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Impact of a Wastewater Treatment Plant on Enterococci Species Distribution in Southwestern Puerto Rico

Armando Román Irizarry [1,†], David Sotomayor-Ramírez [1,*], Luis A. Ríos-Hernández [2], Gustavo Martínez [1], Luis Pérez-Alegría [3] and Elizabeth Padilla-Crespo [4]

[1] Department of Agro-Environmental Sciences, College of Agricultural Sciences, University of Puerto Rico, Mayagüez Campus, Mayagüez, PR 00680, USA; armando.romanir@gmail.com (A.R.I.); gustavo.martinez3@upr.edu (G.M.)
[2] Department of Biology, University of Puerto Rico, Mayagüez, PR 00680, USA; luis.rios5@upr.edu
[3] Department of Agricultural Engineering and Biosystems, University of Puerto Rico, Mayagüez, PR 00680, USA; luisr.perez1@upr.edu
[4] Science and Technology Department, Inter American University of Puerto Rico, Aguadilla, PR 00605, USA; epadilla@aguadilla.inter.edu
* Correspondence: david.sotomayor@upr.edu
† Current address: Department of Homeland Security, Federal Emergency Management Agency, Puerto Rico Recovery Office, Guaynabo, PR 00968, USA.

Abstract

Enterococci are widely used indicators of fecal contamination because they originate in the gastrointestinal tracts of warm-blooded animals, and species-level identification can support source attribution. This study evaluated the temporal abundance and species composition of enterococci in Quebrada Mondongo, southwestern Puerto Rico, a stream influenced by wastewater treatment plant (WWTP) effluent and nonpoint-source inputs. Five sampling campaigns for species distribution and fourteen for population quantification were conducted over approximately one year at the WWTP effluent discharge and at upstream and downstream stations. Enterococci concentrations exceeded the regulatory threshold for surface waters. Among the confirmed isolates, *E. faecium* dominated upstream and in the effluent, occurring approximately twofold more frequently than *E. faecalis*. Downstream, *E. faecalis* increased in relative abundance, shifting the species ratio of *E. faecium*/*E. faecalis* from 2.3–3.2 to 0.89. *E. casseliflavus* was detected at low frequency, and *E. gallinarum* was not observed. Virulence-associated genes (*esp*, *gelE*) were identified in ~75% of *E. faecalis* isolates, consistent with enhanced environmental persistence. Although upstream and effluent patterns reflected a strong human fecal signal, the downstream enrichment of *E. faecalis* suggests additional secondary inputs and/or naturalization. This study provides empirical evidence of species shifts in a tropical stream, with an increase in *E. faecalis* downstream of a WWTP despite *E. faecium* dominance in the effluent highlighting the likely influence of other nonpoint fecal sources within the watershed. Overall, these results suggest that the WWTP effluent did not contribute substantially to enterococci concentrations nor significantly influence the species composition of enterococci downstream in Quebrada Mondongo, highlighting the likely influence of other nonpoint fecal sources within the watershed.

Keywords: enterococci; waste-water; molecular markers

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1. Introduction

A common biological parameter for assessing water quality is the presence of fecal bacteria which have been associated with the presence of specific bacterial organisms such as pathogens or opportunistic bacteria that can affect human health [1]. Epidemiological studies conducted by the United States Environmental Protection Agency (US EPA) demonstrated that enterococci are associated with human illness in sewage-impacted waters [2–4]. Nevertheless, the use of enterococci as fecal indicators has been challenged because they can originate not only from human fecal material but also from other warm-blooded animals [5] and can survive and multiply outside their hosts [6,7], including tropical soils [8] and freshwater sediments [9]. Enterococci have also been shown to persist on planktonic particles and aquatic plants, further complicating their interpretation as fecal indicators [10]. Therefore, high concentrations of enterococci in water may not necessarily imply recent human fecal contamination, leading to potential misclassification of waterbody quality.

Molecular advances for species differentiation within the Enterococcus genus offer new alternatives for fecal source identification [11]. Species-level classification enables more accurate discrimination between human fecal contamination and other sources [12]. For example, *E. faecium* and *E. faecalis* are more prevalent in human feces [13] whereas other species such as *E. mundtii, E. casseliflavus* and *E. gallinarum* are more abundant in other animals or the natural environment [14–17]. This host–species relationship can help link detected enterococci to specific sources such as humans, livestock, domestic pets, or wildlife, particularly when evaluated alongside local land use patterns. Based on this established host–species relationship, we hypothesized that wastewater treatment plant (WWTP) effluent would represent a significant source of enterococci to receiving waters, with *E. faecium* as the dominant species.

