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ORIGINAL ARTICLE

Diversity and antimicrobial resistance of *Enterococcus* from the Upper Oconee Watershed, Georgia

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Abstract

Aim: It is well-known that enterococci are abundant in the environment; however, the role of surface water as a reservoir of antimicrobial-resistant enterococci remains largely undefined. In this study, surface water samples were collected over a 2-year period from the Upper Oconee watershed, Athens, GA to examine enterococci and their antimicrobial resistance.

Methods and Results: Approximately 97% (445/458) of the samples were positive for enterococci and a total of 637 enterococci were isolated. The predominant species were *Enterococcus casseliflavus* (33·6%) followed by *Enterococcus faecalis* (26·5%) and *Enterococcus hirae* (13·2%). Regardless of species, the highest levels of resistance were to lincomycin (88·5%) and tetracycline (13%); isolates also exhibited resistance to newer antimicrobials, daptomycin (8·9%) and tigecycline (6·4%). Multidrug resistance (resistance ≥3 antimicrobial classes) was observed to as many as five classes of antimicrobials. Resistant enterococci appeared to be randomly dispersed over the seasons rather than clustered by species or antimicrobial resistance.

Conclusions: This study demonstrated that surface waters contain a large population of diverse species of antimicrobial-resistant enterococci, including resistance to new antimicrobials.

Significance and Impact of the Study: These results may indicate the potential of human intestinal illness and/or colonization of the human gut with resistant enterococci as enterococci correlate with increased disease risk to humans during recreational exposure to water.

Introduction

Enterococci are a diverse group of bacteria with importance in several areas including clinical medicine, foodborne illness, food processing and microbial risk assessment. In clinical medicine, they are a leading cause of nosocomial infections and have been implicated in bacteraemia, endocarditis and urinary tract infections (Murray 1990; Huycke *et al.* 1991; Jett *et al.* 1994). Treatment of enterococcal infections in clinical medicine is often complicated due to intrinsic and acquired resistance in the bacteria (Murray 1990; Facklam *et al.* 2002; Malani *et al.* 2002; Huijbers *et al.* 2015)). Enterococcal foodborne illness is less severe causing symptoms such as vomiting and headaches as a result of production of biogenic amines from ingestion of enterococci in fermented foods (Tham *et al.* 1990; Gardin *et al.* 2001; Giraffa 2002). On the other hand, enterococci have beneficial qualities that have made them useful as probiotics and as indicators of faecal contamination in water bodies (Foulquie Moreno *et al.* 2006; Byappanahalli *et al.* 2012). As natural inhabitants of the gastrointestinal tract of humans and animals, enterococci have been used as indicators of faecal pollution along with *Escherichia coli* (Roslev and Bukh 2011). Of the two bacteria, enterococci are thought to be better indicators of the possible presence of bacterial pathogens

in surface water as their occurrence, particularly at high levels, is associated with the risk of contracting human gastroenteritis during swimming-related activities (Turbow *et al.* 2003).

As enterococci are generally not considered important pathogens outside the hospitals and healthcare settings, studies reporting enterococci from the environment are scarce. Most studies on water sources are focused either on water quality, human-specific species, *Enterococcus faecalis* and *E. faecium*, or on polluted water environments such as hospital-impacted wastewater and agricultural run-off (Sadowy and Luczkiewicz 2014; Kapoor *et al.* 2015; Nishiyama *et al.* 2015). Less attention has been given to enterococci in natural water environments and on different species of *Enterococcus*. Resistant enterococci present in water sources can potentially transfer resistance genes to bacterial pathogens in the environment or to bacteria in the human gastrointestinal tract after ingestion of contaminated water during recreational activities (Murray 1990; Facklam *et al.* 2002; Malani *et al.* 2002; Huijbers *et al.* 2015). Since resistance in enterococci may vary according to species, species identification is an important factor to consider when characterizing antimicrobial resistance.

Bacterial diversity and prevalence in surface water are subject to change due to both point and nonpoint (e.g. agricultural run-off, storm water routes) sources as well as seasonal influences such as water temperature and rainfall (Singer *et al.* 2006; Lanthier *et al.* 2011). In order to better understand human health risks associated with the presence of enterococci in surface water impacted by nonpoint source contamination, studies are needed to identify the differences in enterococcal species composition, antimicrobial resistance of those species and changes in the composition over time. In this study, the occurrence, species distribution and antimicrobial susceptibility of enterococci from surface water from the Upper Oconee watershed in northeastern Georgia were evaluated. The Oconee River is formed by the convergence of the North Oconee River and the Middle Oconee River just south of Athens, Georgia, and is used for municipal, industrial, agricultural and recreational purposes (Environmental Protection Division 1998). Samples were collected quarterly, once each season, at different locations in the Upper Oconee watershed, for 2 years by the Upper Oconee Watershed Network (UOWN) (http://www.uown.org). Enterococci were isolated from the samples, identified to species and tested for resistance to antimicrobials used in clinical medicine and agriculture. The results of this study will improve the understanding of the dynamics of enterococci in a surface water system which may impact human health.

Materials and methods

Sampling of river water

The rivers and streams sampled in this study were located in the Upper Oconee watershed, Georgia, USA (USGS Cataloging unit 03070101). The sampling sites were located in the Middle Oconee River, North Oconee River and the tributaries that drain into these rivers (Fig. 1). Sample sites were selected to represent a range of land uses, such as forested, agricultural, residential, recreational and industrial. One litre water samples from each site were collected seasonally four times a year with the assistance of the UOWN. Since 2008, UOWN has assembled a team of volunteers to collect water samples from the Upper Oconee watershed on a quarterly basis. The number of locations sampled varied from 80 to 100 during the Spring collection, which is the UOWN's largest collection event, River Rendezvous. The annual River Rendezvous event enlists over 100 volunteers to sample approximately 100 locations in the watershed to evaluate the health of the watershed through chemical and biological sample analysis. During the remaining quarterly collections, 30 to 60 water samples were collected depending on available volunteers and access to the sampling sites. A total of 29 locations were routinely sampled to serve as controls with nine sites sampled all eight times, eight sites sampled seven times and 12 sites sampled six times.

Isolation and identification of bacteria

Filtration and enrichment of water samples were performed as previously described (Cho *et al.* 2018). Briefly, 0·5 g of cellulose filter powder (Aqua Dew™, Lahore, Pakistan) was added to water samples (1 L), which was then filtered onto 47-mm glass fibre filters (Pall Corporation, Ann Arbor, MI). The glass fibre filter and cellulose filter powder were incubated in 25 ml of buffered peptone (BP; BD Difco™, Franklin Lakes, NJ) water for 24 h at 37°C. For *Enterococcus* isolation, 0·1 ml of BP enrichment was streaked on selective agar plates and incubated for 24 h at 37°C. In order to test for the efficacy of the media, multiple media were used until Enterococcosel (BD Difco) was chosen as the media of use from 2015 Fall onward. For 2015 Winter, Enterococcosel and mE agar (BD Difco) were used; for 2015 Spring, mE agar was used; for 2015 Summer, Enterococcosel, mE agar and CHROMagar Enterococcus (not on market, CHROMagar Microbiology, Paris, France) were used. One colony having the typical appearance of *Enterococcus* from each positive plate was confirmed and its species was determined using multiplex PCR as previously described (Jackson *et al.* 2004). An exception was the Summer of 2015 when

