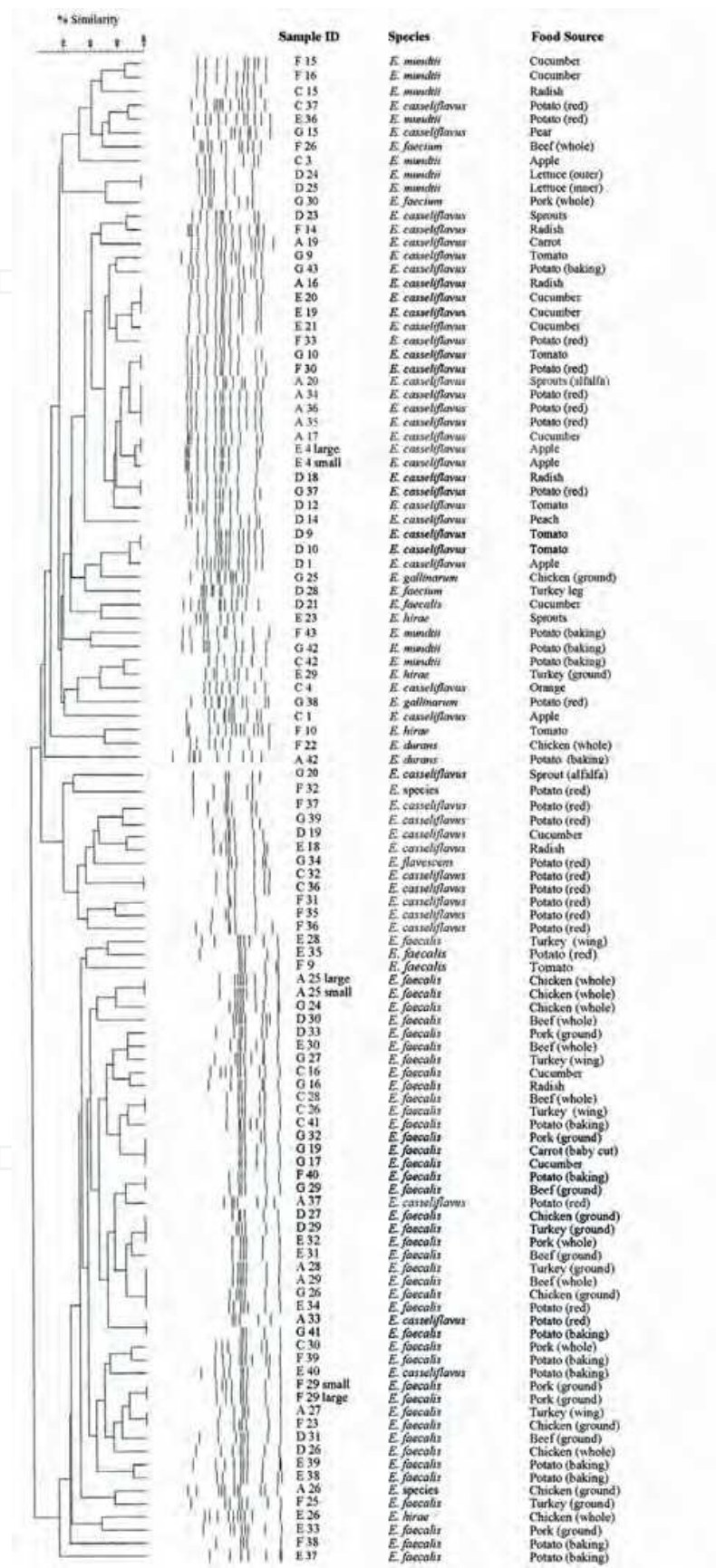
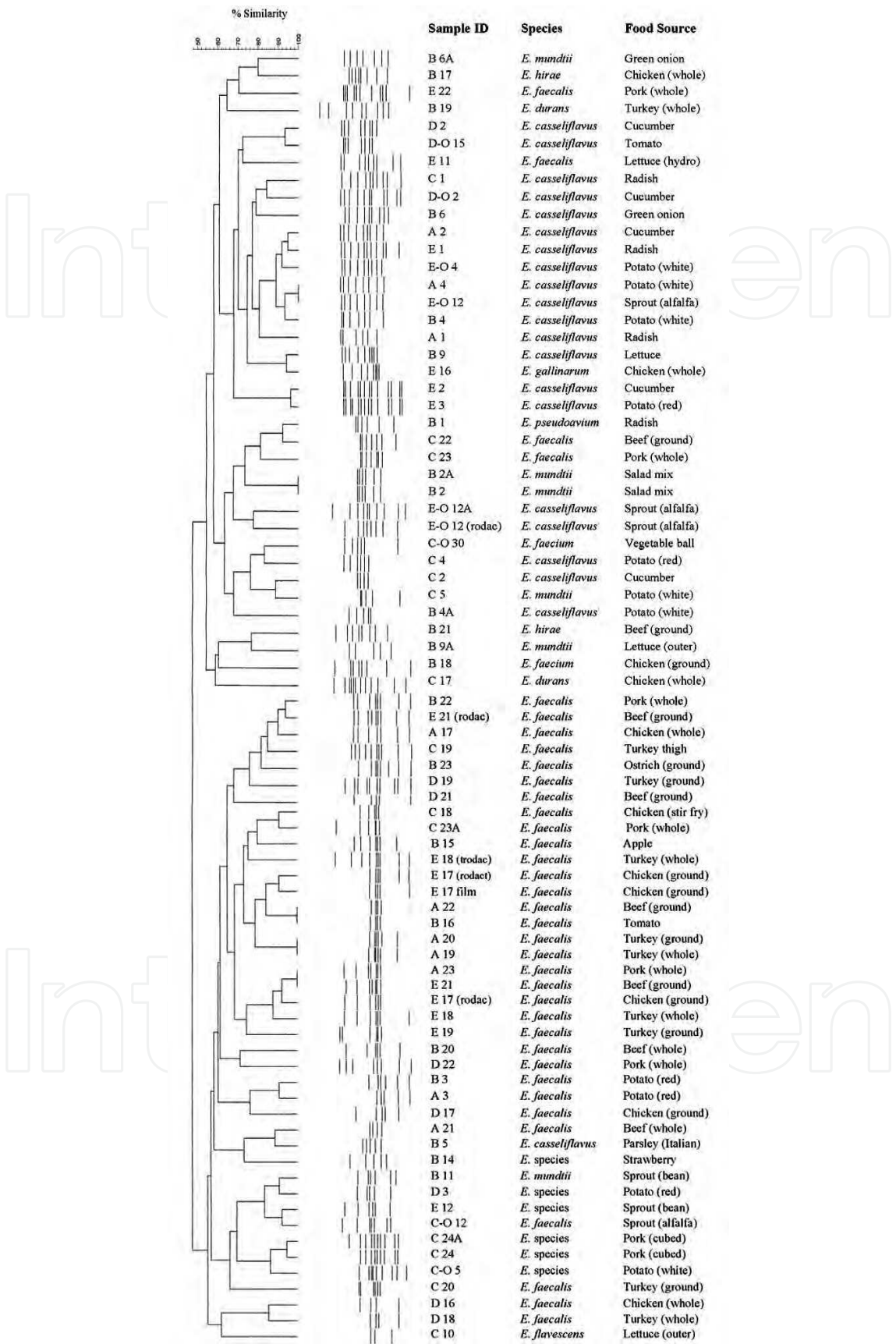Fig. 7. Genetic relatedness of year 2000 enterococci isolates using BOX-PCR.