Molecular tools for species identification and microbial source tracking can reduce misinterpretations stemming from free-living or environmental enterococci, aiding in improving the classification of waterbodies with respect to fecal pollution. Recent advances in quantitative PCR, next-generation sequencing, and marker gene detection have substantially increased the resolution and accuracy of these methods [18,19]. Microbial source tracking (MST) is a suite of techniques in environmental microbiology that is based on the principle that distinct microbial communities dominate specific hosts due to differences in diet and physiology [20,21]. Early MST studies relied primarily on culture-dependent methods, but advances in molecular biology have enabled the combined use of culture-independent techniques, including metagenomics and whole-genome sequencing, which enhance specificity and reduce detection biases [22–26].

Puerto Rico's water quality standards state that enterococci densities shall not exceed 35 colonies/100 mL for SD (SD class waters are surface waters intended for use as a raw source of public water supply, propagation and maintenance of desirable species, including threatened or endangered species, as well as primary and secondary contact recreation) class waters [27]. High fecal coliform concentrations are the primary cause of surface water quality impairment [27]. The Lajas Valley, located in the southwest part of the island, covers approximately 155 square kilometers. Due to low annual precipitation and high evapotranspiration rates, most of its creeks are ephemeral and drainage channels have been modified. The current irrigation–drainage network and infrastructure were constructed in 1952 [28]. The Lajas Valley watershed drains from sub-basins with diverse land uses, including agricultural, urban, and sub-urban areas. Sotomayor-Ramirez et al. [29] reported high enterococci concentrations and nutrient enrichment in the rivers and creeks of these sub-basins, which receive water inputs from sewered and unsewered homesteads as well as a wastewater treatment plant (WWTP).

The objective of this study was to assess the presence and distribution of enterococci species in the drainage channel of Quebrada Mondongo in the Mondongo sub-basin, upstream and downstream of the Lajas WWTP, using a microbial source tracking approach. The findings are intended to support improved water quality monitoring and source attribution in tropical regions, where recent research has emphasized the importance of distinguishing persistent, environmental enterococci from true fecal signals [18,19,30].

2. Materials and Methods

2.1. Site Description and Sampling

The study site was in the upper reaches of Quebrada Mondongo, a second order stream draining Mondongo sub-basin (area of 1543 ha) in Lajas municipality, Puerto Rico. The predominant land uses upstream of the sampling stations were approximately 21% urban, 31% pasture for haylage or grazing, 30% unmanaged shrubland and secondary forest, 18% row crops. The main urban area of Lajas is within the Mondongo sub-basin (Table S1). Mean annual precipitation in the area ranges from 89 to 107 cm.

Quebrada Mondongo receives water input from Lajas WWTP (NPDES Permit No. PR0020575), located along State Road #101, km. 23.7. The WWTP effluent contributes, on average (three-year 2013 to 2015 mean), 1.45×10^6 m^3/yr (or 1.05 million gallons per day, MGD) of secondary/tertiary treated wastewater. The WWTP presumably treats sanitary wastewater through advanced secondary treatment with nutrient removal achieving 85% removal of BOD and TSS. The plant operates as a sequential batch treatment [31], where raw sewage is mixed with the residual sludge in primary holding tanks. After initial treatment, the batch passes a secondary settling tank, followed by chlorination and UV irradiation, before discharge into Quebrada Mondongo.

Raw water volume inputs to the facility fluctuate seasonally, in response to tourist influx in the area. This implies that treatment batches may have heterogeneous chemical or biological composition in the effluent outfall. The WWTP serves between 1736 and 2000 households, or between 6944 and 8000 people, from the urban portion of Lajas municipality as well as the Parguera and Boquerón sectors. Within Mondongo sub-basin, only parts of downtown Lajas are connected to the sewage system and some structures still depend on septic systems for wastewater disposal or discharge gray water through open-pipe outfalls. A ground-truthing survey identified active open-pipe outfalls upstream of the sampling sites in Barrio La Haya and sectors El Tendal and Corillo. The WWTP provides a significant volume of water to Quebrada Mondongo. The mean WWTP contribution to Mondongo stream discharge is about 42% of the mean flow and about 76% during base flow [29]. Thus, any changes in enterococci concentrations in WWTP outfall will significantly impact water quality downstream, either by enrichment or dilution of enterococci concentrations or species distribution.