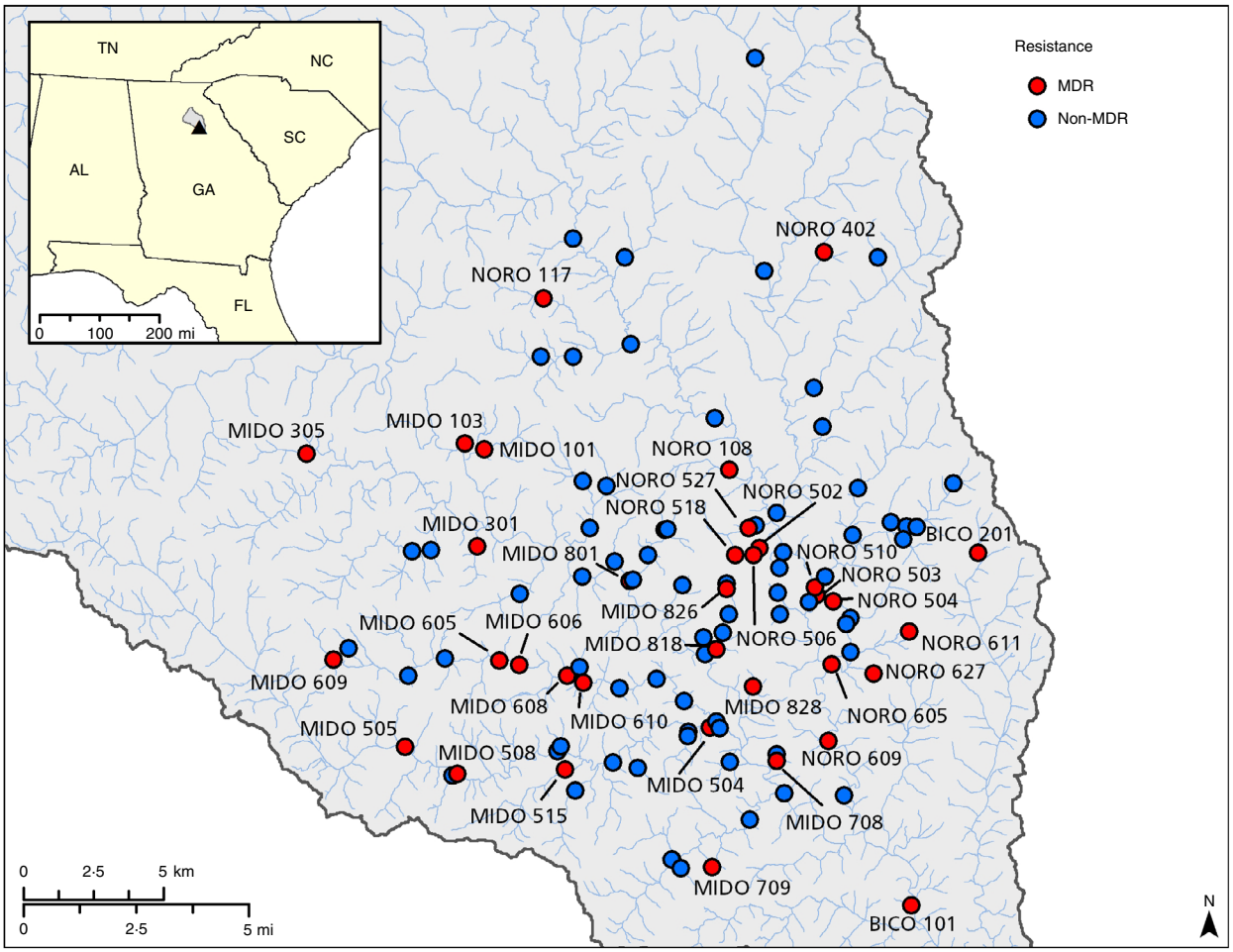

Figure 1 Map of water sampling sites in the Upper Oconee Watershed near Athens, GA. Sampling sites where multidrug-resistant (MDR) *Enterococcus* were isolated are labelled and in red. Other sites, where non-MDR *Enterococcus* were isolated, are in blue. Inset map shows the area covered by the Upper Oconee Watershed in grey and Athens, GA as a black triangle. [Colour figure can be viewed at wileyonlinelibrary.com]

multiple suspect colonies were selected per positive plate and tested for a media comparison purpose. A variant of *Enterococcus casseliflavus* was detected by PCR using the same forward primer (CA1-*E. casseliflavus*) with a different reverse primer 5′-CGATTAAACGGTAGAAAGTGC-3′ (designated CA3). The same PCR condition was used as described by Jackson *et al.* (2004).

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MIC; μg ml^{-1}) for enterococci were determined by broth microdilution using the Sensititre^{TM} semiautomated antimicrobial susceptibility system (Trek Diagnostic Systems Inc., Cleveland, OH) and the Sensititre Gram-Positive Custom Plate CMV3AGPF according to the manufacturer's directions. Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines when defined (CLSI 2018). No CLSI interpretive criteria have been defined for kanamycin, lincomycin and tylosin, and only susceptible breakpoints have been established for daptomycin (≤4 μg ml^{-1}) and tigecycline (≤0·25 μg ml^{-1}). Breakpoints for daptomycin, kanamycin, lincomycin, tigecycline and tylosin were those defined by the National Antimicrobial Resistance Monitoring System (NARMS) (https://www.ars.usda.gov/ARSUserFiles/60400520/NARMS/ABXEntero.pdf). The panel of antimicrobials and breakpoints for classification as resistant were as follows: chloramphenicol (≥32 μg ml^{-1}), ciprofloxacin (≥4 μg ml^{-1}), daptomycin (≥8 μg ml^{-1}), erythromycin (≥8 μg ml^{-1}), gentamicin (≥500 μg ml^{-1}), kanamycin (≥1024 μg ml^{-1}), lincomycin (≥8 μg ml^{-1}), linezolid (≥8 μg ml^{-1}), nitrofurantoin (≥128 μg ml^{-1}), penicillin (≥16 μg ml^{-1}), streptomycin (≥1000 μg ml^{-1}), Synercid (Quinupristin/Dalfopristin; Q/D) (≥4 μg ml^{-1}), tetracycline (≥16 μg ml^{-1}), tigecycline (≥0·5 μg ml^{-1}), tylosin (≥32 μg ml^{-1}) and vancomycin (≥32 μg ml^{-1}).

Enterococcus faecalis ATCC 29212, *Staphylococcus aureus* ATCC 29213, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were quality controls for the determination of MIC.

Statistical methods

Data were analysed using either Microsoft Excel or GraphPad. Student's *t*-test and Pearson correlation coefficients were performed. *P* values of ≤0·05 were considered significant.

Results

Prevalence and seasonal diversity

Over 450 samples were collected in the Upper Oconee watershed (Fig. 1) during eight seasons in 2015 and 2016. The number of sites sampled varied for each of the seasons depending on several factors including the number of volunteers, lack of rainfall resulting in a dry channel and failure to obtain a sample, access to the sampling sites and available resources. The number of sites sampled ranged from 27 in Summer 2016 to 100 in Spring 2015 (Table 1). During the sampling period, enterococci were detected in over 90% of the sites with the percent of positive sites ranging from 93 to 100%; in four seasons (Summer 2015 and Winter, Spring and Summer 2016) all sites were positive for the presence of enterococci.

