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Review

Towards the standardization of *Enterococcus* culture methods for waterborne antibiotic resistance monitoring: A critical review of trends across studies

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ABSTRACT

Antibiotic resistance is a major 21st century One Health (humans, animals, environment) challenge whose spread limits options to treat bacterial infections. There is growing interest in monitoring water environments, including surface water and wastewater, which have been identified as key recipients, pathways, and sources of antibiotic resistant bacteria (ARB). Aquatic environments also facilitate the transmission and amplification of ARB. *Enterococcus* spp. often carry clinically-important antibiotic resistance genes and are of interest as environmental monitoring targets. *Enterococcus* spp. are Gram-positive bacteria that are typically of fecal origin; however, they are also found in relevant environmental niches, with various species and strains that are opportunistic human pathogens. Although the value of environmental monitoring of antibiotic-resistant *Enterococcus* has been recognized by both national and international organizations, lack of procedural standardization has hindered generation of comparable data needed to implement integrated surveillance programs. Here we provide a comprehensive methodological review to assess the techniques used for the culturing and characterization of antibiotic-resistant *Enterococcus* across water matrices for the purpose of environmental monitoring. We analyzed 117 peer-reviewed articles from 33 countries across six continents. The goal of this review is to provide a critical analysis of (i) the various methods applied globally for isolation, confirmation, and speciation of *Enterococcus* isolates, (ii) the different methods for profiling antibiotic resistance among enterococci, and (iii) the current prevalence of resistance to clinically-relevant antibiotics among *Enterococcus* spp. isolated from various environments. Finally, we provide advice regarding a path forward for standardizing culturing of *Enterococcus* spp. for the purpose of antibiotic resistance monitoring in wastewater and wastewater-influenced waters within a global surveillance framework.

1. Introduction

Enterococcus spp. are important members of the natural enteric microbiome of both humans and animals and have emerged as important antibiotic-resistant pathogens in clinical medicine (Arias and Murray, 2012). There are currently 60 classified *Enterococcus* species in the National Center for Biotechnology Information database, most of which are commensal microorganisms, although some act as opportunistic pathogens in humans. *E. faecalis* and *E. faecium* are among the most important etiological agents of nosocomial infections; including urinary tract infections (UTIs), central nervous system infections, endocarditis, bacteremia, neonatal infections, and surgical site infections (Moellering, 1992; Murray, 1990). From 2006 to 2017, *Enterococcus* spp. were responsible for approximately 14% of all healthcare-associated infections in the US, ranking second overall behind *Staphylococcus aureus* (Hidron et al., 2008; Sievert et al., 2013; Weiner-Lastinger et al., 2020; Weiner et al., 2016). *Enterococcus* spp. possess full or partial intrinsic chromosomal resistance to cephalosporins, aminoglycosides, lincosamides, trimethoprim-sulfamethoxazole, and penicillins (Hollenbeck and Rice, 2012). Their rapid development of multi-drug resistance has been attributed in part to their highly malleable genomes that lack CRISPR (clustered regularly interspaced

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palindromic repeats) elements, which has facilitated the ready acquisition of allochthonous mobile DNA (e.g., vancomycin resistance gene clusters) (Palmer and Gilmore, 2010). Nearly 25% of the genomes of many clinical *E. faecalis* and *E. faecium* isolates consist of acquired genetic elements (Hegstad et al., 2010; Paulsen et al., 2003). Recently, over 85% of *E. faecium* and 15% of *E. faecalis* isolates responsible for catheter-associated UTIs and central line-associated bloodstream infections diagnosed in the US have been found to be vancomycin resistant (Weiner-Lastinger et al., 2020). The US Center for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have set VRE to "high" priority and a "serious" threat level (Centers for Disease Control and Prevention, 2019; Tacconelli et al., 2018).

Enterococcus spp. are members of the larger, phenotypically-defined group known as enterococci, which are Gram-positive, catalase-negative, obligately fermentative chemoorganotrophs that can survive over a wide range of temperatures, pH, and salinity (Teixeira et al., 2015). Enterococci are found in many extraenteric environmental niches, including soils and sediments, beach sands, and aquatic and terrestrial vegetation. Enterococci have been extensively isolated from wastewaters, marine waters, and freshwaters (Byappanahalli et al., 2012). Because of their abundance in human and animal feces, their extra-enteric persistence, and the ease with which they are cultured, enterococci have been targeted for decades as fecal indicators for the purpose of water quality monitoring (Jang et al., 2017; Schoen et al., 2011; Sinclair et al., 2012). Enterococci (formerly classified within the larger group known as "fecal streptococci") have been widely used to assess the microbiological safety of surface waters, drinking waters, recreational beaches, and as a target for assessing process removal efficiencies during wastewater treatment. Enterococci have also been found to correlate directly to public health outcomes; for example, across the US, the rate of gastrointestinal illness in swimmers has been correlated with *Enterococcus* spp. levels in recreational beach waters that are impacted by wastewaters (Prüss, 1998; Wade et al., 2006, 2003); however, these illnesses are assumed to be caused mainly by viral pathogens (Soller et al., 2010) rather than *Enterococcus* spp.. Their importance as water quality indicators and their inclusion in governmental regulatory frameworks have led to a great deal of method development for isolation and enumeration from environmental samples (Boehm and Sassoubre, 2014; Health Canada, 2020).

Global and national action plans set in place to combat the spread of antibiotic resistance have generally embraced a One Health approach (humans-animals-environment) (European Commission, 2017; Hernando-amado et al., 2019), but a better understanding of the role of environmental transmission and amplification of antibiotic-resistant *Enterococcus* and their genes to humans, animals, and aquatic environments is needed. Aquatic environments have been identified as a key recipient and transmission pathway of antibiotic resistant bacteria into and out of human and animal populations (Amarasiri et al., 2020; Larsson and Flach, 2021). Multidrug-resistant pathogens and mobile antibiotic resistance genes enter the environment via treated and untreated wastewater across the globe (Alexander et al., 2020; Marathe et al., 2017; Zhang et al., 2020). Recently, the WHO put forth the Tricycle protocol as a standardized method for monitoring the dissemination, transmission, and evolution of antibiotic resistance along the One Health continuum: humans (hospitals and community), the food chain (animal husbandry), and the environment (human and animal fecal contamination) (WHO, 2021). Specifically, the protocol targets Gram-negative extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli*, which display phenotypic resistance to third-generation cephalosporins. While ESBL *E. coli* was selected, in part, to coordinate with global surveillance of *Enterobacterales* (Marano et al., 2020), the extent to which it is truly a representative indicator of resistome dynamics has not been established. Thus, the present is a critical moment to also consider other potential targets.

Antibiotic-resistant enterococci present many advantages as a potential target for monitoring antibiotic resistance in the water environment. The level of standardization for methods targeting *Enterococcus* spp. is arguably second only to *E. coli* in environmental waters. As such, *Enterococcus* spp. could present an attractive complimentary target to *E. coli*. Notably, as Gram-positive organisms, they provide insight into distinct genotypes and phenotypes of antibiotic resistance that would not be captured by monitoring only Gram-negative organisms. In particular, the plasticity of *Enterococcus* genomes and their propensity for horizontal gene transfer and exchange of virulence and antibiotic resistance determinants from clinical strains to environmental reservoirs of enterococci (Ekwanzala et al., 2020b; Gouliouris et al., 2019, 2018) marks them as potentially comprehensive targets for antibiotic resistance monitoring. Like *E. coli,* they are members of the normal flora of human and animal gastrointestinal tracts (Byappanahalli et al., 2012) and are readily quantified in wastewater and environmental waters. Their status as normal human flora creates the disadvantage of extreme difficulty in tracking infection rates from human exposure to contaminated water, another characteristic they share with *E. coli.* A study of individuals engaging in surfing found that surfers were significantly more likely to be colonized with *E. coli* carrying $bla_{\text{CTX-M}}$ compared to non-surfers (Leonard et al., 2018). Analogous studies of antibiotic-resistant *Enterococcus* spp. could shed light on aspects of direct transfer of these bacteria from aquatic environments to humans.

Recent progress has been made in applying culture-based methods for monitoring antibiotic resistant *Enterococcus* spp. in the environment, with emphasis on human and animal wastewater, and hospital wastewater pollution (Gouliouris et al., 2019; Savin et al., 2020; Zaheer et al., 2020). The phenotypic and morphological similarity of other Gram-positive organisms to the enterococci, however, makes isolation on selective media prone to false-positives, with cross-selectivity with other cocci (e.g., *Streptococcus, Pediococcus, Weisella*) (Harwood et al., 2001). False positives are especially problematic for environmental samples (Pagel and Hardy, 1980). Non-selectivity can actually be exacerbated with addition of antibiotics to media, because several members of non-target *Bacillus* and other genera are intrinsically resistant to clinically-relevant concentrations of certain antibiotics (Woodford et al., 1995).

The selection of any monitoring target or strategy entails consideration of the overarching purpose or questions to be addressed, and these may vary depending on the focus across the spectrum from wastewater to surface water. The following are examples of key monitoring goals and considerations addressed by this review:

- Monitoring antibiotic resistance among clinically-relevant strains of *Enterococcus* spp. in sewage as a means of assessing their levels carried in the human population
- Assessing whether clinically-relevant *Enterococcus* spp. or specific resistance phenotypes are effectively removed during wastewater treatment and if they persist in impacted aquatic environments
- Evaluating evidence that clinically-relevant *Enterococcus* spp. acquire antibiotic resistance genes from the environment
- Comparing resistant *Enterococcus* spp. in various water matrices both locally and globally to assess factors that may be contributing to antibiotic resistance in Gram-positive organisms

2. Literature review protocol

This systematic review was conducted in a four-tiered approach using search terms presented in Table S1 to collect studies published between January 2000 and December 2020 (Figure S1). Web of Science, PubMed, and Google Scholar were searched. Briefly, Tier 1 was designed to isolate topic relevant search terms for surface water, wastewater, recycled water, or reclaimed water (3,828,792 articles). Tier 2 was designed to select for those articles addressing antibiotic resistance (15,043). Tier 3 further narrowed the search to culturing techniques (5,439) and Tier 4 specifically to enterococci/*Enterococcus* (682).

Initially, these 682 articles were independently screened by two researchers for containing a complete workflow from environmental sampling through to characterization of individual isolates. Articles were excluded that only used previously collected isolates. Further exclusions were articles that exclusively investigated: biofilms, mesocosms, drinking water, sediments, or digested sludge (e.g., anaerobic digestion). Fecal source tracking articles based on antibiotic resistance analysis were also excluded (Harwood et al., 2000). Articles that used a non-selective media for initial isolation, such as R2A or TSA, were also excluded. Disagreements on article inclusion from the initial screening were presented to a larger group of five researchers to reach a consensus. The resulting 117 peer-reviewed articles were then subject to data extraction using parameters outlined in Table S2. All included articles are listed in Table S4.

3. Collection and analysis of published data

Data relevant to the species and phenotypic distribution of all isolated enterococci was collected from 117 peer-reviewed articles. First, the number of isolates per species was extracted from articles in which libraries were speciated (91 articles) to reveal general population statistics across environments. Second, antibiotic susceptibility testing (AST) data were extracted from all articles that provided the percent of resistant isolates compared to total enterococci isolated in the absence of any antibiotic (77 articles). Studies that summed isolates with "intermediate" or "resistant" classifications of resistance without providing individual statistics, as well as studies that did not cite standardized methodology for classifying resistance (e.g., current CLSI breakpoints at the time of sampling), were excluded.

4. Methods for culturing environmental Enterococci

In the US and Canada, enterococci are recommended for monitoring saline (brackish or marine) and recreational freshwaters. In the EU, enterococci are regulated in both drinking water and recreational water by standardized culture methods (EEA, 2020). Several standardized culture methods have been developed, including the US Environmental Protection Agency (USEPA) Methods 1106.1 and 1600 for ambient waters and wastewaters (USEPA, 2009, 2006), the International Organization for Standardization (ISO) Methods 7899-1 and 7899-2 (ISO, 2000), and Method 9230 (A-D) as part of the American Public Health Association's (APHA) "Standard Methods for the Examination of Water and Wastewater" (APHA, 1999; Rice and Baird, 2017) (Table 1). These methods include three distinct techniques: membrane filtration (MF), multiple tube fermentation (MTF), and defined substrate techniques (e.g., Enterolert). The current "gold standard" for enterococci enumeration from the environment is considered the MF technique (Byappanahalli et al., 2012) and was used by over 90% of articles included in this review (Table 1).

The principal selective and differential solid media used in standard MF assays are Slanetz-Bartley (SB), mEnterococcus (mE), and membrane-Enterococcus indoxyl-β-D-glucoside (mEI). These media use various peptone and yeast extract-based nutrients with the addition of sodium azide and/or nalidixic acid. Sodium azide obstructs the growth of Gram-negative bacteria through the inhibition of cytochrome oxidase. Both SB and mE agars include 2,3,5-triphenyltetrazolium chloride (TTC), which dyes viable colonies red. Differentiated colonies grown on SB or mE are then confirmed as enterococci by their ability to hydrolyze esculin in the presence of bile using either bile esculin azide or esculin iron agars. The hydrolyzed esculin product, esculetin, reacts with iron salt in the media to produce black to reddish colonies for enumeration. mEI is similar to mE medium, but contains the chromogen, indoxyl-β-D-glucoside. When cleaved by β-D-glucosidase positive enterococci, blue halos are formed around positive colonies. mEI is typically used as a standalone media as all colonies with blue halos are considered enterococci.

Table 1
Published standardized methods for the detection and enumeration of enterococci in different water matrices

Organization and Method	Recommended Matrix	Media	Number of Citations[a]	Assay Turnaround (hours)	General Procedure
Membrane Filtration (MF); (CFU/mL); Number of Studies Identified: 111/117					
EPA Method 1600	drinking water; source water; wastewater; marine and freshwater	mEI[b]	18	24	mEI (41°C for 24 hrs); Count blue halos
ISO 7899-2	surface water; wastewater	Slanetz-Bartley; Bile Esculin Azide	15	48	Slanetz-Bartley (36°C for 44 hrs); Bile Esculin Azide Agar (44°C for 2 hrs)
EPA Method 1106.1	marine and freshwater (not applicable to wastewater)	mEnterococcus; Esculin Iron Agar	0	48	mEnterococcus (41°C for 48 hrs); Esculin Iron Agar (41°C for 20 min); Count pink to red colonies
APHA SM 9230C.2a	drinking water; source water; wastewater; marine and freshwater	mEnterococcus; Esculin Iron Agar	3	48	mEnterococcus (41°C for 48 hrs); Esculin Iron Agar (41°C for 20 min); Count pink to red colonies
APHA SM 9230C.2b	drinking water; source water; wastewater; marine and freshwater	mEI[b]	0	24	mEI (41°C for 24 hrs); Count blue halos
APHA SM 9230C.2c	drinking water; source water; wastewater; marine and freshwater	mEnterococcus	10	48	mEnterococcus (35°C for 48 hrs); Count light and dark red colonies
Multiple Tube Fermentation (MTF); (MPN/mL); Number of Studies Identified: 1					
APHA SM 9230B	drinking water; source water; marine and freshwater (not applicable to wastewater)	Azide Dextrose Broth; Bile Esculin Azide Agar	1	48-72	Azide Dextrose Broth (35°C for 24-48hrs); Bile Esculin Azide Agar (35°C for 24 hrs); Compute MPN
Fluorogenic Substrate Test (MTF and MPN); (MPN/mL); Number of Studies Identified: 3					
APHA SM 9230D	drinking water; source water; wastewater; marine and freshwater	Enterolert®	3	24	Enterolert Media (41°C for 24 hrs); Compute MPN
ISO 7899-1	surface water; wastewater	MUD[c] Media	0	36-72	MUD Media in Microtitre Wells (44°C for 36-72 hrs); Compute MPN

[a] The 'Number of Citations' under Membrane Filtration do not correspond to the number of citations in the table as many articles did not follow or cite a standard method. Techniques not listed are direct plating after serial dilutions (8 articles).