Sampling points included the upstream site before the WWTP effluent discharge (Station #34; 18.048087, −67.060913), the WWTP effluent outlet before contact with the creek (Station WWTP; 18.047465, −67.061449) and the downstream site within the creek after the WWTP mixing zone (Station #1; 18.046562, −67.060934) (Figure 1). Sampling stations were selected to allow comparison of background conditions upstream of the wastewater discharge, the treated effluent at the point of release, and conditions downstream after mixing within the stream. This design enabled evaluation of the relative contribution of the WWTP to enterococci concentrations and species composition. Surface water samples were collected at the WWTP effluent outlet fourteen times from 20 September 2016 to 18 January 2018 for enterococci abundance and five times from 30 January 2017 to 18 January 2018 for species distribution in all stations. Although the number of sampling

dates was reasonable the temporal variation in enterococci species distribution may not have been fully characterized.

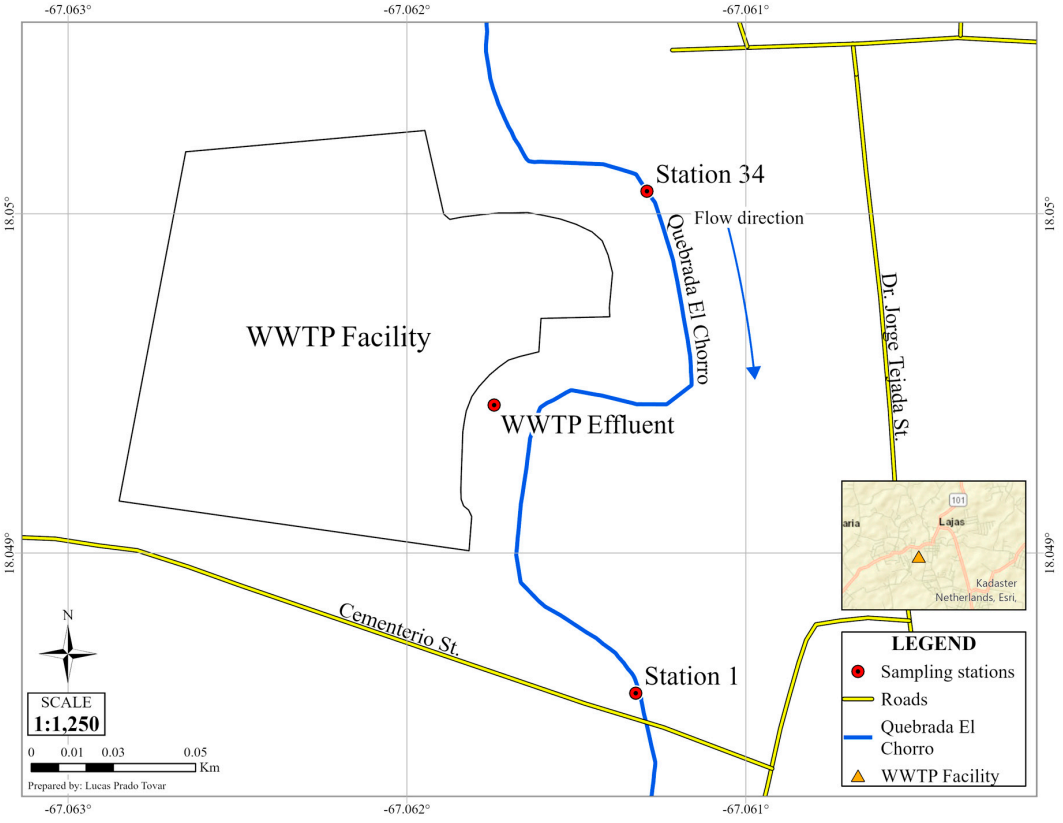


Figure 1. GIS-based rendition of sampling stations: Station #34, upstream of the WWTP (18.048087 N, −67.060913 W; Station WWTP effluent outlet before contact with the creek (18.047465 N, −67.061449 W); and Station #1, the downstream site after the WWTP mixing zone (18.046562 N, −67.060934 W). The inset shows the WWTP in relation to the town of Lajas.

