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ORIGINAL ARTICLE

Effects of season and water type on the distribution and antimicrobial resistance of *Enterococcus faecalis* and *Ent. faecium* from surface and reclaimed water

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Abstract

Aims: To evaluate the safety of irrigation water sources based on phenotypic antimicrobial resistance (AMR) in *Enterococcus* spp., a potential environmental reservoir for AMR determinants.

Methods and Results: Eleven sites representing fresh and brackish water rivers, ponds and reclaimed water, were sampled over 2 years. Samples ($n = 333$) yielded 198 unique isolates of *Ent. faecalis* and *Ent. faecium* which were tested for antimicrobial susceptibility by microbroth dilution. Species distribution was influenced by water type and season. *Enterococcus faecalis* was more likely found in freshwater rivers and in summer, and *Ent. faecium* in reclaimed water and in spring. Only 11% of isolates were pansusceptible, while 48.5% and 26.3% were single (SDR) and multidrug resistant (MDR), respectively. MDR was more likely detected in *Ent. faecium* than *Ent. faecalis*. Winter isolates were more likely than summer isolates to exhibit MDR than SDR.

Conclusions: *Enterococcus faecalis* and *Ent. faecium* in surface and reclaimed water exhibited diverse phenotypic AMR and a low-level resistance to clinically important antimicrobials such as ampicillin, vancomycin and linezolid.

Significance and Impact of the Study: Single and multidrug resistance in *E. faecalis* and *E. faecium* varied by season but not water type. Antimicrobial resistance prevalence can assist decisions on the safety of irrigation water sources for fresh produce crops.

KEYWORDS

antimicrobial resistance, *Enterococcus*, food safety, irrigation water, multidrug resistance, water quality

INTRODUCTION

Changes in climate and rainfall patterns, urban growth and overextraction of groundwater for agriculture and other uses are putting a strain on aquifers, leading to depletion and saltwater intrusion in different parts of the United States (Dong et al., 2019; Moore & Joye, 2021; Scanlon et al., 2012). Identifying alternative, microbially safe sources of irrigation water, including surface and reclaimed water, is critical for sustainable agricultural production. In the mid-Atlantic region of the United States, these water sources can carry faecal bacteria, enteric pathogens, antimicrobials, herbicides and other xenobiotics (Acheamfour et al., 2021; Haymaker et al., 2019; Panthi et al., 2019; Sharma et al., 2020; Solaiman

et al., 2020). Microbiologically contaminated irrigation water is a frequently reported cause of foodborne illness outbreaks (Alegbeleye & Singleton, 2018). Microbial indicators and pathogens in these waters may also harbour antimicrobial resistance (AMR) (Callahan et al., 2019). The presence of AMR in the environment can signal a decline in the environmental health of natural resources as well as pose a threat to the spread of community-acquired AMR infections.

Enterococcus is a widespread genus in many environments including water, plants, food and gastrointestinal tracts of mammals and birds (Lebreton et al., 2014). *Enterococcus* spp. are recommended as bacterial indicators for marine and fresh recreational water quality (USEPA, 2012). They can be opportunistic human pathogens, causing both community acquired and nosocomial infections, and are also well known for the accumulation of AMR (Teixeira & Merquior, 2013). The two most clinically important species are *Ent. faecalis* and *Ent. faecium*, frequently isolated from patients with urinary tract infections, bacteraemia, endocarditis and other hospital acquired infections (Bhardwaj, 2019). The public health concern of *Enterococcus* is directly associated with the tendency for multidrug resistance (MDR) (Kristich et al., 2014) leading to therapeutic treatment failure especially in immunocompromised people and hospitalized patients. Intrinsic resistance against certain antibiotics including some aminoglycosides, cephalosporins, clindamycin and semisynthetic penicillinase-stable penicillins, and acquired resistance to others, such as vancomycin, can make treatment challenging (Byappanahalli et al., 2012; Higuita & Huycke, 2014).

AMR enterococci are frequently found in water environments (Cho et al., 2020; Goldstein et al., 2014; Micallef et al., 2013). The presence of high-risk hospital strains in rivers and reclaimed water discharged into the environment has been reported and may indicate that AMR traits confer some environmental advantage (Sadowy & Luczkiewicz, 2014). AMR enterococci have also been reported in environmental water used as irrigation water (Carey et al., 2016), and it has been suggested that contaminated irrigation water can lead to pre- and postharvest contamination of food crops (Ben Said et al., 2016; Ijabadeniyi et al., 2011). Overhead irrigation of lettuce with water carrying known levels of enterococci resulted in transfer to the crop, where the enterococci persisted for at least 14 days (Xu et al., 2016). With the growing threat of AMR infections, data on the AMR status of enterococci present in surface and reclaimed water will allow for a more comprehensive assessment of water quality for agricultural use. The goal of this study, therefore, was to investigate the spatial and seasonal distribution and AMR of *Ent. faecalis* and *Ent. faecium* in surface and reclaimed irrigation water sources in the mid-Atlantic region. *Enterococcus* spp. isolates for this study were recovered from rivers, ponds and wastewater treatment sites year-round over a 2-year period.