At least 10 different enterococcal species were detected during the sampling period (Table 2). Typically, more than one colony morphology was present on each sample plate, but only one colony was randomly selected for further study. A higher number of isolates than the number of sites was obtained during Winter and Summer of 2015 due to the use of multiple media. Additionally, more than one *Enterococcus* was isolated per positive plate for

Table 1 Prevalence of *Enterococcus* among surface water sampling sites

Sampling season	No. sampling sites	No. positive sampling sites (%)
2015		
Winter	30	29 (96·7)
Spring	100	93 (93)
Summer	33	33 (100)
Fall	59	58 (98·3)
2016		
Winter	41	41 (100)
Spring	87	87 (100)
Summer	27	27 (100)
Fall	81	77 (95·1)

Table 2 Seasonal distribution of enterococci from water samples

	No. of isolates (%)										
Season	*E. avium* (*n* = 3)	*E. casseliflavus* (*n* = 214)	*E. casseliflavus* var. (*n* = 71)	*E. durans* (*n* = 1)	*E. faecalis* (*n* = 169)	*E. faecium* (*n* = 33)	*E. gallinarum* (*n* = 50)	*E. hirae* (*n* = 84)	*E. mundtii* (*n* = 8)	*E. pallens* (*n* = 1)	*E. species* (*n* = 3)
2015											
Winter (*n* = 58)	1 (1·7)	11 (19)	0 (0)	0 (0)	13 (22·4)	12 (20·7)	5 (8·6)	15 (25·9)	1 (1·7)	0 (0)	0 (0)
Spring (*n* = 93)	0 (0)	61 (65·5)	11 (11·8)	0 (0)	13 (14)	0 (0)	7 (7·5)	1 (1·2)	0 (0)	0 (0)	0 (0)
Summer (*n* = 196)	1 (0·5)	40 (20·4)	0 (0)	0 (0)	77 (39·3)	14 (7·1)	26 (13·3)	32 (16·3)	3 (1·5)	1 (0·5)	2 (1)
Fall (*n* = 58)	0 (0)	25 (43·1)	27 (46·6)	0 (0)	1 (1·7)	1 (1·7)	3 (5·2)	1 (1·7)	0 (0)	0 (0)	0 (0)
2016											
Winter (*n* = 41)	0 (0)	5 (12·2)	12 (29·3)	1 (2·4)	4 (9·8)	5 (12·2)	0 (0)	14 (34·1)	0 (0)	0 (0)	0 (0)
Spring (*n* = 87)	0 (0)	31 (35·6)	10 (11·5)	0 (0)	28 (32·2)	1 (1·1)	6 (6·9)	11 (12·6)	0 (0)	0 (0)	0 (0)
Summer (*n* = 27)	0 (0)	7 (25·9)	8 (29·6)	0 (0)	12 (44·4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Fall (*n* = 77)	1 (1·3)	34 (44·2)	3 (3·9)	0 (0)	21 (27·3)	0 (0)	3 (3·9)	10 (13)	4 (5·2)	0 (0)	1 (1·3)

Summer of 2015 as different colony morphologies were selected for species identification. This was done in order to test the efficacy of different media for *Enterococcus* isolation, the comparison results of which will be discussed elsewhere. Accordingly, although 33 sampling sites were positive for enterococci in Summer 2015, 196 enterococci were selected and confirmed as enterococci. Notably, Summer 2015 was the season with the most diverse enterococcal species isolated and the only season in which *E. pallens* was identified; it was also one of only two seasons in which unidentified enterococci were isolated (Table 2). In contrast to Summer 2015, Summer 2016 had the fewest number of species identified ($n = 3$) although all 27 sites sampled were positive for enterococci. Not surprisingly, the season with the highest number of isolates (Summer 2015; $n = 196$) and the lowest number (Summer 2016; $n = 27$) had the highest and lowest diversity of species, eight and three different species respectively. A significantly positive correlation was observed between the number of samples obtained in each season and the number of different species obtained ($r = 0{\cdot}585$, $P < 0{\cdot}5$), but the moderate correlation suggests that other factors may contribute to the diversity of *Enterococcus* in surface water. On average, six different species were identified each sampling period.

Prevalence and seasonal occurrence of *Enterococcus* isolates appeared to relate; as the number of isolates increased, so did the number of seasons in which they were observed ($r = 0{\cdot}870$, $P < 0{\cdot}05$). For example the most prevalent species isolated was *E. casseliflavus* ($n = 214$) and *E. faecalis* ($n = 169$) (Table 2). These two species were also the only ones isolated from all eight sampling events. Fewer *E. hirae* ($n = 84$), *E. casseliflavus* variant ($n = 71$) and *E. gallinarum* ($n = 50$) were isolated and were distributed over fewer sampling seasons (seven seasons for *E. hirae* and six seasons each for *E. casseliflavus* variant and *E. gallinarum*). Only 33 *E. faecium* were isolated in five of the eight sampling events (Table 2). Although *E. avium* ($n = 3$) and *E. mundtii* ($n = 8$) were low in numbers, all isolates of these species were seen in the same three sampling seasons (Winter and Summer 2015 and Fall 2016).

Antimicrobial resistance

Enterococcal isolates were tested against a panel of 16 antimicrobials. Overall, the highest level of resistance was to lincomycin (564/637; 88·5%) followed by tetracycline (83/637; 13%) (Table 3). With the exception of *E. pallens*, which was susceptible to all antimicrobials tested, all the species exhibited resistance to lincomycin ranging from 60% for *E. faecium* (20/33) to 100% for *E. avium* ($n = 3$), *E. durans* ($n = 1$), *E. mundtii* ($n = 8$) and *Ent.* species ($n = 3$). Seven species, mainly *E. gallinarum* (27/83; 32·5%), were resistant to tetracycline. Surprisingly, resistance to the newer antimicrobials daptomycin (57/637; 8·9%) and tigecycline (41/637; 6·4%) was also found. Five enterococcal species were resistant to daptomycin with the majority of resistance exhibited by one species, *E. hirae* (49/57; ~86%). No one species

Table 3 Antimicrobial resistance phenotypes in enterococci isolated from surface water samples

	No. of resistant isolates (%)											
Species	Chl	Cip	Dap	Ery	Kan	Lin	Pen	Syn*	Str	Tet	Tig	Tyl
E. avium ($n = 3$)						3 (100·0)				1 (33·3)		
E. casseliflavus ($n = 214$)		22 (10·3)		1 (0·5)	1 (0·5)	192 (89·7)		14 (6·5)	1 (0·5)	8 (3·7)	13 (6·1)	2 (0·9)
E. casseliflavus var. ($n = 71$)		7 (9·9)				66 (93·0)		5 (7·0)		1 (1·4)	11 (15·5)	
E. durans ($n = 1$)						1 (100·0)						
E. faecalis ($n = 169$)	1 (0·6)		2 (1·2)	3 (1·8)	1 (0·6)	147 (87·0)	1 (0·6)		1 (0·6)	21 (12·4)	9 (5·3)	3 (1·8)
E. faecium ($n = 33$)		7 (21·2)	2 (6·1)	1 (3·0)	3 (9·1)	20 (60·6)	1 (3·0)	1 (3·0)	1 (3·0)	11 (33·3)		1 (3·0)
E. gallinarum ($n = 50$)		7 (14·0)	2 (4·0)	2 (4·0)		44 (88·0)		3 (6·0)		27 (54·0)	4 (8·0)	5 (10·0)
E. hirae ($n = 84$)		1 (1·2)	49 (58·3)			80 (95·2)		1 (1·2)		14 (16·7)	2 (2·4)	
E. mundtii ($n = 8$)			2 (25·0)			8 (100·0)					2 (25·0)	
Ent. species ($n = 3$)						3 (100·0)						
E. pallens ($n = 1$)												
Total ($N = 637$)	1 (0·15)	44 (6·9)	57 (8·9)	7 (1·1)	5 (0·8)	564 (88·5)	2 (0·3)	24 (5·1)	3 (0·5)	83 (13)	41 (6·4)	11 (1·7)

Antimicrobials: chloramphenicol (Chl), ciprofloxacin (Cip), daptomycin (Dap), erythromycin (Ery), kanamycin (Kan), lincomycin (Lin), penicillin (Pen), synercid (Syn), streptomycin (Str), tetracycline (Tet), tigecycline (Tig) and tylosin (Tyl). No resistance to gentamicin, linezolid, nitrofurantoin or vancomycin was detected. *Enterococcus pallens* was susceptible to all antimicrobials tested.