During each visit, surface water samples were manually collected in triplicate using clean, sterile 500 mL polypropylene Nalgene® bottles (Rochester, NY, USA), between 9:00 and 10:00 a.m. during baseflow conditions. Bottles were opened and submerged beneath the water surface, facing upstream against the current. Collected samples were immediately placed on ice at 4 °C and transported to the laboratory, where they were processed within 4 h. For every sampling event, streamflow was measured, considering the cross-sectional area and water depth, using a Flowwatch® flow meter (JDC Electronics, Switzerland). WWTP discharge flow data for the Lajas WWTP (PR0020575) effluent outlet on the sampling dates were obtained from the PRASA Office of Compliance. Total nitrogen (N), total phosphorus (P), suspended sediments, dissolved oxygen and dissolved nitrate-N were quantified as specified by Sotomayor-Ramírez et al. [29].

2.2. Bacteria Enumeration

Enterococci abundance was enumerated using the membrane filtration method USEPA 1600 [32]. Briefly, appropriate sample volumes were filtered through sterile 0.45 µm membrane filters, which were then placed onto mE agar (BD Difco™, Franklin Lakes, NJ, USA), and incubated at 41 °C for 24 h. Typical enterococci colonies presenting a blue halo were counted and reported as colony-forming units (CFUs) per 100 mL. Plates on which colonies could not be reliably counted or distinguished or where counts exceeded 250 counts at up to 10^{-1} dilutions were denoted as "Too Numerous to Count" (TNTC). Reported colony forming unit (CFU) values represent the average of three sub-samples.

2.3. Preliminary Bacterial Identification with Culture-Dependent Biochemical Tests

Tests were conducted to preliminarily identify the species of isolated samples and to group them to enhance the specificity of subsequent molecular methods [33]. Samples that grew on mE selective media and Bile Esculin Agar (BEA) BD Difco™ (Franklin Lakes, NJ, USA), but were catalase positive or did not hydrolyze esculin were designated as false positives. Twenty-four purple colonies from a single mE plate were randomly chosen for isolation (this was done for all 3 locations, and during 5 sampling events for a total of 360 isolates). Individual colonies were transferred to Brain Heart Infusion (BHI) (BD Difco™, Franklin Lakes, NJ, USA) agar to increase biomass. Streak plates were then prepared for each isolate to ensure purity. The isolated colonies were subsequently transferred to BEA to observe esculin hydrolysis. After a positive BEA test, isolates grown in BHI media were tested individually for (i) catalase, (ii) pigmentation, and (iii) motility in Sulfide–Indole–Motility (SIM) medium (Figure S1).

2.4. Molecular Techniques, DNA Extraction and Template Preparation

Isolated enterococci were grouped based on their characteristics, pigmented motile, pigmented non-motile, and non-pigmented non-motile, for DNA extraction following biochemical tests [33]. This grouping and differentiation aided in species identification by PCR using *sodA*-gene-specific primers. DNA extraction was performed using a heat shock cell lysis. The resulting mixture was then subjected to purification using physical methods to separate the DNA from other intracellular and membrane components.

For DNA template preparation, isolates were first grown in Tryptic-soy Broth (TSB) BD Difco™, Franklin Lakes, NJ, USA, media for 24 h. Then, 1.5 mL of the broth culture was centrifuged at 13,000 rpm for 5 min to pellet the bacterial cells. The supernatant TSB medium was carefully discarded without disturbing the pellet. The pellets were then resuspended with 500 µL of molecular-grade distilled water. The tubes were then incubated in a water bath at 95 °C for five minutes, followed by centrifugation at 13,000 rpm for 5 min. The resulting supernatant was transferred to new 1.5 mL tubes and stored at 4 °C. This final liquid suspension was used as the DNA template for subsequent analyses.

