

Influx of Enterococci and Associated Antibiotic Resistance and Virulence Genes from Ready-To-Eat Food to the Human Digestive Tract[∇]

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The influx of enterococcal antibiotic resistance (AR) and virulence genes from ready-to-eat food (RTEF) to the human digestive tract was assessed. Three RTEFs (chicken salad, chicken burger, and carrot cake) were sampled from five fast-food restaurants five times in summer (SU) and winter (WI). The prevalence of enterococci was significantly higher in SU (92.0% of salad samples and 64.0% of burger samples) than in WI (64.0% of salad samples and 24.0% of burger samples). The overall concentrations of enterococci during the two seasons were similar ($\sim 10^3$ CFU/g); the most prevalent were *Enterococcus casseliflavus* (41.5% of isolates) and *Enterococcus hirae* (41.5%) in WI and *Enterococcus faecium* (36.8%), *E. casseliflavus* (27.6%), and *Enterococcus faecalis* (22.4%) in SU. Resistance in WI was detected primarily to tetracycline (50.8%), ciprofloxacin (13.8%), and erythromycin (4.6%). SU isolates were resistant mainly to tetracycline (22.8%), erythromycin (22.1%), and kanamycin (13.0%). The most common *tet* gene was *tet(M)* (35.4% of WI isolates and 11.9% of SU isolates). The prevalence of virulence genes (*gelE*, *asaI*, *cylA*, and *esp*) and marker genes for clinical isolates (EF_0573, EF_0592, EF_0605, EF_1420, EF_2144, and pathogenicity island EF_0050) was low ($\leq 12.3\%$). Genotyping of *E. faecalis* and *E. faecium* using pulsed-field gel electrophoresis revealed that the food contamination likely originated from various sources and that it was not clonal. Our conservative estimate (single AR gene copy per cell) for the influx of *tet* genes alone to the human digestive tract is 3.8×10^5 per meal (chicken salad). This AR gene influx is frequent because RTEFs are commonly consumed and that may play a role in the acquisition of AR determinants in the human digestive tract.

The emergence and spread of antibiotic resistance (AR) determinants are a growing problem in clinical intensive care units, due to the declining number of effective antimicrobial agents for treatment of bacterial diseases (28, 51). The relationship between antibiotic use and the development and spread of AR has been studied extensively in clinical as well as environmental isolates (1, 18, 20, 25, 28, 29, 45).

The microbial community of the human digestive tract, especially of the colon, likely represents an important reservoir of AR genes as well as a site for horizontal intra- and interspecies gene transfer (10, 38, 40). It has been shown that horizontal transfer of AR genes from the ingested bacteria to the microbial community of the human digestive tract is possible and likely represents an important aspect of the ecology of AR determinants (27, 38, 39, 40). The ubiquity of enterococci in mammalian digestive tracts, their medical importance, frequent multiple AR, and great capacity for horizontal gene transfer (16) make the enterococci an ideal bacterial group for investigating the ecology of AR genes. The transfer of the transposon Tn1546 conferring vancomycin resistance from *Enterococcus faecalis* to a clinical isolate of *Staphylococcus aureus* (49) highlighted the importance of horizontal gene

transfer among bacteria from the clinical as well as ecological perspective.

While many studies have assessed the diversity and AR of enterococci in food, the majority have focused on food before preparation and cooking (12, 17, 21, 24, 50), during which many microorganisms and associated genes are likely destroyed. Only a few studies have evaluated enterococcal contamination in ready-to-eat foods (RTEFs), and these included cheese (13, 17), fermented sausages (14), and produce (12, 22, 31). However, RTEFs such as meals from fast-food restaurants that are very commonly consumed in developed countries have not been assessed for the frequency and level of enterococcal contamination nor as a source of a possible influx of AR and virulence genes to the resident microbial community in the human digestive tract.

In this study, the prevalence and diversity of enterococci in RTEFs (chicken salad, chicken burger, and carrot cake) from fast-food restaurants as well as the influx of enterococcal AR and virulence genes to the human digestive tract were evaluated.

MATERIALS AND METHODS

Isolation of enterococci from RTEF. Three types of RTEFs (chicken salad meal, chicken sandwich with French fries, and carrot cake) were purchased five times in both summer (SU; June to August) and winter (WI; November to January) from five fast-food restaurants (R1 to R5) in a town in northeastern Kansas. Each meal was brought to the laboratory, and the total weight of each meal was determined. To screen for enterococcal contamination, each meal was aseptically taken apart to separate the individual ingredients. Overall, meals consisted of (i) chicken salad (chicken meat, lettuce, tomatoes, red cabbage,

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TABLE 1. Prevalence of contamination and concentration (CFU/g) of enterococci in RTEF per meal and per the main group of ingredients^a per meal in five fast-food restaurants in WI and SU