MATERIALS AND METHODS

Sample collection

A total of 333 water samples were collected over 2 years from eight surface water sites and three reclaimed water treatment plants (wastewater treatment plant effluents), within the Cheaspeake Bay watershed in the mid-Atlantic region of the United States (September 2016 to October 2018) (Table 1). Samples were collected

TABLE 1 Total and unique number of *Enterococcus faecalis* and *Ent. faecium* isolated recovered from 11 water collection sites, with details on water type

Sampling site	Water type	Number of samples	Total number of isolates	Unique *Ent. faecalis* and *Ent. faecium* isolates selected for analysis
MA01	Reclaimed	21	43	7
MA02	Reclaimed	17	21	6
MA03	Nontidal freshwater river/creek	33	71	19
MA04	Nontidal freshwater river/creek	34	79	21
MA05	Nontidal freshwater river/creek	32	82	26
MA06	Reclaimed	26	44	12
MA07	Nontidal freshwater river/creek	33	79	19
MA08	Tidal brackish river	34	83	27
MA09	Nontidal freshwater river/creek	34	86	30
MA10	On-farm pond	35	76	18
MA11	On-farm pond	34	67	13
Total		333	731	198

biweekly during late spring, summer and early fall and monthly during early spring, winter and late fall from four different types of water: tertiary treated reclaimed wastewater (RW) (MA01, MA02, MA06), nontidal freshwater river/creek (NF) (MA03, MA04, MA05, MA07, MA09), tidal brackish river water (TB) (MA08) and pond water (PW) (MA10, MA11) (Solaiman et al., 2020). No RW samples were collected in the winter. From each site, 1 L water samples were collected in a sterile polypropylene bottle (Thermo Fisher Scientific) using a handheld sampling pole (Zenport Industries). For reclaimed water, samples were collected from the spigot after letting water run for 1 min. All samples were transported to the laboratory in cooler boxes containing ice-packs. Within 6–12 h of sampling, standard membrane filtration was carried out according to US-EPA method 1600 (EPA 1600, 2006).

Sample processing and *Enterococcus* isolation

Ten millilitre of a series dilution (representing 0.1, 1 and 10 ml of each sample) and a 100 ml aliquot of each water sample were filtered through 0.45 µm, 47 mm cellulose ester membrane filters (Pall Corporation). Membrane filters were placed aseptically onto mEI agar (Becton, Dickinson and Company [BD]) to culture *Enterococcus* species. Colonies ≥0.5 mm in diameter (regardless of colour) with a blue halo on mEI were recorded as enterococci colonies. No more than 3 colonies per sample were randomly selected to subculture on enterococcosel agar (BD) and incubated at 37°C for 24 h. Beige colonies with black halos were grown overnight at 37°C on Brain Heart Infusion (BHI) agar (BD). Isolates were identified with biochemical tests: catalase tests (with 3% H_2O_2) (Millipore-Merck, Darmstadt, Germany) and pyrrolidonyl aminopeptidase tests with a PYR kit following manufacturer's instruction (Hardy Diagnostics).

Enterococcus species identification

Confirmed *Enterococcus* isolates (n = 731) were subcultured on BHI agar and incubated at 35–37°C for 18–20 h. DNA was extracted from the bacterial cultures by rapid heat lysis using 7.5% Chelex 100 solution (Bio-Rad) (Micallef et al., 2012). Aliquots of 2.5 µl DNA suspensions were used as templates for PCR, mixed in a 22.5 µl PCR reaction mix containing 10× PCR buffer (New England Biolabs [NEB]), 1.5 mM $MgCl_2$ (NEB), 0.2 mM dNTPs (VWR), 0.3 µM each of forward and reverse primers (to target gene and 16S rRNA gene as internal control) (IDT technologies) and 0.24 µl of 5 Units of *Taq* DNA Polymerase (NEB). D-alanine: D-alanine ligase encoding gene *ddl* and superoxide dismutase encoding gene *sodA* were used as primer for both *Ent. faecalis* and *Ent. faecium* as described in Micallef et al. (2013) and modified from Kariyama et al. (2000) and Jackson et al. (2004). Initial PCR included targeting of the *ddl* genes and an internal control targeting a 352 base pair section of the 16S rRNA gene (Table 2). For isolates that were not able to be differentiated by *ddl* amplification, a second PCR amplification targeting the *sodA* gene was performed. Amplification was done with an initial denaturation step of 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 20 s and elongation at 72°C for 45 s, with a final elongation of 7 min. PCR products were electrophoresed on 2% agarose (Lonza). *Enterococcus faecalis* ATCC 51299 and *Ent. faecium* ATCC 29212 were used as reference strains.

TABLE 2 PCR primers used in this study

Primer	Gene	Size (bp)	Primer sequences	References
ddl *Ent. faecalis*	D-alanine: D-alanine ligase	941	5'-ATCAAGTACAGTTAGTCTTTATTAG-3' 5'-ACGATTCAAAGCTAACTGAATCAGT-3'	Kariyama et al. (2000)
ddl *Ent. faecium*	D-alanine: D-alanine ligase	658	5'-TTGAGGCAGACCAGATTGACG-3' 5'-TATGACAGCGACTCCGATTCC-3'	Kariyama et al. (2000)
sodA *Ent. faecalis*	superoxide dismutase	360	5'-ACTTATGTGACTAACTTAACC-3' 5'-TAATGGTGAATCTTGGTTTGG-3'	Jackson et al. (2004)
sodA *Ent. faecium*	superoxide dismutase	215	5'-GAAAAAACAATAGAAGAATTAT-3' 5'-TGCTTTTTTGAATTCTTCTTTA-3'	Jackson et al. (2004)
16S rRNA	Housekeeping	352	5′-AGAGTTTGATCCTGGCTCAG-3′ 5′-CTGCTGCCTCCCGTAGG-3′	Edwards et al. (1989) and Motta et al. (2007)