**Enterococcus faecalis* are intrinsically resistant to Synercid (Quinupristin/Dalfopristin) and were not included for this antimicrobial.

contributed primarily to tigecycline resistance as six different species were resistant at levels of <25% each. Low resistance was observed for two of the aminoglycosides, kanamycin (5/637; 0·8%) and streptomycin (3/637; 0·5%), whereas none of the isolates were resistant to gentamicin (Table 3). The same three species (*E. casseliflavus*, *E. faecalis* and *E. faecium*) were resistant to both kanamycin and streptomycin although two additional *E. faecium* were resistant to kanamycin, but not to streptomycin. The same five species of isolates (*E. casseliflavus*, *E. faecalis*, *E. faecium* and *E. gallinarum*) were resistant at similar levels for the macrolides, erythromycin and tylosin, 1·1% (7/637) and 1·7% (11/637) respectively. In addition to gentamicin, none of the isolates were resistant to linezolid, nitrofurantoin or vancomycin. Statistical analysis revealed a significant correlation in the distribution of certain AR among different *Enterococcus* species. Very strong positive correlations were observed between daptomycin and *E. hirae* as well as tetracycline and *E. gallinarum*, *E. faecalis* and *E. faecium* ($P < 0{\cdot}05$).

For some species, higher numbers of isolates resulted in resistance to a greater number of antimicrobials. *E. casseliflavus* was the predominant species isolated and was resistant to nine antimicrobials, whereas *E. faecalis*, with the second highest number of isolates, was resistant to 10 of 16 antimicrobials tested including the only resistance detected to chloramphenicol (Table 3). However, 33 *E. faecium* isolates were also resistant to 10 antimicrobials; 21% (7/33) of those isolates were resistant to ciprofloxacin, whereas none of the *E. faecalis* were ciprofloxacin resistant. Compared to *E. casseliflavus*, the *E. casseliflavus* variants were resistant to only five antimicrobials (ciprofloxacin, lincomycin, Q/D, tetracycline and tigecycline), four fewer than *E. casseliflavus*. The differences between the two groups were primarily due to susceptibility to the aminoglycoside and macrolide classes for the *E. casseliflavus* variant. Other enterococcal species with resistant isolates distributed over a range of antimicrobials included *E. gallinarum* ($n = 50$) and *E. hirae* ($n = 84$) exhibiting resistance to eight and six antimicrobials respectively (Table 3).

Multidrug resistance (MDR), defined as resistance to three or more antimicrobial classes and resistance to multiple antimicrobials is shown in Table 4. Isolates were resistant to three to six different antimicrobials and up to five antimicrobial classes. One isolate, an *E. faecalis*, was resistant to six antimicrobials (ChlDapEryLinTetTyl) and five antimicrobial classes. Eighteen different patterns were observed among 51 MDR enterococcal isolates. The most common MDR group by drug class was the three drug combination which was composed of 11 different resistance patterns (Table 4), whereas the fewest different patterns ($n = 1$) was for the six drug combination. Due to the prevalence of resistance to lincomycin in the isolates, lincomycin was found in all resistance patterns with the exception of CipDapTet which was represented by *E. faecium* (Table 4).

Table 4 Multidrug resistance patterns in enterococci from surface water

Pattern*	No. resistances	No. classes	No. isolates	Species (no.)
CipDapTet	3	3	2	*E. faecium* (2)
CipLinSyn	3	3	5	*E. casseliflavus* (4) *E. hirae* (1)
CipLinTet	3	3	8	*E. casseliflavus* (1) *E. faecium* (3) *E. gallinarum* (4)
CipLinTig	3	3	5	*E. casseliflavus* (1) *E. casseliflavus var.* (4)
DapLinTet	3	3	6	*E. hirae* (6)
DapLinTig	3	3	2	*E. hirae* (1) *E. mundtii* (1)
KanLinTet	3	3	1	*E. faecalis* (1)
LinPenTet	3	3	1	*E. faecalis* (1)
LinStrTet	3	3	2	*E. faecalis* (1) *E. faecium* (1)
LinSynTig	3	3	5	*E. casseliflavus* var. (3) *E. gallinarum* (2)
LinTetTig	3	3	2	*E. faecalis* (2)
CipLinSynTig	4	4	1	*E. casseliflavus* (1)
CipLinTetTig	4	4	3	*E. casseliflavus* (1) *E. gallinarum* (2)
EryLinTetTyl	4	3	4	*E. casseliflavus* (1) *E. faecalis (2),* *E. gallinarum* (1)
CipEryLinTetTyl	5	4	1	*E. faecium* (1)
EryLinSynTetTyl	5	4	1	*E. gallinarum* (1)
KanLinStrTetTyl	5	4	1	*E. casseliflavus* (1)
ChlDapEryLinTetTyl	6	5	1	*E. faecalis* (1)

Antimicrobials: chloramphenicol (Chl), ciprofloxacin (Cip), daptomycin (Dap), erythromycin (Ery), kanamycin (Kan), lincomycin (Lin), Penicillin (Pen), Synercid (Syn), streptomycin (Str), tetracycline (Tet), tigecycline (Tig) and tylosin (Tyl).

**Enterococcus faecalis* are intrinsically resistant to Synercid (Quinupristin/Dalfopristin) and were not included for this antimicrobial.

Analysis of distribution of resistant enterococci from the sampling events did not reveal the differences in species, the clustering of species or the antimicrobial resistance over the seasons. Only two sampling events (Fall 2015 and Spring 2016) had identical groups of species (*E. casseliflavus*, *E. casseliflavus* variant, *E. faecalis*, *E. faecium*, *E. gallinarum* and *E. hirae*), but those isolates were resistant to different antimicrobials (Table 5). Resistance to the fewest number of antimicrobials ($n = 3$) also occurred during Summer 2016 with the lowest number

Table 5 Seasonal distribution of antimicrobial resistance phenotypes in enterococci isolated from surface water samples

