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Diversity and health risk potentials of the *Enterococcus* population in tropical coastal water impacted by Hurricane Lane

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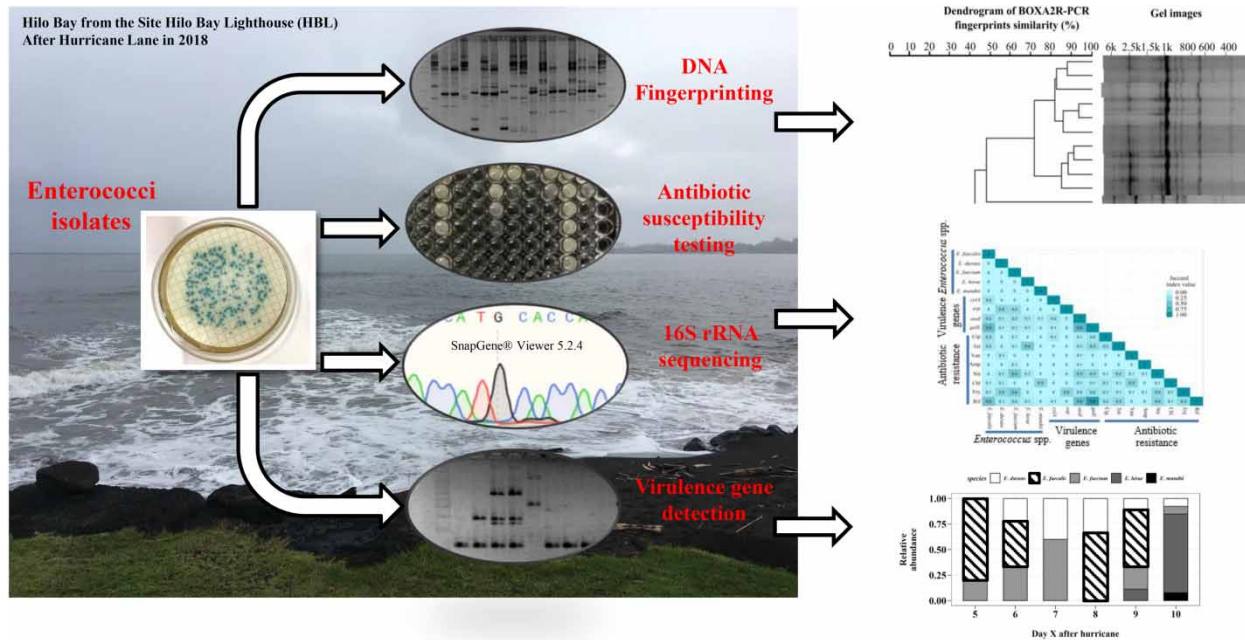
ABSTRACT

Hurricane-caused stormwater runoffs transport diverse terrestrial pollutants, adversely impact microbiological water quality, and introduce fecal and other pathogens to coastal water environments. This study investigated the genotypic diversity, phylogenetic composition, anti-biotic resistance patterns, and virulence gene repertoire of the *Enterococcus* population in the Hilo Bay coastal water after the immediate impact of Hurricane Lane. DNA fingerprinting of *Enterococcus* isolates exhibited large genotypic diversity, while 16S rRNA gene sequencing identified four major species, including *E. faecalis* (34.7%), *E. faecium* (22.4%), *E. hirae* (22.4%), and *E. durans* (18.4%). Four common enter-ococcal virulence genes (*cylA*, *esp*, *asa1*, and *gelE*) were detected in the *Enterococcus* population, with significant portions of *E. durans* (33.3%), *E. faecalis* (41.2%), *E. faecium* (36.4%), and *E. hirae* (27.3%) isolates possessing two or more virulence genes. Considerable antibiotic resistance to rifampin, erythromycin, tetracycline, and nitrofurantoin was detected in the *Enterococcus* population, with one *E. durans* isolate showing vancomycin resistance. The results indicate considerable health implications associated with *Enterococcus* spp. in the hurricane-impacted tropical coastal water, illustrating the needs for more comprehensive understanding of the microbiological risks associated with storm-impacted coastal water.

Key words: coastal, diversity, *Enterococci*, Hurricane, risks, water

HIGHLIGHTS

- The study investigated the *Enterococcus* population in a tropical coastal water immediately after the impact of a major hurricane.
- Genotypic diversity and phylogenetic composition of the *Enterococcus* population were determined.
- A significant portion of the *Enterococcus* population contains various virulence factors.
- Considerable antibiotic resistance was detected in the *Enterococcus* population.

GRAPHICAL ABSTRACT

INTRODUCTION

Hilo town area (Hawaii island, Hawaii), which has a tropical rainforest climate (NOAA 2021), is populated with dense sewer networks and a large number of on-site wastewater treatment systems (e.g. 8,700 cesspools; Silvius et al. 2005) and is also located next to the mouth of the Wailuku River. Hurricane Lane, a category five hurricane that affected the area during August 15–29, 2018, caused heavy rainfall over the Hilo town area and resulted in its wettest 3-day period on record in precipitation (80.9 cm) (Beven II & Wroe 2019). This unprecedented rainfall resulted in significant stormwater runoff and flooding, and sanitary sewer overflows due to rainfall-derived infiltration and inflow into the aging sewer infrastructures in the area. As a result, the microbiological quality of coastal water in Hilo Bay was deteriorated according to the high levels of fecal indicator bacteria (FIB) Enterococcus observed in the water samples collected after the heavy rains (Saingam et al. 2021).