Antimicrobial susceptibility testing

To avoid clonality, one *Ent. faecalis* and one *Ent. faecium* from each water sample (where possible) were selected for antimicrobial susceptibility testing using the Gram-positive plate GPN3F (Thermo Scientific™) on a Sensitire® system (Thermo Scientific™). The plate included the antimicrobials erythromycin (ERY, 0.25–4 μg ml^{-1}), clindamycin (CLI, 0.12–2 μg ml^{-1}), gentamicin (GEN, 2–16 and 500 μg ml^{-1}), streptomycin (STR, 1000 μg ml^{-1}), quinupristin/dalfopristin (Synercid, SYN, 0.12–4 μg ml^{-1}), daptomycin (DAP, 0.25–8 μg ml^{-1}), vancomycin (VAN, 1–128 μg ml^{-1}), tetracycline (TET, 2–16 μg ml^{-1}), ampicillin (AMP, 0.12–16 μg ml^{-1}), rifampicin (RIF, 0.5–4 μg ml^{-1}), levofloxacin (LEVO, 0.25–8 μg ml^{-1}), linezolid (0.5–8 μg ml^{-1}), penicillin (PEN, 0.06–8 μg ml^{-1}), ciprofloxacin (CIP, 0.5–2 μg ml^{-1}), trimethoprim/sulfamethoxazole (SXT, 1/19–4/76 μg ml^{-1}), ceftriaxone (AXO, 8–64 μg ml^{-1}) and gatifloxacin (1–8 μg ml^{-1}). Since cephalosporins (ceftriaxone), clindamycin, trimethoprim-sulfamethoxazole and low-concentration aminoglycosides (gentamicin) are not clinically effective against *Enterococcus* (CLSI, 2020), results were not reported. High-concentration gentamicin and streptomycin were included due to their negative effect on Gram-positive bacteria in combination with penicillin/aminoglycoside (CLSI, 2020). Antimicrobial susceptibility testing and data interpretation were performed according to the Clinical and Laboratory Standard Institute guidelines (CLSI, 2020). The minimum inhibitory concentrations (MICs) were recorded as the lowest concentration (μg ml^{-1}) of an antimicrobial that completely inhibited bacterial growth for all but linezolid. For linezolid, 80–90% growth inhibition compared with positive control was used. MDR was defined as resistance to two or more classes of antibiotics.

Statistical analysis

For statistical analysis, 373 *Enterococcus* spp. isolates were selected and data were pooled by four water types (five NF, one TB, two PW and three RW) and four season (March 01 to May 30 as spring, June 01 to August 31 as summer, September 01 to November 30 as fall, December 01 to February 28 as winter). Multinomial logistic regression followed by the chi-squared test with $\alpha = 0.05$ were used to assess the effect of season or water type variation on *Enterococcus* species distribution (*Ent. faecalis, Ent. faecium* and other species) and binomial logistic regression followed by the chi-squared test with $\alpha = 0.05$ were used to assess antibiotic susceptibility. Effect of water type and season on AMR and resistance pattern (MDR and single drug resistance (SDR)) were also assessed using multinomial logistic regression. Correspondence analysis (CA) was used to explore associations between species classification and water type or season. Multiple correspondence analysis (MCA) was conducted to explore associations between resistance pattern and species classification, season and water type. *Enterococcus faecalis* has intrinsic resistance against quinupristin/dalfopristin (Duh et al., 2001) and was therefore omitted from statistical analysis. Statistical analysis was conducted in R studio v. 4.0 using "multinom" package and "glm" function and in JMP Pro 15.2.0.

RESULTS

Enterococcus faecalis and *Ent. faecium* distribution is affected by water type and season

Enterococcus spp. were isolated from 299 out of 333 (89.8%) water samples and bacterial dynamics fully described in Solaiman et al. (2020), from which 731 *Enterococcus* isolates were archived. To avoid clonality, only 373 isolates with a unique combination of collection site, date and species designation were selected for further analysis. Out of 373 *Enterococcus* isolates, 150 were identified as *Ent. faecalis* (40.2%), 48 as *Ent. faecium* (12.9%) and 175 (46.9%) as species other than *Ent. faecalis* and *Ent. faecium* (not classified).

Species distribution was significantly affected by water type (likelihood ratio χ^2 [df = 6, $N = 373$] = 18.55, $p < 0.01$). Freshwater river samples ($n = 204$) yielded 93 *Ent. faecalis* isolates (45.6%) and 22 (10.8%) *Ent. faecium* (Figure 1a). Similarly, 23 (54.7%) and 4 (9.5%) isolates out of 42 tidal brackish river isolates were identified as *Ent. faecalis* and *Ent. faecium*, respectively. In fact, *Ent. faecalis* was significantly more likely to be found in freshwater river ($p < 0.05$) than *Ent. faecium*, and in tidal brackish water ($p < 0.05$) than other species (Figure 1b). Pond water had the highest proportion of "other" *Enterococcus* spp. (57.5%), and only 21 (28.8%) and 10 (13.7%) out of 73 pond water isolates were *Ent. faecalis* and *Ent. faecium*, respectively. Reclaimed water isolates had the highest proportion of *Ent. faecium* (12/54; 22.2%) of any water type and the lowest proportion of *Ent. faecalis* (13/54; 24.1%). *Enterococcus faecium* was significantly more likely to be found in reclaimed water than other water type ($p < 0.01$) than *Ent. faecalis* (Figure 1a,b).