Season (sample no.)	Species	No. of resistant isolates (%)*											
		Chl	Cip	Dap	Ery	Kan	Lin	Pen	Syn†	Str	Tet	Tig	Tyl
2015													
Winter (*n* = 58)	*E. avium* (*n* = 1)						1 (100·0)						
	Enterococcus casseliflavus (*n* = 11)		4 (36·4)				11 (100·0)		5 (45·5)				
	E. faecalis (*n* = 13)			1 (7·7)			13 (100·0)				3 (23·1)		
	E. faecium (*n* = 12)		6 (50·0)	2 (16·7)	1 (8·3)	1 (8·3)	7 (58·3)		1 (8·3)	1 (8·3)	6 (50·0)		1 (8·3)
	E. gallinarum (*n* = 5)		1 (20·0)				5 (100·0)				1 (20·0)		3 (60·0)
	E. hirae (*n* = 15)			11 (73·3)			15 (100·0)				1 (6·7)		
	E. mundtii (*n* = 1)												
Spring (*n* = 93)	*E. casseliflavus* (*n* = 61)		3 (4·9)			1 (1·6)	59 (96·7)		4 (6·6)	1 (1·6)	2 (3·3)	4 (6·6)	1 (1·6)
	E. casseliflavus var. (*n* = 11)						10 (90·9)		2 (18·2)				
	E. faecalis (*n* = 13)	1 (7·7)		1 (7·7)	1 (7·7)		12 (92·3)				3 (23·1)	2 (15·4)	1 (7·7)
	E. gallinarum (*n* = 7)		1 (14·3)				7 (100·0)				3 (42·9)		
	E. hirae (*n* = 1)						1 (100·0)						
Summer (*n* = 196)	*E. avium* (*n* = 1)						1 (100·0)						
	E. casseliflavus (*n* = 40)		1 (2·5)				24 (60·0)				1 (2·5)		
	E. faecalis (*n* = 77)				2 (2·6)		72 (93·5)	1 (1·3)			8 (10·4)		2 (2·6)
	E. faecium (*n* = 14)					2 (14·3)	8 (57·1)	1 (7·1)			2 (14·3)		
	E. gallinarum (*n* = 26)		2 (7·7)	2 (7·7)	2 (7·7)		26 (100·0)		1 (3·8)		19 (73·1)		2 (7·7)
	E. hirae (*n* = 32)			22 (68·8)			31 (96·9)				5 (15·6)		
	E. mundtii (*n* = 3)						3 (100·0)	†					
	Ent species (*n* = 2)						2 (100·0)						
Fall (*n* = 58)	*E. casseliflavus* (*n* = 25)		4 (16·0)				24 (96)				2 (8·0)	5 (20·0)	
	E. casseliflavus var. (*n* = 27)		6 (22·2)				27 (100·0)		3 (11·1)		1 (3·7)	11 (40·7)	
	E. faecalis (*n* = 1)												
	E. faecium (*n* = 1)												
	E. gallinarum (*n* = 3)						3 (100·0)		2 (66·7)			2 (66·7)	
	E. hirae (*n* = 1)						1 (100)						
2016													
Winter (*n* = 41)	*E. casseliflavus* (*n* = 5)						5 (100·0)						
	E. casseliflavus var. (*n* = 12)						8 (66·7)						
	E. durans (*n* = 1)						1 (100·0)						
	E. faecalis (*n* = 4)						4 (100·0)			1 (25·0)	1 (25·0)		
	E. faecium (*n* = 5)		1 (20·0)				4 (80·0)				2 (40·0)		
	E. hirae (*n* = 14)			7 (50·0)			12 (85·7)				3 (21·4)		

(Continued)

Table 5 *(Continued)*

Season (sample no.)	Species	No. of resistant isolates (%)*											
		Chl	Cip	Dap	Ery	Kan	Lin	Pen	Syn†	Str	Tet	Tig	Tyl
Spring (*n* = 87)	*E. casseliflavus* (*n* = 31)		5 (16·1)		1 (3·2)		31 (100·0)		2 (6·5)		2 (6·5)		1 (3·2)
	E. casseliflavus var. (*n* = 10)		1 (10·0)				10 (100·0)						
	E. faecalis (*n* = 28)						27 (96·4)				1 (3·6)		
	E. faecium (*n* = 1)						1 (100·0)				1 (100·0)		
	E. gallinarum (*n* = 6)		1 (16·7)								1 (16·7)		
	E. hirae (*n* = 11)			4 (36·4)			10 (90·9)				3 (27·3)		
Summer (*n* = 27)	*E. casseliflavus* (*n* = 7)		2 (28·6)				7 (100·0)				1 (14·3)		
	E. casseliflavus var. (*n* = 8)						8 (100·0)						
	E. faecalis (*n* = 12)										1 (8·3)		
Fall (*n* = 77)	*E. avium* (*n* = 1)						1 (100·0)				1 (100·0)		
	E. casseliflavus (*n* = 34)		3 (8·8)				31 (91·2)		3 (8·8)			4 (11·8)	
	E. casseliflavus var. (*n* = 3)						3 (100·0)						
	E. faecalis (*n* = 21)					1 (4·8)	19 (90·5)				4 (19·0)	7 (33·3)	
	E. gallinarum (*n* = 3)		2 (66·7)				3 (100·0)				3 (100·0)	2 (66·7)	
	E. hirae (*n* = 10)		1 (10·0)	5 (50·0)			10 (100·0)		1 (10·0)		2 (20·0)	2 (20·0)	
	E. mundtii (*n* = 4)			2 (50·0)			4 (100·0)					2 (50·0)	
	Ent species (*n* = 1)						1 (100·0)						
	Total (*N* = 637)	1	44	57	7	5	564	2	24	3	83	41	11

Antimicrobials: Chloramphenicol (Chl), Ciprofloxacin (Cip), Daptomycin (Dap), Erythromycin (Ery), Kanamycin (Kan), Lincomycin (Lin), Penicillin (Pen), Synercid (Syn), Streptomycin (Str), Tetracycline (Tet), Tigecycline (Tig) and Tylosin (Tyl). No resistance to gentimicin, linezolid, nitrofurantoin or vancomycin was detected.

*Percentage by species.

†*E. faecalis* are intrinsically resistant to Synercid (Quinupristin/Dalfopristin) and were not included for this antimicrobial.

of isolates. Spring 2015, with lower number of isolates analysed ($n = 93$) than Summer 2015, during which the highest number of isolates were obtained, had a low diversity of species ($n = 4$) but the most resistance detected (i.e. resistance to the highest number of antimicrobials; $n = 11$) (Table 5). Like the prevalence of lincomycin resistance among MDR patterns, lincomycin resistance was detected in enterococci isolated during all seasons in both 2015 and 2016. In contrast, although lower numbers of isolates resistant to ciprofloxacin ($n = 44$) and tetracycline ($n = 83$) were detected when compared to lincomycin ($n = 564$), resistance to both ciprofloxacin and tetracycline was found in all seasons as well.

Discussion

The Upper Oconee watershed, located in the Piedmont region of northeast Georgia, is one of 14 river basins in the state (Meyer and Loeffler 2018). The headwaters of the river originate in an area dominated by forest, but forested areas decrease as areas of agriculture increase along the river. Agricultural use of the land has greatly affected the quality of the water within the watershed. Additionally, the watershed has also been impacted by other factors including pollutants from urban run-off, municipal sources, storm and combined sewer systems. As a mixed-use watershed that is utilized for numerous purposes including recreational activity as well as for municipal, commercial and industrial purposes, assessment of the condition of the watershed is important. In 2005, the Upper Oconee watershed was surveyed to obtain a general description (prevalence, antimicrobial resistance) of the bacterial composition focusing primarily on enteric bacteria, including *Enterococcus* (Meinersmann *et al.* 2008). Although results from the study provided much needed information, no data were generated on possible seasonal changes in the occurrence and diversity of *Enterococcus* in the watershed. This study aimed to provide a description of prevalence, species distribution and antimicrobial resistance profile of enterococci over different seasons and years to more clearly assess this population of bacteria in the Upper Oconee watershed, which may indicate potentially negative impact on the communities utilizing the water.