The Enterococcus genus is a group of Gram-positive bacteria with 58 known species (Parte 2014), which are commensals in human and various animal guts (Layton et al. 2010) and are used as an important FIB in monitoring coastal marine water quality (USEPA 2012). Certain Enterococcus spp. are pathogenic and cause endocarditis, bacteremia, neonatal, central nervous system, urinary tract, abdominal, and pelvis infections (Lewis & Zervos 1990), which is estimated to cause more than $539 million per year in healthcare costs (CDC 2019). E. faecalis and E. faecium are the two major species of opportunistic pathogens prevalent in health clinical, whereas E. durans, E. gallinarum, E. avium, E. hirae, and E. mundtii can also be responsible for infections (Blaimont et al. 1995). Pathogenicity of enterococci is contributed by several common virulence factors encoded by the asa1, cylA, gelE, esp, and hyl genes (Jett et al. 1994). The asa1 gene encodes aggregation substance proteins that assist in attachment and colonization during renal tissue infection (Waters et al. 2004). The cylA gene encodes cytolysin that aggravates the endocarditis infection (Huycke & Gilmore 1995). The gelE encodes extracellular gelatinase which mediates the virulence by modulating host immune response and degrading tissues (Park et al. 2008). The esp gene encodes the enterococcal surface proteins that are involved in biofilm formation and urinary tract infection (Shankar et al. 1999). The hyl gene encodes hyaluronidase that is linked to increased gastrointestinal colonization in mouse models (Rice et al. 2003).

The presence of antibiotic-resistant genes (ARGs) could further increase and complicate health risks caused by the pathogenic enterococci in coastal water (Kristich et al. 2014). Enterococci were reported to be inherently tolerant to several

antibiotics within the classes of penicillin and aminoglycosides (Robbins & Tompsett 1951). Enterococci from environmental water also showed resistance to several other antibiotics, including tetracyclines, erythromycin, or gentamicin, likely through horizontal gene transfer (Macedo et al. 2011). Among them, vancomycin-resistant *Enterococcus* (VRE) are resistant to one of the last-resort antimicrobial agents (i.e. vancomycin) and have contributed to approx. 30% of enterococcal infections and about 5,400 mortalities each year in the USA (CDC 2019).

Previous studies on enterococcal isolates in coastal waters after the impact of major storms were largely focused on the abundance of *Enterococcus* population (Roca et al. 2019; Jiang et al. 2020). The phylogenetic composition of the population in Lake Pontchartrain, a fresh water lake, after the impact of Hurricane Katrina was also assessed by sequencing the 23S rRNA gene of the enterococcal isolates (Bae & Hou 2013). In a previous study, Saingam et al. (2021) reported that the Hilo Bay Lighthouse (HBL) site was the most contaminated site in Hilo Bay after the stormwater impact of Hurricane Lane, which had a mean enterococcal density of 144 CFU/100 mL and exceeded the water quality criteria statistical threshold value. Bae & Hou (2013) reported a large portion of classical fecal origins *E. faecalis*, *E. faecium*, *E. durans*, *E. hirae*, and *E. mundtii* were detected in freshwater as the impact of Hurricane Katrina-derived urban runoff. A study done by Jiang et al. (2020) reported that *E. faecalis* were detected in 60% of coastal waters and 67% of street surface runoff at a concentration of <10 MPN/L by the MPN-LAMP assay after the back-to-back hurricane strikes by Irma and Maria. High concentrations of enterococci (mean concentration of gene marker of 5.05×10^5 copies/100 mL) were detected in 40–100% of surface water samples collected from flooded regions of the river impacted by Hurricane Harvey which was also reported by Kapoor et al. (2018). However, the pathogenic potentials, genotypic diversity, and ARG repertoire of the *Enterococcus* population after major storms in coastal marine water have not been adequately investigated.

The aim of this study was to understand microbial risks of the *Enterococcus* population in coastal water immediately after the impact of a major hurricane. Enterococcal isolates were obtained from near-shore coastal water at the HBL site, where the high level of FIB *Enterococcus* after Hurricane Lane was detected (Saingam et al. 2021). The *Enterococcus* spp. distribution was determined by 16S rRNA gene sequencing of the enterococcal isolates. The *Enterococcus* spp. genotypic diversity was determined by BOX-PCR fingerprinting and then compared with their 16S rRNA gene-based phylogeny. The prevalence of virulence genes in the *Enterococcus* population was determined by multiplex PCR detection, and their antimicrobial susceptibility was determined by microbroth dilution assays.

MATERIALS AND METHODS

Water sampling and enterococcal isolation

Coastal water sampling immediately after Hurricane Lane and sample processing were described in detail in a previous publication (Saingam et al. 2021). Briefly, approximately 2 L of the water samples ($n = 7$) were collected at knee-depth at the HBL sampling site from Day 5 to Day 11 when field sampling became feasible immediately after the peak hurricane impact (i.e. Day 0). The HBL site (GPS coordinates: 19°43′38.0″N 155°05′11.0″W) is located at the shoreline of Downtown Hilo area and approximately 100 and 2,000 m from the mouths of the Wailuku River and Wailoa River, respectively. Water samples (100 mL) were filtered through 0.45 μm GN-6 filter membranes, which were then incubated on the mEI agar to selectively grow enterococci colonies according to the EPA Method 1600 (USEPA 2006). Presumptive enterococcal colonies (i.e. colonies with blue halo) were picked and further isolated via streak plating on tryptic soy agar. The presumptive enterococcal isolates were subsequently confirmed based on growth in both Brain Heart Infusion Broth (BHIB) (35 °C ± 0.5 °C, 48 h) and BHIB supplemented with 6.5% NaCl (45 °C ± 0.5 °C, 48 h). A total of 117 confirmed enterococcal isolates were obtained and cultured in BHIB at 35 °C for 24 h and then stored at −80 °C in the presence of 18% glycerol.