RTEF meal and ingredient(s)	WI				SU			
	Total no. of samples	% Prevalence ^b	No. of positive samples	Mean concn \pm SEM (CFU/g)	Total no. of samples	% Prevalence ^b	No. of positive samples	Mean concn \pm SEM (CFU/g)
Chicken salad	25	64 A ^c	16	$(2.1 \pm 1.5) \times 10^3$	25	92 B	23	$(2.1 \pm 0.5) \times 10^3$
Vegetables	60	28 (40)	17	$(1.2 \pm 3) \times 10^3$	60	43 (84)	26	$(1.9 \pm 6.2) \times 10^3$
Meat	25	20 (20)	5	$(7.5 \pm 4.5) \times 10^2$	25	28 (28)	7	$(2.8 \pm 1.8) \times 10^2$
Miscellaneous	45	25 (36)	11	$(8.0 \pm 1.5) \times 10^3$	45	20 (32)	9	$(4.2 \pm 3.9) \times 10^2$
Chicken burger	25	24 A	6	$(1.6 \pm 0.9) \times 10^3$	25	64 B	16	$(5.8 \pm 1.8) \times 10^2$
Vegetables	50	18 (24)	9	$(0.4 \pm 3.0) \times 10^3$	60	17 (28)	10	$(0.7 \pm 1.2) \times 10^3$
Meat	25	12 (12)	3	$(4.8 \pm 4.5) \times 10^2$	25	24 (24)	6	$(1.5 \pm 1.1) \times 10^2$
Miscellaneous	65	12 (24)	8	$(0.6 \pm 1.5) \times 10^3$	65	14 (16)	9	$(1.6 \pm 1.8) \times 10^2$
Carrot cake	15	20	3	$(8.4 \pm 4.2) \times 10^3$ A	15	7	1	$(4.1 \pm 1.6) \times 10^1$ B

^a Vegetables, two to three ingredients per meal; meat, one ingredient per meal; miscellaneous, two to four ingredients per meal (chips, fries, sauce, bread, croutons, dressing, and cheese).

^b Values in parentheses show percent meals (salads and burgers) contaminated due to contamination of a main ingredient.

^c Different superscript capital letters within the same row indicate significant differences.

peppers, onions, dressing, croutons, cheese, and chips), (ii) chicken burger/sandwich (chicken fillet, lettuce, tomatoes, pickles, bread, cheese, French fries, and barbecue sauce), or (iii) carrot cake (cake with frosting). Some minor ingredients such as croutons, chips, dressing, and type of vegetables varied between restaurants depending on the recipe as well as within restaurants based on availability. Five to 10 grams of each ingredient was separately homogenized in 100 ml of phosphate-buffered saline (pH 7.2; ICN Biomedicals, OH) for 5 min using a stomacher (Stomacher 400 Circulator). From this sample, 100 μ l was plated in triplicate on mEnterococcus Agar (Becton Dickinson, MA) and incubated at 37°C for 48 h. Red, pink, and burgundy colonies were counted, and the concentration of enterococci was calculated in CFU per gram of RTEF (meal and its main ingredients) (Table 1). Up to four presumptive enterococcal colonies with different colony morphologies per RTEF sample were streaked on Trypticase soy agar (Becton Dickinson, MA), incubated at 37°C for 24 h, and stored at 4°C for further analysis. The genus-level identification was confirmed by the esculin hydrolysis test using Enterococcosel broth (Becton Dickinson, MA) and by growth at 44.5°C in Trypticase soy broth (Becton Dickinson, MA) with 5% sodium chloride. Results for prevalence and concentration of enterococci in RTEF were analyzed and presented as values per each meal (the meal was considered positive if it contained one or more contaminated individual ingredients) as well as per its three main groups of ingredients: vegetables, meat, and miscellaneous ingredients (chips, fries, sauce, bread, croutons, dressing, and cheese) (Table 1). The groups of ingredients were created because of the variation in availability of some ingredients (vegetables and miscellaneous) between and within restaurants over time (resulting in a low number of replicates). The diversity of enterococci is reported per each of the main ingredient groups of three different RTEF meals (chicken salad, chicken burger, and carrot cake) (Fig. 1).

Isolate identification and screening for AR by phenotype. Multiplex PCR was used to identify four common species: *E. faecalis*, *Enterococcus faecium*, *Enterococcus casseliflavus*, and *Enterococcus gallinarum* (23). PCR amplification and sequencing of the manganese-dependent superoxide dismutase gene *sodA* (36) were used for those isolates which could not be identified by multiplex PCR. The strains used as positive and negative controls and the primer sequences have been reported previously (30).

Identified isolates were screened for AR by the disc diffusion method on Mueller-Hinton agar (Becton Dickinson, MA) using six antibiotics: tetracycline, 30 μ g/ml; chloramphenicol, 30 μ g/ml; ciprofloxacin, 5 μ g/ml; erythromycin, 15 μ g/ml; vancomycin, 30 μ g/ml; and ampicillin, 10 μ g/ml. High-level resistance to aminoglycosides was assessed by the agar dilution technique using 2,000 μ g/ml of streptomycin and 2,000 μ g/ml kanamycin in brain heart infusion agar (Becton Dickinson, MA). *E. faecalis* ATCC 19433 was used as a quality control strain. The protocols followed the guidelines of the Clinical and Laboratory Standards Institute (34).