As enterococci are ubiquitous in the environment, the high number of positive sampling sites across seasons was not unexpected. Concentrations of enterococci were not recorded in this study, but peak numbers of enterococci have been reported to be frequently detected in late Winter and early Spring in coastal waters (Turbow *et al.* 2003). In this study, no significant difference was observed in the number of *Enterococcus*-positive sites based on season ($P > 0{\cdot}05$), with all seasons exhibiting a high prevalence of enterococci. The lowest percent positive (93%) was higher than that from the 2005 study in which 85·5% (71/83) sites tested were positive for enterococci using the same isolation method (Meinersmann *et al.* 2008). While the two studies of the Upper Oconee watershed occurred 10 years apart, the average temperature varied very little between those two sampling months with 60°F and 65·1°F recorded in April 2005 and April 2015 respectively (https://www.usclimatedata.com/climate/athens/georgia/united-states/usga0027/2016/11). However, the differences in precipitation may have affected the detected enterococcal population. April 2005 had significantly less total precipitation recorded (0·22 inches) than April 2015 (8·01 inches) or the lowest amount of any sampling month of this study (2·24 inches recorded for November, 2016).

Previous studies have shown that surface water contains a wide variety of enterococcal species (Svec and Sedlacek 1999; Svec *et al.* 2001; Niemi *et al.* 2012). The spectrum of species isolated may be influenced by the surrounding environment or human/animal contact with the water as many sources of enterococci exist. Commonly isolated enterococcal species include *E. faecalis* and *E. faecium* as well as *E. casseliflavus*, *E. durans*, *E. gallinarum*, *E. hirae* and *E. mundtii*. Of the 10 species of enterococci identified in this study, *E. casseliflavus* was the predominant species paralleling the results from the previous study of enterococci in the Upper Oconee watershed (Meinersmann *et al.* 2008). A variant of *E. casseliflavus* was also identified. Sequencing of the *sod*A gene has shown that the variant had the same nucleotide changes in the superoxide dismutase gene (C377T, T381C, C384T and T390G) as an *Enterococcus* isolate obtained from a park in Athens, GA from a previous study at the USDA-ARS conducted in 2001 (P. J. Fedorka-Cray, C.R. Jackso, & S.L. House, unpublished data), indicating the prevalence and persistence of this particular *E. casseliflavus* variant in the environment in this area. The detection of high numbers of *E. casseliflavus* in this surface water is similar to findings in some studies but differs from most other studies, as either *E. faecalis* or *E. faecium* was reported as the dominant species from surface or other environmental waters (Pinto *et al.* 1999; Moore *et al.* 2008; Lata *et al.* 2009; Luczkiewicz *et al.* 2010; Lanthier *et al.* 2011; de *et al.* 2013). This difference in species distribution could be due to a difference in the isolation method as studies have shown that difference in media and incubation temperatures could select for certain *Enterococcus* species (Jackson *et al.* 2005; Ferguson *et al.* 2013). The difference in species distribution could also be the result of the regional difference as Moore *et al.* (2008) detected

different *Enterococcus* species distribution at separate locations based on the structure of the waterbody and the presence of potential contamination sources. *E. faecalis* and *E. faecium* have been documented to be more resistant to stress caused by environmental conditions and thus may survive longer in harsh environments, such as water, where nutrients are scarce (Leclerc *et al.* 1996).

Definitive contributing sources of *Enterococcus* in the environment are still unresolved. For example some studies attribute the presence of *E. faecalis* to nonhuman sources (livestock, poultry, wildlife) (Lanthier *et al.* 2011), whereas other reports point to clinical specimens, hospital patients and hospital sewage (Kuhn *et al.* 2003). Contamination of water with *E. faecium* usually suggests human or wastewater sources (Niemi *et al.* 1993; Lanthier *et al.* 2011). Both of these scenarios are plausible, as *E. faecalis* has historically accounted for more human infections in the community and hospital settings, whereas *E. faecium* is associated with increased healthcare-acquired infections (Hidron *et al.* 2008). On the other hand, *E. casseliflavus* has been primarily associated with plants and, to a lesser degree, as an animal-derived or food-associated species (Luczkiewicz *et al.* 2010).

The clinical importance of *E. faecalis* and *E. faecium* is well-known; however, other enterococcal species can cause disease in humans, including *E. casseliflavus. E. casseliflavus* has been known to cause invasive infection in humans, but the true clinical significance of this species has not been fully determined (Reid *et al.* 2001). One other enterococcal species identified in this study was *E. pallens*. This species has not been previously found in surface water but has primarily been described as a cause of peritonitis in humans (Tyrrell *et al.* 2002; Levesque *et al.* 2016). The significance of detecting this species in surface water remains unknown, but could be a possible source for human infections.

Antimicrobial resistance in enterococci from surface water varies with the geographical area under study and is most likely due to the type of polluting source the water is receiving as antimicrobial use in humans and animals differ. Resistance to aminoglycosides, β-lactams, fluoroquinolones, lincosamides, macrolides, phenicols and tetracycline has been described with erythromycin, lincomycin and tetracycline resistance observed most often (Moore *et al.* 2008; Servais and Passerat 2009; Luczkiewicz *et al.* 2010; Lanthier *et al.* 2011; de *et al.* 2013). Those same patterns of resistance were seen in this study with a vast majority of isolates displaying resistance to lincomycin; at least one of every species exhibited resistance to this drug. None of the isolates were resistant to vancomycin, although other studies have detected vancomycin resistance in enterococci from surface water (Pinto *et al.* 1999; Luczkiewicz *et al.* 2010; Lanthier *et al.* 2011; Santiago-Rodriguez *et al.* 2013; Molale and Bezuidenhout 2016). Schwartz *et al.* (2003) identified vancomycin-resistant *E. faecium* with an identified vancomycin resistance gene, but only from hospital wastewater biofilms. *Enterococcus casseliflavus* and *E. gallinarum* are known to be intrinsically resistant to vancomycin as they carry *vanC* genes; however, their level of resistance is lower than the CLSI breakpoint of 32 μg $ml^{-1}$and not considered clinically relevant (Gold 2001). Because the NARMS susceptibility panel was used to phenotype the isolates, estimation of resistance to the newer drugs (daptomycin and tigecycline) used to treat human infections was possible. Neither those drugs nor their analogs are used in food animal production in the US suggesting that those isolates originated from a human source. Alternatively, those isolates could also either be intrinsically resistant, especially as *E. hirae* is highly associated with resistance to daptomycin, or have acquired resistance to the antimicrobials. Nevertheless, resistance in environmental isolates to drugs used in human medicine is a cause for concern due to the potential for horizontal transfer of genes responsible for these resistances to clinical isolates.

The high number of MDR profiles detected in this study was notable; 18 different MDR profiles were observed for the enterococci with resistance to a maximum of five classes of antimicrobials. Direct comparison of MDR pattern prevalence between studies may be difficult due to the differences in antimicrobials and antimicrobial classes tested as well as the susceptibility testing method used (Servais and Passerat 2009). However, a high number of MDR profiles was also reported in a study by Carvalho *et al.* (2014) in which 24 MDR profiles with resistance to six classes of antimicrobials in enterococci from a marine outfall in Brazil were seen. Resistance genes carried on plasmids accounted for some of the MDR phenotypes in that study. Although analysis of plasmids was not performed in this study, MDR profiles in isolates from this study could also be due to the presence of mobile genetic elements harbouring antimicrobial resistance genes.