Enterococcus species distribution was also significantly affected by season (likelihood ratio χ^2 [df = 6, $N = 373$] = 13.43, $p < 0.05$) (Figure 1c). Comparing

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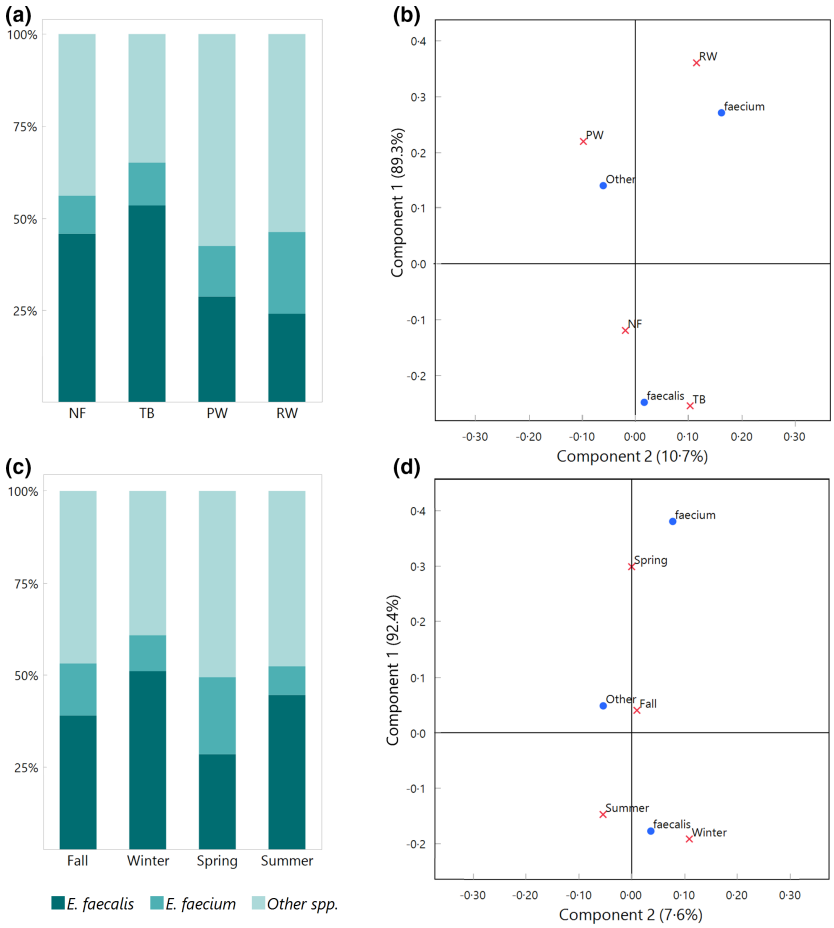

FIGURE 1 Distribution plots (a, c) and correspondence analysis plots (b, d) for *Enterococcus* species, by water type (a, b) and season (c, d). Plots a and c show percent proportion. NF, nontidal freshwater river ($n = 204$); PW, pond water ($n = 73$); RW, reclaimed water ($n = 54$); TB, tidal brackish river ($n = 42$). Sample sizes for season are 113, 51, 81, 128 for fall, winter, spring and summer, respectively

seasons, the highest proportion of *Ent. faecalis* isolates were found in winter (26/51; 51.0%) and summer (57/128; 44.5%). By contrast, *Ent. faecium* incidence was highest in spring (17/81; 21.0%) and lowest in summer and winter (10/128; 7.8%% and 5/51; 9.8%, respectively). *Enterococcus faecalis* was significantly more likely to be found in summer ($p < 0.05$) than *Ent. faecium*, and *Ent. faecium* was more likely to be found in spring ($p < 0.05$; Figure 1d). "Other" *Enterococcus* spp. were more homogenously distributed (Figure 1c).

Antimicrobial resistance in *Enterococcus* is species dependent and affected by season

All the *Ent. faecalis* and *Ent. faecium* isolates ($n = 198$; 150 *Ent. faecalis* and 48 *Ent. faecium*) were further subjected to antimicrobial susceptibility testing. Only 11 out of 198 isolates (5.6%), including 9/150 (6.0%) *Ent. faecalis* and 2/48 (4.2%) *Ent. faecium*, were found to be pansusceptible (Figure 2a), and pansusceptible isolates were the most different from all isolates in the MCA plot (Figure 3). SDR was identified in 96/198 (48.5%) isolates, including 82/150 (54.7%) *Ent. faecalis* and 14/48 (29.2%) *Ent. faecium*. On the other hand, MDR was detected in 52/198 (26.3%) isolates, comprising 26/150 (17.3%) *Ent. faecalis* and 26/48 (54.2%) *Ent. faecium*. Regression analysis showed that resistance patterns were dependent on species (likelihood ratio χ^2 [df = 3, $N = 198$] = 21.76, $p < 0.001$), with MDR more likely to be detected in *Ent. faecium* than *Ent. faecalis* and vice versa for SDR ($p < 0.001$) (Figures 2a and 3). While water type was not found to be a significant factor in resistance, season had an effect (likelihood ratio χ^2 [df = 3, $n = 198$] = 26.23, $p < 0.01$) (Figure 2a,b). Summer and winter were the two most unrelated seasons, with summer being the second highest contributor to inertia (20%) in dimension 1 (species: *faecalis* was highest) and winter contributing the most to inertia (29%) in dimension 2 on the MCA plot (Figure 3). Summer ($p < 0.01$) and spring isolates were more likely to exhibit SDR than MDR, represented by 42/67 (62.7%) and 22/40 (55%) of summer and spring isolates, respectively (Figure 3). Winter isolates ($p = 0.001$) were more likely than summer isolates to exhibit MDR than SDR (13/31; 41.9%). Analysing the data by species, however, revealed that this dependence only held for *Ent. faecalis* (likelihood ratio χ^2 [df = 9, $n = 150$] = 21.53, $p = 0.01$).