Screening for AR and virulence determinants by PCR. Multiplex or single PCR was used to screen all identified isolates for tetracycline and erythromycin resistance genes. The group I multiplex reaction included *tet(A)*, *tet(C)*, and *tet(Q)* genes; group II covered *tet(M)*, *tet(S)*, *tet(K)*, and *tet(O)* as described

previously (30). Each reaction mixture consisted of 25 μ l Master Mix (Promega, Madison, WI) with 4 mM MgCl₂ (group I), 3 mM MgCl₂ (group II), and 3 μ l of supernatant from freshly boiled cells. The PCR conditions were described previously (35, 47). Single PCRs were used to screen *tet(W)* (3) and *erm(B)* (43). All identified isolates were screened for four putative virulence determinants including *gelE* (gelatinase), *asa1* (aggregation substance), *cylA* (cytolysin), and *esp* (enterococcus surface protein) using multiplex PCR as described previously (46). To confirm the identity of the determinants, one randomly selected PCR product for each resistance and virulence determinant was purified, sequenced using either the PCR primers or the T7 primer after cloning of the PCR product using the pGEM-T Easy Vector System (Promega, Madison, WI), and compared to the sequences in GenBank using BLAST (Basic Local Alignment Search Tool) (2). Manual sequence alignment was done with the CodonCode Aligner (version 1.5.2; CodonCode Corporation, Dedham, MA). The same positive-control strains were used as reported previously (30).

All *E. faecalis* isolates ($n = 21$) were screened for six marker genes (EF_0573, EF_0592, EF_0605, EF_1420, EF_2144, and pathogenicity island EF_0050) for potentially clinically relevant strains. In addition, we analyzed 50 isolates of *E. faecalis* isolated in the previous study (30) from houseflies collected from the same five fast-food restaurants for these marker genes. PCRs were conducted under conditions described previously (26).

Screening for virulence genes by phenotype. Trypticase soy agar with 3% skim milk was used for detection of gelatinase activity. All isolates were streaked and after 24 h of incubation at 37°C were examined for a clearance zone surrounding the colonies (15).

For the phenotypic expression of the *asa1* gene, *E. faecalis* JH2-2 was grown for 6 h at 37°C in Todd-Hewitt broth (Becton Dickinson, MA). Broth was then centrifuged at 6,000 rpm for 10 min on a Sorvall RC-5B refrigerated (4°C) Superspeed centrifuge with the SS-34 rotor, and the pheromone-containing supernatant that induces pheromone-responsive plasmids was removed and autoclaved for 15 min. Tested isolates were grown in Todd-Hewitt broth (5 ml) for 6 h at 37°C. After incubation, 1 ml of the supernatant from *E. faecalis* JH2-2 was added to each tube and incubated at 37°C overnight on a shaker (150 rpm). Isolates that showed clumping (examined by naked eye and under a compound microscope) were considered positive for aggregation substance expression (7). *E. faecalis* OG1RF:pCF10 was used as a positive control.

Phenotypic assays for cytolysin were conducted using Columbia blood agar base (Becton Dickinson, MA) with 5% cattle blood. Isolates were streaked and incubated at 37°C for 48 to 72 h. Isolates showing a complete clearance zone around the colonies (β -hemolysis) were considered positive for cytolysin expression (15).

Genotyping by PFGE. Relationships of *E. faecalis* and *E. faecium* were analyzed by pulsed-field gel electrophoresis (PFGE) using the CHEF Mapper (BioRad, Hercules, CA) and the restriction enzyme AluI as previously described (33, 44). Cluster analysis was performed with BioNumerics (Applied Maths, Kortrijk, Belgium) by using the Dice correlation coefficient (6) and the unweighted-pair group mathematical average algorithm (41).