A total of 51 MDR *Enterococcus* isolates were isolated from 35 sampling sites. The site locations where MDR enterococci were recovered are indicated in Fig. 1 and the exact locations of the sites, along with the GPS coordinates, are in Table S1. Interestingly, a complimentary study conducted on the same sampling sites showed that Enteropathogenic *E. coli* and AR *E. coli* isolates were recovered from 22 of the 35 MDR *Enterococcus*-positive sites (Cho *et al.* 2018). This finding shows that there are sites within the Upper Oconee watershed that contribute towards the spread of pathogenic and AR bacteria. Two or more MDR isolates were recovered from 14 of 35 sites,

most of which were residential areas, just as AR *E. coli* were mostly isolated from sites draining residential areas (Cho *et al.* 2018). Additionally, McNutt Creek, a creek previously identified as a source of AR *E. coli*, served as the source of six MDR *Enterococcus* isolates at three distinct locations along the creek.

Seasonal effects on species distribution and antimicrobial resistance have been examined previously (Lanthier *et al.* 2011). In that study, significant differences in species were found between seasons with *E. faecalis* having a more dominant presence in Summer and Fall, whereas *E. faecium* was more frequently identified in Winter and Spring. That study also found a higher proportion of antimicrobial susceptible isolates in Fall compared to Winter and Spring, but not to Summer. Data from this study differed from that of Lanthier *et al.* as *E. faecalis* seemed to be predominant in Summer and *E. faecium* in Winter; however, none of these differences were significant ($P > 0{\cdot}05$). On the other hand, the frequency of *E. casseliflavus* was significantly higher during Fall than during Winter and Summer ($P < 0{\cdot}05$). Unknown environmental variations between studies may exist, rendering significant assumptions about species diversity and antimicrobial resistance very difficult to interpret. Additional collections over time need to be done in order to provide statistically sound data for seasonal diversity and antimicrobial resistance of enterococci from surface water.

For this study, one typical, well-isolated colony was randomly selected per positive plate, according to NARMS methodology (https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/resources), in order to represent the enterococcal species distribution of the watershed as a whole and not of individual water samples. Furthermore, different selective media were used for the first few samplings for *Enterococcus* isolation in order to test for the efficacy of the media used (S. Cho, L.M. Hiott, T.A. Woodley, H. Ramadan, J.G. Frye, & C.R. Jackson, manuscript in preparation). As the purpose of this study was to examine the different enterococcal species composition of the watershed and their antimicrobial resistance, isolates recovered from all the media were included in this study.

This study has demonstrated that surface waters of the Upper Oconee watershed contain a high population of diverse species of enterococci. The detection of antimicrobial resistance and MDR in the isolates, especially to newer drugs used to treat human infections, was concerning and may have resulted from human impacts in the area, including excretion of antibiotics, as these antibiotics are only used in human medicine and not in food animals. Resistant enterococcal species were consistently isolated over the sampling period indicating that the results were not a transient observation, but that those isolates maintain a presence in surface water of the Upper Oconee watershed. As some of the resistance phenotypes were present in species of enterococci implicated as leading causes of nosocomial infections, those isolates may be an additional source of contact for humans during recreational and other surface water use. Colonization of the human gut with resistant enterococci could limit therapeutic options during human infections posing a risk to human health.

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Conflict of Interest

No conflict of interest declared.

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References

Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R. and Harwood, V.J. (2012) Enterococci in the environment. *Microbiol Mol Biol Rev* **76**, 685–706.

Carvalho, E.M.R., Costa, R.A., Araujo, A.J.G., Carvalho, F.C.T., Pereira, S.P., Sousa, O.V. and Vieira, R.H.S.F. (2014) Multiple antibiotic-resistance of *Enterococcus* isolated from coastal water near an outfall in Brazil. *African J Microbiol* **8**, 1825–1831.

Cho, S., Hiott, L.M., Barrett, J.B., McMillan, E.A., House, S.L., Humayoun, S.B., Adams, E.S., Jackson, C.R. *et al.* (2018) Prevalence and characterization of *Escherichia coli* isolated from the Upper Oconee Watershed in Northeast Georgia. *PLoS ONE* **13**, e0197005.

CLSI (2018) *Performance Standards for Antimicrobial Susceptibility Testing*. Wayne, PA: Clinical and Laboratory Standards Institute.

De Niederhäusern, S., Bondi, M., Anacarso, I., Iseppi, R., Sabia, C., Bitonte, F. and Messi, P. (2013) Antibiotics and heavy metals resistance and other biological characters in enterococci isolated from surface water of Monte Cotugno Lake (Italy). *J Environ Sci Health A Tox Hazard Subst Environ Eng* **48**, 939–946.

Environmental Protection Division (1998) *Oconee River Basin Management Plan*. Atlanta, GA: Environmental Protection Division.

Facklam, R.R., Carvalho, M.G.S. and Teixeiram, L.M. (2002) History, taxonomy, biochemical characteristics, and antibiotic susceptibility testing of enterococci. In *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance* ed. Gilmore, M.S., Clewell, D.B., Courvalin, P., Dunny, G.M., Murray, B.E. and Rice, L.B. pp. 1–54. Washington, DC: ASM Press.

Ferguson, D.M., Griffith, J.F., McGee, C.D., Weisberg, S.B. and Hagedorn, C. (2013) Comparison of *Enterococcus* species diversity in marine water and wastewater using Enterolert and EPA Method 1600. *J Environ Public Health* **2013**, 848049.

Foulquie Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E. and De Vuyst, L. (2006) The role and application of enterococci in food and health. *Int J Food Microbiol* **106**, 1–24.

Gardin, F., Martuscelli, M., Caruso, M.C., Galgano, F., Crudele, M.A., Favati, F., Guerzoni, M.E. and Suzzi, G. (2001) Effects of pH, temperature and NaCl concentration on the growth kinetics, proteolytic activity and biogenic amine production of *Enterococcus faecalis*. *Int J Food Microbiol* **64**, 105–117.

Giraffa, G. (2002) Enterococci from foods. *FEMS Microbiol Rev* **26**, 163–171.

Gold, H.S. (2001) Vancomycin-resistant enterococci: mechanisms and clinical observations. *Clin Infect Dis* **33**, 210–219.

Hidron, A.I., Edwards, J.R., Patel, J., Horan, T.C., Sievert, D.M., Pollock, D.A. and Fridkin, S.K. (2008) NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infect Control Hosp Epidemiol* **29**, 996–1011.

Huijbers, P.M., Blaak, H., de Jong, M.C., Graat, E.A., Vandenbroucke-Grauls, C.M. and de Roda Husman, A.M. (2015) Role of the environment in the transmission of antimicrobial resistance to humans: a review. *Environ Sci Technol* **49**, 11993–12004.

Huycke, M.M., Spiegel, C.A. and Gilmore, M.S. (1991) Bacteremia caused by hemolytic, high-level gentamicin-resistant *Enterococcus faecalis*. *Antimicrob Agents Chemother* **35**, 1626–1634.

Jackson, C.R., Fedorka-Cray, P.J. and Barrett, J.B. (2004) Use of a genus- and species-specific multiplex PCR for identification of enterococci. *J Clin Microbiol* **42**, 3558–3565.

Jackson, C.R., Fedorka-Cray, P.J., Jackson-Hall, M.C. and Hiott, L.M. (2005) Effect of media, temperature and culture conditions on the species population and antibiotic resistance of enterococci from broiler chickens. *Lett Appl Microbiol* **41**, 262–268.

Jett, B.D., Huycke, M.M. and Gilmore, M.S. (1994) Virulence of enterococci. *Clin Microbiol Rev* **7**, 462–478.