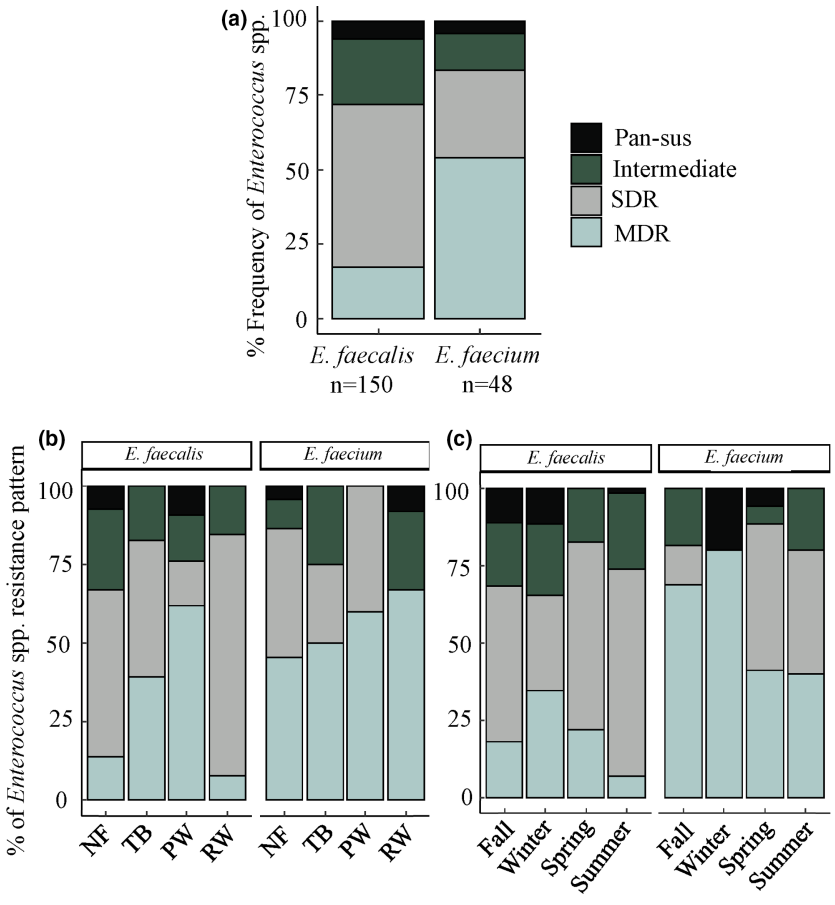

FIGURE 2 Distribution of pansusceptible, intermediate, single drug resistant (SDR) and multidrug resistant (MDR) isolates of *Enterococcus faecalis* and *Ent. faecium* in (a) all water samples, and (b) by water type. NF, nontidal freshwater river ($n = 204$); PW, pond water ($n = 73$); RW, reclaimed water ($n = 54$); TB, tidal brackish river ($n = 42$). (c) Season of sample collection, including fall ($n = 113$), winter ($n = 51$), spring ($n = 81$) and summer ($n = 128$)

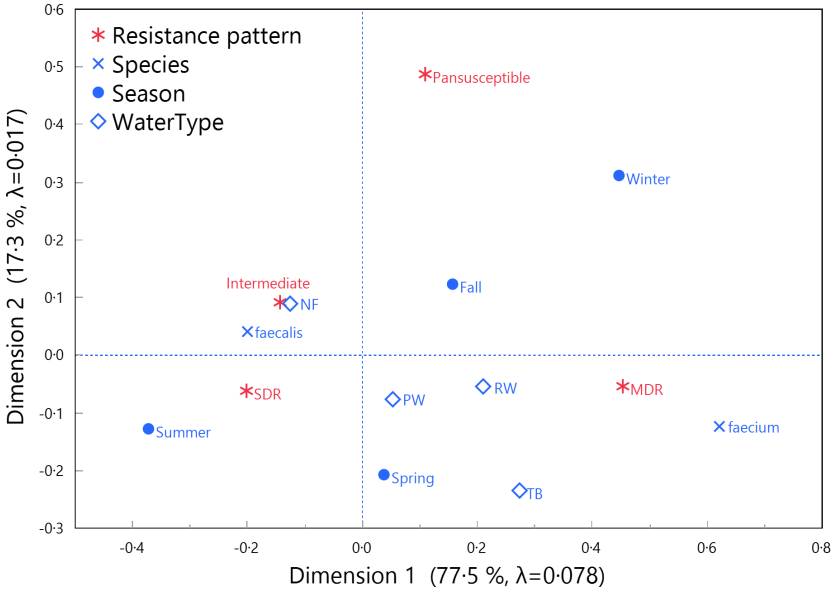

FIGURE 3 Multiple correspondence analysis plot exploring the association between antimicrobial resistance pattern (pansusceptibility or intermediate, single drug, (SDR) or multidrug (MDR) resistance) and *Enterococcus* spp., season of collection and water type (nontidal freshwater river (NF), tidal brackish river (TB), pond water (PW) and reclaimed water (RW))