[b] membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI)

[c] 4-methylumbelliferyl-β-D-glucoside (MUD), Environmental Protection Agency (EPA), International Organization for Standardization (ISO), American Public Health Association (APHA)

4.1. Performance of standard Enterococci culture assays

Several comparative studies have been conducted over recent decades to assess selectivity of culture media for *Enterococcus* (Table 2). Pagel et al. 1980 used pure cultures to assess PSE (Pfizer), KF Streptococcus, mE, and SB agars against over 100 pure cultures of clinical and environmental isolates of various cocci. The highest selectivity for enterococci was observed for PSE (94%) and mE (94%) agars, with the lowest being KF Streptococcus (80%) and SB (78%) (Pagel and Hardy, 1980). Compared to mE, however, PSE was found to yield lower recovery efficiencies from wastewaters with much higher rates of background colony growth. Other comparative studies found enterococci selectivity on PSE and KF Streptococcus agars as low as 86% and 54%, respectively (Brodsky and Schiemann, 1976). The original mE agar formulation study found a false positive rate of 10% and false negative rate of 11.2% for surface water isolates (Levin et al., 1975). Subsequent studies have confirmed false positive rates for mE agar as low as 2.5% when testing pure *Enterococcus* cultures (Dionisio and Borrego, 1995) and 1.7% in marine, riverine, and treated wastewater effluent (Adcock and Saint, 2001). The inclusion of the indoxyl-β-D-glucoside chromogen to mE agars resulted in an increase in specificity of *Enterococcus* to upwards of 99.7% in ambient freshwaters (Adcock and Saint, 2001). In a recent benchmarking study, ISO method 7899-2 (SB media) was found to have false positive rates as high as 18% and false negative rates as high as 57.1%, depending on the colony count on the filter membrane of recreational marine water (Tiwari et al., 2018). Differences in *Enterococcus* selectivity have also been documented between MF and defined substrate techniques, where *E. faecalis* is differentially selected for in wastewater using Enterolert, leading to the conclusion that these methods should not be used interchangeably for regulatory purposes (Ferguson et al., 2013, 2010; Kinzelman et al., 2003; Maheux et al., 2009).

Significant media-dependent differences in *Enterococcus* concentrations have also been reported. For instance, several studies were conducted in the wake of the advent of Enterolert assays in the mid-1990s to compare its efficacy against established MF techniques for water quality monitoring (Fricker and Fricker, 1996). Significant differences in concentrations were reported between Enterolert and SB agar in marine and recreational freshwaters (Valente et al., 2010), while no significant differences were found between mE agar and Enterolert concentrations across surface water, wastewater, or marine waters (Abbott et al., 1998; Budnick et al., 1996; Eckner, 1998; Fricker and Fricker, 1996). No significant differences in enterococci concentrations were identified between mE and mEI agar (Adcock and Saint, 2001). Importantly, no studies were identified that directly compared the specificities and concentrations derived from SB and mE or mEI across water matrices. Such a comparison should be considered in future studies that assess their utility for regulatory frameworks for antibiotic resistance monitoring internationally. Any biases in species distributions and total enterococci concentrations originating from the selective media could skew downstream distributions in resistance frequencies and introduce bias if the data are used for risk assessment.

5. Workflows for antibiotic-resistant *Enterococcus* monitoring

A useful method for culturing and enumerating both generic and antibiotic resistant environmental *Enterococcus* would strike a balance between sensitivity (i.e., detect all *Enterococcus* spp. that are present), specificity (i.e., avoid detecting other genera), and the high-throughput needed for large-scale environmental monitoring. Aquatic matrices display a large and dynamic range of enterococci concentrations, and a method for their enumeration would also need an appropriately low limit of detection for “cleaner” samples and a sufficiently high limit of quantification for matrices like wastewater where enterococci are concentrated. Specific logistical considerations are also warranted, such as the ability to perform the assay in low-tech laboratories using materials, techniques, and media that are economically feasible for AMR monitoring in low- and middle-income countries. In this instance, standard methods that have been developed for enumerating generic enterococci can be leveraged for their extensive vetting with respect to quality assurance/quality control and adapted to the increased throughput needs of AMR monitoring projects.

Culture-based approaches for investigating antibiotic resistance amongst environmental *Enterococcus* must be modified based on the aquatic matrix being investigated and the purpose of the assessment. Here we delineate these approaches into three general categories: population-level surveys, targeted monitoring for specific antibiotic resistant phenotypes, and recovery of low concentration or viable but non-culturable (VBNC) populations (Figure 1), each with their own benefits and limitations.

For population-level monitoring (73 articles) (Figure 1A), where the objective is to achieve an unbiased snapshot of the distribution of resistance phenotypes, a collection of isolates can be generated using an *Enterococcus* selective method (e.g., Table 1). After colonies have been counted, isolates with the specified morphologies can then be selected randomly off plates for phenotypic antibiotic susceptibility testing, generating an antibiotic resistance profile as a function of the total number of isolates subsampled. The disadvantage of this approach is

Table 2
Performance of Enterococcus selective media used in standard membrane filtration assays.

Medium	Matrix Tested	Presumptive Colonies	Specificity (%)[1]	Selectivity (%)[2]	Reference
mEI	Marine	1361	-	82.4	(Ferguson et al., 2005)
mEI	Pure Cultures	101	97.3	100	(Maheux et al., 2009)
mEI	Surface	54	-	100	(Nishiyama et al., 2015)
mEI	Surface; Wastewater; Marine	1279	-	94.9	(Ferguson et al., 2013)
mEI	Surface; Wastewater; Marine	641	-	94.5	(Ferguson et al., 2010)
mEI	Surface; Wastewater; Marine	361	-	93.9	(Messer and Dufour, 1998)
mEnterococcus	Marine	80	-	97.5	(Dionisio and Borrego, 1995)
mEnterococcus	Marine	624	-	94.2	(de Oliveira and Watanabe Pinhata, 2008)
mEnterococcus	Pure Cultures	93	91.0	88.2	(Pagel and Hardy, 1980)
mEnterococcus	Surface	2231	-	88.5	(Levin et al., 1975)
mEnterococcus	Surface; Wastewater; Marine	1043	-	90.2	(Adcock and Saint, 2001)
Slanetz-Bartley	Marine	97	-	93.8	(Audicana et al., 1995)
Slanetz-Bartley	Marine	234	-	92.7	(Tiwari et al., 2018)
Slanetz-Bartley	Pure Cultures	82	78	74.4	(Pagel and Hardy, 1980)
Slanetz-Bartley	Surface	321	-	95.3	(Łuczkiewicz et al., 2010)
Slanetz-Bartley	Surface; Wastewater	385	-	93.8	(Fricker and Fricker, 1996)

[1] . Specificity = (True Negatives)/(True Negatives + False Positives)
[2] . Selectivity = True Positives/(True Positives + False Positives) or (Colonies Confirmed to Enterococcus Genus)/(Total Presumptive Enterococcus Colonies in Collection)

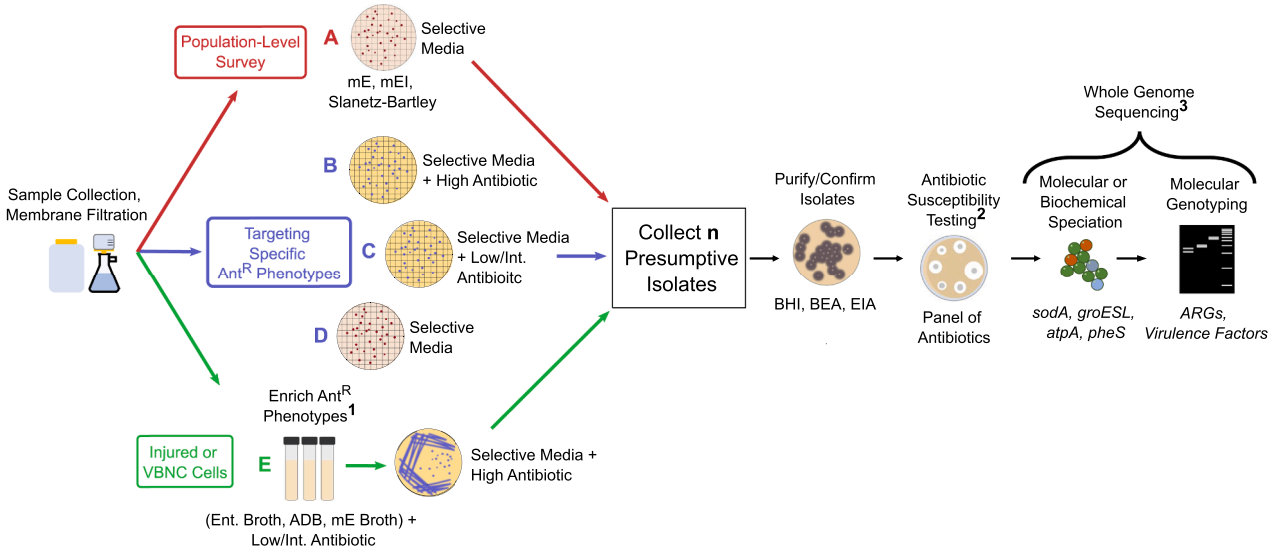

Fig. 1. Workflows for monitoring antibiotic-resistant Enterococcus in the environment. A) Unbiased ecological survey of ant^R phenotypes. B) Interest in only high-level resistance (i.e., clinical relevance). C) Capture broad range of ant^R phenotypes. D) Add selective media without antibiotic to workflow B or C if interested in proportion of ant^R in total enterococci population (WHO Tricycle program). E) Interest in low concentration/injured cells (e.g., disinfected water). [1]Note that pre-enrichment for resistance phenotypes (injured or VBNC cells) prevents their quantification. [2]Antibiotic susceptibility testing of subsampled colonies often includes original selective antibiotic to confirm full "resistant" classification. [3]Whole genome sequencing is recommended for the most accurate speciation and comprehensive genotyping for global isolate comparisons. Ant^R = antibiotic resistance, VBNC = viable but non-culturable, ADB = azide dextrose broth, BEA=bile esculin azide, EIA=esculin iron agar, ARGs = antibiotic resistance genes

that most colonies screened may not be antibiotic resistant and finding colonies with the resistant phenotypes of interest, and to achieve required statistical power, can be akin to "searching for a needle in a haystack". The advantage to this approach is that it provides a denominator for total *Enterococcus* in the sample and an unbiased distribution of both enterococcal species and their genotypes and phenotypes, resulting in an ecologically-relevant analysis (Cho et al., 2019).

If the phenotype of interest is already known (e.g., high-level VRE), targeted monitoring approaches may be more efficient for in-depth characterizations of sub-populations of *Enterococcus*. Such approaches use an antibiotic at clinically-relevant breakpoints to select for specific resistance phenotypes (Figure 1B). The use of low/intermediate breakpoints of antibiotics may be useful for capturing a broad range of phenotypes in the environment but will frequently capture clinically-irrelevant organisms, especially in the case of glycopeptide resistance (Figure 1C). The sample can also be plated in tandem on the selective media without the antibiotic (Figure 1D), thus allowing the quantification of the resistant population as a fraction of the total enterococci measured in CFU/unit volume, a universally comparable monitoring value (e.g., see WHO Tricycle Program recommendations (WHO, 2021)). Studies utilizing targeted approaches often screen the identified resistant colonies against a panel of antibiotics, which can include the original selective antibiotic to confirm clinically-relevant levels of resistance. This approach requires the choice of initial selective antibiotic and therefore will exclude strains that are not resistant to the primary selective antibiotic. Using a selective antibiotic will also skew the distribution of *Enterococcus* spp. away from the true distribution, often selecting for closely related genera that share the same resistance phenotype, such as *Lactobacillus, Leuconostoc, Weissella,* and *Pediococcus* in the case of high levels of glycopeptide resistance (Harwood et al., 2001; Nishiyama et al., 2017, 2015).

In some scenarios, the recovery of very dilute phenotypes (rare targets) or stressed cells is desirable, for instance in advanced treated wastewater intended for reuse or other disinfected waters. Pre-enrichment of samples in concentrated selective broth (e.g., Enterococcosel or Azide Dextrose Broth) amended with the selected antibiotic at low/intermediate concentrations can greatly increase the detection limit of rare phenotypes by helping to recover VBNC colonies (Blanch et al., 2003; Vilanova and Blanch, 2006). These recovered, resistant colonies can then be streaked on high-levels of the antibiotic to recover clinically-relevant phenotypes of interest. However, any protocol employing a target enrichment step will preclude the ability to quantify the resistant *Enterococcus* population or normalize to the total population, a necessity for universally comparable datatypes (Figure 1E).

After the collection of isolates is generated, purification of isolates can be performed on the isolation medium or on other selective-differential media such as bile esculin azide (BEA), or esculin iron agar (EIA). Ideally, all isolates should be confirmed to genus by a method such as qPCR for the 23S rRNA gene (US Environmental Protection Agency, 2012). It is crucial that at least a sub-set be confirmed to understand the false-positive rate of the isolation method, and so that false-positive isolates can be removed from the dataset. Kirby-Bauer disc diffusion testing can be used to confirm the level of resistance to the primary isolation antibiotic and multi-drug resistance. Further isolate characterization may be desirable, including speciation and genotyping for ARGs and virulence factors. Whole genome sequencing (WGS) is recommended when resources allow, as it is the most accurate and comprehensive method for speciation and genotyping and enables global isolate comparisons. A comprehensive evaluation of the suite of methods used for isolate characterization is presented in the following sections.