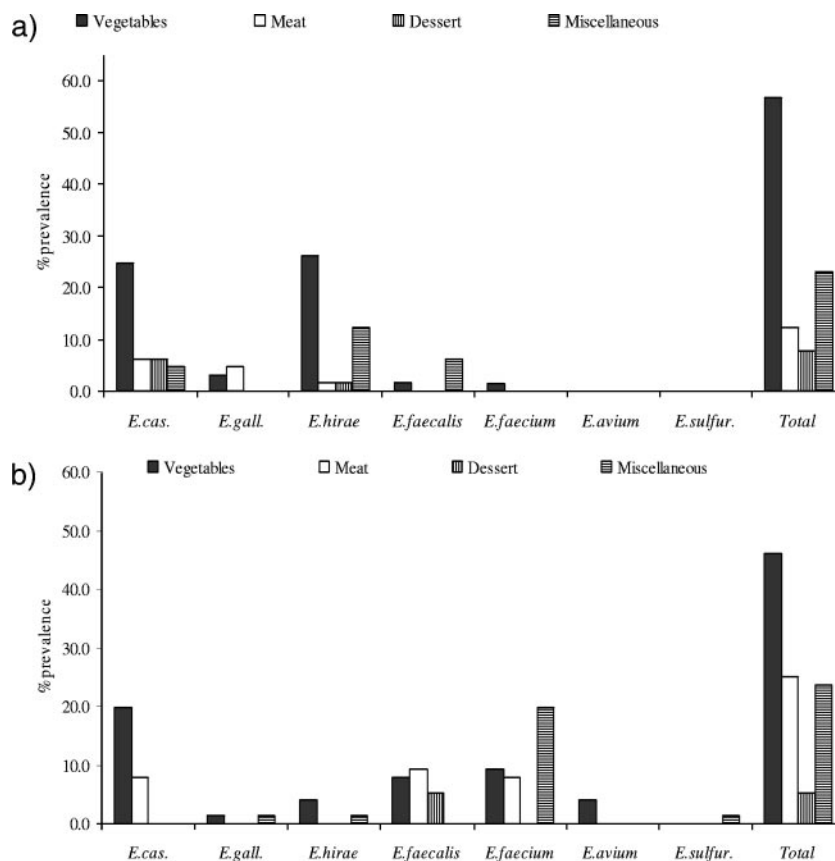


FIG. 1. Prevalence and diversity of enterococci from RTEF from five fast-food restaurants in WI (a) and SU (b). *E. cas.*, *E. casseliflavus*; *E. gall.*, *E. gallinarum*; *E. sulfur.*, *E. sulfureus*. The “miscellaneous” group includes chips, fries, sauce, bread, croutons, dressing, and cheese.

Statistical analysis. Data for the prevalence of enterococci in different RTEFs were analyzed by *t* test (bacterial concentration) and chi-square test (frequency of contamination) using the PopTools plug-in for Excel (G. Hood, 2000; <http://www.cse.csiro.au/poptools/>).

RESULTS

Prevalence, concentration, and diversity of enterococci in RTEF. The prevalence of contaminated RTEF in SU was significantly higher than that in WI, increasing from 64 to 92% in chicken salads and from 24 to 64% in chicken burgers ($P = 0.001$) (Table 1). More specifically, the increase in frequency of contamination in chicken salads in SU was mainly due to contaminated vegetables (from 28% in WI to 43% in SU per vegetable ingredients), making 84% of chicken salads contaminated in SU due to one or more contaminated vegetable ingredients (Table 1). In contrast, the contamination frequency increase in chicken burgers in SU was due to higher prevalence of contaminated meat (from 12% in WI to 24% in SU per ingredient and meal) and also because of a relatively even distribution of contaminated ingredients (17% vegetables, 24% meat, and 14% miscellaneous) across meals, making 64% of chicken burgers contaminated (Table 1). The frequency of contamination in miscellaneous ingredients was similar between the seasons within different meals (Table 1), although the frequency of contamination in chicken burgers during WI (12% of 65 miscellaneous ingredients) was more evenly spread

across the meals (making 24% of chicken burgers contaminated due to one or more miscellaneous ingredients) than that in SU (Table 1). The prevalence of enterococci in carrot cakes decreased from 20% in WI to 7% in SU; however, the number of samples was lower than that of other foods due to low availability of carrot cakes in two of the selected restaurants (Table 1).

The overall concentrations of enterococci in contaminated RTEF (CFU/g) during the two seasons were similar (chicken salad, $2.1 \times 10^3 \pm 1.5 \times 10^3$ [WI] and $2.1 \times 10^3 \pm 0.5 \times 10^3$ [SU]; chicken burger, $1.6 \times 10^3 \pm 0.9 \times 10^3$ [WI] and $5.8 \times 10^2 \pm 1.9 \times 10^2$ [SU]) while enterococcal concentration in carrot cakes decreased in SU from 8.4×10^3 (WI) to 4.1×10^1 CFU/g (Table 1). In salads and burgers, the concentrations of enterococci in WI and SU were not significantly different ($P = 0.56$ and $P = 0.96$, respectively), while the concentration in carrot cakes decreased significantly in SU ($P = 0.03$) (Table 1). This represents a relatively high concentration of enterococci per each meal from both sampling seasons (total weight of each meal): 7.6×10^5 CFU/chicken salad (362.2 g), 4.8×10^5 CFU/chicken burger (443.1 g), and 6.6×10^5 CFU/carrot cake (156.2 g).

A total number of 145 enterococcal colonies from RTEF were isolated and further characterized. After the identification by sequencing of the *sodA* gene, four isolates were found not to be enterococci and were not analyzed further. Thus, 65 WI and 76 SU isolates were selected for further analysis (Table 2) (3).

TABLE 2. AR profiles of identified enterococcal isolates from WI and SU sampling of RTEFs

Season and identification	No. (%) of isolates								
	Total	Resistant to drug ^a :							
		CIP	CHL	VAN	ERY	AMP	TET	KAN	STR
WI									
<i>E. casseliflavus</i>	27 (41.5)	9 (33.4)	2 (7.4)	0	1 (3.7)	0	2 (7.4)	1 (3.7)	1 (3.7)
<i>E. gallinarum</i>	5 (7.7)	0	0	0	0	0	1	0	0
<i>E. hirae</i>	27 (41.5)	0	2 (7.4)	0	1 (3.7)	0	27 (100)	1 (3.7)	0
<i>E. faecalis</i>	5 (7.7)	0	1 (20)	0	1 (20)	0	3 (60)	0	0
<i>E. faecium</i>	1 (1.5)	0	0	0	0	0	0	0	0
Total	65	9 (13.8)	4 (6.1)	0	3 (4.6)	0	33 (50.8)	2 (3.1)	1 (1.5)
SU									
<i>E. casseliflavus</i>	21 (27.7)	0	1 (4.8)	0	3 (14.2)	0	2 (10)	0	1 (4.8)
<i>E. gallinarum</i>	2 (2.6)	0	0	0	0	0	2 (100)	1 (50)	0
<i>E. hirae</i>	4 (5.3)	0	0	0	1 (25)	0	4 (100)	0	0
<i>E. faecalis</i>	17 (22.4)	0	0	0	0	0	0	0	0
<i>E. faecium</i>	28 (36.8)	0	0	0	11 (39.2)	0	4 (14.3)	8 (28.5)	0
<i>E. sulfureus</i>	1 (1.3)	0	0	0	0	0	0	1 (100)	0
<i>E. avium</i>	3 (3.9)	0	0	0	2 (66.7)	0	1 (33.3)	0	0
Total	76	0	1 (1.3)	0	17 (22.3)	0	13 (17.1)	10 (13.2)	1 (1.3)