Resistance to specific antimicrobials

None of the isolates exhibited resistance to daptamycin, and resistance to gatifloxacin was only intermediate in two *Ent. faecalis* isolates (Figure 4). Resistance to ampicillin, vancomycin and linezolid was only detected in a very small number of *Ent. faecalis* isolates. Antimicrobials with very low rates of resistance (<5%) detected in both species included high-concentration gentamicin and levofloxacin, although a higher frequency of intermediate resistance was detected for the latter (Figure 4). In both species, resistance frequency was highest for rifampicin, 60% (90 out of 150) and 56.3% (27 out of 48) for *Ent. faecalis* and *Ent. faecium*, respectively. The intermediate resistance detected in 22% of *Ent. faecalis* isolates was mostly to two or more antimicrobial classes, with the most common

Journal of Applied Microbiology

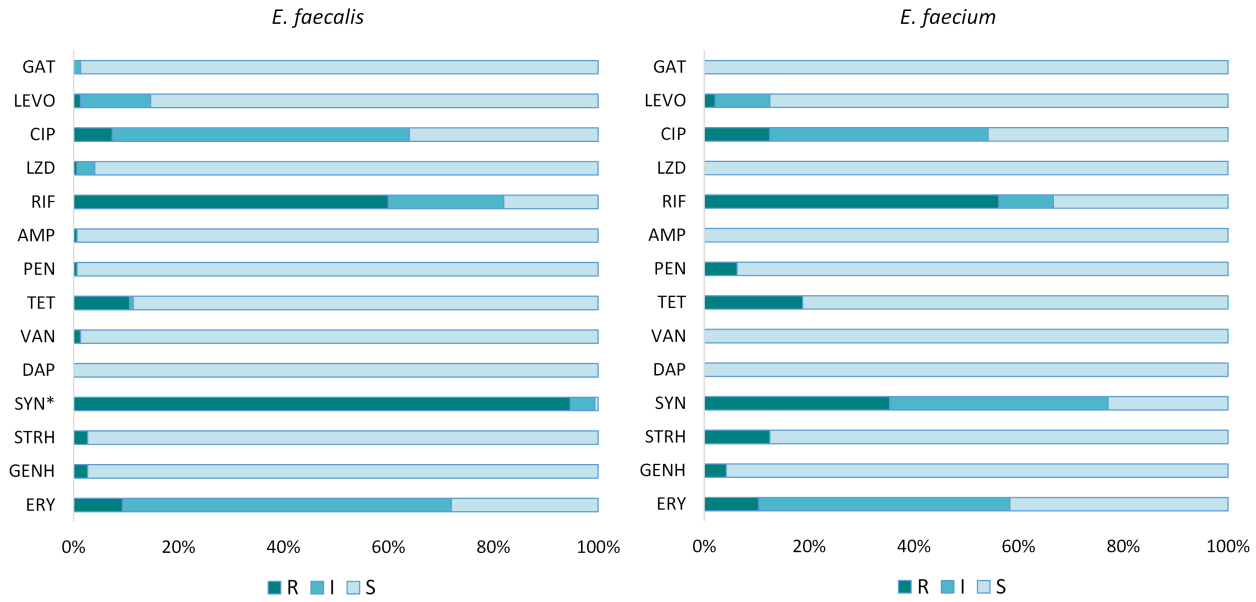

FIGURE 4 Frequency of antimicrobial resistance (resistant, R; intermediate, I and susceptible S) to specific antibiotics tested among *Enterococcus faecalis* ($n = 150$) and *Ent. faecium* ($n = 48$) isolates. Abbreviations are as follows: ampicillin (AMP), ciprofloxacin (CIP), erythromycin (ERY), high-concentration gentamicin (GENH), levofloxacin (LEVO), linezolid (LZD), penicillin (PEN), rifampicin (RIF), high-concentration streptomycin (STRH), tetracycline (TET), vancomycin (VAN), quinupristin/dalfopristin (SYN). * *Ent. faecalis* has intrinsic resistance to SYN

combinations including erythromycin-rifampicin-ciprofloxacin. One river isolate recovered in the fall had intermediate resistance to linezolid.

Species designation was a significant factor only for high-concentration streptomycin, with resistance more likely to be detected in *Ent. faecium* than *Ent. faecalis* ($p < 0.05$). A weak effect of species was detected for rifampicin and penicillin, with resistance more likely in *Ent. faecalis* and *Ent. faecium*, respectively ($p < 0.1$ for both). Season appeared to have the most effect on resistance. Resistance to erythromycin was more likely to be detected in winter isolates compared to summer ($p < 0.05$). Tetracycline-resistant isolates were more likely to be recovered in winter rather than summer ($p < 0.01$) or fall ($p = 0.01$), and in spring than fall ($p < 0.05$). Resistance to rifampicin was more likely found in summer ($p = 0.01$) and fall ($p < 0.05$) than winter. Water type was not found to have much of an effect, and only a weak association was uncovered, with ciprofloxacin resistance being more likely to be detected in brackish river isolates compared to freshwater river ($p < 0.01$) and pond water ($p < 0.05$) isolates.