5.1. *Antibiotic susceptibility testing methods*

Several techniques and automated platforms exist for generalized antibiotic susceptibility testing (AST) or the determination of minimum inhibitory concentrations (MICs) of isolate libraries; including Kirby-Bauer disk diffusion assays (~ 47% of articles), various commercial automated systems (24%), manual broth or agar dilutions (20%), or strip test methods (9%) (Figure 2A). Over 90% of antimicrobial sensitivity tests (AST) were performed on either Mueller-Hinton agar or in Mueller-Hinton broth. Concentrations of antibiotics chosen for AST were predominantly determined by referencing the Clinical and Research Standards Institute (CLSI; 70%) breakpoints for *Enterococcus*, according

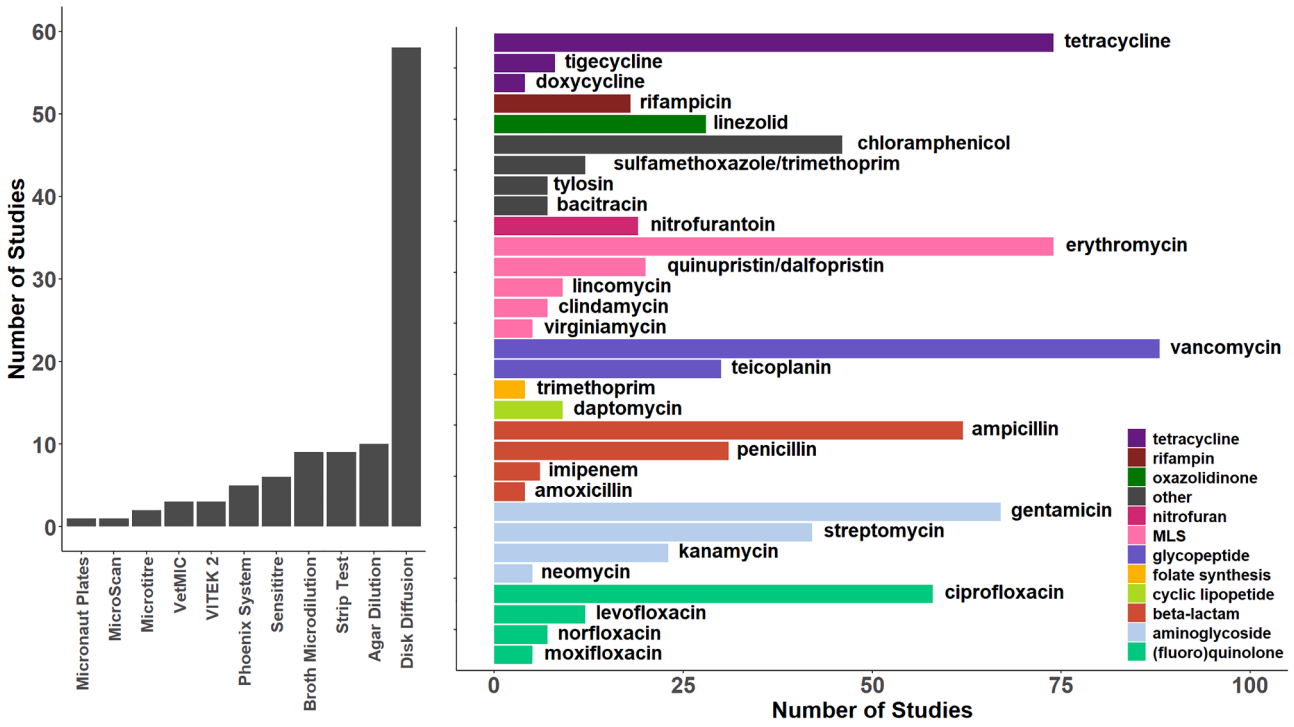

Fig. 2. Summary of antibiotic susceptibility testing (AST) methods applied across the reviewed articles. A) Distribution of assays and commercialized platforms used for AST. B) Distribution of antibiotics used for screening enterococci isolate collections.

to the most currently available guidelines. Other standardized breakpoint concentrations were specific to a particular nation or governmental body, e.g., including the European Committee on Antimicrobial Susceptibility Testing (EUCAST; 7%), the National Antimicrobial Resistance Monitoring System (NARMS; USA; 2%), and the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS; 1%). Approximately 15% of studies utilizing AST that also differentiated degrees of resistance (i.e., "susceptible", "intermediate", or "resistant") did not cite a justification for antibiotic concentrations nor a standardized method for determining the level of resistance observed.

The panel of antibiotics used to screen isolates varied across studies, but overarching trends were apparent. VRE was mentioned in the title of ~35% of articles identified, and vancomycin was included in the screening panel in over 75% of the articles (Figure 2B). The number of observed phenotypes among clinical and environmental isolates of *Enterococcus* are wide-ranging, which warrants a diverse range of antibiotics included in the panels. The antibiotics tested were further categorized into twelve distinct classes by activity. The most prominent classes across all studies were glycopeptides (76%), macrolide-lincosamide-streptogramin (MLS; 72%), tetracyclines (72%), beta-lactams (especially penicillins) (70%), and aminoglycosides (64%). Interestingly, antibiotics that are either approved by the FDA to treat VRE infections or are commonly used to treat VRE (Arias and Murray, 2012) were less commonly included in panels. These compounds include linezolid (27%), quinupristin/dalfopristin (21%), daptomycin (8%), and the synthetic glycopeptide, teicoplanin (28%). High-level aminoglycoside resistance (e.g., gentamicin, streptomycin, and kanamycin) in enterococci isolated from the environment was the focus of small subset of studies.

5.2. Multidrug resistance profiling

Because enterococci are intrinsically resistant to several antibiotics; including cephalosporins, penicillins, clindamycin, and aminoglycosides, resistant phenotypes are commonly found in environmental samples. Plasmid- and transposon-mediated resistance to tetracyclines, erythromycin, chloramphenicol, trimethoprim, vancomycin, and clindamycin; however, have further allowed the genus to become a leading cause of multidrug resistant nosocomial infections, particularly in the US (Murray, 1998). Modern nosocomial *E. faecium* isolates, for example, are commonly resistant to ampicillin, vancomycin, and high levels of aminoglycosides (Miller et al., 2014). Recently, the emergence of multidrug-resistant VRE to newer, last-resort antibiotics; including oxazolidinone-linezolid, daptomycin, quinupristin/dalfopristin, and tigecycline, have caused frequent treatment failures and are of global concern (Ahmed and Baptiste, 2018). Thus, screening for multidrug resistance amongst isolated environmental enterococci is essential for monitoring the evolution of the genus over time, specifically as a function of anthropogenic pollution, as well as assessing the relative hazard posed by the isolate. The choice of antibiotics to include in screening panels is crucial, as most are not useful from a risk-based monitoring framework, although some may highlight ecological relevance. For instance, screening for phenotypes that are intrinsic to the genus may not have clinical relevance but may be useful in determining the distribution of endemic phenotypes to contextualize the relative frequency of VRE detection.

5.3. Biochemical and molecular confirmation and speciation

Because the virulence and resistance characteristics across different *Enterococcus* spp. vary substantially, speciation of resistant *Enterococcus* isolates is desirable. A suite of biochemical tests has been developed by clinical microbiologists to confirm *Enterococcus* to the genus level. These tests include Gram staining, catalase testing, thermal growth range/thermotolerance (growth at 10 and 45°C), halotolerance (6.5% NaCl), growth at pH 9.7, pyrrolidonylarylamidase activity, and the ability to hydrolyze esculin in the presence of bile salts (e.g. growth on bile esculin agar) (Facklam and Collins, 1989; Teixeira et al., 2015). These tests were common features of nearly three quarters of articles and served as a prerequisite for inclusion in downstream characterization, including further speciation (Figure S2). Studies that did not confirm isolates to the genus level either relied on chromogenic agar (e.g., mEI,

CHROMagar VRE) to select presumptive enterococci or speciated their library without screening for characteristic metabolisms or morphologies. Genus-specific primers based on the 16S rRNA gene (Deasy et al., 2000), 23S rRNA gene (EPA Method 1611) (EPA, 2012), or the elongation factor EF-Tu (*tuf*) (Ke et al., 1999) gene have also been used for rapid identification of the genus *Enterococcus*. However, 16S rRNA primer sets are known to fail to capture all *Enterococcus* spp. (Botina and Sukhodolets, 2006), while EPA Method 1611 has proven to be very reliable in our experience.

Speciation of enterococci libraries was common and performed in 91/117 articles, the most common approach of which was PCR. There are several conserved proteins and corresponding genes that are targeted in these assays. The simultaneous detection of enterococcal species and glycopeptide resistance was the first molecular approach to improve diagnostic speeds for clinical enterococci and was based on the detection of genes encoding D-alanine:D-alanine (*ddl*) ligases and other glycopeptide resistance determinants. A reduced affinity for glycopeptides in VanA- and VanB-type resistance in enterococci are due to the integration of D-alanyl:D-lacate into peptidoglycan precursors by the chromosomally-encoded *ddl* ligases (Dutka-Malen et al., 1995a). The *ddl* enzymes in *E. faecium* ($ddl_{E.\ faecium}$) and *E. faecalis* ($ddl_{E.\ faecalis}$) are conserved, and in resistant strains, these enzymes are present in addition to *vanA* or *vanB*. Similarly conserved ligases, *vanC1* and *vanC2-3* are highly specific for *E. gallinarum* (Dutka-Malen et al., 1992) and *E. casseliflavus* (Navarro and Courvalin, 1994), respectively. The primers published by Dutka-Malen et al. (1995) for $ddl_{E.\ faecium}$, $ddl_{E.\ faecalis}$, $vanC1_{E.\ gallinarum}$, and $vanC2\text{-}3_{E.\ casseliflavus}$ have been the most widely used for the speciation of environmental enterococci, as many researchers are specifically concerned with the identification of these four most common and clinically-relevant species (Table S3). (Kariyama et al., 2000) and (Depardieu et al., 2004) provided additional multiplex PCR assays for more high-throughput approaches to VRE surveillance. A multiplex PCR assay based on species-specific superoxide dismutase (*sodA*) genes developed by Jackson et al. (2004) includes primers for 23 different enterococcal species (Jackson et al., 2004).

The PCR primers described above were in part developed due to a lack of consensus between commercially-available systems and kits, such as the Analytical Profile Index (API; bioMérieux), PhenePlate (PhPlate Microplate Techniques AB), Phoenix Microbiology Systems (BD Phoenix), VITEK (bioMérieux), Micronaut-Strep2 (MERLIN), MicroScan Walk Away (Beckman Coulter), and BBL Crystal (MG Scientific) manual or automated rapid identification systems. The principles behind these higher-throughput systems are derived from conventional biochemical phenotyping of enterococci which involve differentiating carbohydrate fermentation of mannitol, sorbitol, sorbose, inulin, arabinose, melibiose, sucrose, raffinose, trehalose, lactose, glycerol, salicin, and maltose, among others (Facklam and Collins, 1989; Teixeira et al., 2015). The commercial methods employ a panel of biochemical tests in parallel to reduce the labor costs of manual phenotyping. However, if atypical species are present, these systems will struggle to identify the organism with acceptable levels of certainty (Castillo-Rojas et al., 2013). This issue is especially problematic in matrices outside of the clinical setting, as these systems were developed and validated targeting common clinical strains and reference cultures with distinguishable biochemical characteristics and not the wide phenotypic diversity of environmental samples. A comprehensive survey of these systems has been reviewed previously (Emery et al., 2016).

An emerging technology for the rapid identification of microorganisms is matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Singhal et al., 2015). This method uses lasers to generate singly protonated ions from analytes in the sample. In the process of identifying unknown microbes, these analytes are primarily housekeeping and ribosomal proteins, given that they constitute a large portion of the dry weight of microbial cells. The ionized proteins are then separated by their mass-to-charge ratio and depending on their time of flight through a channel, a peptide mass fingerprint is generated that can be compared to openly sourced databases. MALDI-TOF MS for the purpose of enterococci speciation emerged in the literature in 2017, as this is an emerging technique in environmental studies. MALDI-TOF MS systems are considerably more expensive than most molecular or phenotypic methods, but the throughput is generally larger (thousands of isolates) and can better accommodate the demand in clinical laboratories.

16S rRNA gene sequencing was less frequently used than other methods due its non-specificity. Other genes may be sequenced, including the *sodA, rpoA*, and *pheS* genes, which have shown to be more discriminatory than the 16S rRNA gene for closely-related species, such as *E. casseliflavus* and *E. flavescens* (Naser et al., 2005; Poyart et al., 2000). In a recent comparative study of various loci to differentiate closely-related *Enterococcus* spp., it was determined that the alpha subunits of ATP synthase (*atpA*), chaperonins (*groESL*), and phenylalnyl-tRNA synthase alpha subunits (*pheS*) performed equally well or better than 16S rRNA gene sequencing against 308 enterococci isolates from untreated urban wastewater (Sanderson et al., 2019). The rate of false identification of consensus reference strains based on loci sequencing was approximately 2%, much lower than the parallelized carbohydrate phenotyping systems discussed above, such as RapID STR, which had error rates of 15.9% for *E. faecalis*, 21.5% for *E. faecium*, and 56.9% for *E. casseliflavus/ gallinarum*. The gold standard for speciating enterococci, and any organism for that matter, is WGS (Sanderson et al., 2019).

5.4. Genotyping resistant Enterococci

Numerous genetic determinants confer antibiotic resistance across the genus *Enterococcus*. Co-occurrence of resistance genes and virulence factors is of particular concern from a clinical standpoint and is common among nosocomial strains (Guzman Prieto et al., 2016; Pöntinen et al., 2021). Most genetic determinants of antibiotic resistance in *Enterococcus* spp. are intrinsically encoded, i.e., they exist on the chromosome within the core genome of the genus. Typically, acquired resistance, i.e., a product of horizontal gene transfer, is of greater interest for monitoring, where the purpose is to examine trends in resistance patterns and if they are changing in time and space. Acquired resistance genes are of primary concern as drivers of failure of antibiotic treatment in clinical infections. Canonical mobile resistance determinants within enterococci include those that confer resistance to glycopeptides (*van* gene clusters), aminoglycosides (aac(6')-Ie-aph(2'')-Ia and aph(3')-IIIa), MLS (*ermB*), and tetracyclines (*tetM* and *tetL*) (Figure S3).

Just over a third of analyzed articles (41/117) genotyped colonies for antibiotic resistance determinants after they had been isolated on antibiotic-containing media, of which 37 used PCR and 4 used WGS. The *van* operon was commonly targeted, with an emphasis on *vanA* and *vanB* within VRE isolates themselves (Figure S3). There are nine distinct gene clusters conferring glycopeptide resistance in enterococci (VanA, B, C, D, E, G, L, M, N) (Hancock et al., 2014; Teixeira et al., 2015) and these determinants differ both genetically and phenotypically based on their physical location (encoded on mobile genetic elements or chromosomal), whether resistance is inducible or constitutive, the type of peptidoglycan precursor that is produced, and ultimately the level of resistance conferred. VanA gene clusters are the most common in clinical isolates and are typically found on Tn*1546*-like transposons, are frequently integrated into a wide range of plasmids, and produce clinical levels of resistance to vancomycin (MIC 64-1,000 μg/mL) and teicoplanin (MIC 16-512 μg/mL) (Teixeira et al., 2015). Similar to VanA, VanB gene clusters are also typically found in clinical isolates and are present on transposons (Tn*1547* or Tn*1549* to Tn*5382*), but differ from *van*A due their inability to recognize teicoplanin, allowing strains with the VanB phenotype to remain susceptible (Miller et al., 2014). These two gene clusters are the most significant genetic determinants in clinically-resistant enterococci and several PCR assays have been developed for their detection (Dutka-Malen et al., 1995b; Kariyama

et al., 2000; Nam et al., 2013; Rathnayake et al., 2011). Enterococci displaying susceptible to intermediate resistance are typically attributed to chromosomally encoded *van* clusters, like *vanC1* in *E. gallinarum* and *vanC2/3* in *E. casseliflavus*, which are commonly detected in environmental samples. The much more rare *vanD-N* genotypes were not detected in any articles that screened for them (Kotzamanidis et al., 2009; Taučer-Kapteijn et al., 2016; Zdragas et al., 2008).

5.5. Virulence factors and pathogenesis

The pathogenesis of infections caused by enterococci is still poorly understood (Teixeira et al., 2015). However, several PCR assays have been developed for the detection of virulence factors common to *Enterococcus*, including: surface adhesion proteins (*esp*), aggregation substances (*agg*), cytolysin (*cyl*) and hemolysin (*hyl*) secretion operons, collagen adhesion (*ace*), and gelatinase secretion proteins that are predominantly found in endocarditis isolates (*gelE*) (Eaton and Gasson, 2001; Mannu et al., 2003; Vankerckhoven et al., 2004). These virulence factors were primarily screened for in the studied articles due to their implications in pathogenesis routes from environmental reservoirs back into humans and animals. Only 18 of the studies identified screened for virulence factors, the most common being *esp* (all 18 articles), *cyl,* and *gelE* (Figure S3). The first vancomycin-resistant *E. faecalis* strain documented in the US was revealed to carry a large, transmissible pathogenicity island containing both *esp* and a complete *cyl* operon, and several other functions that are non-essential to commensal behavior of the organism (Shankar et al., 2002). The *E. faecalis* pathogenicity island is an integrative conjugative element that can be mobilized between plasmids and chromosomes in *E. faecalis* and *E. faecium,* transferring virulence factors and antibiotic resistance determinants (Laverde Gomez et al., 2011; Manson et al., 2010). The co-occurrence of virulence with antibiotic resistance is a key consideration when screening the environment for the emergence of potentially hyper-virulent strains. Rathnayake et al. (2012) found significant correlations between the presence of virulence factors and phenotypic antibiotic resistance among both *E. faecium* and *E. faecalis* isolates in surface waters and regional clinical isolates in Australia (Rathnayake et al., 2012). Similarly, Lata et al. (2016) documented widespread co-occurrence of *vanA* and *vanB* genotypes with *gelE, ace, efaA,* and *esp* virulence factors in both *E. faecalis* and *E. faecium* in impacted surface waters in northern India (Lata et al., 2016). Such studies demonstrate the value of monitoring both antibiotic resistance and virulence, particularly for gaining insight into ecological factors at play in observed resistance patterns.