Fig. 8. Genetic relatedness of year 2001 enterococci isolates using BOX-PCR.
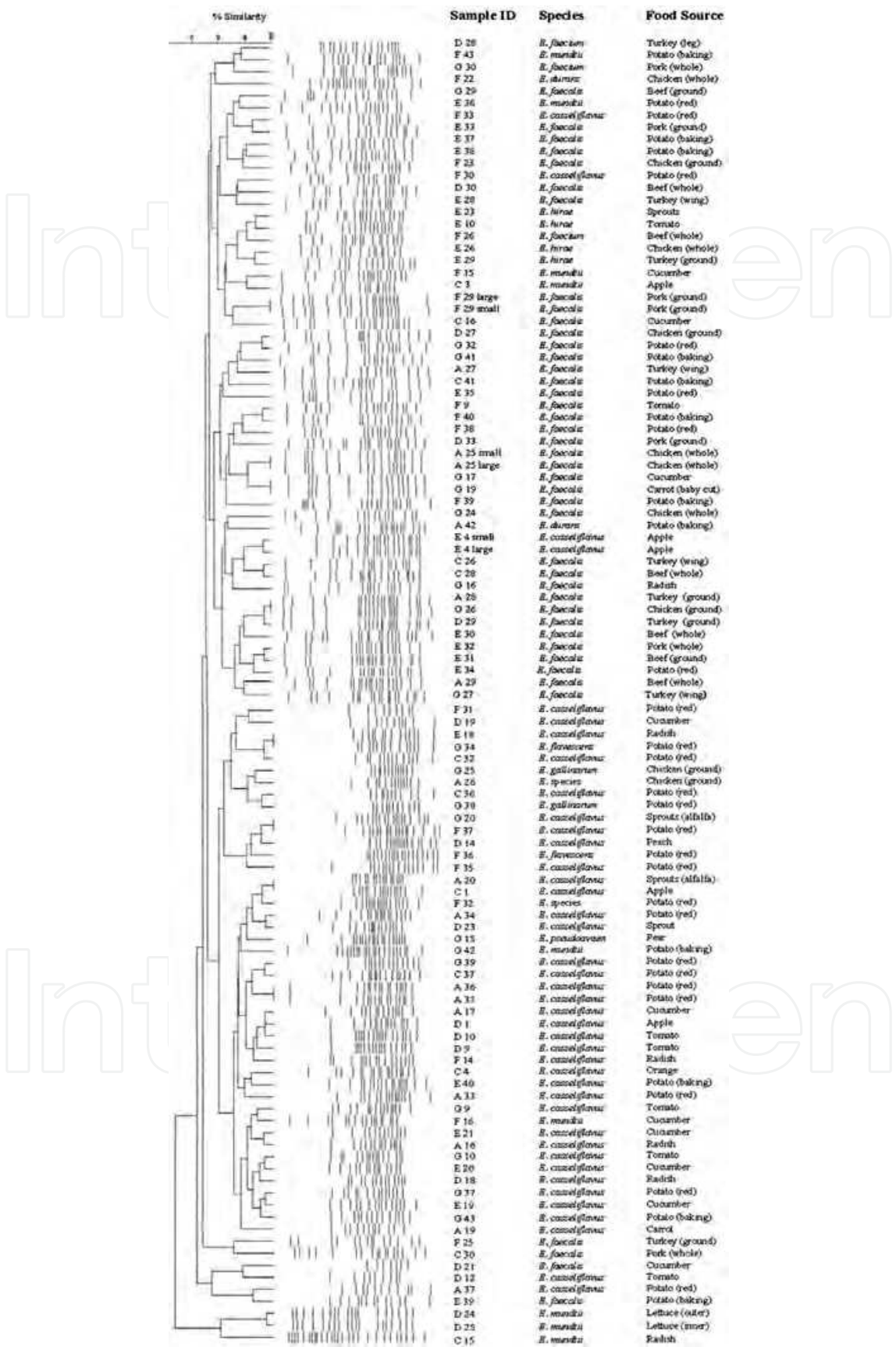
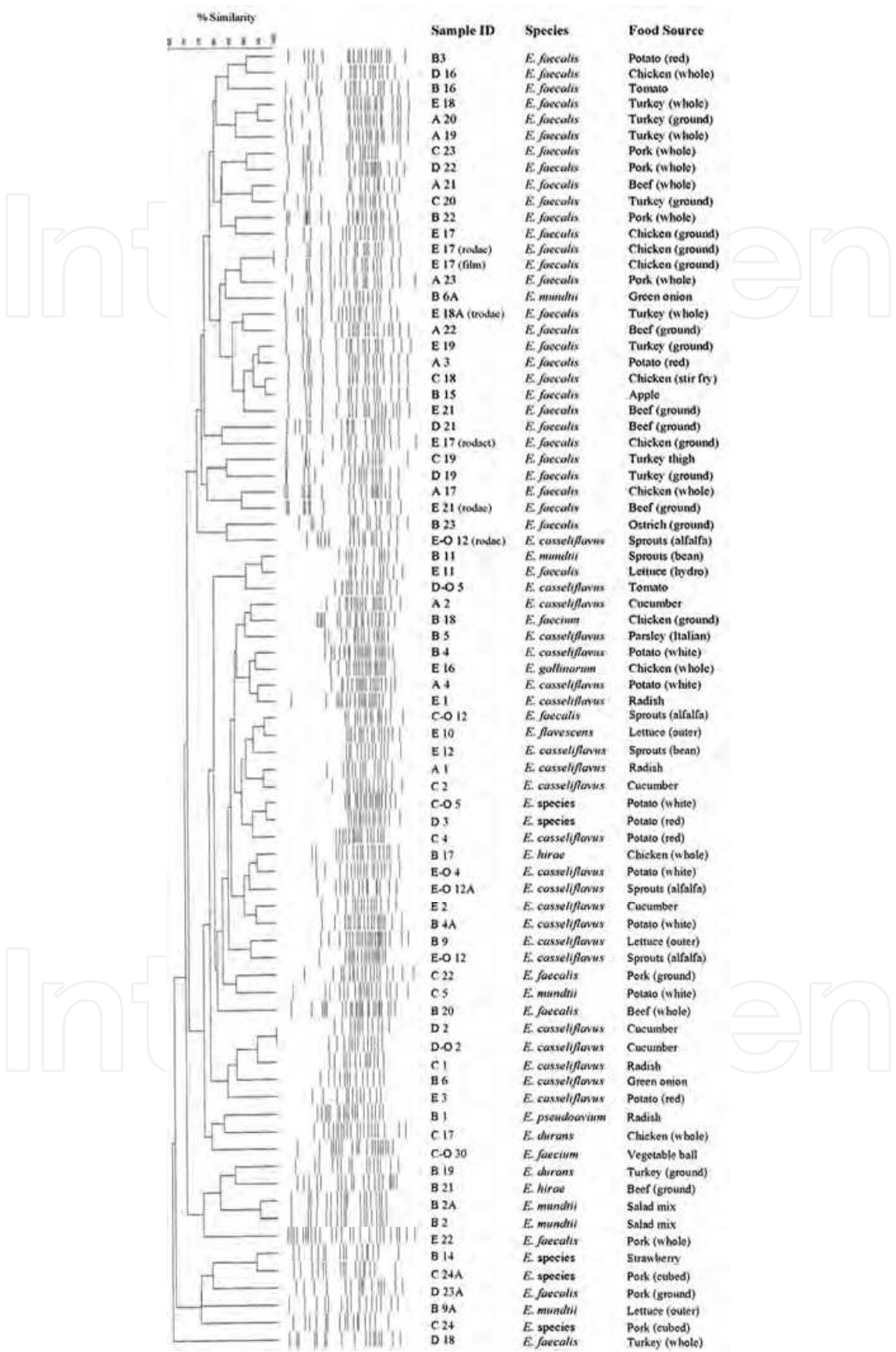Fig. 9. Genetic relatedness of year 2000 enterococci isolates using PFGE.