Kapoor, V., Pitkanen, T., Ryu, H., Elk, M., Wendell, D. and Santo Domingo, J.W. (2015) Distribution of human-specific bacteroidales and fecal indicator bacteria in an urban watershed impacted by sewage pollution, determined using RNA- and DNA-based quantitative PCR assays. *Appl Environ Microbiol* **81**, 91–99.

Kuhn, I., Iversen, A., Burman, L.G., Olsson-Liljequist, B., Franklin, A., Finn, M., Aarestrup, F., Seyfarth, A.M. *et al.* (2003) Comparison of enterococcal populations in animals, humans, and the environment–a European study. *Int J Food Microbiol* **88**, 133–145.

Lanthier, M., Scott, A., Zhang, Y., Cloutier, M., Durie, D., Henderson, V.C., Wilkes, G., Lapen, D.R. *et al.* (2011) Distribution of selected virulence genes and antibiotic resistance in *Enterococcus* species isolated from the South Nation River drainage basin, Ontario, Canada. *J Appl Microbiol* **110**, 407–421.

Lata, P., Ram, S., Agrawal, M. and Shanker, R. (2009) Enterococci in river Ganga surface waters: propensity of species distribution, dissemination of antimicrobial-resistance and virulence-markers among species along landscape. *BMC Microbiol* **9**, 140.

Leclerc, H., Devriese, L.A. and Mossel, D.A. (1996) Taxonomical changes in intestinal (faecal) enterococci and streptococci: consequences on their use as indicators of faecal contamination in drinking water. *J Appl Bacteriol* **81**, 459–466.

Levesque, S., Longtin, Y., Domingo, M.C., Masse, C., Bernatchez, H., Gaudreau, C. and Tremblay, C. (2016) *Enteroccocus pallens* as a potential novel human pathogen: three cases of spontaneous bacterial peritonitis. *JMM Case Rep* **3**, e005024.

Luczkiewicz, A., Jankowska, K., Kurlenda, J. and Olanczuk-Neyman, K. (2010) Identification and antimicrobial resistance of *Enterococcus spp*. isolated from surface water. *Water Sci Technol* **62**, 466–473.

Malani, P.N., Kauffman, C.A. and Zervos, M.J. (2002) Enterococcal disease, epidemiology, and treatment. In *The Enterococci: Pathogenesis, Molecular Biology, and Antibiotic Resistance* ed. Gilmore, M.S., Clewell, D.B., Courvalin, P., Dunny, G.M., Murray, B.E. and Rice, L.B. pp. 385–408. Washington, DC: ASM Press.

Meinersmann, R.J., Berrang, M.E., Jackson, C.R., Fedorka-Cray, P., Ladely, S., Little, E., Frye, J.G. and Mattsson, B. (2008) *Salmonella*, *Campylobacter* and *Enterococcus* spp.: their antimicrobial resistance profiles and their spatial

relationships in a synoptic study of the Upper Oconee River basin. *Microb Ecol* **55**, 444–452.

Meyer, J.L. and Loeffler, G.L. (2018) River basins. In *New Georgia Encyclopedia*. Available at georgiaencyclopedia.org/articles/geography-environment/river-basins. Accessed May 29, 2018.

Molale, L.G. and Bezuidenhout, C.C. (2016) Antibiotic resistance, efflux pump genes and virulence determinants in *Enterococcus* spp. from surface water systems. *Environ Sci Pollut Res Int* **23**, 21501–21510.

Moore, D.F., Guzman, J.A. and McGee, C. (2008) Species distribution and antimicrobial resistance of enterococci isolated from surface and ocean water. *J Appl Microbiol* **105**, 1017–1025.

Murray, B.E. (1990) The life and times of the *Enterococcus*. *Clin Microbiol Rev* **3**, 46–65.

Niemi, R.M., Niemela, S.I., Bamford, D.H., Hantula, J., Hyvarinen, T., Forsten, T. and Raateland, A. (1993) Presumptive fecal streptococci in environmental samples characterized by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Appl Environ Microbiol* **59**, 2190–2196.

Niemi, R.M., Ollinkangas, T., Paulin, L., Svec, P., Vandamme, P., Karkman, A., Kosina, M. and Lindstrom, K. (2012) *Enterococcus rivorum* sp. nov., from water of pristine brooks. *Int J Syst Evol Microbiol* **62**, 2169–2173.

Nishiyama, M., Iguchi, A. and Suzuki, Y. (2015) Identification of *Enterococcus faecium* and *Enterococcus faecalis* as vanC-type Vancomycin-Resistant Enterococci (VRE) from sewage and river water in the provincial city of Miyazaki, Japan. *J Environ Sci Health A Tox Hazard Subst Environ Eng* **50**, 16–25.

Pinto, B., Pierotti, R., Canale, G. and Reali, D. (1999) Characterization of 'faecal streptococci' as indicators of faecal pollution and distribution in the environment. *Lett Appl Microbiol* **29**, 258–263.

Reid, K.C., Cockerill, I.F. and Patel, R. (2001) Clinical and epidemiological features of *Enterococcus casseliflavus/flavescens* and *Enterococcus gallinarum* bacteremia: a report of 20 cases. *Clin Infect Dis* **32**, 1540–1546.

Roslev, P. and Bukh, A.S. (2011) State of the art molecular markers for fecal pollution source tracking in water. *Appl Microbiol Biotechnol* **89**, 1341–1355.

Sadowy, E. and Luczkiewicz, A. (2014) Drug-resistant and hospital-associated *Enterococcus faecium* from wastewater, riverine estuary and anthropogenically impacted marine catchment basin. *BMC Microbiol* **14**, 66.

Santiago-Rodriguez, T.M., Rivera, J.I., Coradin, M. and Toranzos, G.A. (2013) Antibiotic-resistance and virulence genes in *Enterococcus* isolated from tropical recreational waters. *J Water Health* **11**, 387–396.

Schwartz, T., Kohnen, W., Jansen, B. and Obst, U. (2003) Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiol Ecol* **43**, 325–335.

Servais, P. and Passerat, J. (2009) Antimicrobial resistance of fecal bacteria in waters of the Seine river watershed (France). *Sci Total Environ* **408**, 365–372.

Singer, R.S., Ward, M.P. and Maldonado, G. (2006) Can landscape ecology untangle the complexity of antibiotic resistance? *Nat Rev Microbiol* **4**, 943–952.

Svec, P. and Sedlacek, I. (1999) Occurrence of *Enterococcus* spp. in waters. *Folia Microbiol (Praha)* **44**, 3–10.

Svec, P., Devriese, L.A., Sedlacek, I., Baele, M., Vancanneyt, M., Haesebrouck, F., Swings, J. and Doskar, J. (2001) *Enterococcus haemoperoxidus* sp. nov. and *Enterococcus moraviensis* sp. nov., isolated from water. *Int J Syst Evol Microbiol* **51**, 1567–1574.

Tham, W., Karp, G. and Danielsson-Tham, M.-L. (1990) Histamine formation by enterococci in goat cheese. *Int J Food Microbiol* **11**, 225–229.

Turbow, D.J., Osgood, N.D. and Jiang, S.C. (2003) Evaluation of recreational health risk in coastal waters based on *Enterococcus* densities and bathing patterns. *Environ Health Perspect* **111**, 598–603.

Tyrrell, G.J., Turnbull, L., Teixeira, L.M., Lefebvre, J., Carvalho Mda, G., Facklam, R.R. and Lovgren, M. (2002) *Enterococcus gilvus* sp. nov. and *Enterococcus pallens* sp. nov. isolated from human clinical specimens. *J Clin Microbiol* **40**, 1140–1145.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Masterfile of multidrug resistant enterococci from surface water.