5.6. Multilocus sequence typing

Multilocus sequence typing (MLST) is a technique by which multiple loci, or specific internal DNA fragments within an organism's genome, are amplified by PCR, sequenced, and then compared across multiple isolates of that species. The loci are typically housekeeping genes common to the genus, and the allelic composition of the set of targets determines the "sequence type", allowing for the determination of clonal complexes (CCs; isolates with differences of no more than 2 loci) and potential source attribution of isolates. Sequence types can then be compared to publicly available and curated databases, such as PubMLST (Jolley et al., 2018), where global isolate comparisons can be made. Pulse-field gel electrophoresis is a similar technique to MLST and was previously known as the "gold standard" for source attribution and epidemiological linkages of bacterial isolates, but low interlaboratory reproducibility and inability to perform phylogenetic or population structure studies makes it unsuitable for global, long-term epidemiological studies (Nemoy et al., 2005). MLST profiles of *Enterococcus* have only been developed for *faecium* (Homan et al., 2002) and *faecalis* (Ruiz-Garbajosa et al., 2006) and are therefore the only two species present in the PubMLST database. The two profiles are derived from a mixed set of 11 housekeeping genes: *gdh* (glucose-6-phosphate dehydrogenase), *purK* (phosphoribosylaminoimidazol carboxylase ATPase subunit), *pstS* (phosphate ATP-binding cassette transporter), *atpA* (ATP synthase, alpha subunit), *gyd* (glyceraldehyde-3-phosphate dehydrogenase), *adk* (adenylate kinase), *ddl, gki* (glucokinase), *aroE* (shikimate 5-dehydrogenase), *xpt* (shikimate 5-dehydrogenase), and *yqiL* (acetyl-coenzyme A acetyltransferase), which were chosen for their low ratios of nonsynonymous to synonymous mutations and their dispersed locations on the chromosomes. MLST is useful when exploring potential clonal relationships between *Enterococcus* spp. isolated from the environment and those from critical AMR monitoring points such as hospital wastewaters and pharmaceutical production waste. The clonal complex 17 (CC17) of *Enterococcus faecium*, for example, is a nosocomial strain associated with outbreaks worldwide and is generally ampicillin and quinolone resistant and contains the *esp* surface adhesion protein (Top et al., 2008). CC17 has been detected in several environmental samples and is an indication of the interconnectedness of the environment and clinical wastewater streams (Caplin et al., 2008).

5.7. Whole genome sequencing

Despite the development of several sophisticated molecular biological assays for the complete characterization of enterococcal isolates over the last few decades, the advent and proliferation of next-generation sequencing techniques has allowed for comprehensive and high-throughput functionality of all the previous assays in a singular method (Figure 1). WGS of isolates allows for the simultaneous detection of ARGs, virulence factors, plasmids, bacteriophages, insertion sequences, transposons, and the sequence type and cladal relatedness of isolates that can be comparted with enterococcal libraries globally. Only four articles reviewed here performed WGS (Ekwanzala et al., 2020a; Gouliouris et al., 2019, 2018; Zaheer et al., 2020), indicating that comprehensive epidemiological analysis of antibiotic resistant, virulent, hospital-adapted enterococcal clades is largely absent from the environmental literature. In WGS workflows, resistant enterococci are initially screened for on selective media supplemented with antibiotics (in this case ampicillin or vancomycin) and then isolated and speciated before they are subject to sequencing. Genomic surveillance of *E. faecium* isolates from retail meat, patients with bloodstream infections, and wastewater treatment plants revealed distinct clades with limited sharing of ARGs between livestock and humans in the UK (Gouliouris et al., 2018). There was, however, extensive overlap between isolates from bloodstream infections and those from the influents and effluents of 17 different wastewater treatment plants (WWTPs) in the region, which could indicate the emergence of new lineages of *E. faecium* that are both hospital-adapted and persist in the environment (Gouliouris et al., 2019). Similarly, a South African study investigated the prevalence of VRE in hospital wastewater, municipal wastewater, and the receiving surface water (Ekwanzala et al., 2020a).Thirty-five percent of the enterococci exiting the wastewater treatment plant were vancomycin resistant, leading to the greatest VRE loadings in the downstream sediment. Subsequent comparative genomics found that ST40, a human pathogenic *E. faecalis* sequence type, and CC17 of *E. faecium* were found persisting in downstream sediments, posing a risk to human health, and demonstrating the need for more advanced wastewater treatment in this scenario. Although WGS is more expensive and difficult to perform than PCR-based genotyping, its high-throughput and robust analysis is quickly becoming commonplace as sequencing costs continue to fall. Also, the storage and sharing of sequenced genomes to public databases allows for longitudinal, phylogenetic tracking of problematic clones as they are transmitted globally (van Hal et al., 2021).

6. Trends in total and antibiotic-resistant *Enterococcus* found in water environments

A comprehensive data extraction and analysis was performed to

identify overarching trends in the species distribution of generic and antibiotic-resistant *Enterococcus* spp. to illuminate general trends and inform sampling priorities, extracting data from all articles that reported resistance as a percentage of the total number of isolates in a collection. Together, this collection consisted of 42,459 isolates extracted from 95/117 articles. To reduce the amount of bias introduced by sampling and enrichment procedures, only *Enterococcus* AST data that was generated in the absence of an initial selective antibiotic were used. This reduced the collection size for AST data to 18,729 isolates extracted from 77 articles but allowed for an estimation of the "true" phenotypic diversity of environmental antibiotic-resistant *Enterococcus*. This approach also allows for the empirical prioritization of monitoring targets for *Enterococcus* resistant to critical antibiotics as well as a baseline for further studies across different water matrices without a bias towards resistant populations.

6.1. Total Enterococci

Only 62 of the 91 articles in which libraries were speciated were the data provided in a format that could be extracted unambiguously (e.g., in tabular format) or detailed population statistics reported, resulting in a collective of 28,343 speciated isolates for analysis (Figure 3). The three most common *Enterococcus* spp. identified across the 62 articles were *E. faecium* (34.0%), *E. faecalis* (29.0%), and *E. hirae* (13.0%). Minor species such as *E. raffinosus, E. avium,* and *E. pseudoavium* (Table S3) each represented less than 2% of the total isolates, although this is likely influenced by underrepresentation of these species in common PCR confirmation assays. In fact, in many articles, only *E. faecalis, E. faecium, E. gallinarum*, and *E. casseliflavus* were screened, as these species represent the most encountered resistant clinical isolates. Despite the uneven representation of the number isolates from each water matrix, there were clear trends among enterococcal populations. In WWTP and hospital wastewater samples, the combined proportions of *E. faecium, E. faecalis,* and *E. hirae* were nearly identical, making up approximately 90% of all isolates (Figure 3). These proportions are consistent with the natural distributions of *Enterococcus* spp. in the gastrointestinal tract of healthy human adults and animals (Silva et al., 2012). The proportions of non-fecal related or undefined *Enterococcus* spp. were greater in surface water and coastal water samples, consistent with dilution of fecal contamination with environmental strains. A large proportion *E. hirae*, the dominant species excreted by cattle (Jackson et al., 2011), was found in cattle feedlot drains (Zaheer et al., 2020). The even proportions of species across comparable water matrices (e.g., WWTPs and hospital wastewater) observed, despite extensive heterogeneity in methodology, suggests that there is a general lack of systematic errors or biases in either media selectivity or speciation techniques.

6.2. Surface water

The majority of isolates obtained across studies were derived from surface water (fresh water), which included both urban and rural watersheds and riverine sites directly impacted by municipal or hospital wastewater discharge (7,146 isolates) (Figure 4). Compared to the other environments examined, surface waters displayed some of the lowest percentages of resistant isolates to all 18 antibiotics and antibiotic classes. It should be noted that, despite dilution effects and environmental attenuation, 3.5% of 7,146 *Enterococcus* spp. isolates were fully resistant to vancomycin. The vast majority of these isolates were either *E. faecium* or *E. faecalis.* By contrast, full vancomycin resistance was virtually undetected in *E. casseliflavus, E. gallinarum* and the other less dominant species (Figure 4; Table S3). Despite intrinsic resistance of *E. casseliflavus* and *E. gallinarum* via *vanC* genes, their phenotypes rarely exceeded CLSI breakpoints for full clinical resistance. Łuczkiewicz et al. (2010) examined *Enterococcus* resistance to 13 different antibiotics in an urban river system in Poland in the absence of wastewater treatment plant discharge and found that resistance to erythromycin, ciprofloxacin, and tetracycline was common among all isolates (Łuczkiewicz et al., 2010). They also found multidrug resistance (some to all 13 antibiotics tested), including vancomycin and high-level aminoglycoside resistance, among *E. faecalis* and *E. faecium* isolates in the two main tributaries feeding the coastal waters. The authors suggested that riverine enterococci should be considered as a potential risk for downstream recreational bathers, even in the absence of point-source wastewater pollution. In contrast, studies of rural watersheds (< 1 % urban) in Ontario (Canada) and Georgia (US) found that the diversity and distribution of antibiotic resistance among *Enterococcus* were strikingly different than in more anthropogenically-impacted water bodies (Cho et al., 2019; Lanthier et al., 2011). These two studies, together comprising 2,195 isolates, indicate that the *Enterococcus* species and their phenotypes were stochastically distributed and sparse, with few multidrug (< 6 antibiotics) resistant strains and no isolates reaching the CLSI breakpoints for vancomycin, teicoplanin, or linezolid. They

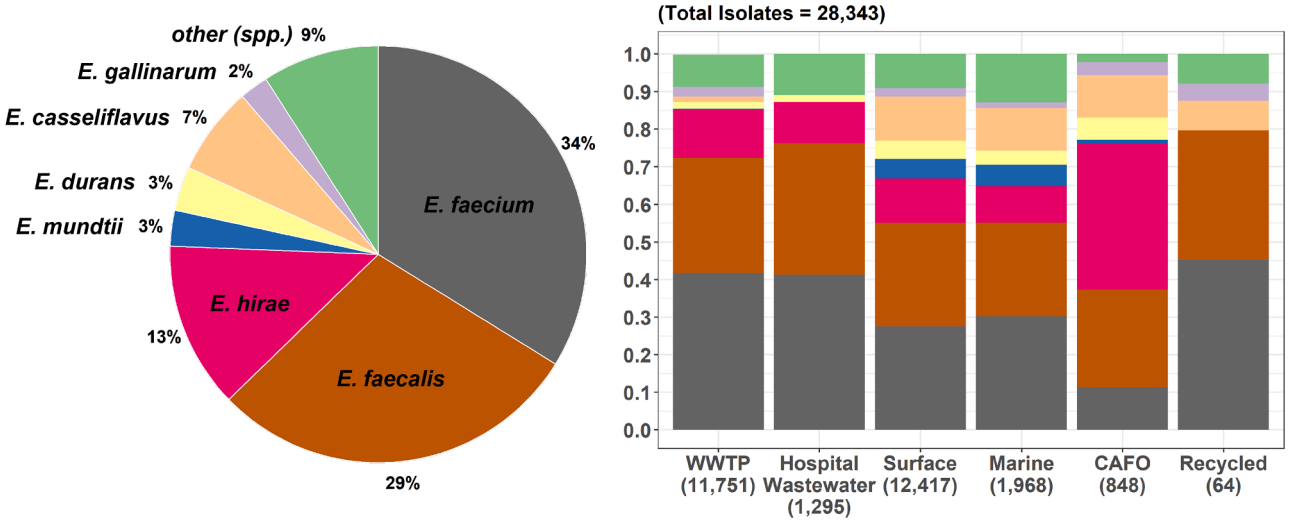

Fig. 3. Distribution of Enterococcus spp. isolates across all studies. A) Species distribution of the total speciated meta-library across all water types (27,464 isolates). "other (spp.)" is any species that was not detected at >2% of the total meta-library abundance or was reported only as Enterococcus spp. B) The distribution of generic enterococcal isolates by water matrix. The total number of isolates representing each matrix is indicated parenthetically. Concentrated animal feeding operation (CAFO) samples encompass wastewater lagoons, feedlot drains, and any on-site treatment systems.

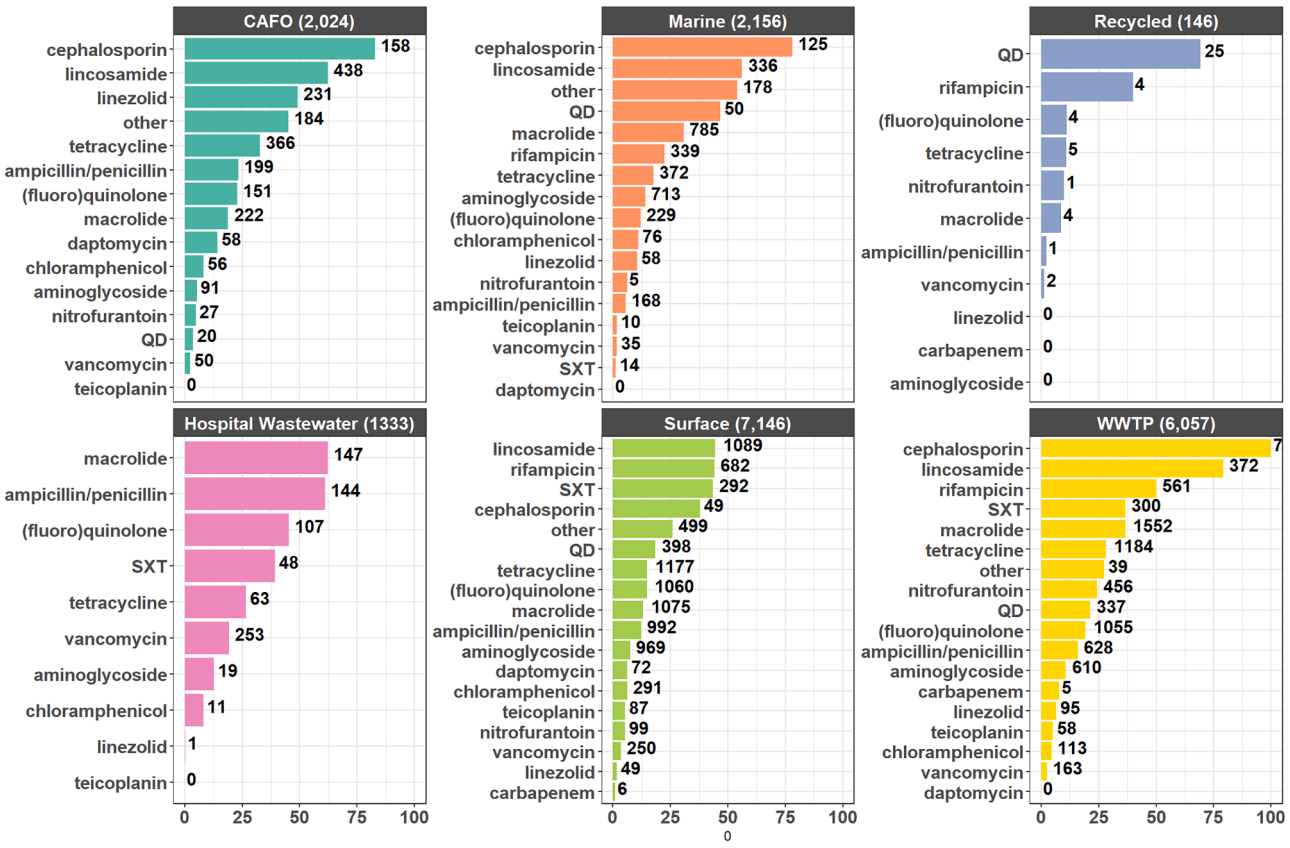

Fig. 4. Distribution of percent of Enterococcus isolates resistant to each antibiotic by sample matrix. Isolates were only included if they were initially isolated in the absence of a selective antibiotic. The number of unique isolates representing each matrix is in parentheses in the headers but note that not every isolate was tested for every antibiotic. The number of resistant isolates per antibiotic tested is represented adjacent to each bar in each panel. CAFO = concentrated animal feeding operation, WWTP = wastewater treatment plant. QD = quinupristin/dalfopristin; SXT = sulfamethoxazole/trimethoprim

attributed the dispersion of the resistant fecal indicators to domesticated animal and wildlife fecal pollution and their potential for dissemination to be limited. The highest rates of resistance among *Enterococcus* isolates from surface water studies came from the North West province of South Africa where 86/124 *Enterococcus* spp. were phenotypically resistant to vancomycin. These were isolated in the absence of a selective antibiotic and displayed multidrug resistance to ampicillin, amoxicillin, penicillin, ciprofloxacin, erythromycin, and tetracycline. Interestingly, a single isolate of *E. sulfureus* was found to be resistant to ampicillin, amoxicillin, penicillin, streptomycin, vancomycin, chloramphenicol, ciprofloxacin, erythromycin, and tetracycline (Molale and Bezuidenhout, 2016).