16S rRNA gene sequencing

Forty-nine of the 117 enterococcal isolates were randomly selected and subjected to 16S rRNA gene sequencing to determine the phylogenetic composition of the *Enterococcus* population. Total genomic DNA from the enterococcal isolates was prepared by using the freeze–thaw method (Ran et al. 2013). The universal 16S rRNA gene primers (27F: 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R: 5′-GGTTACCTTGTTACGACTT-3′) were used for PCR amplification. The reaction mixture contained 200 μM dNTPs, 0.125 U/μL Taq polymerase, and 0.4 μM primers in a final volume of 25 μL. The amplification program included initial denaturation at 94 °C for 5 min, 30 cycles of denaturation (94 °C for 30 s), annealing (53 °C for 45 s), and extension (72 °C for 1 min), and a final extension step (72 °C for 10 min). PCR products were purified using a Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). The sequencing reaction was performed

using the ABI 3730XL sequencer at the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB), University of Hawaii at Manoa. The sequence reads were quality trimmed and checked manually against the chromatograms by using the Sequence Scanner (Version 2.0; Thermo Fisher, Waltham, MA, USA), and the species identification was confirmed by using BLASTn. The 49 16S rRNA gene sequences were deposited in the GenBank database (accession no. MZ668452–MZ668500).

Multiplex PCR for virulence gene detection

The presence of enterococcal virulence genes, including *asa1*, *gelE*, *cylA*, *esp*, and *hyl*, were tested on the 49 *Enterococcus* spp. by following a multiplex PCR assay (Vankerckhoven *et al.* 2004). The reaction mixture contained 0.16 mg/mL BSA, 200 μM dNTPs, 1 mM MgCl₂, 0.1 U/μL Taq polymerase, 0.1 μM of *asa1* (F: 5′-GCACGCTATTACGAACTATGA-3′; R: 5′-TAAGAAAGAACATCACCACGA-3′), *hyl* (F: 5′-ACAGAAGAGCTGCAGGAAATG-3′; R: 5′-GACTGACGTCCAAGTTTC-CAA-3′), and *gelE* (F: 5′-TATGACAATGCTTTTTGGGAT-3′; R: 5′-AGATGCACCCGAAATAATATA-3′) primer pairs each, and 0.2 μM of *esp* (F: 5′-AGATTTCATCTTTGATTCTTGG-3′; R: 5′-AATTGATTCTTTAGCATCTGG-3′) and *cylA* (F: 5′-ACTCGGGGATTGATAGGC-3′; R: 5′-GCTGCTAAAGCTGCGCTT-3′) primers each. The amplification steps included the initial denaturation at 95 °C for 7 min, 30 cycles of 94 °C for 1 min, 1 min annealing at 56 °C, 1 min extension at 72 °C, and 10 min final extension at 72 °C. PCR amplicons were separated by 3.0% agarose gel electrophoresis for 3 h at 80 V in 1× TBE. Gel with PCR amplicons was stained in 1× GelRed solution (Biotium; Hayward, CA, USA) and visualized using a *GelDoc* imager (Bio-Rad, Hercules, CA, USA). *E. faecalis* MMH594 (*gelE*⁺ *asa1*⁺ *cylA*⁺ *esp*⁺) and *E. faecium* U0317 (*esp*⁺ *hyl*⁺) were used as positive controls. The *Enterococcus* isolates that carried more than two virulence genes were considered multi-virulence gene carrier.

Antibiotic susceptibility testing

Antimicrobial susceptibility of the *Enterococcus* isolates ($n = 49$) was determined by the microbroth dilution method according to the Clinical and Laboratory Standards Institute (CLSI) protocol (CLSI 2012). Briefly, stock cultures were revived in the cation-adjusted Mueller Hinton broth (CAMHB) (BD; MD, USA) in 96-well microtiter plates at 35 °C until OD₆₂₀ reached 0.4, which was equivalent of 0.5 McFarland standard (~5×10^8 cells/mL). Approximately 5×10^5 cells were transferred to wells of CAMHB supplemented with different antibiotics at different concentrations. The antibiotics tested included ciprofloxacin (Cip) (0.5–8 μg/mL), ampicillin (Amp) (4–32 μg/mL), chloramphenicol (Chl) (4–64 μg/mL), tetracycline (Tet) (2–32 μg/mL), vancomycin (Van) (2–64 μg/mL), erythromycin (Ery) (0.25–16 μg/mL), rifampin (Rif) (0.5–8 μg/mL), and nitrofurantoin (Nit) (16–256 μg/mL). The microbroth dilution plates were incubated at 35 °C for 20 h. The minimal inhibitory concentrations were recorded, and the strains that showed growth at the resistant breakpoints were recorded as 'Resistant' per CLSI criteria (CLSI 2012). *E. faecalis* ATCC 29212 was used as a quality control for the test media and antibiotics. Isolates that showed resistance to equal or more than two antibiotics were categorized in a group named as multi-antibiotic resistance (MAR) carrier.

Co-occurrence of virulence factors and AR

Co-occurrence of virulence genes and antibiotics resistance in the *Enterococcus* isolates were calculated by using the Jaccard index, which is the number of isolates carrying both entities (A ∩ B) divided by the total number of isolates carrying either entity (A ∪ B). The Jaccard index ranged from 0 (no co-occurrence) to 1 (always co-occurrence). The analysis and plotting were performed in the R environment (Version 3.6.1).