Fig. 10. Genetic relatedness of year 2001 enterococci isolates using PFGE.

For BOX-PCR 2001 clusters and chloramphenicol, cluster 3 clearly had a larger percentage of isolates with higher MIC values than the rest of the isolates (Table 5). This same cluster also had the highest percentage of isolates with the highest MIC value for flavomycin, with 100% with resistance of 32 μg/ml. Clusters 3 and 10 contained 100% of isolates resistant to bacitracin. For kanamycin, cluster 13 showed the highest percent resistance, with 60% isolates with an MIC=1024 μg/ml. This cluster also had 80% of isolates with high-level resistance (MIC=2048) to streptomycin. Cluster 10 contained the highest percentage of isolates with the highest MIC values for tetracycline, with 100% of isolates exhibiting resistance (MIC=32 μg/ml), as well as to vancomycin, with 83% with an MIC=2 μg/ml.

In the PFGE 2001 Sigma Plot analysis, cluster 2 contained more isolates with higher MIC values to chloramphenicol, ciprofloxacin, flavomycin, and vancomycin. For chloramphenicol and flavomycin, 39% and 89% of isolates were resistant, respectively. For ciprofloxacin and vancomycin, 30% had MIC=2 μg/ml and 44% had MIC=8 μg/ml, respectively.

Antimicrobial	Type	Chi-Square Probability	Result
Bacitracin	Year	0.0324	Significant difference
	Species	0.0041	Significant difference
Ciprofloxacin	Food	0.0042	Significant difference
Flavomycin	Food	0.0014	Significant difference
	Species	<0.0001	Significant difference
Kanamycin	Species	0.0016	Significant difference
Linezolid	Species	0.0199	Significant difference
Streptomycin	Species	0.0037	Significant difference
Synercid	Year	0.0227	Significant difference
	Food	0.0562	Possible difference
	Species	0.0177	Significant difference
Tetracycline	Food	0.0002	Significant difference
	Year	0.0894	Possible difference
Vancomycin	Year	0.0148	Significant difference
	Species	<0.0001	Significant difference

Table 2. SAS results for antimicrobial susceptibility data for all isolates.