Upstream and downstream sampling of municipal wastewater discharge was carried out in several studies. The detection of enterococci with resistance to clinically-relevant antibiotics downstream of municipal wastewater discharge was regionally dependent and linked to the degree of treatment employed by the WWTP. A Tunisian study found no ampicillin-resistant *Enterococcus* (ARE) or VRE in the influent, effluent, or receiving water, suggesting the regional variation in their distribution (Ben Said et al., 2015). Another study (Bessa et al., 2014) found *vanA* positive *E. faecium* mostly upstream of WWTP discharge in Portugal, a region where VRE is prevalent in the population.

6.4. Municipal and hospital wastewater

Municipal and hospital wastewater were commonly sampled across the identified articles. Together they accounted for 7,400 isolates in the collection and displayed high percentages of clinically-relevant antibiotic resistant phenotypes (Figure 4). *Enterococcus* spp. isolates from untreated hospital wastewater displayed the highest percentages of resistance to fluoroquinolones, ampicillin/penicillin, macrolides, and vancomycin.

WWTPs, which encompassed raw influent, activated sludge, secondary effluents, and variably treated final effluents across 15 different countries, yielded *Enterococcus* spp. isolates with the highest percentages of resistance to cephalosporins, lincosamides, nitrofurantoin, and teicoplanin (Figure 4). Within the "WWTP" category, only 2.7% of 6,057isolates were resistant to vancomycin in the collection. The highest percentages of full vancomycin resistance across studies of WWTP influents came from Germany (19%; 16/84 isolates) (Gallert et al., 2005), Portugal (3.4%; 17/499 isolates) (Araújo et al., 2010), Iran (3.3%; 19/577) (Talebi et al., 2007), and Poland (3.2%; 6/185 isolates) (Luczkiewicz et al., 2013). The 2005 paper (Gallert et al., 2005), is the oldest of the group, and reveals the challenges of accurately assessing antibiotic-resistant *Enterococcus,* particularly VRE, whose phenotypic characteristics are mimicked by genera such as *Leuconostoc, Pediococcus* and *Weisella* (Harwood et al., 2001). In this case bacteria were isolated on relatively non-selective bile esculin agar, and were confirmed only by ability to grow at 6.5% NaCl and by microscopy for cell morphology. Furthermore, the method for assessing antibiotic resistance relied on a set zone of inhibition of 2 mm for all antibiotics, rather than standardized zone diameters.

Although they represented a smaller fraction of isolates, hospital wastewater environments showed the most definitive contributions of phenotypic resistance to receiving waters. For example, Novais et al. (2005) found statistically significant increases in phenotypic resistance exceeding CLSI breakpoints among *E. faecium* and *E. faecalis* for vancomycin, teicoplanin, ciprofloxacin, and ampicillin in urban sewers receiving hospital wastewater discharge in Portugal (Novais et al., 2005). Clonal analysis, Tn*1546* typing, and virulence factor assays were also consistent with local clinics being the source of downstream surface

water isolates. Similar observations were made along a medical center-retirement home-wastewater treatment plant-river continuum in France in 2013, where the hospital-adapted clonal complex, CC17 *E. faecium*, was culturable along the continuum and into receiving waters (Leclercq et al., 2013). The CC17 concentration, though, was attenuated by the WWTP and the proportion of CC17 became outweighed by environmental strains. Further, epidemiological source tracking using WGS of VRE isolated from 20 WWTPs in the UK in 2019 determined that there was widespread dissemination of hospital-adapted *E. faecium* in WWTP effluents across eastern England (Gouliouris et al., 2019). They found that WWTPs receiving hospital wastewater had significantly higher VRE and ARE concentrations than non-hospital associated treatment plants and found highly similar isolates shared between the local teaching hospital and those emitted from surrounding WWTPs. Together, these studies provide strong support of *Enterococcus* spp. as both a clinically- and environmentally-relevant target for waterborne monitoring. Hospital wastewaters, the municipal wastewater infrastructure responsible for treatment, and receiving waters are key monitoring points for tracking their dissemination.

6.5. Pharmaceutical wastewater

Pharmaceutical wastewater is a critical monitoring point in the dissemination of resistant microorganisms and is currently understudied in the field. Only two such articles focused on resistant *Enterococcus* were encountered in this review. Guardabassi et al. (2002) documented intermediate resistance of presumptive *Enterococcus* to 20 ug/mL vancomycin and evidence of *vanA* and *vanD* isolates in the waste biomass from the fermentation tanks used in the production of vancomycin (Guardabassi et al., 2002). The WWTP treating the waste was also enriched with presumptive, intermediate-level VRE, and quantifiable CFUs were routinely emitted from the plant after secondary clarification without disinfection. PFGE analysis found identical VRE patterns between vancomycin production fermentation waste and the final effluents of the WWTP, suggesting that pharmaceutical production waste can be a direct contributor to the dissemination of VRE into the environment. Further, Guardabassi et al. (2004) investigated the relationship of Tn*1546*-like elements in *Enterococcus* spp. isolated from municipal sewage, activated sludge, vancomycin production waste, human feces, mussels, and soil using long PCR-restriction fragment length polymorphism and found indistinguishable elements shared across the ecologically distinct locations and between enterococcal species, suggesting ready transferability of the *vanA* genotype between clinical and environmental strains (Guardabassi and Dalsgaard, 2004).

6.6. Recycled water

Due to the increased pressures on freshwater around the world, wastewaters are increasingly being treated and reintroduced into water and food cycles as the recycled water is used for crop irrigation, groundwater recharge, and even direct potable reuse. Only three studies in the current review examined resistant *Enterococcus* in wastewaters intended for reuse. Goldstein et al. (2014) sampled two WWTPs in the Mid-Atlantic and two from the Midwest regions of the US that reuse their treated effluents and detected VRE in 27% of wastewater samples, with higher rates in the Mid-Atlantic plants. VRE were only detected in final treated effluents when there was lack of chlorination (Goldstein et al., 2014). Subsequent studies from the same WWTPs found that VRE are detectable at low concentrations at the point of use after recovery from UV disinfection, although other phenotypes are more prevalent (Carey et al., 2016). Both WWTPs studied receive hospital wastewater and their effluents were used for spray irrigation (Goldstein et al., 2012).

6.7. Marine waters and recreational beaches

Freshwater and marine water environments used for recreational bathing are at the direct interface between environmental fecal pollution and human exposure and are therefore important monitoring points. Studies of enterococcal populations of marine and freshwater beaches from Spain, Puerto Rico, Poland, Greece, Malaysia, Brazil, Italy, and Michigan confirmed that many drug resistant strains are readily culturable in recreational marine water and sand (Alm et al., 2014; Arvanitidou et al., 2001; Dada et al., 2013; de Oliveira and Watanabe Pinhata, 2008; Monticelli et al., 2019; Sadowy and Luczkiewicz, 2014; Santiago-Rodriguez et al., 2013; Tejedor Junco et al., 2001). High phenotypic and phylogenetic diversity was observed across all studies, and many environmental-associated enterococci dominated local populations, including *E. casseliflavus* (Monticelli et al., 2019), *E. hirae* (Sadowy and Luczkiewicz, 2014), and *E. avium* and *E. raffinosus* (Arvanitidou et al., 2001). Beach sands are of particular interest as they represent a niche environment for enterococci where horizontal gene transfer can occur at higher frequencies than among planktonic bacteria, accelerating the rate at which fecal microbiota exchange genes with pathogens and facilitating human-pathogen interactions (Alm et al., 2014; Oravcova et al., 2017).

6.8. CAFOs and irrigation water

Studies of the effects of cattle and swine concentrated animal feeding operation (CAFO) wastewater on downstream environments were also prevalent in the literature. The enterococcal isolates from CAFO studies in the collection showed the highest resistance prevalence to tetracyclines, which is not surprising given that tetracyclines make up nearly 40% of all antimicrobials used in animal husbandry in the US. (CDC, 2013). As many CAFOs contain and treat their wastewater on-site, their direct impact on groundwater or downstream surface water (e.g., due to runoff or unintentional discharge) was a concern. The animal products themselves also represent a potential direct line of exposure from animals to humans through the food chain.

Sapkota et al. (2007) sampled upstream and downstream of a high-density swine operation in the Mid-Atlantic region of the US and found higher MICs for clindamycin and tetracycline amongst the *Enterococcus* spp. in both downstream groundwater and surface water (Sapkota et al., 2007). Similarly, Stine et al. (2007) sampled the waste lagoons, surface waters, and well water of a swine CAFO in the US. that had been administering tetracycline-containing feed for over 20 years and found that 68% of all enterococci were resistant to tetracyclines, and a total of 60 different species displayed phenotypic resistance to tetracyclines across the sites (Stine et al., 2007). Further evidence for the direct dissemination of clinically-relevant enterococci into ambient surface waters by CAFOs was documented by Jahne et al. (2015) (Jahne et al., 2015). They documented a cattle CAFO and its on-site wastewater treatment system comprising of an infiltration basin with subsequent sequestration by a constructed wetland. Enterococci that displayed co-resistance to vancomycin, linezolid, and daptomycin were common in the wastewater and, during rain events, the increased hydraulic loading on the infiltration basin and constructed wetland resulted in the direct emission of these organisms into downstream surface waters.

7. Conclusion and recommendations

In this review we recounted the last 20 years of research assessing antibiotic-resistant enterococci in various water environments. Because *Enterococcus* has been shown to be a reliable indicator of fecal contamination of water bodies, several nationally and internationally recognized standard culture methods have been developed for their enumeration. Various *Enterococcus* spp. are both clinically-relevant and survive and persist in the environment. The studies surveyed here lay the groundwork for considering *Enterococcus* spp. as a standardized target for waterborne monitoring of antibiotic resistance.

Recently, the WHO put forward a standardized, comprehensive surveillance program for One Health-inspired monitoring, i.e., the

Tricycle protocol (WHO, 2021), which targets the Gram-negative ESBL *E. coli*. Here, *Enterococcus* spp., as Gram-positive organisms, represent a compelling target to consider as a complement to such monitoring programs. *Enterococcus* spp. display resistance to critically-important antibiotics that would not be captured by an *E. coli*-targeted monitoring program alone. As observed in this critical review, *Enterococcus* spp. also display sensitive responses to anthropogenic pollution, including hospitals and CAFOs, that are apparent in their distinct geographical occurrence patterns.

The recommended path forward for standardizing environmental antibiotic-resistant *Enterococcus* monitoring should ensure the comparability of monitoring points, methods employed, and reporting metrics. Accessibility and ease of application are also important considerations. Ideally, *Enterococcus* spp. monitoring for the purpose of antibiotic resistance surveillance could be incorporated into existing monitoring programs, especially considering the high level of existing standardization and regulatory requirements. The existence of nationally and internationally recognized standard culture methods is of great value in ensuring comparability of the data gathered in space and time, however, further standardization is needed for the purpose of antibiotic resistance surveillance specifically. The conclusions and recommendations based on this critical review are as follows:

- mEI is a prime candidate for a standard selective media, given that it yields the highest selectivity over mEnterococcus and Slanetz-Bartley and is integrated into existing regulatory recommendations in the US. Still, the findings here were encouraging that studies are generally consistent, even if different media were employed, but confirmation of the genus is critical because no *Enterococcus* media is 100% selective. The addition of a selective antibiotic to the media can decrease specificity by selecting for intrinsically-resistant, non-target genera.
- In line with recommendations made in the WHO Tricycle protocol, plating environmental samples on the selective media with and without a primary selective antibiotic produces both a percent resistance of the enterococci population and a CFU/unit volume measurement (e.g., CFU/ml). A CFU/mL measurement represents a universally comparable magnitude of antibiotic-resistant bacteria.
- Depending on the research question, vancomycin and other antibiotics used for primary selection may be added to media at the breakpoint for full, intermediate, or low resistance. The full resistance breakpoint is the most useful for clinical relevance, but in some environments, one may be interested in intermediate resistance. Where one expects bacteria to be compromised (injured), use of low-level antibiotic in enrichment cultures may be useful for lowering detection limits. The use of low/intermediate breakpoints may complicate human-health risk assessments due to the isolation of intrinsically-resistant species that are common in the environment unless further testing is done.
- A defined set of key antibiotics aimed at treating VRE infections could also be employed for monitoring emerging phenotypes and multidrug resistance. These include ampicillin, teicoplanin, oxazolidinone-linezolid, daptomycin, quinupristin/dalfopristin, and tigecycline. The emergence of multidrug resistant VRE that are co-resistant to these 6 antibiotics are of great concern.
- Antimicrobial sensitivity testing using disk diffusion on Mueller-Hinton agar or agar dilutions in Mueller-Hinton broth should be followed according to the most up to date CLSI guidelines.
- Speciation and virulence typing of resistant enterococci is of interest for risk assessment and longitudinally tracking changing genotypes and phenotypes across the genus. PCR-based approaches are appropriate for low-tech labs, specific loci sequencing, and emerging technologies such as MALDI-TOF MS will increase throughput without sacrificing accuracy.
- Key monitoring points to consider for the dissemination of resistant enterococci are the hospital-municipal wastewater continuum and their receiving water bodies, especially where they impact recreational waters. Pharmaceutical wastewater and recycled water are critically understudied for resistant *Enterococcus*. The surrounding areas of CAFOs are of particular concern after storm events.
- Whenever possible, isolation of antibiotic-resistant colonies for WGS will aid in determining the sequence type, virulence genotype, plasmid type, acquired AMR genes, and chromosomal point mutations. Public sharing of WGS data will help to advance understanding of the ecology, epidemiology, and global transmission of this important pathogen.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Amy Pruden and Valerie J. Harwood reports financial support was provided by Water Research Foundation.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2022.100161.