BOX-PCR fingerprint clustering

The DNA fingerprinting of environmental *Enterococcus* isolates from the HBL site followed the procedures described previously (Saingam *et al.* 2021). Briefly, BOX-PCR fingerprints of the 49 *Enterococcus* isolates that had their 16S rRNA gene sequenced were generated and analyzed through clustering analysis in BioNumerics v.7.01 (Applied Maths Inc., Sint-Martens-Latem, Belgium). The level of similarity between fingerprints was calculated using the Dice coefficient at 1.0% optimization and 1.0% band position tolerance. Bands of up to 6 kb were included in the analysis. A representative dendrogram was constructed in BioNumerics using the unweighted pair group method with arithmetic mean. The relationship between phylogenetic clusters of DNA fingerprinting and species identification of enterococci isolates was evaluated by using Fisher's exact test. Comparisons of species among cluster groups were achieved by the post-hoc test, and the *p*-value

was adjusted by the Benjamini–Hochberg false discovery rate method. The analysis was performed by using the rcompanion package (Mangiafico & Mangiafico 2017) in R.

RESULTS

Diversity of *Enterococcus* in hurricane-impacted coastal water

Forty unique genotypes out of total 49 *Enterococcus* isolates were identified based on the similarities of the BOX A2R fingerprinting patterns, which were grouped into four major clusters (Figure 1). The 16S rRNA gene sequencing showed that the *Enterococcus* isolates belong to five different species, including *E. faecalis* ($n = 17$, 34.7%), *E. faecium* ($n = 11$, 22.4%), *E. hirae* ($n = 11$, 22.4%), *E. durans* ($n = 9$, 18.4%), and *E. mundtii* ($n = 1$, 2.0%) (Figure 2). Fisher's exact test showed that there was a significant difference ($p < 0.05$) between clusters and *Enterococcus* spp. proportion in each clusters. In cluster A, the proportion of *E. hirae* is significantly different with other clusters, especially clusters B ($p < 0.001$) and C ($p < 0.001$). In cluster B, the proportion of *E. faecalis* is significantly different ($p < 0.001$) from all other clusters. There were 11 genotypes of *E. faecalis* ($n = 13$), all of which clustered in group B and possessed *gelE* gene and were resistant to rifampin. In cluster C, the proportions of *E. faecium* ($p = 0.006$) and *E. durans* ($p = 0.012$) were significantly different from other clusters, and the post-hoc test showed that *E. faecium* and *E. durans* in cluster C were specifically different
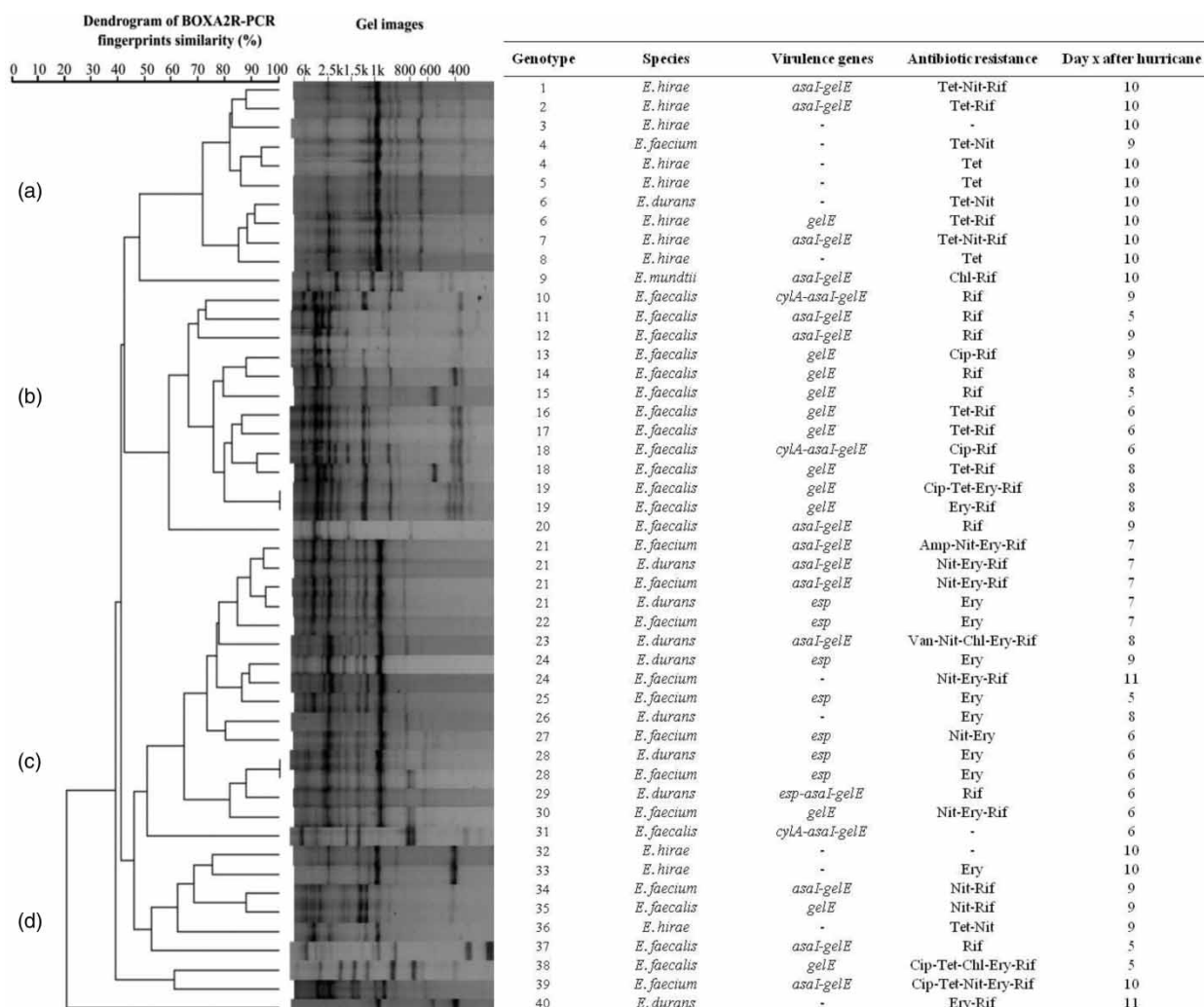
Genotype	Species	Virulence genes	Antibiotic resistance	Day x after hurricane
1	E. hirae	asaI-gelE	Tet-Nit-Rif	10
2	E. hirae	asaI-gelE	Tet-Rif	10
3	E. hirae	-	-	10
4	E. faecium	-	Tet-Nit	9
4	E. hirae	-	Tet	10
5	E. hirae	-	Tet	10
6	E. durans	-	Tet-Nit	10
6	E. hirae	gelE	Tet-Rif	10
7	E. hirae	asaI-gelE	Tet-Nit-Rif	10
8	E. hirae	-	Tet	10
9	E. mundtii	asaI-gelE	Chl-Rif	10
10	E. faecalis	cylA-asaI-gelE	Rif	9
11	E. faecalis	asaI-gelE	Rif	5
12	E. faecalis	asaI-gelE	Rif	9
13	E. faecalis	gelE	Cip-Rif	9
14	E. faecalis	gelE	Rif	8
15	E. faecalis	gelE	Rif	5
16	E. faecalis	gelE	Tet-Rif	6
17	E. faecalis	gelE	Tet-Rif	6
18	E. faecalis	cylA-asaI-gelE	Cip-Rif	6
18	E. faecalis	gelE	Tet-Rif	8
19	E. faecalis	gelE	Cip-Tet-Ery-Rif	8
19	E. faecalis	gelE	Ery-Rif	8
20	E. faecalis	asaI-gelE	Rif	9
21	E. faecium	asaI-gelE	Amp-Nit-Ery-Rif	7
21	E. durans	asaI-gelE	Nit-Ery-Rif	7
21	E. faecium	asaI-gelE	Nit-Ery-Rif	7
21	E. durans	esp	Ery	7
22	E. faecium	esp	Ery	7
23	E. durans	asaI-gelE	Van-Nit-Chl-Ery-Rif	8
24	E. durans	esp	Ery	9
24	E. faecium	-	Nit-Ery-Rif	11
25	E. faecium	esp	Ery	5
26	E. durans	-	Ery	8
27	E. faecium	esp	Nit-Ery	6
28	E. durans	esp	Ery	6
28	E. faecium	esp	Ery	6
29	E. durans	esp-asaI-gelE	Rif	6
30	E. faecium	gelE	Nit-Ery-Rif	6
31	E. faecalis	cylA-asaI-gelE	-	6
32	E. hirae	-	-	10
33	E. hirae	-	Ery	10
34	E. faecium	asaI-gelE	Nit-Rif	9
35	E. faecalis	gelE	Nit-Rif	9
36	E. hirae	-	Tet-Nit	9
37	E. faecalis	asaI-gelE	Rif	5
38	E. faecalis	gelE	Cip-Tet-Chl-Ery-Rif	5
39	E. faecium	asaI-gelE	Cip-Tet-Nit-Ery-Rif	10
40	E. durans	-	Ery-Rif	11