4. Discussion

Typing methods for the enterococci can be either phenotypic, genotypic or a combination of both methods (Facklam et al. 2002). Phenotypic methods include bacteriocin typing, phage typing, serotyping, biotyping, and antimicrobial susceptibility testing. These methods are useful for characterizing various isolates, but may not be definitive for epidemiological outbreaks or identification of clones (Miranda et al. 1991). This is mainly due to the commonality of certain phenotypic traits such as the same or similar antimicrobial resistance profiles. Genotypic methods generally provide more discrimination between bacterial isolates (Facklam et al. 2002). PFGE is considered one of the more discriminatory genotypic typing techniques for *Enterococcus* (Turabelidze et al. 2000). It provides consistent results as the protocol is essentially standardized, especially for other bacteria. Other band-based methods employing gel electrophoresis used for typing enterococci include plasmid typing and typing based upon repetitive sequences (REP-PCR) dispersed throughout the bacterial genome (Olive and Bean 1999). One type of Rep-PCR is BOX-PCR (van Belkum and Hermans 2001).

One purpose of the present study was to characterize enterococci isolated from various foods purchased from different retail stores in the Athens, Georgia area, during fall 2000 and spring 2001. Enterococcal species identification among food types were investigated using an agarose gel-based genus and species specific multiplex PCR. Enterococcal isolates were sub-typed

using BOX-PCR and PFGE. The profiles for each method were then compared to determine if related isolates were contaminating more than one food type or retail store. Genotypic data obtained from BOX-PCR and PFGE analysis were combined with phenotypic analysis using antimicrobial susceptibility testing to determine if there was any correlation between antimicrobial resistance and isolates in common BOX-PCR and PFGE clusters. Plasmid profiling was also conducted to elucidate the plasmid content of bacterial isolates.

4.1 Identification and antimicrobial susceptibility of enterococci in retail food

Conventional methods for identification of species of the genus *Enterococcus* incorporate phenotypic characteristics on different media and morphologic characteristics including motility and pigmentation (Murray 1990). These methods are often time-consuming and do not guarantee that non-human enterococci will conform to the conventional identification methods (Facklam et al. 2002). To avoid these problems, molecular methods such as enterococcal species PCR have been developed. PCR protocols have been designed to distinguish enterococcal species including the *ddl* (D-Ala:D-Ala ligase) and *van* (vancomycin resistance) genes (Dutka-Malen et al. 1995), 16S rRNA gene (Monstein et al. 1998;Monstein et al. 2001), and the *tuf* (elongation factor EF-Tu) gene (Ke et al. 1999). Enterococci were identified in this study using a multiplex PCR designed to amplify a genus specific band based upon the 16S rRNA gene (Deasy et al. 2000) and species specific primers based upon the *sodA* (superoxide dismutase) gene simultaneously (Jackson et al. 2004a). A few primer pairs of this multiplex PCR have been recently modified to amplify enterococcal DNA beneficial for microbial source tracking (Layton et al. 2010).

Possible reservoirs for transmission of antimicrobial resistant enterococci include water, food, or food animals and may provide a source for horizontal transfer of antimicrobial resistant enterococci (Aarestrup et al. 2002). Results from sampling fruits, vegetables and meats in this study clearly showed that enterococci were present on the food items. Specifically, enterococcal species appeared to inhabit certain food items more so than others. For example, *E. faecalis* was the most common species isolated from meat samples, while *E. casseliflavus* was the most common species found on fruit and vegetable samples. *E. faecalis* and *E. faecium* are two enterococcal species most often associated with fecal contamination of water sources, while *E. casseliflavus* is considered a plant-associated species (Aarestrup et al. 2002). Thus, the presence of *E. faecalis* predominantly on meat products may suggest cross-contamination of the processed meat with animal waste, while *E. casseliflavus* was occupying its most common environmental niche. Results from other studies have indicated that *E. faecium* is a common contaminant on fruits, vegetables, and poultry products (Butaye et al. 2000;Hayes et al. 2003;Johnston and Jaykus 2004;Klein et al. 1998;Simjee et al. 2002). These differences may be accounted for by the collection methods used in the different studies including geographical location, variety and seasonality of the fruits and vegetables and slaughter conditions and age of the food animals for meats. Presently, there is a vast variety of food available in the markets, many of which are imported to the U.S.

Antimicrobial susceptibility testing was conducted on all enterococcal isolates and included those commonly used as therapeutic agents and growth promoters. Consistent by year and by store, the highest numbers of resistant isolates were to bacitracin, lincomycin, and flavomycin. High levels of bacitracin resistance have been previously observed in enterococci from poultry samples (Butaye et al. 2003), whereas enterococci isolated from produce from a separate study exhibited low resistance to lincomycin (Johnston and Jaykus 2004). With few exceptions, regardless of antimicrobial, most of the resistance was from

enterococci from meat samples. None of the isolates from the current study were resistant to linezolid or vancomycin. Linezolid is a newer antimicrobial approved to treat methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) while vancomycin is used as a last line antimicrobial for susceptible enterococci in serious human infections and in cases of antimicrobial allergies (Malani et al. 2002).