References

Abbott, S., Caughley, B., Scott, G., 1998. Evaluation of Enterolert® for the enumeration of enterococci in the marine environment. New Zeal. J. Mar. Freshw. Res. 32, 505–513. https://doi.org/10.1080/00288330.1998.9516839.

Adcock, P.W., Saint, C.P., 2001. Development of glucosidase agar for the confirmation of water-borne Enterococcus. Water Res. 35, 4243–4246. https://doi.org/10.1016/S0043-1354(01)00125-7.

Ahmed, M.O., Baptiste, K.E., 2018. Vancomycin-resistant Enterococci: A review of antimicrobial resistance mechanisms and perspectives of human and animal health. Microb. Drug Resist. 24, 590–606. https://doi.org/10.1089/mdr.2017.0147.

Alexander, J., Hembach, N., Schwartz, T., 2020. Evaluation of antibiotic resistance dissemination by wastewater treatment plant effluents with different catchment areas in Germany. Sci. Rep. 10, 1–9. https://doi.org/10.1038/s41598-020-65635-4.

Alm, E.W., Zimbler, D., Callahan, E., Plomaritis, E., 2014. Patterns and persistence of antibiotic resistance in faecal indicator bacteria from freshwater recreational beaches. J. Appl. Microbiol. 117, 273–285. https://doi.org/10.1111/jam.12512.

Amarasiri, M., Sano, D., Suzuki, S., 2020. Understanding human health risks caused by antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in water environments: Current knowledge and questions to be answered. Crit. Rev. Environ. Sci. Technol. 50, 2016–2059. https://doi.org/10.1080/10643389.2019.1692611.

APHA, 1999. Standard Methods for the Examination of Water and Wastewater, 20th Edition.

Araújo, C., Torres, C., Silva, N., Carneiro, C., Gonçalves, A., Radhouani, H., Correia, S., Da Costa, P.M., Paccheco, R., Zarazaga, M., Ruiz-Larrea, F., Poeta, P., Igrejas, G., 2010. Vancomycin-resistant enterococci from Portuguese wastewater treatment plants. J. Basic Microbiol. 50, 605–609. https://doi.org/10.1002/jobm.201000102.

Arias, C.A., Murray, B.E., 2012. The rise of the Enterococcus: Beyond vancomycin resistance. Nat. Rev. Microbiol. 10, 266–278. https://doi.org/10.1038/nrmicro2761.

Arvanitidou, M., Katsouyannopoulos, V., Tsakris, A., 2001. Antibiotic resistance patterns of enterococci isolated from coastal bathing waters. J. Med. Microbiol. 50, 1001–1005. https://doi.org/10.1099/0022-1317-50-11-1001.

Audicana, A., Perales, I., Borrego, J.J., 1995. Modification of kanamycin-esculin-azide agar to improve selectivity in the enumeration of fecal streptococci from water

samples. Appl. Environ. Microbiol. 61, 4178–4183. https://doi.org/10.1128/aem.61.12.4178-4183.1995.
Ben Said, L., Klibi, N., Lozano, C., Dziri, R., Ben Slama, K., Boudabous, A., Torres, C., 2015. Diversity of enterococcal species and characterization of high-level aminoglycoside resistant enterococci of samples of wastewater and surface water in Tunisia. Sci. Total Environ. 530–531, 11–17. https://doi.org/10.1016/j.scitotenv.2015.05.091.
Bessa, L.J., Barbosa-Vasconcelos, A., Mendes, Â., Vaz-Pires, P., Da Costa, P.M., 2014. High prevalence of multidrug-resistant Escherichia coli and Enterococcus spp. in river water, upstream and downstream of a wastewater treatment plant. J. Water Health 12, 426–435. https://doi.org/10.2166/wh.2014.160.
Blanch, A.R., Caplin, J.L., Iversen, A., Kühn, I., Manero, A., Taylor, H.D., Vilanova, X., 2003. Comparison of enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions. J. Appl. Microbiol. 94, 994–1002. https://doi.org/10.1046/j.1365-2672.2003.01919.x.
Boehm, A.B., Sassoubre, L.M., 2014. Enterococci as indicators of environmental fecal contamination. Enterococci from commensals to lead. Causes Drug Resist. Infect. 1–21.
Botina, S.G., Sukhodolets, V.V., 2006. Speciation in bacteria: comparison of the 16S rRNA gene for closely related Enterococcus species. Russ. J. Genet. 42, 247–251. https://doi.org/10.1134/S1022795406030033.
Brodsky, M.H., Schiemann, D.A., 1976. Evaluation of Pfizer selective enterococcus and KF media for recovery of fecal streptococci from water by membrane filtration. Appl. Environ. Microbiol. 31, 695–699. https://doi.org/10.1128/aem.31.5.695-699.1976.
Budnick, G.E., Howard, R.T., Mayo, D.R., 1996. Evaluation of enterolert for enumeration of enterococci in recreational waters. Appl. Environ. Microbiol. 62, 3881–3884. https://doi.org/10.1128/aem.62.10.3881-3884.1996.
Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R., Harwood, V.J., 2012. Enterococci in the Environment. Microbiol. Mol. Biol. Rev. 76, 685–706. https://doi.org/10.1128/mmbr.00023-12.
Caplin, J.L., Hanlon, G.W., Taylor, H.D., 2008. Presence of vancomycin and ampicillin-resistant Enterococcus faecium of epidemic clonal complex-17 in wastewaters from the south coast of England. Environ. Microbiol. 10, 885–892. https://doi.org/10.1111/j.1462-2920.2007.01507.x.
Carey, S.A., Goldstein, R.E.R., Gibbs, S.G., Claye, E., He, X., Sapkota, A.R., 2016. Occurrence of vancomycin-resistant and -susceptible Enterococcus spp. in reclaimed water used for spray irrigation. Environ. Res. 147, 350–355. https://doi.org/10.1016/j.envres.2016.02.030.
Castillo-Rojas, G., Mazari-Hiríart, M., Ponce de León, S., Amieva-Fernández, R.I., Agis-Juárez, R.A., Huebner, J., López-Vidal, Y., 2013. Comparison of Enterococcus faecium and enterococcus faecalis strains isolated from water and clinical samples: antimicrobial susceptibility and genetic relationships. PLoS One 8, 1–10. https://doi.org/10.1371/journal.pone.0059491.
CDC, 2013. Antibiotic Resistance Threats in the United States, 37, pp. 828–831. https://doi.org/10.1016/j.medmal.2007.05.006, 2013.
Centers for Disease Control and Prevention, 2019. Vacomycin-Resistant Enterococci.
Cho, S., Hiott, L.M., McDonald, J.M., Barrett, J.B., McMillan, E.A., House, S.L., Adams, E. S., Frye, J.G., Jackson, C.R., 2019. Diversity and antimicrobial resistance of Enterococcus from the upper oconee watershed. Georgia. J. Appl. Microbiol. 128, 1221–1233. https://doi.org/10.1111/jam.14550.
Dada, A.C., Ahmad, A., Usup, G., Heng, L.Y., Hamid, R., 2013. High-level aminoglycoside resistance and virulence characteristics among Enterococci isolated from recreational beaches in Malaysia. Environ. Monit. Assess. 7427–7443. https://doi.org/10.1007/s10661-013-3110-x.
de Oliveira, A.J.F.C., Watanabe Pinhata, J.M., 2008. Antimicrobial resistance and species composition of Enterococcus spp. isolated from waters and sands of marine recreational beaches in Southeastern Brazil. Water Res 42, 2242–2250. https://doi.org/10.1016/j.watres.2007.12.002.
Deasy, B.M., Rea, M.C., Fitzgerald, G.F., Cogan, T.M., Beresford, T.P., 2000. A rapid PCR based method to distinguish between Lactococcus and Enterococcus. Syst. Appl. Microbiol. 23, 510–522. https://doi.org/10.1016/S0723-2020(00)80025-9.
Depardieu, F., Perichon, B., Courvalin, P., 2004. Detection of the van alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. J. Clin. Microbiol. 42, 5857–5860. https://doi.org/10.1128/JCM.42.12.5857-5860.2004.
Dionisio, L.P.C., Borrego, J.J., 1995. Evaluation of media for the enumeration of faecal streptococci from natural water samples. J. Microbiol. Methods 23, 183–203. https://doi.org/10.1016/0167-7012(95)00014-C.
Dutka-Malen, S., Evers, S., Courvalin, P., 1995a. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. 33, 24–27.
Dutka-Malen, S., Evers, S., Courvalin, P., 1995b. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. 33, 24–27. https://doi.org/10.1128/jcm.33.1.24-27.1995.
Dutka-Malen, S., Molinas, C., Arthur, M., Courvalin, P., 1992. Sequence of the vanC gene of Enterococcus gallinarum BM4174 encoding a d-alanine:d-alanine ligase-related protein necessary for vancomycin resistance. Gene 112, 53–58. https://doi.org/10.1016/0378-1119(92)90302-6.
Eaton, T.J., Gasson, M.J., 2001. Molecular screening of Enterococcus virulence determinants and potential for genetic exchange between food and medical isolates. Appl. Environ. Microbiol. 67, 1628–1635. https://doi.org/10.1128/AEM.67.4.1628-1635.2001.
Eckner, K.F., 1998. Comparison of membrane filtration and multiple-tube fermentation by the Colilert and Enterolert methods for detection of waterborne coliform bacteria, Escherichia coli, and Enterococci used in drinking and bathing water quality monitoring in southern Swed. Appl. Environ. Microbiol. 64, 3079–3083. https://doi.org/10.1128/aem.64.8.3079-3083.1998.
EEA, 2020. Bathing water management in Europe: Successes and challenges.
Ekwanzala, M.D., Dewar, J.B., Kamika, I., Momba, M.N.B., 2020a. Comparative genomics of vancomycin-resistant Enterococcus spp. revealed common resistome determinants from hospital wastewater to aquatic environments. Sci. Total Environ. 719, 137275 https://doi.org/10.1016/j.scitotenv.2020.137275.
Ekwanzala, M.D., Dewar, J.B., Momba, M.N.B., 2020b. Environmental resistome risks of wastewaters and aquatic environments deciphered by shotgun metagenomic assembly. Ecotoxicol. Environ. Saf. 197, 110612 https://doi.org/10.1016/j.ecoenv.2020.110612.
Emery, C.L., Appleman, M.D., Siders, J.A., Davis, T.E., 2016. Rapid devices and instruments for the identification of anaerobic bacteria. Man. Commer. Methods Clin. Microbiol. 56–86. https://doi.org/10.1002/9781119021872.ch4.
EPA, 2012. Method 1611 : Enterococci in Water by TaqMan ® Quantitative Polymerase Chain Reaction (qPCR) Assay.
European Commission, 2017. A European One Health Action Plan against Antimicrobial Resistance (AMR).
Facklam, R.R., Collins, M.D., 1989. Identification of enterococcus species isolated from human infections by a conventional test scheme. J. Clin. Microbiol. 27, 731–734. https://doi.org/10.1128/jcm.27.4.731-734.1989.
Ferguson, D.M., Griffith, J.F., McGee, C.D., Weisberg, S.B., 2010. Comparison of Enterococcus Species Selectivity using Enterolert and EPA Method 1600.
Ferguson, D.M., Griffith, J.F., McGee, C.D., Weisberg, S.B., Hagedorn, C., 2013. Comparison of enterococcus species diversity in marine water and wastewater using enterolert and EPA method 1600. J. Environ. Public Health 2013. https://doi.org/10.1155/2013/848049.
Ferguson, D.M., Moore, D.F., Getrich, M.A., Zhowandai, M.H., 2005. Enumeration and speciation of enterococci found in marine and intertidal sediments and coastal water in southern California. J. Appl. Microbiol. 99, 598–608. https://doi.org/10.1111/j.1365-2672.2005.02660.x.
Fricker, E.J., Fricker, C.R., 1996. Use of defined substrate technology and a novel procedure for estimating the numbers of enterococci in water. J. Microbiol. Methods 27, 207–210. https://doi.org/10.1016/S0167-7012(96)00950-5.
Gallert, C., Fund, K., Winter, J., 2005. Antibiotic resistance of bacteria in raw and biologically treated sewage and in groundwater below leaking sewers. Appl. Microbiol. Biotechnol. 69, 106–112. https://doi.org/10.1007/s00253-005-0033-7.
Goldstein, R.E., Micallef, S.A., Gibbs, S.G., Davis, J.A., He, X., George, A., Kleinfelter, L. M., Schreiber, N.A., Mukherjee, S., Sapkota, A., Joseph, S.W., Sapkota, A.R., 2012. Methicillin-resistant Staphylococcus aureus (MRSA) detected at four U.S. wastewater treatment plants. Environ. Health Perspect. 120, 1551–1558.
Goldstein, R.E., Micallef, S.A., Gibbs, S.G., George, A., Claye, E., Sapkota, A., Joseph, S. W., Sapkota, A.R., 2014. Detection of vancomycin-resistant enterococci (VRE) at four U.S. wastewater treatment plants that provide effluent for reuse. Sci. Total Environ. 466–467, 404–411. https://doi.org/10.1016/j.scitotenv.2013.07.039.
Gouliouris, T., Raven, K.E., Ludden, C., Blane, B., Corander, J., Horner, C.S., Hernandez-Garcia, J., Wood, P., Hadjirin, N.F., Radakovic, M., Holmes, M.A., de Goffau, M., Brown, N.M., Parkhill, J., Peacock, S.J., 2018. Genomic surveillance of enterococcus faecium reveals limited sharing of strains and resistance genes between livestock and humans in the United Kingdom. MBio 9, 1–15. https://doi.org/10.1128/mBio.01780-18.
Gouliouris, T., Raven, K.E., Moradigaravand, D., Ludden, C., Coll, F., Blane, B., Naydenova, P., Horner, C., Brown, N.M., Corander, J., Limmathurotsakul, D., Parkhill, J., Peacock, S.J., 2019. Detection of vancomycin-resistant Enterococcus faecium hospital-adapted lineages in municipal wastewater treatment plants indicates widespread distribution and release into the environment. Genome Res. 29, 626–634. https://doi.org/10.1101/gr.232629.117.
Guardabassi, L., Brønnum, P.T., Danø, R., Forslund, A., Dalsgaard, A., 2002. Dissemination of vancomycin-resistant enterococci harboring vanA through disposal of waste derived from industrial production of vancomycin. Microb. Drug Resist. 8, 401–406. https://doi.org/10.1089/10766290260469688.
Guardabassi, L., Dalsgaard, A., 2004. Occurrence, structure, and mobility of Tn1546-like elements in environmental isolates of vancomycin-resistant Enterococci. Appl. Environ. Microbiol. 70, 984–990. https://doi.org/10.1128/AEM.70.2.984-990.2004.
Guzman Prieto, A.M., van Schaik, W., Rogers, M.R.C., Coque, T.M., Baquero, F., Corander, J., Willems, R.J.L., 2016. Global emergence and dissemination of enterococci as nosocomial pathogens: attack of the clones? Front. Microbiol. 7, 1–15. https://doi.org/10.3389/fmicb.2016.00788.
Hancock, L.E., Murray, B.E., Sillanpää, J., 2014. Enterococci: from commensals to leading causes of drug resistant infection. Boston: Massachusetts Eye and Ear Infirmary.
Harwood, V.J., Brownell, M., Perusek, W., Whitlock, J.E., 2001. Vancomycin-resistant Enterococcus spp. isolated from wastewater and chicken feces in the United States. Appl. Environ. Microbiol. 67, 4930–4933. https://doi.org/10.1128/AEM.67.10.4930-4933.2001.
Harwood, V.J., Whitlock, J., Withington, V., 2000. Classification of antibiotic resistance patterns of indicator bacteria by discriminant analysis: use in predicting the source of fecal contamination in subtropical waters. Appl. Environ. Microbiol. 66, 3698–3704.
Health Canada, 2020. Guidance on the use of Enterococci as an Indicator in Canadian Drinking Water Supplies.
Hegstad, K., Mikalsen, T., Coque, T.M., Werner, G., Sundsfjord, A., 2010. Mobile genetic elements and their contribution to the emergence of antimicrobial resistant Enterococcus faecalis and Enterococcus faecium. Clin. Microbiol. Infect. 16, 541–554. https://doi.org/10.1111/j.1469-0691.2010.03226.x.