Figure 1 | Cluster analysis of *Enterococci* isolates ($n = 49$) based on the similarity between BOX A2R-PCR fingerprints of each strains, cutoff threshold of 89%. The detailed information of assigned genotype, species, virulence genes, AR profiles, and date of isolation were shown next to the dendrogram.
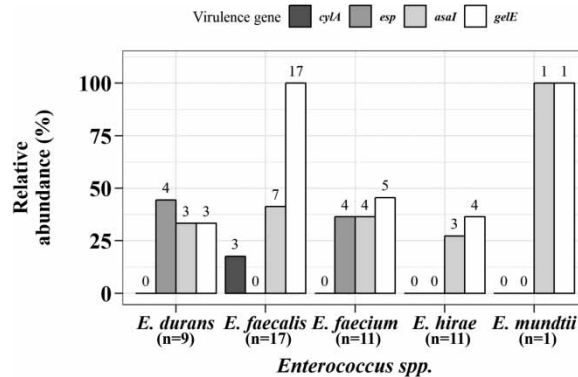
Figure 2 | Relative abundance of virulence genes detected among 49 isolates of *Enterococcus* spp. identified Four virulence genes of *cylA*, *esp*, *asa1*, and *gelE* were identified among *E. durans* ($n = 9$), *E. faecalis* ($n = 17$), *E. faecium* ($n = 11$), *E. hirae* ($n = 11$), and *E. mundtii* ($n = 1$). The numbers of each virulence genes detected in the specific *Enterococcus* spp. were shown on top of the bars.

from cluster B (*E. faecium*: $p = 0.019$; *E. durans*: $p = 0.012$). Eleven genotypes comprised of *E. faecium* ($n = 8$), *E. durans* ($n = 7$), and *E. faecalis* ($n = 1$) were grouped in cluster C, where one strain of *E. durans* which was resistant to vancomycin and one strain of *E. faecium* which was resistant to ampicillin were grouped together in this cluster.

Genotypes 4, 6, 18, 19, 21, 24, and 28 were found to have more than one *Enterococcus* isolate grouped in the same genotype, with Genotype 21 which has the highest number of strains (*E. faecium*, $n = 2$; *E. durans*, $n = 2$) and the strains in this genotype were isolated on Day 7 after Hurricane Lane. Three of the strains in this genotype contained *asa1* and *gelE*, but only one strain contained an *esp* gene. One *E. faecium* in this genotype was found to be resistant to ampicillin and MAR (Amp–Nit–Ery–Rif). Genotypes 4, 6, 24, and 28 contained two different *Enterococcus* species in each genotype, but both species have different virulence genes and AR gene structures. Although, genotypes 18 and 19 contained the two strains of the same species that is *E. faecalis*, both strains in each genotype possess different characteristics of virulence genes and AR gene profiles. Genotype 23 has a single strain of *E. durans* which was found to be resistant to vancomycin and also to MAR (Van–Nit–Chl–Ery–Rif).