Resistance patterns

Out of 82 SDR *Ent. faecalis*, 72 (87.8%) exhibited resistance to rifampicin (Table 3). Other SDR was to tetracycline, ciprofloxacin, erythromycin, high-concentration streptomycin and vancomycin. SDR in *Ent. faecium* ($n = 14$) was also diverse but excluded vancomycin (Table 3). There were 26 isolates each of *Ent. faecalis* and *Ent. faecium* that exhibited MDR. Most of these, 19 and 20 isolates, respectively, carried resistance to two classes of antibiotics. The most frequent resistance pattern in *Ent. faecalis* was to rifampicin-ciprofloxacin ($n = 5$), and to erythromycin-rifampicin ($n = 4$). In *Ent. faecium*, the most frequent resistance pattern was to quinupristin/dalfopristin-rifampicin. Some drug resistance combinations were unique to each species, including vancomycin-ampicillin ($n = 1$), erythromycin-tetracycline ($n = 2$) and rifampicin-levofloxacin ($n = 1$) or -linezolid ($n = 1$), detected in *Ent. faecalis* only. Rifampicin-penicillin ($n = 1$) was detected in *Ent. faecium* only. Seven *Ent. faecalis* isolates (7/150; 4.7%) and six *Ent. faecium* isolates (6/48; 12.5%) showed resistance to three or more antibiotics (Table 1). The most recurrent resistance was to tetracycline, erythromycin and rifampicin. None of these 13 isolates had resistance to ampicillin or vancomycin, but 3 had resistance to penicillin (Table 3).

DISCUSSION

The threat of AMR is currently one of the most pressing public health concerns. Wider dissemination of AMR into the environment could augment the intra- and interspecies exchange of AMR traits and increase community acquired infections. Despite this situation, AMR in the

TABLE 3 Antimicrobial resistance patterns for single (SDR) and multidrug resistance (MDR) for *Enterococcus faecalis* and *Ent. faecium* isolates

SDR (96)	*Ent. faecalis* (82)	*Ent. faecalis* (14)
	CIP (2)	CIP (1)
	ERY (2)	ERY (1)
		GENH (1)
	RIF (72)	RIF (5)
	STRH (1)	STRH (2)
	TET (4)	TET (1)
	VAN (1)	
	—	SYN (3)
MDR (52)	***Ent. faecalis* (26)**	***Ent. faecalis* (26)**
Resistance to 2 antibiotics	AMP-VAN (1)	
	ERY-RIF (4)	ERY-RIF (2)
	ERY-TET (2)	
		ERY-SYN (1)
	GESH-RIF (1)	GESH-RIF (1)
	GESH-TET (1)	
		PEN-RIF (1)
	STRH-CIP (1)	
		STRH-SYN (1)
		SYN-RIF (9)
	TET-CIP (1)	TET-CIP (1)
	TET-RIF (1)	TET-RIF (3)
	RIF-CIP (5)	RIF-CIP (1)
	RIF-LEVO (1)	
	RIF-LZD (1)	
Resistance to 3+ antibiotics	ERY-GESH-TET (1)	
	ERY-TET-RIF (1)	
		STRH-TET-PEN (1)
		SYN-RIF-LEVO (1)
		TET-RIF-CIP (1)
	ERY-GESH-STRH-TET (1)	
	ERY-STRH-TET-RIF (1)	
	ERY-TET-PEN-RIF (1)	
	ERY-TET-RIF-CIP (1)	
		STRH-SYN-RIF-CIP (1)
		TET-PEN-RIF-CIP (1)
	TET-RIF-CIP-LEVO (1)	
		ERY-STRH-SYN-TET-RIF (1)

Numbers in parentheses indicate number of isolates in that group. Abbreviations are as follows: ampicillin (AMP), ciprofloxacin (CIP), erythromycin (ERY), high-concentration gentamicin (GENH), levofloxacin (LEVO), linezolid (LZD), penicillin (PEN), rifampicin (RIF), high-concentration streptomycin (STRH), tetracycline (TET), vancomycin (VAN), quinupristin/dalfopristin (SYN, *Ent. faecium* only).

environment is not closely monitored, and data on AMR are lacking for various habitats, including agricultural environments. Surface and reclaimed water that serve as potential irrigation water sources for fruit and vegetables are one possible route of transmission of AMR enterococci being found on fresh produce crops (Allen et al., 2013; Ben Said et al., 2016; Johnston & Jaykus, 2004; Schwaiger et al., 2011). In turn, this provides a channel for AMR bacteria to humans, since these crops are often eaten raw. Monitoring AMR accumulating in bacteria commonly found in irrigation water can be an additional criterion by which water quality is assessed. In this study, we evaluated the distribution and AMR profiles of two important enterococci, *Ent. faecalis* and *Ent. faecium*, isolated from surface and reclaimed water in the mid-Atlantic region. These two species are of particular importance to public health as they are the most frequently isolated species from humans with enterococcal infections (García-Solache & Rice, 2019). Isolates were retrieved over a 2-year period, allowing us to assess differences in species and AMR distribution by water type and season. As expected, *Enterococcus* was a widespread bacterial taxon in our water samples, with almost 90 percent of the water samples testing positive. *Enterococcus faecalis* comprised 40% of the isolates tested, occurring at three times the rate of *Ent. faecium*. Seasonally, the two species were mutually exclusively dominant, with *Ent. faecalis* more frequent in summer and winter when *Ent. faecium* was low, and vice versa in spring. This alternating seasonal dominance was also apparent in a Georgia watershed (Cho et al., 2020). Species distribution was also affected by water type, with *Ent. faecium* more likely to be isolated from reclaimed water and *Ent. faecalis* from rivers. Within each water type, *Ent. faecalis* was dominant in all surface water sources, but the two species were distributed evenly in reclaimed water. In a previous study, *Ent. faecalis* was more frequently isolated from irrigation ponds on mid-Atlantic farms than *Ent. faecium* (Micallef et al., 2013). While no single *Enterococcus* spp. is a known indicator of specific animal or human hosts, enterococcal species distribution varies across hosts, and a 100% isolation rate of *Ent. faecium* was reported from human faeces compared to 78% for *Ent. faecalis* (Layton et al., 2010). Fingerprints based on six *Enterococcus* spp. grouped human faeces, sewage and dog samples in a cluster separate from assemblages containing wildlife samples (Layton et al., 2010). The association of *Ent. faecium* with reclaimed water and *Ent. faecalis* with freshwater river identified in this study could be associated with the proportion of these species in human versus wildlife populations.