4.2 Genotypic characterization of enterococci using BOX-PCR and PFGE

Compared to PFGE, BOX-PCR offers a cheaper and quicker method of generating banding patterns to differentiate bacterial isolates. BOX-PCR primers for *Enterococcus* were designed from highly conserved interspersed repetitive sequences that were initially identified in *S. pneumoniae* (van Belkum and Hermans 2001). A PFGE procedure for typing *Enterococcus* was developed in the early 1990's by Murray et al. (Murray et al. 1990). The procedure has evolved as an efficient tool for discriminating multi-drug resistant strains of enterococci. For example, vancomycin resistant enterococci (VRE) strains can be better distinguished in less time than before (Turabelidze et al. 2000). A study comparing BOX-PCR to PFGE was conducted in which BOX-PCR and PFGE patterns were generated using *E. faecalis* (Malathum et al. 1998). Results indicated that reproducibility of the PCR patterns were found to be challenging, although when stricter criteria were used, the interpretation of Rep-PCR results were more similar to those obtained by PFGE (Malathum et al. 1998). To overcome some of the concerns with reproducibility of BOX-PCR, numerous steps were taken to ensure that banding patterns could be reproduced gel to gel. First, the same internal control was used on all gels to ensure that the banding pattern was consistent. To make sure that extraneous DNA was not a contaminating factor, a no DNA control (consisting of water only) was used. Gels containing bands in the negative control lane were discarded and not included in the analysis. Furthermore, gel thickness and electrophoresis times were standardized as thinner gels produced sharper bands that could be more easily distinguished than in thicker gels. Longer electrophoresis times also tended to distort the bands making it more difficult to differentiate them on the gel. Finally, in this study, *S. cerevisiae* chromosomes were used as DNA standards. DNA standards prepared from *Salmonella enterica* serotype Braenderup H9812 are now used to provide improved sizing of DNA fragments and analysis using BioNumerics (Davis et al. 2011).

PFGE in conjunction with a PCR-based method or PFGE using two different restriction enzymes is highly recommended in order to verify genetic clones (Facklam et al. 2002). In addition to selection of a typing procedure, interpretation of molecular typing data is an ongoing problem (Duck et al. 2003). Currently, computer based analysis programs such as BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium) are being utilized to interpret results from band-based methods (Duck et al. 2003). Software packages can perform sophisticated similarity calculations and cluster analyses of the patterns in the database and from that generate analytical data in the form of dendrograms (Gerner-Smidt et al. 1998). These programs are now being used progressively more with epidemiological typing. These tools are helpful in establishing intralab and interlab similarity of interpretation (Gerner-Smidt et al. 1998).

From the study, a wide range of genetic variability was found among isolates tested. Dendrograms generated using BOX-PCR and PFGE were each divided into two large clusters. One cluster contained mostly *E. faecalis* isolates while the other consisted of mostly *E. casseliflavus* isolates. In a few cases, isolates were found outside their respective clusters; a few *E. faecalis* isolates had identical or nearly identical genetic banding patterns with some *E.*

casseliflavus isolates. Several pair of isolates (i.e. A28 and G26; G20 and F37; E18 and G34 from year 2001 samples) that exhibited identical banding patterns were identified as the same species by multiplex PCR, but were isolated from different food sources from different stores. This suggested possible environmental dissemination of enterococcal clones from a common source, possibly from cross-contamination by shoppers or workers. Another possibility was that different stores may obtain their foods from the same vendor/warehouse. Samples of water taken from vegetable sprayers at one retail store contained enterococci as well. If the stores received their water from the same contaminated source, this could also account for the genetic relatedness among isolates from different stores.

BOX-PCR Clusters								
BOX 2000								
Cluster 1	Isolate	Species	**Cluster 4**	Isolate	Species	**Cluster 11**	Isolate	Species
	F 15	*E. mundtii*		D 9	*E. casseliflavus*		C 28	*E. faecalis*
	F 16	*E. mundtii*		D 10	*E. casseliflavus*		C 26	*E. faecalis*
	C 15	*E. mundtii*		D 1	*E. casseliflavus*		C 41	*E. faecalis*
	C 37	*E. casseliflavus*		G 25	*E. gallinarum*		G 32	*E. faecalis*
	E 36	*E. mundtii*		D 28	*E. faecium*		G 19	*E. faecalis*
	G 15	*E. casseliflavus*					G 17	*E. faecalis*
	F 26	*E. faecium*	**Cluster 9**	G 20	*E. casseliflavus*		F 40	*E. faecalis*
	C 3	*E. mundtii*		F 32	*E. species*		G 29	*E. faecalis*
				F 37	*E. casseliflavus*		A 37	*E. casseliflavus*
Cluster 3	D 23	*E. casseliflavus*		G 39	*E. casseliflavus*		D 27	*E. faecalis*
	F 14	*E. casseliflavus*		D 19	*E. casseliflavus*		D 29	*E. faecalis*
	A 19	*E. casseliflavus*		E 18	*E. casseliflavus*		E 32	*E. faecalis*
	G 9	*E. casseliflavus*		G 34	*E. flavescens*		E 31	*E. faecalis*
	G 43	*E. casseliflavus*		C 32	*E. casseliflavus*		A 28	*E. faecalis*
	A 16	*E. casseliflavus*		C 36	*E. casseliflavus*		A 29	*E. faecalis*
	E 20	*E. casseliflavus*		F 31	*E. casseliflavus*		G 26	*E. faecalis*
	E 19	*E. casseliflavus*		F 35	*E. casseliflavus*		E 34	*E. faecalis*
	E 21	*E. casseliflavus*		F 36	*E. casseliflavus*		A 33	*E. casseliflavus*
	F 33	*E. casseliflavus*					G 41	*E. faecalis*
	G 10	*E. casseliflavus*	**Cluster 11**	E 28	*E. faecalis*		C 30	*E. faecalis*
	F 30	*E. casseliflavus*		E 35	*E. faecalis*		F 39	*E. faecalis*
	A 20	*E. casseliflavus*		F 9	*E. faecalis*		E 40	*E. casseliflavus*
	A 34	*E. casseliflavus*		A 25 large	*E. faecalis*		F 29 small	*E. faecalis*
	A 36	*E. casseliflavus*		A 25 small	*E. faecalis*		F 29 large	*E. faecalis*
	A 35	*E. casseliflavus*		G 24	*E. faecalis*		A 27	*E. faecalis*
	A 17	*E. casseliflavus*		D 30	*E. faecalis*		F 23	*E. faecalis*
	E 4 large	*E. casseliflavus*		D 33	*E. faecalis*		D 31	*E. faecalis*
	E 4 small	*E. casseliflavus*		E 30	*E. faecalis*		D 26	*E. faecalis*
	D 18	*E. casseliflavus*		G 27	*E. faecalis*		E 39	*E. faecalis*
	G 37	*E. casseliflavus*		C 16	*E. faecalis*		E 38	*E. faecalis*
	D 12	*E. casseliflavus*		G 16	*E. faecalis*			
	D 14	*E. casseliflavus*						
BOX 2001								
Cluster 3	Isolate	Species	**Cluster 12**	Isolate	Species			
	C 1	*E. casseliflavus*		E 17 rodact	*E. faecalis*			
	D-O 2	*E. casseliflavus*		E 17 film	*E. faecalis*			
	B 6	*E. casseliflavus*		A 22	*E. faecalis*			
	A 2	*E. casseliflavus*		B 16	*E. faecalis*			
	E 1	*E. casseliflavus*		A 20	*E. faecalis*			
	E-O 4	*E. casseliflavus*		A 19	*E. faecalis*			
	A 4	*E. casseliflavus*						
	E-O 12	*E. casseliflavus*	**Cluster 13**	A 23	*E. faecalis*			
	B 4	*E. casseliflavus*		E 21	*E. faecalis*			
	A 1	*E. casseliflavus*		E 17 rodac	*E. faecalis*			
				E 18	*E. faecalis*			
Cluster 6	B 1	*E. pseudoavium*		E 19	*E. faecalis*			
	C 22	*E. faecalis*						
	C 23	*E. faecalis*						
	B 2A	*E. mundtii*						
	B2	*E. mundtii*						
Cluster 10	B 22	*E. faecalis*						
	E 21 rodac	*E. faecalis*						
	A 17	*E. faecalis*						
	C 19	*E. faecalis*						
	B 23	*E. faecalis*						
	D 19	*E. faecalis*						