Detection and distribution of virulence genes

Four virulence genes (*cylA*, *esp*, *asa1*, and *gelE*) were detected in the *Enterococcus* species isolated from the HBL site ([Figure 2](#)). Most of the *Enterococcus* isolates contained the *asa1* gene (*E. durans*, 3/9; *E. faecalis*, 7/17; *E. faecium*, 4/11; *E. hirae*, 3/11; *E. mundtii*, 1/1) and/or *gelE* gene (*E. durans*, 3/9; *E. faecalis*, 17/17; *E. faecium*, 5/11; *E. hirae*, 4/11; *E. mundtii*, 1/1). Interestingly, the *cylA* gene was only detected in *E. faecalis* (3/17) and the *esp* gene in *E. durans* (4/9) and *E. faecium* (4/11), respectively. Overall, there were 33.3% of *E. durans*, 41.2% of *E. faecalis*, 36.4% of *E. faecium*, and 27.3% of *E hirae* isolates possessing two or more virulence genes. Among the strains, three *E. faecalis* isolates possess *cylA–asa1-gelE* and one *E. durans* possess *esp–asa1-gelE* simultaneously. The rest of the species which possess more than one virulence genes were comprised of *asa1–gelE* that was distributed among the five species.

Antibiotic resistance

AR profiles of the *Enterococcus* isolates to eight different antibiotics are shown in [Figure 3](#). *E. durans*, *E. faecalis*, and *E. faecium* exhibited AR to a maximum of six different kinds of antibiotic. The first, second, third, and fourth highest numbers of isolates resistant to rifampin (31/49 or 63.3%; *E. faecalis* $= 16$, *E. faecium* $= 6$, *E. durans* $= 4$, *E. hirae* $= 4$, *E. mundtii* $= 1$), erythromycin (20/49 or 40.8%; *E. faecium* $= 9$, *E. durans* $= 7$, *E. faecalis* $= 3$, *E. hirae* $= 1$), tetracycline (16/49 or 32.7%; *E. hirae* $= 8$; *E. faecalis* $= 5$; *E. faecium* $= 2$, *E. durans* $= 1$), and nitrofurantoin (15/49 or 30.6%; *E. faecium* $= 8$, *E. durans* $= 3$, *E. hirae* $= 3$, *E. faecalis* $= 1$) were enumerated. Only one *E. durans* isolate was detected to confer resistant to vancomycin, which, however, indicates significance due to VRE being an urgent threat of AR designed by the US CDC ([CDC 2019](#)). This *E. durans* isolate exhibited multiple resistant to five antibiotics (Van–Nit–Chl–Ery–Rif). Only one *E. faecium* was found to be resistant to ampicillin and was multiple resistant to four antibiotics (Amp–Nit–Ery–Rif). Two isolates were also shown to be resistant to five different antibiotics, including one *E. faecalis* isolate (Cip–Tet–Chl–Ery–Rif) and one *E. faecium* isolate
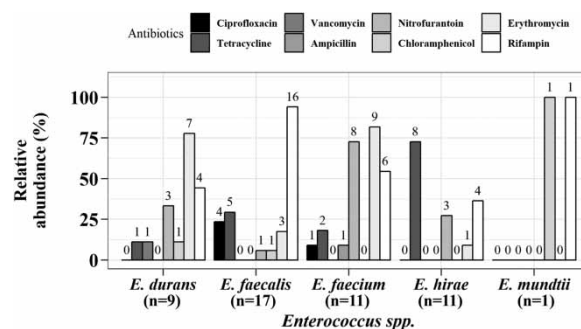
Figure 3 | AR profile of 49 *Enterococcus* spp. to eight different kinds of antibiotic tested (ciprofloxacin, tetracycline, vancomycin, ampicillin, nitrofurantoin, chloramphenicol, erythromycin, and rifampin). The numbers of each *Enterococcus* spp. resistant to each antibiotics were shown by the number on top of the bars.

(Cip–Tet–Nit–Ery–Rif). In total, 44.4% of *E. durans*, 52.9% of *E. faecalis*, 72.7% of *E. faecium*, 45.5% of *E. hirae*, and 100% of *E. mundtii* were found to be resistant to MAR (i.e. resistant to at least two antibiotics).