^a CIP, ciprofloxacin; CHL, chloramphenicol; VAN, vancomycin; ERY, erythromycin; AMP, ampicillin; TET, tetracycline; KAN, kanamycin; STR, streptomycin.

The diversity of isolates from RTEF is shown in Fig. 1a and b. Overall, five *Enterococcus* species were found in WI and seven species in SU. The most prevalent species among WI isolates were *E. casseliflavus* and *Enterococcus hirae* (41.5% each), followed by *E. gallinarum* (7.7%), *E. faecalis* (7.7%), and *E. faecium* (1.5%) (Fig. 1a). *E. faecium* (36.8%), *E. casseliflavus* (27.6%), and *E. faecalis* (22.4%) were the most common species among isolates from RTEF in SU, followed by *E. hirae* (5.3%), *E. avium* (3.9%), *E. gallinarum* (2.6%), and *Enterococcus sulfureus* (1.3%) (Fig. 1b).

E. casseliflavus and *E. hirae* were detected primarily from vegetables (24.6% WI and 19.7% SU and 26.2% WI and 3.9% SU, respectively) (Fig. 1a and b). In WI, *E. faecalis* was isolated mainly from miscellaneous ingredients while the contamination with *E. faecalis* increased in SU in meat and vegetables to 9.2% and 7.9%, respectively (Fig. 1a and b). Similarly, *E. faecium* was more common in SU months, mainly in the miscellaneous group (19.7%), vegetables (9.2%), and meat (7.9%). The prevalence of *E. gallinarum* was low during both sampling seasons, and *Enterococcus avium* and *Enterococcus sulfuricans* were detected at a low frequency in SU only (Fig. 1a and b).

Prevalence and diversity of AR and virulence factors by phenotype and genotype. Phenotypic analysis of WI isolates showed resistance to several antibiotics including tetracycline (50.8%), ciprofloxacin (13.8%), erythromycin (4.6%), chloramphenicol (6.1%), kanamycin (3.1%), and streptomycin (1.5%) (Table 2). Isolates from SU RTEF were resistant to erythromycin (22.3%), tetracycline (17.1%), and kanamycin (13.2%), followed by chloramphenicol and streptomycin (both 1.3%). Several isolates (7.7%) in WI were resistant to two or more antibiotics (one *E. casseliflavus* isolate was resistant to six antibiotics), while isolates from RTEF in SU were resistant to a maximum of two antibiotics.

The most common determinant coding for tetracycline resistance was *tet*(M) (35.4% prevalence in WI and 4.0% in SU). *tet*(M) was frequent in *E. faecalis* (80%), followed by *E. hirae*

(66.7%) and *E. casseliflavus* (3.7%) in WI and *E. faecium* (3.6%) and *E. hirae* (50%) in SU (Table 3). The diversity of the *tet* genes was greater in SU isolates, where *tet*(S) (3.6%) and *tet*(O) (3.6%) were detected. The *erm*(B) gene was detected at low frequency (9.2% in WI and 4.0% in SU).

The overall prevalence of the putative virulence genes was low: 12.3% for *gelE*, 3.1% for *asa1*, and 0% for *cylA* (Table 3). The *gelE* gene was found in WI, primarily in *E. faecalis* (80% of isolates), *E. gallinarum* (40%), and *E. casseliflavus* (7.4%). Many of these isolates expressed this gene in the phenotypic assay for gelatinase (Table 3). No isolates from the SU were positive for *gelE*. Interestingly, one isolate of *E. hirae* and two of *E. faecalis* were positive for gelatinase by phenotype but negative for *gelE* by PCR (Table 3). The gene for the aggregation substance of *E. faecalis* was detected in 40.0% of WI isolates and 47.1% of SU isolates, but it was not expressed phenotypically (Table 3). The *cylA* gene was not detected among any of the isolates; however, several isolates of *E. casseliflavus* and *E. gallinarum* from WI tested positive for β -hemolysis on cattle blood (Table 3). The identity of selected AR and virulence genes from multiplex PCRs was confirmed by sequencing (data not shown).