Table 3. BOX-PCR clusters used for Sigma Plot analysis.

PFGE Clusters

PFGE 2000

Cluster 1	Isolate	Species	Cluster 2	Isolate	Species	Cluster 3	Isolate	Species
	D 28	*E. faecium*		G 17	*E. faecalis*		A 34	*E. casseliflavus*
	F 43	*E. mundtii*		G 19	*E. faecalis*		D 23	*E. casseliflavus*
	G 30	*E. faecium*		F 39	*E. faecalis*		G 15	*E. casseliflavus*
	F 22	*E. durans*		G 24	*E. faecalis*		G 42	*E. mundtii*
	G 29	*E. faecalis*		A 42	*E. durans*		G 39	*E. casseliflavus*
	E 36	*E. mundtii*		E 4 small	*E. casseliflavus*		C 37	*E. casseliflavus*
	F 33	*E. casseliflavus*		E 4 large	*E. casseliflavus*		A 36	*E. casseliflavus*
	E 33	*E. faecalis*		C 26	*E. faecalis*		A 35	*E. casseliflavus*
	E 37	*E. faecalis*		C 28	*E. faecalis*		A 17	*E. casseliflavus*
	E 38	*E. faecalis*		G 16	*E. faecalis*		D 1	*E. casseliflavus*
	F 23	*E. faecalis*		A 28	*E. faecalis*		D 10	*E. casseliflavus*
	F 30	*E. casseliflavus*		G 26	*E. faecalis*		D 9	*E. casseliflavus*
	D 30	*E. faecalis*		D 29	*E. faecalis*		F 14	*E. casseliflavus*
	E 28	*E. faecalis*		E 30	*E. faecalis*		C 4	*E. casseliflavus*
	E 23	*E. hirae*		E 32	*E. faecalis*		E 40	*E. casseliflavus*
	F 10	*E. hirae*		E 31	*E. faecalis*		A 33	*E. casseliflavus*
	F 26	*E. faecium*		E 34	*E. faecalis*		G 9	*E. casseliflavus*
	E 26	*E. hirae*		A 29	*E. faecalis*		F 16	*E. mundtii*
	E 29	*E. hirae*		G 27	*E. faecalis*		E 21	*E. casseliflavus*
	F 15	*E. mundtii*					A 16	*E. casseliflavus*
	C 3	*E. mundtii*	**Cluster 3**	F 31	*E. casseliflavus*		G 10	*E. casseliflavus*
	F 29 large	*E. faecalis*		D 19	*E. casseliflavus*		E 20	*E. casseliflavus*
	F 29 small	*E. faecalis*		E 18	*E. casseliflavus*		D 18	*E. casseliflavus*
	C 16	*E. faecalis*		G 34	*E. flavescens*		G 37	*E. casseliflavus*
				C 32	*E. casseliflavus*		E 19	*E. casseliflavus*
Cluster 2	D 27	*E. faecalis*		G 25	*E. gallinarum*		G 43	*E. casseliflavus*
	G 32	*E. faecalis*		A 26	*E. species*		A 19	*E. casseliflavus*
	G 41	*E. faecalis*		C 36	*E. casseliflavus*				
	A 27	*E. faecalis*		G 38	*E. gallinarum*				
	C 41	*E. faecalis*		G 20	*E. casseliflavus*				
	E 35	*E. faecalis*		F 37	*E. casseliflavus*				
	F 9	*E. faecalis*		D 14	*E. casseliflavus*				
	F 40	*E. faecalis*		F 36	*E. casseliflavus*				
	F 38	*E. faecalis*		F 35	*E. casseliflavus*				
	D 33	*E. faecalis*		A 20	*E. casseliflavus*				
	A 25 small	*E. faecalis*		C 1	*E. casseliflavus*				
	A 25 large	*E. faecalis*		F 32	*E. species*				