Co-occurrence frequency of virulence genes and AR

A co-occurrence pyramid based on the Jaccard index value (JIV) was constructed to illustrate the correlation of virulence genes and AR in the *Enterococcus* isolates (Figure 4). Most of the *E. faecalis* isolates possessed the *gelE* gene (JIV: 0.6) while resistant to rifampin (JIV: 0.5). *E. hirae* isolates have a high JIV of 0.4 to tetracycline that is shown in Figure 3 where this *Enterococcus* species has the highest number of isolates resistant to tetracycline. *E. faecium* isolates also have a high JIV to nitrofurantoin (0.4) and erythromycin (0.4) in which the highest number of isolates from this species were found to be resistant to nitrofurantoin ($n = 8$) and erythromycin ($n = 9$). Overall, there were high JIV numbers of the co-
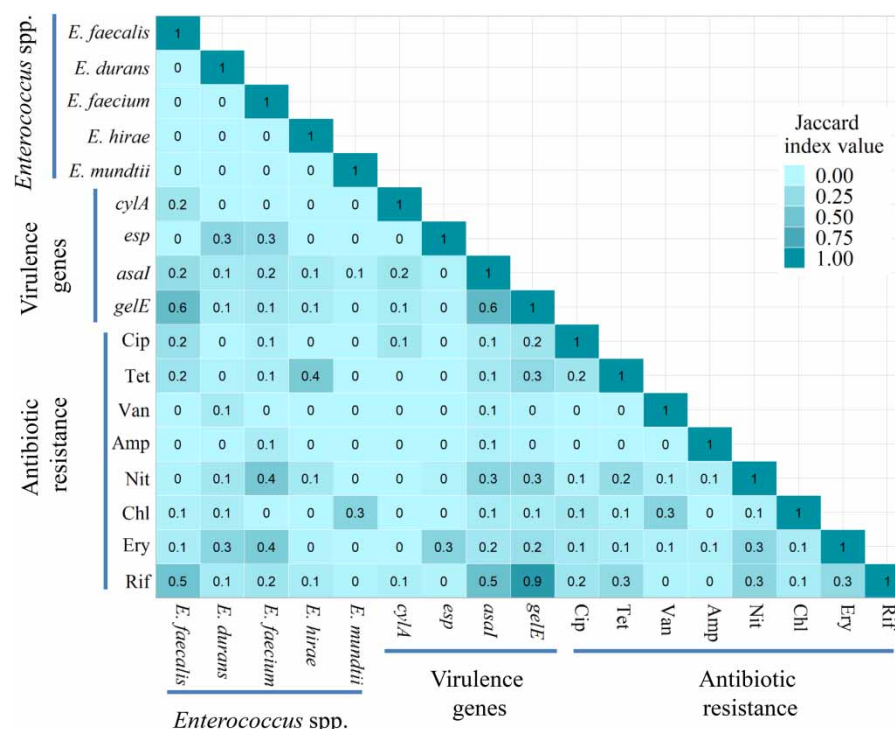

Figure 4 | Matrix showing the co-occurrence frequency of virulence genes ($n = 4$) and the AR profile ($n = 8$) with the five *Enterococcus* spp. calculated by the JIV. A score near 1.0 indicates that the variables are perfectly correlated, whereas a score near 0 indicates that the variables are perfectly inversely correlated. The higher the number, the higher the frequency of co-occurrences.

occurrence of the *gelE* gene (0.9) when the presence of rifampin resistance (0.5) and the *asa1* gene (0.5) was detected simultaneously.

Temporal changes of pathogenic *Enterococcus* spp. after hurricane impact

A temporal change of *Enterococcus* spp. from the fifth day to the tenth day right after Hurricane Lane impact was observed (Figure 5). Overall, *E. faecalis*, *E. faecium*, and *E. durans* were commonly detected in the *Enterococcus* population after the impact of Hurricane Lane. *E. hirae*, on the other hand, was not detected from Day 5 to Day 8, but became more predominant starting on Day 9 (11.1%) and Day 10 (76.9%). The isolates detected on Day 11 were removed from this analysis because the sample number was below 5 ($n = 2$) and was considered as the lack of statistical significance.

DISCUSSION

E. faecalis and *E. faecium* are the two most common *Enterococcus* species causing approximately 87.7 and 8.6% enterococcal infections in clinical settings (Ruoff *et al.* 1990). Other *Enterococcus* species, including *E. durans* (Kenzaka *et al.* 2013), *E. hirae* (Bourafa *et al.* 2015), and *E. mundtii* (Higashide *et al.* 2005), typically contribute to much smaller percentage of enterococcal infections. In this study, 57.1% of the *Enterococcus* isolates obtained in the Hilo Bay water immediately after the Hurricane Lane impact were *E. faecalis* and *E. faecium*, and the remaining 42.9% were *E. durans*, *E. hirae*, and *E. mundtii*. A similar distribution of *Enterococcus* species were also isolated from fresh river water after the storm event, including *E. faecalis* (43%), *E. faecium* (18%), *E. mundtii* (14%), *E. casseliflavus* (12%), *E. durans* (6%), *E. hirae* (7%), and *E. avium* (2%) (Sidhu *et al.* 2014). Furthermore, *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. mundtii* have been identified as the most commonly isolated enterococcal species from urban runoff and receiving water (Moore *et al.* 2008).

Stormwater has been shown to increase the number of enterococcal isolates in the runoff, and the sources of the *Enterococcus* spp. were related to fecal sources. A study done by Hou *et al.* (2006) showed that the discharged floodwaters after the impact of Hurricane Katrina had an elevated number of enterococci counts (170 CFU/100 mL) at stations near the mouth of a canal compared to stations 5 km from the mouth of the canal where the number of enterococci was 5 times lower. Sinigalliano *et al.* (2007) found that after the impacts of Hurricane Katrina and Rita, the number of enterococci was 380 CFU/100 mL at the middle of the main canal discharging water into a nearby lake. They also found that the source of enterococci was from within the city where the number of enterococci in the canal water increased from 93 to 1,400 CFU/100 mL before and after the pumping of the effluent water. Bae & Hou (2013) determined that the sources of enterococci in the Hurricane Katrina floodwater discharges were from fecal contamination by the sequencing results of typical fecal *Enterococcus* spp. isolated from the impacted water.

Enterococcus spp. are an important group of opportunistic pathogens capable of spreading AR at intra- and interspecies levels as demonstrated by the high prevalence of acquired AR (Da Silva *et al.* 2006). The dissemination of AR in environmental *Enterococcus* strains has increased concerns when AR gene transfer was reported in sewage water treatment plants (Tejedor Junco *et al.* 2001). The abundance of enterococcal isolates from different sources has been found to possess AR, including stormwater (Saingam *et al.* 2021), bay and ocean (Moore *et al.* 2008), municipal wastewater (Da Silva *et al.* 2006; Abriouel *et al.* 2008; Moore *et al.* 2008), vegetables and produce (Johnston & Jaykus 2004; Abriouel *et al.* 2008; Igbinosa & Beshiru 2019), and various other water bodies (Tejedor Junco *et al.* 2001; Abriouel *et al.* 2008; Macedo *et al.*
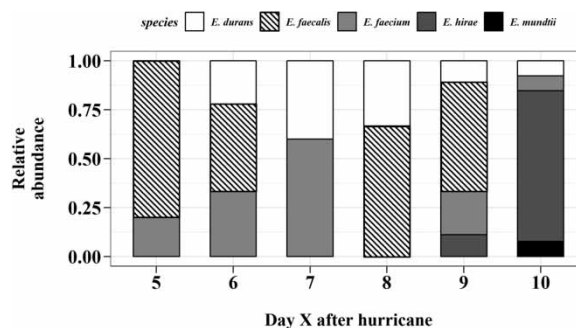

Figure 5 | Temporal changes of *Enterococcus* spp. after the hurricane impact. Sampling started on the fifth day after the heaviest impact of Hurricane Lane where the stream flow was recorded as the highest.