Hernando-amado, S., Coque, T.M., Baquero, F., Martínez, J.L., 2019. Defining and combating antibiotic resistance from One Health and Global Health perspectives. Nat. Microbiol. 4 https://doi.org/10.1038/s41564-019-0503-9.

Hidron, A.I., Edwards, J.R., Patel, J., Horan, T.C., Sievert, D.M., Pollock, D.A., Fridkin, S. K., 2008. 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. https://doi.org/10.1086/591861.

Hollenbeck, B.L., Rice, L.B., 2012. Intrinsic and acquired resistance mechanisms in enterococcus. Virulence 3, 421–569. https://doi.org/10.4161/viru.21282.

Homan, W.L., Tribe, D., Poznanski, S., Li, M., Hogg, G., Spalburg, E., Van Embden, J.D. A., Willems, R.J.L., 2002. Multilocus sequence typing scheme for Enterococcus faecium. J. Clin. Microbiol. 40, 1963–1971. https://doi.org/10.1128/JCM.40.6.1963-1971.2002.

ISO, 2000. ISO 7899-2 2000 Water Quality – Detection and Enumeration of Intestinal Enterococci – Part 2: Membrane Filtration Method. Geneva, Switzerland.

Jackson, C.R., Fedorka-Cray, P.J., Barrett, J.B., 2004. Use of a genus- and species-specific multiplex PCR for identification of enterococci. J. Clin. Microbiol. 42, 3558–3565. https://doi.org/10.1128/JCM.42.8.3558-3565.2004.

Jackson, C.R., Lombard, J.E., Dargatz, D.A., Fedorka-Cray, P.J., 2011. Prevalence, species distribution and antimicrobial resistance of enterococci isolated from US dairy cattle. Lett. Appl. Microbiol. 52, 41–48. https://doi.org/10.1111/j.1472-765X.2010.02964.x.

Jahne, M.A., Rogers, S.W., Ramler, I.P., Holder, E., Hayes, G., 2015. Hierarchal clustering yields insight into multidrug-resistant bacteria isolated from a cattle feedlot wastewater treatment system. Environ. Monit. Assess. 187 https://doi.org/10.1007/s10661-014-4168-9.

Jang, J., Hur, H.G., Sadowsky, M.J., Byappanahalli, M.N., Yan, T., Ishii, S., 2017. Environmental Escherichia coli: ecology and public health implications—a review. J. Appl. Microbiol. 123, 570–581. https://doi.org/10.1111/jam.13468.

Jolley, K.A., Bray, J.E., Maiden, M.C.J., 2018. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications [version 1; referees: 2 approved]. Wellcome Open Res 3, 1–20. https://doi.org/10.12688/wellcomeopenres.14826.1.

Kariyama, R., Mitsuhata, R., Chow, J.W., Clewell, D.B., Kumon, H., 2000. Simple and reliable multiplex PCR assay for surveillance isolates of vancomycin-resistant enterococci. J. Clin. Microbiol. 38, 3092–3095. https://doi.org/10.1128/jcm.38.8.3092-3095.2000.

Ke, D., Picard, F.J., Martineau, F., Ménard, C., Roy, P.H., Ouellette, M., Bergeron, M.G., 1999. Development of a PCR assay for rapid detection of enterococci. J. Clin. Microbiol. 37, 3497–3503. https://doi.org/10.1128/jcm.37.11.3497-3503.1999.

Kinzelman, J., Ng, C., Jackson, E., Gradus, S., Bagley, R., 2003. Enterococci as indicators of Lake Michigan recreational water quality: Comparison of two methodologies and their impacts on public health regulatory events. Appl. Environ. Microbiol. 69, 92–96. https://doi.org/10.1128/AEM.69.1.92-96.2003.

Kotzamanidis, C., Zdragas, A., Kourelis, A., Moraitou, E., Papa, A., Yiantzi, V., Pantelidou, C., Yiangou, M., 2009. Characterization of vanA-type enterococcus faecium isolates from urban and hospital wastewater and pigs. J. Appl. Microbiol. 107, 997–1005. https://doi.org/10.1111/j.1365-2672.2009.04274.x.

Lanthier, M., Scott, A., Zhang, Y., Cloutier, M., Durie, D., Henderson, V.C., Wilkes, G., Lapen, D.R., Topp, E., 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. https://doi.org/10.1111/j.1365-2672.2010.04893.x.

Larsson, D.G.J., Flach, C.-F., 2021. Antibiotic resistance in the environment. Nat. Rev. Microbiol. 0123456789. https://doi.org/10.1038/s41579-021-00649-x.

Lata, P., Ram, S., Shanker, R., 2016. Multiplex PCR based genotypic characterization of pathogenic vancomycin resistant Enterococcus faecalis recovered from an Indian river along a city landscape. Springerplus 5. https://doi.org/10.1186/s40064-016-2870-5.

Laverde Gomez, J.A., Hendrickx, A.P.A., Willems, R.J., Top, J., Sava, I., Huebner, J., Witte, W., Werner, G., 2011. Intra- and interspecies genomic transfer of the Enterococcus faecalis pathogenicity Island. PLoS One 6. https://doi.org/10.1371/journal.pone.0016720.

Leclercq, R., Oberlé, K., Galopin, S., Cattoir, V., Budzinski, H., Petit, F., 2013. Changes in enterococcal populations and related antibiotic resistance along a medical center-wastewater treatment plant-river Continuum. Appl. Environ. Microbiol. 79, 2428–2434. https://doi.org/10.1128/AEM.03586-12.

Leonard, A.F.C., Zhang, L., Balfour, A.J., Garside, R., Hawkey, P.M., Murray, A.K., Ukoumunne, O.C., Gaze, W.H., 2018. Exposure to and colonisation by antibiotic-resistant E. coli in UK coastal water users: Environmental surveillance, exposure assessment, and epidemiological study (Beach Bum Survey). Environ. Int. 114, 326–333. https://doi.org/10.1016/j.envint.2017.11.003.

Levin, M.A., Fischer, J.R., Cabelli, V.J., 1975. Membrane Filter Technique for Enumeration of Enterococci in Marine Waters. Appl. Microbiol. 30, 66–71. https://doi.org/10.1128/am.30.1.66-71.1975.

Luczkiewicz, A., Felis, E., Ziembinska, A., Gnida, A., Kotlarska, E., Olanczuk-Neyman, K., Surmacz-Gorska, J., 2013. Resistance of Escherichia coli and Enterococcus spp. to selected antimicrobial agents present in municipal wastewater. J. Water Health 11, 600–612. https://doi.org/10.2166/wh.2013.130.

Łuczkiewicz, A., Jankowska, K., Kurlenda, J., Olańczuk-Neyman, K., 2010. Identification and antimicrobial resistance of Enterococcus spp. isolated from surface water. Water Sci. Technol. 62, 466–473. https://doi.org/10.2166/wst.2010.909.

Maheux, A.F., Picard, F.J., Boissinot, M., Huppé, V., Bissonnette, L., Bernier, J.L.T., Cantin, P., Huletsky, A., Bergeron, M.G., 2009. Analytical limits of three β-glucosidase-based commercial culture methods used in environmental microbiology, to detect enterococci. Water Sci. Technol. 60, 943–955. https://doi.org/10.2166/wst.2009.428.

Mannu, L., Paba, A., Daga, E., Comunian, R., Zanetti, S., Duprè, I., Sechi, L.A., 2003. Comparison of the incidence of virulence determinants and antibiotic resistance between Enterococcus faecium strains of dairy, animal and clinical origin. Int. J. Food Microbiol. 88, 291–304. https://doi.org/10.1016/S0168-1605(03)00191-0.

Manson, J.M., Hancock, L.E., Gilmore, M.S., 2010. Mechanism of chromosomal transfer of Enterococcus faecalis pathogenicity island, capsule, antimicrobial resistance, and other traits. Proc. Natl. Acad. Sci. U. S. A. 107, 12269–12274. https://doi.org/10.1073/pnas.1000139107.

Marano, R.B.M., Fernandes, T., Manaia, C.M., Nunes, O., Morrison, D., Berendonk, T.U., Kreuzinger, N., Telson, T., Corno, G., Fatta-Kassinos, D., Merlin, C., Topp, E., Jurkevitch, E., Henn, L., Scott, A., Heß, S., Slipko, K., Laht, M., Kisand, V., Di Cesare, A., Karaolia, P., Michael, S.G., Petre, A.L., Rosal, R., Pruden, A., Riquelme, V., Agüera, A., Esteban, B., Luczkiewicz, A., Kalinowska, A., Leonard, A., Gaze, W.H., Adegoke, A.A., Stenstrom, T.A., Pollice, A., Salerno, C., Schwermer, C. U., Krzeminski, P., Guilloteau, H., Donner, E., Drigo, B., Libralato, G., Guida, M., Bürgmann, H., Beck, K., Garelick, H., Tacão, M., Henriques, I., Martínez-Alcalá, I., Guillén-Navarro, J.M., Popowska, M., Piotrowska, M., Quintela-Baluja, M., Bunce, J. T., Polo-López, M.I., Nahim–Granados, S., Pons, M.N., Milakovic, M., Udikovic-Kolic, N., Ory, J., Ousmane, T., Caballero, P., Oliver, A., Rodriguez-Mozaz, S., Balcazar, J.L., Jäger, T., Schwartz, T., Yang, Y., Zou, S., Lee, Y., Yoon, Y., Herzog, B., Mayrhofer, H., Prakash, O., Nimonkar, Y., Heath, E., Baraniak, A., Abreu-Silva, J., Choudhury, M., Munoz, L.P., Krizanovic, S., Brunetti, G., Maile-Moskowitz, A., Brown, C., Cytryn, E., 2020. A global multinational survey of cefotaxime-resistant coliforms in urban wastewater treatment plants. Environ. Int. 144, 106035 https://doi.org/10.1016/j.envint.2020.106035.

Marathe, N.P., Pal, C., Gaikwad, S.S., Jonsson, V., Kristiansson, E., Larsson, D.G.J., 2017. Untreated urban waste contaminates Indian river sediments with resistance genes to last resort antibiotics. Water Res. 124, 388–397. https://doi.org/10.1016/j.watres.2017.07.060.

Messer, J.W., Dufour, A.P., 1998. A rapid, specific membrane filtration procedure for enumeration of enterococci in recreational water. Appl. Environ. Microbiol. 64, 678–680. https://doi.org/10.1128/aem.64.2.678-680.1998.

Miller, W.R., Munita, J.M., Arias, C.A., 2014. Mechanisms of antibiotic resistance in enterococci. Expert Rev. Anti. Infect. Ther. 12, 1221–1236. https://doi.org/10.1586/14787210.2014.956092.

Moellering, R.C., 1992. Emergence of Enterococcus as a Significant Pathogen. Clin. Infect. Dis. 14, 1173–1176.

Molale, L.G., Bezuidenhout, C.C., 2016. Antibiotic resistance, efflux pump genes and virulence determinants in Enterococcus spp. from surface water systems. Environ. Sci. Pollut. Res. 23, 21501–21510. https://doi.org/10.1007/s11356-016-7369-7.

Monticelli, L.S., Decembrini, F., Bergamasco, A., Caruso, G., 2019. Water quality assessment of transitional and coastal marine Sicilian waters (Italy): Ecological and epidemiological significance of multiple antimicrobial resistant Enterococcus spp. Estuar. Coast. Shelf Sci. 217, 173–184. https://doi.org/10.1016/j.ecss.2018.11.021.

Murray, B.E., 1998. Diversity among multidrug-resistant enterococci. Emerg. Infect. Dis. 4, 37–47. https://doi.org/10.3201/eid0401.980106.

Murray, B.E., 1990. The life and times of the enterococcus. Clin. Microbiol. Rev. 3, 46–65. https://doi.org/10.1128/CMR.3.1.46.

Nam, S., Kim, M.J., Park, C., Park, J.G., Maeng, P.J., Lee, G.C., 2013. Detection and genotyping of vancomycin-resistant Enterococcus spp. by multiplex polymerase chain reaction in Korean aquatic environmental samples. Int. J. Hyg. Environ. Health 216, 421–427. https://doi.org/10.1016/j.ijheh.2012.12.004.

Naser, S.M., Thompson, F.L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M., Swings, J., 2005. Application of multilocus sequence analysis (MLSA) for rapid identification of Enterococcus species based on rpoA and pheS genes. Microbiology 151, 2141–2150. https://doi.org/10.1099/mic.0.27840-0.

Navarro, F., Courvalin, P., 1994. Analysis of genes encoding D-alanine-D-alanine ligase-related enzymes in Enterococcus casseliflavus and Enterococcus flavescens. Antimicrob. Agents Chemother. 38, 1788–1793. https://doi.org/10.1128/AAC.38.8.1788.

Nemoy, L.L., Kotetishvili, M., Tigno, J., Keefer-Norris, A., Harris, A.D., Perencevich, E.N., Johnson, J.A., Torpey, D., Sulakvelidze, A., Morris, J.G., Stine, O.C., 2005. Multilocus sequence typing versus pulsed-field gel electrophoresis for characterization of extended-spectrum beta-lactamase-producing Escherichia coli isolates. J. Clin. Microbiol. 43, 1776–1781. https://doi.org/10.1128/JCM.43.4.1776-1781.2005.

Nishiyama, M., Iguchi, A., 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. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng. 50, 16–25. https://doi.org/10.1080/10934529.2015.964599.

Nishiyama, M., Ogura, Y., Hayashi, T., Suzuki, Y., 2017. Antibiotic resistance profiling and genotyping of vancomycin-resistant enterococci collected from an urban river basin in the provincial city of Miyazaki, Japan. Water. https://doi.org/10.3390/w9020079.

Novais, C., Coque, T.M., Ferreira, H., Sousa, C., Peixe, L., Cie, F., 2005. Environmental contamination with vancomycin-resistant Enterococci from hospital sewage in Portugal. Appl. Environ. Microbiol. 71, 3364–3368. https://doi.org/10.1128/AEM.71.6.3364.

Oravcova, V., Mihalcin, M., Zakova, J., Pospisilova, L., Masarikova, M., Literak, I., 2017. Vancomycin-resistant enterococci with vanA gene in treated municipal wastewater and their association with human hospital strains. Sci. Total Environ. 609, 633–643. https://doi.org/10.1016/j.scitotenv.2017.07.121.

Pagel, J.E., Hardy, G.M., 1980. Comparison of selective media for the enumeration and identification of fecal streptococci from natural sources. Can. J. Microbiol. 26, 1320–1327. https://doi.org/10.1139/m80-219.