Pansusceptibility to the tested antimicrobials was very low for both species. On the other hand, MDR was more prevalent in *Ent. faecium* than *Ent. faecalis* while the latter

had a higher tendency to accumulate intermediate resistance. MDR was highest in winter and lowest in summer and vice versa for SDR, possibly attributable to seasonal fluctuations in the levels of antimicrobials present in these waters (Panthi et al., 2019). On the other hand, AMR was not influenced by water type, although the study limitation of not including winter-collected reclaimed water samples could be a confounding factor. Together, these findings suggest that the source of AMR in environmental *Enterococcus* in this region is likely diffuse and not traceable to a point source. The AMR profiles we describe in our study differ from what is reported for a Georgia watershed, where *Ent. faecalis* and *Ent. faecium* isolates exhibited some resistance to daptamycin, higher resistance to tetracycline, but lower resistance to erythromycin compared to our study (Cho et al., 2020). In this study, although some phenotypic resistance was commonly detected, resistance was most prevalent to rifampicin in both species (59–64%) and quinupristin/dalfopristin in *Ent. faecium*, followed by resistance to other antibiotics at lower rates (8–20%). This included tetracycline, erythromycin and ciprofloxacin in both species. Resistance to ampicillin, a common first line of treatment for enterococcal infections (Said et al., 2021), and vancomycin, which is administered against ampicillin resistant strains (Said et al., 2021), was very low. Only two *Ent. faecalis* isolate were resistant to vancomycin, one of which was also resistant to ampicillin. Both these isolates originated from river water. Other resistance to note was to linezolid, used to treat ampicillin- and vancomycin-resistant infections (Said et al., 2021), detected in one isolate from a pond. Susceptibility to daptamycin is encouraging as this antibiotic is also used as a last resort antibiotic, although for certain infections it can be in combination with rifampicin (Said et al., 2021), to which resistance was widespread. Overall, the profiles of these water isolates exhibited substantially less problematic resistance to what is reported for clinical isolates cultured from hospital-acquired infections (García-Solache & Rice, 2019; Kristich et al., 2014). However, they did share some traits with feedlot enterococci which predominantly build up resistance against antibiotics used in animal operations, including tetracycline and macrolides such as erythromycin (Zaheer et al., 2020), as seen in our study. This suggests that selective pressures resulting in AMR in environmental water isolates in the mid-Atlantic may be more similar to drivers of AMR in feedlots than nosocomial settings.

Using replenishable surface and reclaimed water sources for agriculture should be a sustainability goal for the mid-Atlantic area of the United States as a way to reduce pressures on groundwater sources that are experiencing decline in many parts of the region (Dong et al., 2019). However, groundwater was reported as the primary source of irrigation of fresh produce in this region, while a reduction from 49% to 23% in growers using surface water was noted between 2010 and 2013 (Marine et al., 2016). New water quality standards in the Produce Safety Rule (Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR; 21 CFR 112) (FSMA PSR, 2011), that apply to water used in fresh fruit and vegetable production, may be discouraging some growers from tapping into surface water sources due to the lower microbial quality of these water types (Pagadala et al., 2015). Our study does not provide evidence of enhanced risk of using reclaimed water compared to surface water in terms of AMR enterococci. Reclaimed water is not used for irrigation of fresh produce crops in the mid-Atlantic. However, growers were open to the idea of using tertiary treated reclaimed water, especially when armed with knowledge about alternative water sources, but still cited food safety concerns as a main barrier for application (Suri et al., 2019). Therefore, characterizing the hazards associated with using surface and reclaimed water for production of fresh produce is of utmost importance to garner grower acceptance and support policies regarding reclaimed water use, which could help conserve groundwater resources.

This study provides data on the AMR profiles of the two most commonly cultured enterococci from humans, *Ent. faecalis* and *Ent. faecium*. AMR enterococci were detected in all water types but were affected by season, with higher likelihood of MDR detected in winter compared to summer. This is an encouraging finding since in the mid-Atlantic region, irrigation does not occur in open fields in winter, being outside of the vegetable crop growing season. Resistance to antibiotics that are mostly reserved for human use (e.g., vancomycin and linezolid) was detected, suggesting that AMR could be spreading from human activity to the environment but in general remains relatively infrequent. Resistance to clinically important antibiotics such as ampicillin, vancomycin and linezolid, although rare, was detected in isolates that originated from surface water. Enterococci could easily spread to vegetable crops via irrigation water, potentially posing a long-term threat to food safety following consumption of affected crops. Data on AMR capabilities of bacteria that may be used to evaluate microbial water quality can provide an additional metric by which to evaluate irrigation water safety and adequacy for use in fresh produce crop production.

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CONFLICT OF INTEREST

The authors declare that no competing interests exist.

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