2011). Particularly, VRE is of concern because it is a leading cause of healthcare-associated infection that particularly affects critically ill and immunocompromised patients (Gouliouris *et al.* 2019). The detection of VRE is not limited to the clinical settings, but has been widely found in wastewater treatment plants (Beier *et al.* 2008; Varela *et al.* 2013; Rosenberg Goldstein *et al.* 2014; Oravcova *et al.* 2017; Gouliouris *et al.* 2019). In this study, an isolate of *E. durans* was found to be resistant to vancomycin and four other antibiotics. Vancomycin-resistant *E. durans* was not as common as *E. faecalis* and *E. faecium*, but it has been isolated from stool samples from patients (Cercenado *et al.* 1995) and sewage (Torres *et al.* 1994). *E. durans* were commonly found in human and animal fecal sources (Layton *et al.* 2010), which suggests that the source of the vancomycin-resistant *E. durans* isolated from Hilo Bay could be originated from non-environmental sources impacted by stormwater from Hurricane Lane.

While enterococci in the environment are generally considered to be benign, they can become opportunistic pathogens due to the possession or acquisition of virulent genes and antibiotic-resistant determinants through horizontal gene transfer (Ferguson *et al.* 2016). *E. faecalis* and *E. faecium* in human waste foster higher numbers of virulence genes compared to other sources (Ferguson *et al.* 2016). In this study, 75.5% of *Enterococcus* isolates were found to possess at least one virulence genes. Studies have shown that the most frequently detected virulent genes among environmental enterococcal species impacted by storm events were *gelE* (gelatinase) and *asa1* (aggregation substance) (Macedo *et al.* 2011; Sidhu *et al.* 2014), with *gelE* commonly associated in *E. faecalis* (Creti *et al.* 2004; Sidhu *et al.* 2014; Ferguson *et al.* 2016). However, *gelE* can also be detected in clinical isolates of *E. faecium* and *E. mundtii* (Biswas *et al.* 2016; Shokoohizadeh *et al.* 2018). Our findings indicated that *E. durans*, *E. faecalis*, *E. faecium*, *E. hirae*, and *E. mundtii* isolated from hurricane-impacted water possessed both *gelE* and *asa1*. The *asa1* gene was detected in various clinical enterococcal isolates (Biswas *et al.* 2016) and environmental isolates, except for *E. hirae* (Sidhu *et al.* 2014). The *asa1* gene, which was carried by a sex-phero-mone-containing plasmid, could spread among enterococci through conjugative transfer (Galli *et al.* 1990), and suggests the potential occurrence of horizontal gene transfer in the environment or when there are input of virulence genes from other sources through natural impacts.

The cytolysin gene *cylA*, which can be carried by either chromosome or plasmid elements, was reported primarily in *E. faecalis* isolates from clinical and food origins (Semedo *et al.* 2003; Sidhu *et al.* 2014), but rarely detected in environmental enterococcal isolates or only present in very low abundance (Semedo *et al.* 2003; Lanthier *et al.* 2011; Ahmad *et al.* 2014). Similarly, in this study, the *cylA* gene was only detected in *E. faecalis* isolates from the water environment (17.6%). The common clinically identified enterococcal surface protein, *esp*, was mostly found in *E. faecalis* and *E. faecium* (Willems *et al.* 2001; Biswas *et al.* 2016). However, in this study, the *esp* gene was not found in *E. faecalis*, but instead in *E. faecium* (36.4%) and *E. durans* (44.4%). A study revealed that *esp* genes were more frequently identified in *E. faecalis* (45%) than *E. faecium* (21%) among clinical isolates (Di Rosa *et al.* 2006). On the contrary, as much as 84% of clinical *E. faecium* were confirmed to possess the *esp* gene as compared to *E. faecalis* (33%) (Ferguson *et al.* 2016). A study done by Ferguson *et al.* (2016) found that the *esp* gene was not detected in any of the *E. faecium* isolated from beach water, but the genes are detected in 12% of *E. faecalis* (Ferguson *et al.* 2016). Since the *esp* genes were typically detected in the clinical samples, the *E. faecium* and *E. durans* isolated in this study could suggest the possibility of clinical input via excessive runoff as a result of hurricane impact.

CONCLUSIONS

In this study, we were able to isolate sufficient number of putative pathogenic *Enterococcus* spp. from coastal water immediately impacted by hurricane event, especially those responsible for human enterococcal infections such as vancomycin-resistant *E. durans*. The *Enterococcus* spp. isolated were found to possess prevalent virulence genes and multiple AR profiles that are related to non-environmental sources, suggesting considerable input from urban runoff nearby and from diffused sources of pollution. Temporal changes of *Enterococcus* spp. days after the hurricane impact suggested the ability of these bacteria to survive in the environment for a sustained period of time. Further monitoring of the coastal water quality was needed as the presence of these opportunistic human pathogenic *Enterococcus* spp. could adversely impact the human community health.

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DATA AVAILABILITY STATEMENT

All relevant data are available from an online repository or repositories.

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