Six marker genes for potentially virulent *E. faecalis* were screened in 21 isolates of *E. faecalis* from RTEF and an additional 50 isolates of *E. faecalis* from the houseflies collected from the same five fast-food restaurants in our previous study (30). A single isolate, RTEF59, from the chicken salad (lettuce) in SU was positive for the four of the six marker genes (EF_0573, EF_0592, EF_0605, and EF_0050) (overall prevalence, 4.7%). One *E. faecalis* isolate, RTEF15, was positive for the EF_1420 gene. From the housefly gut isolates from restaurants R1 and R5, two *E. faecalis* isolates were positive for EF_0592, another two isolates from R1 were positive for EF_0050, and one isolate from R1 was positive for EF_1420.

Genotypic diversity of *E. faecalis* and *E. faecium* assessed by PFGE. *E. faecalis* ($n = 20$) and *E. faecium* ($n = 23$) isolates from RTEF were genotyped by PFGE. *E. faecalis* repre-

TABLE 3. Prevalence of tetracycline and erythromycin resistance genes and virulence determinants (by genotype and phenotype) in identified enterococcal isolates from RTEF

Season and identification	No. (%) of isolates									
	Total	With genotype:						With phenotype determined by assay:		
		<i>tet</i> gene(s)	<i>erm</i> (B)	<i>gelE</i>	<i>asaI</i>	<i>esp</i>	<i>cyLA</i>	Gelatinase	Clumping assay	Hemolysis ^a
WI										
<i>E. casseliflavus</i>	27	1 (3.7) [<i>tet</i> (M)]	2 (7.4)	2 (7.4)	NA ^b	0	0	1 (3.7)	NA	11 (40.7)
<i>E. gallinarum</i>	5	0	2 (40)	2 (40)	NA	0	0	1	NA	4 (80)
<i>E. hirae</i>	27	18 (66.7) [<i>tet</i> (M)]	0	0	NA	0	0	1 (3.7)	NA	0
<i>E. faecalis</i>	5	4 (80) [<i>tet</i> (M)]	2 (40)	4 (80)	2 (40)	0	0	3 (60)	0	0
<i>E. faecium</i>	1	0	0	0	NA	0	0	0	NA	0
Total	65	23 (35.4)	6 (9.2)	8 (12.3)	2 (3.1)	0	0	6 (9.2)	0	15 (23.1)
SU										
<i>E. casseliflavus</i>	21	2 (9.5) [<i>tet</i> (S)]	0	0	NA	0	0	0	NA	0
<i>E. gallinarum</i>	2	1 (50) [<i>tet</i> (S)]	0	0	NA	0	0	0	NA	0
<i>E. hirae</i>	4	2 (50) [<i>tet</i> (M)]; 1 (25) [<i>tet</i> (O)]	0	0	NA	0	0	0	NA	0
<i>E. faecalis</i>	17	0	1 (5.9)	0	8 (47.1)	0	0	2 (11.8)	0	0
<i>E. faecium</i>	28	1 (3.6) [<i>tet</i> (M)]; 1 (3.6) [<i>tet</i> (S)]; 1 (3.6) [<i>tet</i> (O)]	0	0	NA	0	0	0	NA	0
<i>E. sulfureus</i>	1	0	0	0	NA	0	0	0	NA	0
<i>E. avium</i>	3	0	2 (66.7)	0	NA	0	0	0	NA	0
Total	76	9 (11.9)	3 (4.0)	0	8 (10.5)	0	0	2 (2.6)	0	0

^a Cattle blood.^b NA, not applicable.

sented four different genotypes ($\leq 85\%$ similarity) with type I containing the majority of the isolates divided into two subtypes ($>85\%$ similarity) (Fig. 2a). Subtype Ia contained isolates from the chicken salad from three different restaurants (R4, meat and lettuce; R3, croutons; and R5, cheese). This subtype clustered together with the positive-control strains *E. faecalis* ATCC 19433 and *E. faecalis* MMH583. Subtype Ib contained isolates from restaurants R2 and R3. R2 isolates originated from the chicken salad (meat) and cake frosting, and R3 isolates came from the chicken salad (meat, peppers, tomatoes, and lettuce). The type II isolates were from lettuce and barbecue sauce from restaurant R3. The type III cluster represented one isolate from the chicken salad (meat) from R2. The type IV isolates were from different restaurants: the chicken salad (lettuce) isolate was from R4 and the cake frosting isolate was from R2. Identical or very closely related strains ($\geq 95\%$ similarity) were detected from two restaurants: R2 (chicken salad and carrot cake) and R3 (chicken salad) (Fig. 2a).

E. faecium isolates were more divergent and grouped in five genotypes with the closest similarity index being 75.5 (Fig. 2b). Type I, II, and III isolates clustered (81.3% similarity) and originated from R4 and R5 (chicken salad; cheese, lettuce, and meat), and one isolate came from R1 chicken salad (tomato). Types IV and V clustered together (84.4% similarity) and represented chicken salad (vegetables and cheese); chicken burger (meat and bread) from restaurants R5, R1, and R4 (type IV); and the chicken salad cheese from R5 (type V). Identical or very closely related strains ($\geq 95\%$ similarity) were detected from R1 (chicken salad and chicken burger) and R5 (chicken salad) from the SU sampling (Fig. 2b).