PFGE 2001

Cluster 1	Isolate	Species	Cluster 1	Isolate	Species	Cluster 2	Isolate	Species
	B 3	*E. faecalis*		E 17 rodact	*E. faecalis*		B 17	*E. hirae*
	D 16	*E. faecalis*		C 19	*E. faecalis*		E-O 4	*E. casseliflavus*
	B 16	*E. faecalis*		D 19	*E. faecalis*		E-O 12A	*E. casseliflavus*
	E 18	*E. faecalis*		A 17	*E. faecalis*		E 2	*E. casseliflavus*
	A 20	*E. faecalis*		E 21 rodac	*E. faecalis*		B 4A	*E. casseliflavus*
	A 19	*E. faecalis*					B 9	*E. casseliflavus*
	C 23	*E. faecalis*	**Cluster 2**	B 11	*E. mundtii*		E-O 12	*E. casseliflavus*
	D 22	*E. faecalis*		E 11	*E. faecalis*		C 22	*E. faecalis*
	A 21	*E. faecalis*		D-O 15	*E. casseliflavus*		C 5	*E. mundtii*
	C 20	*E. faecalis*		A 2	*E. casseliflavus*		B 20	*E. faecalis*
	B 22	*E. faecalis*		B 18	*E. faecium*		D 2	*E. casseliflavus*
	D 17	*E. faecalis*		B 5	*E. casseliflavus*		D-O 2	*E. casseliflavus*
	E 17 rodac	*E. faecalis*		B 4	*E. casseliflavus*		C 1	*E. casseliflavus*
	E17 film	*E. faecalis*		E 16	*E. gallinarum*		B 6	*E. casseliflavus*
	A 23	*E. faecalis*		A 4	*E. casseliflavus*		E 3	*E. casseliflavus*
	B 6A	*E. faecalis*		E 1	*E. casseliflavus*		B 1	*E. pseudoavium*
	E 18 trodac	*E. faecalis*		C-O 12	*E. faecalis*		C 17	*E. durans*
	A 22	*E. faecalis*		C 10	*E. flavescens*		C-O 30	*E. faecium*
	E 19	*E. faecalis*		E 12	*E. casseliflavus*				
	A 3	*E. faecalis*		A 1	*E. casseliflavus*	**Cluster 3**	B 19	*E. durans*
	C 18	*E. faecalis*		C 2	*E. casseliflavus*		B 21	*E. hirae*
	B 15	*E. faecalis*		C-O 5	*E. casseliflavus*		B 2A	*E. mundtii*
	E 21	*E. faecalis*		D 3	*E. species*		B 2	*E. mundtii*
	D 21	*E. faecalis*		C 4	*E. casseliflavus*		E 22	*E. mundtii*

Table 4. PFGE clusters used for Sigma Plot analysis.

Cluster	Antimicrobial	Bac (MIC=128) Resistant	Chl (MIC=8) Susceptible	Chl (MIC=16) Intermediate	Cip (MIC=1) Susceptible	Cip (MIC=2) Intermediate	Cip (MIC=4) Resistant	Fla (MIC=1) Susceptible	Fla (MIC=2) Susceptible	Fla (MIC=4) Susceptible	Fla (MIC=32) Resistant	Kan (MIC=128) Susceptible	Kan (MIC=1024) Resistant
Box 2000 Cluster 1		75	75	-	50	-	-	-	-	-	75	-	50
Box 2000 Cluster 3		91	65	-	-	35	39	-	-	-	83	96	-
Box 2000 Cluster 4		100	60	-	-	40	40	-	-	-	100	80	-
Box 2000 Cluster 9		83	75	-	-	75	-	-	-	-	92	92	-
Box 2000 Cluster 11		69	76	-	52	-	-	31	38	-	-	57	43
PFGE 2000 Cluster 1		79	58	-	42	-	-	-	-	-	62	31	-
PFGE 2000 Cluster 2		64	71	-	58	-	-	29	39	-	-	58	-
PFGE 2000 Cluster 3		86	64	-	-	43	-	-	-	-	80	89	-
Box 2001 Cluster 3		100	-	70	-	-	-	-	-	-	100	100	-
Box 2001 Cluster 6		80	40	40	80	-	-	-	40	-	40	80	-
Box 2001 Cluster 10		100	50	-	50	-	-	50	-	-	-	67	-
Box 2001 Cluster 12		83	83	-	83	-	-	33	33	33	-	83	-
Box 2001 Cluster 13		60	100	-	60	-	-	60	-	-	-	-	60
PFGE 2001 Cluster 1		86	79	-	59	-	-	52	-	-	-	72	-
PFGE 2001 Cluster 2		92	47	39	44	30	-	-	-	-	89	92	-

Cluster	Antimicrobial	Str (MIC=512) Susceptible	Str (MIC=2048) Resistant	Syn (MIC=2) Susceptible	Syn (MIC=4) Resistant	Syn (MIC=32) Resistant	Tet (MIC=4) Susceptible	Tet (MIC=32) Resistant	Van (MIC=1) Susceptible	Van (MIC=2) Susceptible	Van (MIC=4) Susceptible	Van (MIC=8) Intermediate
Box 2000 Cluster 1		50	50	50	-	-	100	-	75	-	-	-
Box 2000 Cluster 3		87	-	-	61	-	91	-	-	-	-	70
Box 2000 Cluster 4		80	-	-	60	-	80	-	-	-	-	60
Box 2000 Cluster 9		92	-	75	-	-	100	-	-	-	-	83
Box 2000 Cluster 11		62	38	-	-	57	57	40	45	-	-	45
PFGE 2000 Cluster 1		62	-	-	-	38	67	-	54	-	-	-
PFGE 2000 Cluster 2		58	-	-	-	52	58	42	52	-	-	-
PFGE 2000 Cluster 3		82	-	45	-	-	86	-	-	-	-	60
Box 2001 Cluster 3		80	-	50	-	-	90	-	-	-	40	40
Box 2001 Cluster 6		60	-	-	-	40	60	-	60	-	-	-
Box 2001 Cluster 10		83	-	-	-	67	-	100	-	83	-	-
Box 2001 Cluster 12		83	-	-	-	83	-	67	-	67	-	-
Box 2001 Cluster 13		-	80	-	-	80	-	60	40	40	-	-
PFGE 2001 Cluster 1		62	-	-	-	59	-	59	-	55	-	-
PFGE 2001 Cluster 2		80	-	58	-	-	78	-	-	-	28	44