Palmer, K.L., Gilmore, M.S., 2010. Multidrug-resistant enterococci lack CRISPR-cas. MBio 1, 1–10. https://doi.org/10.1128/mBio.00227-10.

Paulsen, I.T., Banerjei, L., Hyers, G.S.A., Nelson, K.E., Seshadri, R., Read, T.D., Fouts, D. E., Eisen, J.A., Gill, S.R., Heidelberg, J.F., Tettelin, H., Dodson, R.J., Umayam, L., Brinkac, L., Beanan, M., Daugherty, S., DeBoy, R.T., Durkin, S., Kolonay, J., Madupu, R., Nelson, W., Vamathevan, J., Tran, B., Upton, J., Hansen, T., Shetty, J., Khouri, H., Utterback, T., Radune, D., Ketchum, K.A., Dougherty, B.A., Fraser, C.M., 2003. Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis. Science 299, 2071–2074. https://doi.org/10.1126/science.1080613, 80-.

Pöntinen, A.K., Top, J., Arredondo-Alonso, S., Tonkin-Hill, G., Freitas, A.R., Novais, C., Gladstone, R.A., Pesonen, M., Meneses, R., Pesonen, H., Lees, J.A., Jamrozy, D., Bentley, S.D., Lanza, V.F., Torres, C., Peixe, L., Coque, T.M., Parkhill, J., Schürch, A. C., Willems, R.J.L., Corander, J., 2021. Apparent nosocomial adaptation of Enterococcus faecalis predates the modern hospital era. Nat. Commun. 12 https://doi.org/10.1038/s41467-021-21749-5.

Poyart, C., Quesnes, G., Trieu-Cuot, P., 2000. Sequencing the gene encoding manganese-dependent superoxide dismutase for rapid species identification of enterococci. J. Clin. Microbiol. 38, 415–418. https://doi.org/10.1128/JCM.38.1.415-418.2000.

Prüss, A., 1998. Review of epidemiological studies on health effects from exposure to recreational water. Int. J. Epidemiol. 27, 1–9. https://doi.org/10.1093/ije/27.1.1.

Rathnayake, I.U., Hargreaves, M., Huygens, F., 2012. Antibiotic resistance and virulence traits in clinical and environmental Enterococcus faecalis and Enterococcus faecium isolates. Syst. Appl. Microbiol. 35, 326–333. https://doi.org/10.1016/j.syapm.2012.05.004.

Rathnayake, I.U., Hargreaves, M., Huygens, F., 2011. Genotyping of Enterococcus faecalis and Enterococcus faecium isolates by use of a set of eight single nucleotide polymorphisms. J. Clin. Microbiol. 49, 367–372. https://doi.org/10.1128/JCM.01120-10.

Rice, E.W., Baird, A.D., 2017. Method 9230 Fecal Enterococcus/Streptococcus Groups. Standard Methods for the Examination of Water and Wastewater, 23rd Edition, pp. 3–6.

Ruiz-Garbajosa, P., Bonten, M.J.M., Robinson, D.A., Top, J., Nallapareddy, S.R., Torres, C., Coque, T.M., Cantón, R., Baquero, F., Murray, B.E., Del Campo, R., Willems, R.J.L., 2006. Multilocus sequence typing scheme for Enterococcus faecalis reveals hospital-adapted genetic complexes in a background of high rates of recombination. J. Clin. Microbiol. 44, 2220–2228. https://doi.org/10.1128/JCM.02596-05.

Sadowy, E., Luczkiewicz, A., 2014. Drug-resistant and hospital-associated Enterococcus faecium from wastewater, riverine estuary and anthropogenically impacted marine catchment basin. BMC Microbiol 14. https://doi.org/10.1186/1471-2180-14-66.

Sanderson, H., Ortega-Polo, R., McDermott, K., Zaheer, R., Brown, R.S., Majury, A., McAllister, T., Liss, S.N., 2019. Comparison of biochemical and genotypic speciation methods for vancomycin-resistant enterococci isolated from urban wastewater treatment plants. J. Microbiol. Methods 161, 102–110. https://doi.org/10.1016/j.mimet.2019.04.019.

Santiago-Rodriguez, T.M., Rivera, J.I., Coradin, M., Toranzos, G.A., 2013. Antibiotic-resistance and virulence genes in Enterococcus isolated from tropical recreational waters. J. Water Health 11, 387–396. https://doi.org/10.2166/wh.2013.005.

Sapkota, A.R., Curriero, F.C., Gibson, K.E., Schwab, K.J., 2007. Antibiotic-resistant enterococci and fecal indicators in surface water and groundwater impacted by a concentrated swine feeding operation. Environ. Health Perspect. 115, 1040–1045. https://doi.org/10.1289/ehp.9770.

Savin, M., Bierbaum, G., Hammerl, J.A., Heinemann, C., Parcina, M., Sib, E., Voigt, A., Kreyenschmidt, J., 2020. Antibiotic-resistant bacteria and antimicrobial residues in wastewater and process water from German pig slaughterhouses and their receiving municipal wastewater treatment plants. Sci. Total Environ. 727, 138788 https://doi.org/10.1016/j.scitotenv.2020.138788.

Schoen, M.E., Soller, J.A., Ashbolt, N.J., 2011. Evaluating the importance of faecal sources in human-impacted waters. Water Res 45, 2670–2680. https://doi.org/10.1016/j.watres.2011.02.025.

Shankar, N., Baghdayan, A.S., Gilmore, M.S., 2002. Modulation of virulence within a pathogenicity island in vancomycin-resistant Enterococcus faecalis. Nature 417, 746–750. https://doi.org/10.1038/nature00802.

Sievert, D.M., Ricks, P., Edwards, J.R., Schneider, A., Patel, J., Srinivasan, A., Kallen, A., Limbago, B., Fridkin, S., 2013. Antimicrobial-resistant pathogens associated with healthcare-associated infections summary of data reported to the national healthcare safety network at the centers for disease control and prevention, 2009–2010. Infect. Control Hosp. Epidemiol. 34, 1–14. https://doi.org/10.1086/668770.

Silva, N., Igrejas, G., Gonçalves, A., Poeta, P., 2012. Commensal gut bacteria: Distribution of Enterococcus species and prevalence of Escherichia coli phylogenetic groups in animals and humans in Portugal. Ann. Microbiol. 62, 449–459. https://doi.org/10.1007/s13213-011-0308-4.

Sinclair, R.G., Rose, J.B., Hashsham, S.A., Gerba, C.P., Haase, C.N., 2012. Criteria for selection of surrogates used to study the sate and control of pathogens in the environment. Appl. Environ. Microbiol. 78, 1969–1977. https://doi.org/10.1128/AEM.06582-11.

Singhal, N., Kumar, M., Kanaujia, P.K., Virdi, J.S., 2015. MALDI-TOF mass spectrometry: An emerging technology for microbial identification and diagnosis. Front. Microbiol. 6, 1–16. https://doi.org/10.3389/fmicb.2015.00791.

Soller, J.A., Schoen, M.E., Bartrand, T., Ravenscroft, J.E., Ashbolt, N.J., 2010. Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination. Water Res. 44, 4674–4691. https://doi.org/10.1016/j.watres.2010.06.049.

Stine, O.C., Johnson, J.A., Keefer-Norris, A., Perry, K.L., Tigno, J., Qaiyumi, S., Stine, M. S., Morris, J.G., 2007. Widespread distribution of tetracycline resistance genes in a confined animal feeding facility. Int. J. Antimicrob. Agents 29, 348–352. https://doi.org/10.1016/j.ijantimicag.2006.11.015.

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D.L., Pulcini, C., Kahlmeter, G., Kluytmans, J., Carmeli, Y., Ouellette, M., Outterson, K., Patel, J., Cavaleri, M., Cox, E.M., Houchens, C.R., Grayson, M.L., Hansen, P., Singh, N., Theuretzbacher, U., Magrini, N., Aboderin, A.O., Al-Abri, S.S., Awang Jalil, N., Benzonana, N., Bhattacharya, S., Brink, A.J., Burkert, F.R., Cars, O., Cornaglia, G., Dyar, O.J., Friedrich, A.W., Gales, A.C., Gandra, S., Giske, C.G., Goff, D.A., Goossens, H., Gottlieb, T., Guzman Blanco, M., Hryniewicz, W., Kattula, D., Jinks, T., Kanj, S.S., Kerr, L., Kieny, M.P., Kim, Y.S., Kozlov, R.S., Labarca, J., Laxminarayan, R., Leder, K., Leibovici, L., Levy-Hara, G., Littman, J., Malhotra-Kumar, S., Manchanda, V., Moja, L., Ndoye, B., Pan, A., Paterson, D.L., Paul, M., Qiu, H., Ramon-Pardo, P., Rodríguez-Baño, J., Sanguinetti, M., Sengupta, S., Sharland, M., Si-Mehand, M., Silver, L.L., Song, W., Steinbakk, M., Thomsen, J., Thwaites, G.E., van der Meer, J.W., Van Kinh, N., Vega, S., Villegas, M. V., Wechsler-Fördös, A., Wertheim, H.F.L., Wesangula, E., Woodford, N., Yilmaz, F. O., Zorzet, A., 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect. Dis. 18, 318–327. https://doi.org/10.1016/S1473-3099(17)30753-3.

Talebi, M., Rahimi, F., Katouli, M., Kühn, I., Möllby, R., Eshraghi, S., Pourshafie, M.R., 2007. Prevalence and antimicrobial resistance of enterococcal species in sewage treatment plants in Iran. Water. Air. Soil Pollut. 185, 111–119. https://doi.org/10.1007/s11270-007-9435-8.

Taučer-Kapteijn, M., Hoogenboezem, W., Heiliegers, L., de Bolster, D., Medema, G., 2016. Screening municipal wastewater effluent and surface water used for drinking water production for the presence of ampicillin and vancomycin resistant enterococci. Int. J. Hyg. Environ. Health 219, 437–442. https://doi.org/10.1016/j.ijheh.2016.04.007.

Teixeira, L.M., Siqueira, G., Shewmaker, P.L., Facklam, R.R., 2015. Enterococcus. Manual of Clinical Microbiology. https://doi.org/10.1128/9781555817381.ch23.

Tejedor Junco, M.A.T., Martin, M.G., Pita Toledo, M.A.L., Gómez, P.L., Barrasa, J.L.M., 2001. Identification and antibiotic resistance of faecal enterococci isolated from water samples. Int. J. Hyg. Environ. Health 203, 363–368. https://doi.org/10.1078/1438-4639-00043.

Tiwari, A., Hokajärvi, A.M., Santo Domingo, J.W., Kauppinen, A., Elk, M., Ryu, H., Jayaprakash, B., Pitkänen, T., Domingo, J.W.S., 2018. Categorical performance characteristics of method ISO 7899-2 and indicator value of intestinal enterococci for bathing water quality monitoring. J. Water Health 16, 711–723. https://doi.org/10.2166/wh.2018.293.

Top, J., Willems, R., Van Der Velden, S., Asbroek, M., Bonten, M., 2008. Emergence of clonal complex 17 Enterococcus faecium in the Netherlands. J. Clin. Microbiol. 46, 214–219. https://doi.org/10.1128/JCM.01351-07.

USEPA, 2009. Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl- B -D-Glucoside Agar (mEI).

USEPA, 2006. Method 1106.1 : Enterococci in Water by Membrane Filtration Using membrane- Enterococcus-Esculin Iron Agar.

Valente, M.S., Pedro, P., Alonso, M.C., Borrego, J.J., Dionísio, L., 2010. Are the defined substrate-based methods adequate to determine the microbiological quality of natural recreational waters? J. Water Health 8, 11–19. https://doi.org/10.2166/wh.2009.220.

van Hal, S.J., Willems, R.J.L., Gouliouris, T., Ballard, S.A., Coque, T.M., Hammerum, A. M., Hegstad, K., Westh, H.T., Howden, B.P., Malhotra-Kumar, S., Werner, G., Yanagihara, K., Earl, A.M., Raven, K.E., Corander, J., Bowden, R., Pinholt, M., Loens, K., Xavier, B.B., Matheeussen, V., Goossens, H., 2021. The global dissemination of hospital clones of Enterococcus faecium. Genome Med. 13, 1–12. https://doi.org/10.1186/s13073-021-00868-0.

Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Jabes, D., Goossens, H., 2004. Development of a multiplex PCR for the detection of asaI, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among european hospital isolates of Enterococcus faecium. J. Clin. Microbiol. 42, 4473–4479. https://doi.org/10.1128/JCM.42.10.4473-4479.2004.

Vilanova, X., Blanch, A.R., 2006. Distribution and persistence of fecal bacterial populations in liquid and dewatered sludge from a biological treatment plant. J. Gen. Appl. Microbiol. 51, 361–368. https://doi.org/10.2323/jgam.51.361.

Wade, T.J., Calderon, R.L., Sams, E., Beach, M., Brenner, K.P., Williams, A.H., Dufour, A. P., 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ. Health Perspect. 114, 24–28. https://doi.org/10.1289/ehp.8273.

Wade, T.J., Pai, N., Eisenberg, J.N.S., Colford, J.M., 2003. Do U.S. Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. Environ. Health Perspect. 111, 1102–1109. https://doi.org/10.1289/ehp.6241.

Weiner-Lastinger, L.M., Abner, S., Edwards, J.R., Kallen, A.J., Karlsson, M., Magill, S.S., Pollock, D., See, I., Soe, M.M., Walters, M.S., Dudeck, M.A., 2020. Antimicrobial-resistant pathogens associated with adult healthcare-associated infections: Summary of data reported to the National Healthcare Safety Network, 2015–2017. Infect. Control Hosp. Epidemiol. 41, 1–18. https://doi.org/10.1017/ice.2019.296.

Weiner, L.M., Webb, A.K., Limbago, B., Dudeck, M.A., Patel, J., Kallen, A.J., Edwards, J. R., Sievert, D.M., 2016. Antimicrobial-Resistant Pathogens Associated with Healthcare-Associated Infections: Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011-2014. Infect. Control Hosp. Epidemiol. 37, 1288–1301. https://doi.org/10.1017/ice.2016.174.

WHO, 2021. Global Tricycle Surveillance: ESBL E. coli, WHO integrated global surveillance on ESBL-producing E. coli using a "One Health" approach: Implementation and opportunities.

Woodford, N., Johnson, A.P., Morrison, D., Speller, D.C.E., 1995. Current perspectives on glycopeptide resistance. Clin. Microbiol. Rev. 8, 585–615. https://doi.org/10.1128/cmr.8.4.585.

Zaheer, R., Cook, S.R., Barbieri, R., Goji, N., Cameron, A., Petkau, A., Polo, R.O., Tymensen, L., Stamm, C., Song, J., Hannon, S., Jones, T., Church, D., Booker, C.W., Amoako, K., Van Domselaar, G., Read, R.R., McAllister, T.A., 2020. Surveillance of Enterococcus spp. reveals distinct species and antimicrobial resistance diversity across a One-Health continuum. Sci. Rep. 10, 1–16. https://doi.org/10.1038/s41598-020-61002-5.

Zdragas, A., Partheniou, P., Kotzamanidis, C., Psoni, L., Koutita, O., Moraitou, E., Tzanetakis, N., Yiangou, M., 2008. Molecular characterization of low-level vancomycin-resistant enterococci found in coastal water of Thermaikos Gulf, Northern Greece. Water Res 42, 1274–1280. https://doi.org/10.1016/j.watres.2007.09.022.

Zhang, S., Huang, J., Zhao, Z., Cao, Y., Li, B., 2020. Hospital wastewater as a reservoir for antibiotic resistance genes: a meta-analysis. Front. Public Heal. 8, 1–12. https://doi.org/10.3389/fpubh.2020.574968.