DISCUSSION

The recent metagenomic analysis of microbes from the human intestine has shown that the microbial community in this organ, primarily in the colon, is dominated by two bacterial divisions, the *Bacteroidetes* and the *Firmicutes* (8, 9, 52), and outnumbers human cells 10-fold and human genes 100-fold (4, 10). This enormous bacterial community represents an important site for gene exchange (intra- and interspecies) (38, 39, 40) among permanent and transient colonizers as well as bacteria ingested with food and drinks and passing through the digestive tract (10, 27, 42). While several studies have assessed the potential for ingested bacteria to colonize the gut of adults and/or horizontally transfer selected genes to the resident microbial community (27, 32, 42), the extent of the actual influx of bacteria and associated resistance and virulence genes from RTEF to the digestive tract has not been extensively studied.

Investigations of the level of contamination of food with antibiotic-resistant strains have been focused on raw food (pork, beef, and poultry) before preparation and cooking (17, 19, 20, 31, 48, 50), during which most of the bacteria and their genes are likely destroyed; milk and milk products (cheese and other fermented products) (12, 13, 17, 48); fresh produce (12, 22, 31, 48); and probiotic strains (11, 12).

Our data showed that RTEFs, mainly chicken salads and chicken burgers (Table 1) from fast-food restaurants, were frequently contaminated with enterococci at relatively high concentrations ($\sim 10^3$ CFU per gram of food) and the contamination frequency increased in SU months. While *E. casseliflavus* and *E. hirae* were the dominant species in RTEFs (mainly vegetables) in WI, the enterococcal community was different in RTEF in SU months with an increased diversity and elevated population of *E. faecalis* (in vegetables and meat) and *E. fae-*

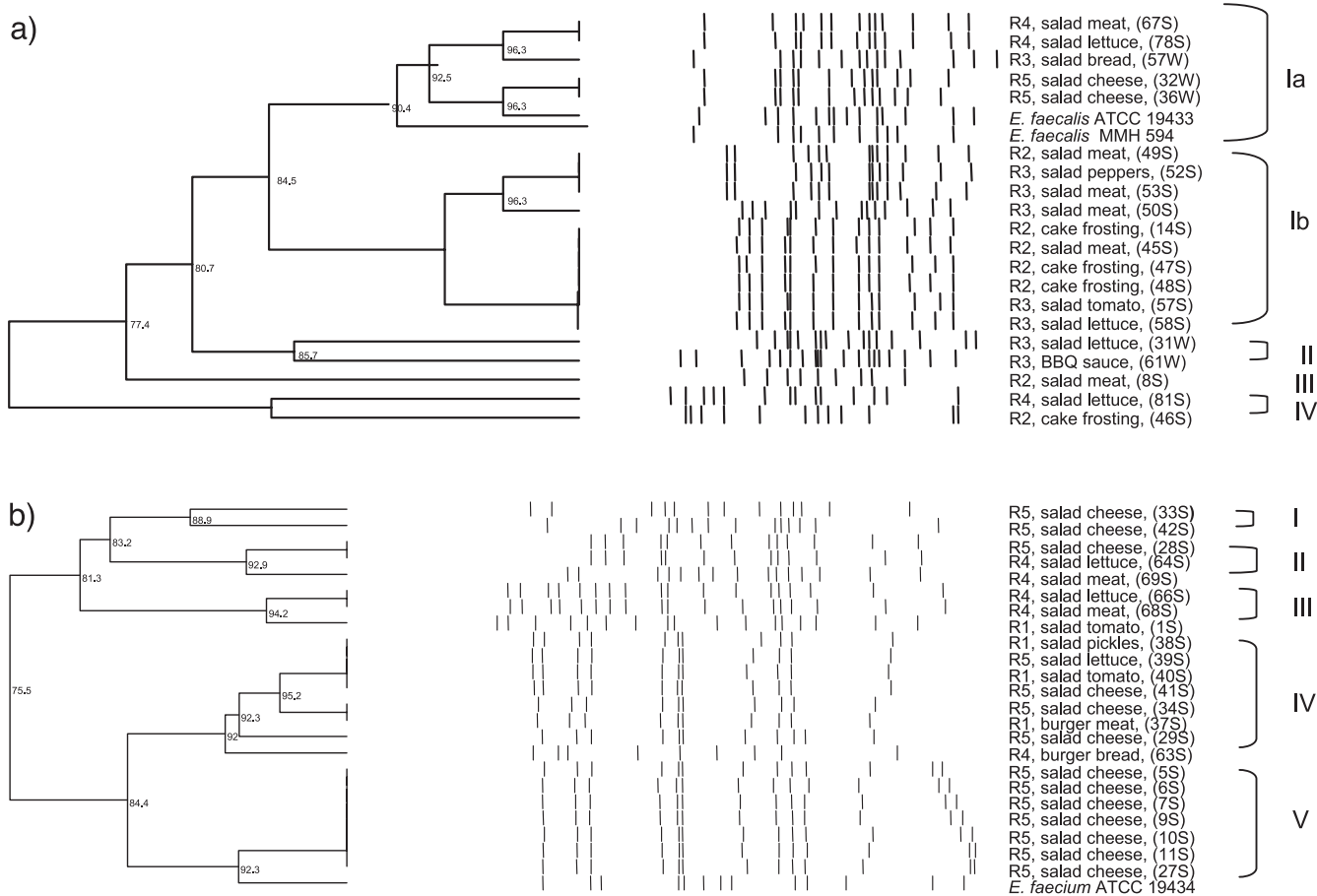


FIG. 2. Dendrogram illustrating relatedness of *E. faecalis* (a) and *E. faecium* (b) strains isolated from RTEF based on PFGE patterns of AluI-digested DNA. R, restaurant; S, SU isolate; W, WI isolate.