Table 5. Results of Sigma Plot analysis. Clusters are separated by year and typing method and include only those with $\geq$75% homology containing $\geq$5 isolates. Bac=Bacitracin, Chl=Chloramphenicol, Cip=Ciprofloxacin, Fla=Flavomycin, Kan=Kanamycin, Str=Streptomycin, Syn=Synercid (quinupristin/dalfopristin), Tet=Tetracycline, Van=Vancomycin.

Three isolates could not be compared by PFGE because their bands were smeared. Some isolates produced faint bands and were analyzed as best as possible for entry into BioNumerics. Though most isolates produced clear bands, some fainter ones may have been missed during analysis. The fainter bands could be caused by incomplete digestion of DNA in the plugs with *Sma*I (Maslow et al. 1993). Endogenous endonucleases that "chew" up the DNA may also have resulted in unclear banding patterns. Degraded DNA during PFGE analysis has been previously described in both gram-negative and gram-positive bacteria (Corkill et al. 2000;Hollis et al. 1999). Although not utilized in this study, the addition of thiourea in the running buffer during PFGE has been reported to aide in decreasing DNA degradation and subsequent smearing (Corkill et al. 2000;Romling and Tummler 2000;Silbert et al. 2003). Inability to accurately visualize the faint bands may account for the mixing of species in particular clusters (Maslow et al. 1993).

Some of the problems associated with identifying clear bands may also be due to plasmids in the isolates (Miranda et al. 1991;Ogle et al. 1987). Enterococci contain small, high-copy number plasmids as well as large, low-copy number plasmids that may range in size up to ~100 kb (Weaver et al. 2002). Plasmid analysis of the isolates in this study also revealed numerous plasmids of varying sizes. Faint bands in the PFGE patterns may be indicative of large, low-copy number plasmids containing few *Sma*I restriction sites rather than smaller, high-copy number plasmids. Smaller plasmids would be less likely to contribute to band changes due to their size and odds against multiple *Sma*I restriction sites producing one or more bands. Plasmids in enterococci have been previously shown to influence PFGE patterns (Werner et al. 2003).

Some sets of isolates that were identified as 100% homologous using BOX-PCR were genetically distinct using PFGE. Four sets of isolates from the year 2000 (F29 small and F29 large; G17 and G19; A28 and G36; and A35 and A36) were homologous using BOX-PCR and PFGE. F29 small and large were expected to be identical, as they were isolated from the same freezer stock. When streaked from freezer stock to BHI for testing with multiplex PCR, a few colonies appeared smaller than others and were treated as different strains. Two other isolates, A25 and E4, also contained small and large colonies when preparing templates from freezer stocks. A25 small and large were identified as *E. faecalis*; E4 small and large were identified as *E. casseliflavus*. Each pair of isolates showed 100% homology by either BOX-PCR or PFGE, but not both. Malathum (Malathum et al. 1998) found more agreement in clustering of isolates using BOX-PCR and PFGE than the present study. Fewer numbers of isolates with identical banding patterns were noted in the present study because the parameters for analysis used in BioNumerics were stringent for comparing banding patterns. Malathum (Malathum et al. 1998) observed banding patterns manually. For example, three isolates A17, D1, and D10 in Figure 8 were visually very similar in banding patterns, ~95% similarity between all three. Only 14 bands were identified in isolate A17 using BioNumerics; D1 had 15 bands; and D10 had 17 bands. Manual classification of these isolates given the guidelines by Tenover *et al.* (1995) would consider these isolates as less similar.

5. Conclusion

Different enterococcal species were prevalent on fruits, vegetables, and meat from retail grocery stores. Specific species were predominant on certain food products, but were also found in lower numbers on other food items. The majority of enterococcal isolates from the

retail food items were resistant primarily to bacitracin, flavomycin, and lincomycin. Resistance of enterococci to penicillin, salinomycin, and nitrofurantoin was low and none of the isolates were resistant to linezolid or vancomycin. Enterococcal isolates with identical banding patterns were identified using BOX-PCR and PFGE, including isolates from different stores, food types, and of different species. Increased prevalence of antimicrobial resistance, the ability of antimicrobial resistant bacteria to persist in the environment, and possible transfer of resistance genes to bacterial pathogens is cause for concern for human health. Additional studies on enterococci from retail food including their resistance to antimicrobials used in human medicine and their genetic relatedness are needed.

6. Note

The mention of trade names or commercial products in this manuscript is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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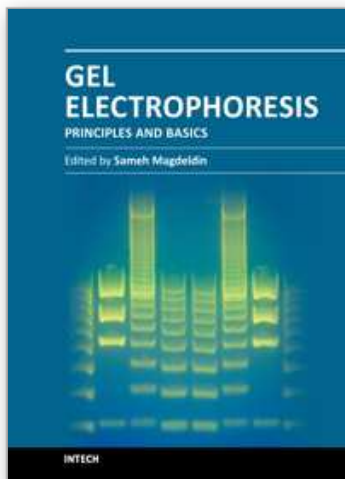
Gel Electrophoresis - Principles and Basics

Edited by Dr. Sameh Magdeldin

Most will agree that gel electrophoresis is one of the basic pillars of molecular biology. This coined terminology covers a myriad of gel-based separation approaches that rely mainly on fractionating biomolecules under electrophoretic current based mainly on the molecular weight. In this book, the authors try to present simplified fundamentals of gel-based separation together with exemplarily applications of this versatile technique. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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