cium (in vegetables, meat, and miscellaneous ingredients such as cheese, fries, and croutons). The presence of *E. casseliflavus* and *E. hirae* on vegetables is not surprising, as they are commonly found associated with plants (12). The source(s) of contamination of RTEF in SU with *E. faecalis* and *E. faecium* is unknown; however, the low prevalence of putative virulence genes and virulence markers suggests that the enterococcal contamination in RTEF is environmental and not of clinical origin. It is possible that enterococci originated from people handling the food in restaurants or during harvesting and processing. One of the rationales for screening RTEF in WI and SU in this study was to assess indirectly the potential role of insects, primarily houseflies, in contamination of food with enterococci in SU months. In a previous study (30), we characterized enterococci from houseflies collected in the same five fast-food restaurants and showed that these insects commonly carried a high population of antibiotic-resistant and potentially virulent enterococci. Results from the current study do not directly implicate flies as a source of RTEF contamination, although the frequency of enterococcal contamination as well as the prevalence of *E. faecalis* (the dominant species detected in houseflies) (30) increased in RTEF in SU months when flies

in fast-food restaurants are common. Other factors likely playing a role in the seasonal differences include, for example, possible different geographical origins of RTEF ingredients, various growing practices (e.g., using animal manure as fertilizer), and longer refrigeration and storage times in WI.

Taking into account the total weight of each meal, the concentration of enterococci that would be ingested in each contaminated meal was relatively high (7.6×10^5 CFU/chicken salad, 4.8×10^5 CFU/chicken burger, and 6.6×10^5 CFU/carrot cake), and considering, for example, that 51% of WI isolates were phenotypically resistant to tetracycline, this represents (using a conservative estimate of only a single copy of the *tet* resistance gene per cell) the influx of 3.8×10^5 , 2.5×10^5 , and 3.4×10^5 *tet* genes from the chicken salad, chicken burger, and carrot cake, respectively. Since RTEFs are very popular and commonly consumed in the United States and other parts of the world, the influx of resistance genes is very frequent and may play a role in the acquisition and spread of AR determinants in the human digestive tract.

The most prevalent tetracycline resistance determinants in our food isolates were *tet*(M), *tet*(O), and *tet*(S) genes coding for ribosomal protection proteins (5) which are common in

tetracycline-resistant strains reported from various environments including food products (21, 48). No efflux pump genes were identified in our isolates. A seasonal shift in the AR profile was observed: *tet*(M) dominated in the WI isolates, while SU isolates carried *tet*(S), *tet*(M), and *tet*(O). These genes are commonly carried on mobile genetic elements such as plasmids and conjugative transposons (37); therefore, it is possible that they can be transferred to the resident bacterial microbiota of the human digestive tract by horizontal gene transfer.

The prevalence of putative virulence genes including *gelE*, *asa1*, *cylA*, and *esp* in our isolates was low, indicating that these isolates were not likely virulent and the influx of virulence genes was not high. Interestingly, a large percentage of *E. casseliflavus* isolates (40.7%) lysed red blood cells on cattle blood agar but the *cylA* gene was not detected in any of those isolates. In contrast, several *E. faecalis* isolates were positive for *asa1* but this was not expressed phenotypically. In addition, RTEFs as well as houseflies from restaurants carried enterococcal isolates with a very low prevalence of genes (EF_0573, EF_0592, EF_0605, EF_0050, EF_1420, and EF_2144) predicted to be associated with clinically important isolates (26). This supports the suggestion that these genes are potentially good markers for virulent enterococci and also that our isolates can be considered environmental.

Genotyping with PFGE showed that both *E. faecalis* and *E. faecium* overall clustered based on the source (restaurant) and season. *E. faecalis* represented several types and subtypes from RTEFs across different restaurants, but with the exception of restaurants R2 and R3, no common strains were detected, indicating that the contamination originated from various sources and did not represent a clonal spread. Genotypes of *E. faecium* were more diverse and similarly grouped by the restaurant, food ingredient, and season. A few common strains were detected from restaurants R4 and R5 (from chicken salad lettuce) as well as R1 and R5 (chicken salad pickles, lettuce, tomatoes, and cheese), indicating a common source of one or more of these ingredients. Clearly, the likelihood of cross-contamination of ingredients within the same meal due to their close physical contact was high.

In conclusion, the results of this study showed that RTEFs such as chicken salads and chicken burgers in fast-food restaurants are frequently contaminated with a relatively high concentration of enterococci. This represents a frequent influx of AR genes (primarily tetracycline and erythromycin resistance genes) to the human digestive tract that can potentially spread by horizontal transfer to the resident microbial community and result in formation of the AR gene reservoir without any selective pressure from antibiotic use. Additional studies are needed to quantify the influx of resistance genes from enterococci and other common food bacterial contaminants and their potential to be transferred to the resident microbial community of the human digestive tract.

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