2.5. Multiplex PCR for the Superoxide Dismutase (sodA) Gene

A multiplex PCR approach developed by Jackson et al. [34] was employed to provide simultaneous genus and species identification of four enterococci species, with some modifications. The PCR master mix consisted of 18.75 µL of ddH_2O, 10 µL of 5× Taq polymerase Buffer (Promega, Madison, WI, USA), 5 µL of 25 mM $MgCl_2$, 5 µL of 16 mM ammonium sulfate, 2 µL of 2.5 mM dNTP's, 3.75 µL total of selected *Enterococcus* species primers (16 µM), and 0.5 µL of Taq polymerase (5 U/µL, Promega, Madison, WI, USA,) for a total of 45 µL. Specifically, 0.625 µL of each primer targeting *E. faecalis* and *E. casseliflavus* was included, and 1.25 µL of each primer targeting *E. faecium* and *E. gallinarum* was added. The final PCR reaction volume was brought to 50 µL with 5 uL of DNA template. Only two of the seven reactions established in this procedure were performed, and only two primers of each group were used (a total of four primers), because not all *Enterococcus* species were of interest. The selected primers for Group 1 differentiated between *E. faecalis* and *E. faecium* (Table S2), and for Group 2 between *E. casseliflavus* and *E. gallinarum* (Table S3).

2.6. Genus-Specific Tuf Gene and Virulence Factors (Esp and gelE) PCR

Samples that did not amplify with the four selected *sodA* gene primers were subjected to *tuf* gene PCR to confirm whether they belonged to the *Enterococcus* genus [35]. These primers (Table S4) amplify an *Enterococcus*-specific region that is 112 bp long. Additionally,

a virulence factors assay targeting the *esp* gene and *gelE* gene was performed using primers developed by Vankerckhoven et al. [36] (Table S5).

2.7. PCR Product Analysis

Polymerase chain reaction products were observed in 1.8% agarose gels stained with ethidium bromide. PCR reaction products (6 µL) were deposited in the agarose gel wells and electrophoresed at 100 volts for 1 h. Agarose gels were visualized under UV light and photographed using a Molecular Imager VersaDoc™ MP 5000 System (Bio-Rad, Hercules, CA, USA). The molecular marker used was 100 bp DNA ladder (New England Biolabs®, Ipswich, MA, USA) mixed with Purple Gel Loading Dye (6×). *E. faecalis* ATCC 19,433 and *E. faecium* ATCC 19,434 were used as positive controls for the Jackson Multiplex Group #1. *E. casseliflavus* ATCC 49,573 and *E. gallinarum* CDRR005 were used as controls for Group #2. *E. faecalis* ATCC 19,433 was used as a positive control for *tuf* gene PCR.

2.8. Data Analysis for Enterococci Concentrations

Statistical analyses were performed using Infostat® (Version 2014) [37]. In some cases, enterococci enumeration exceeded the quantification limit, which could underestimate CFU concentration values. In these instances, the maximum concentration (>2500 CFU/100 mL) was recorded. As the data did not exhibit normality using Shapiro–Wilk test, and because maximum values recorded may underestimate the true concentration, the data were analyzed using the non-parametric Kruskal–Wallis H-test to test the effect of sampling station. *Enterococci* species data were analyzed using a one-way ANOVA, with sampling stations considered as treatment and sampling dates as replicates, to compare species abundance between the two sampling sites (upstream and downstream of the WWTP) and to evaluate the effects of the WWTP effluent direct discharge. Mean separation was performed using Fishers' LSD test, with a probability value of <0.05 considered statistically significant. Enterococci concentrations from the periodic sampling in the WWTP effluent and stream water at specific sampling stations were correlated with streamflow and one-, two-, or three-day antecedent precipitation [38]) using Spearman rank correlation.

3. Results and Discussion

In general, water quality deteriorated downstream of the WWTP. Water temperature, specific conductance, hydrologic discharge, total N, total P, and suspended sediments were higher and dissolved oxygen and dissolved nitrate-N were lower downstream of the WWTP (Table 1).

Table 1. Water quality parameters of upstream station (Station #34) and downstream station (Station #1) relative to the WWTP in Quebrada Mondongo. Values are the means of fourteen measurements.

Indicator	Units	Upstream (Station #34)	Downstream (Station #1)
Temperature	°C	25.4	27.8
pH		7.8	7.8
Specific conductance	µS/m	724.4	2570.4
Hydrologic discharge	10^3 m^3/d	0.78	1.64
Dissolved oxygen	mg/L	5.9	5.4
Total N	mg/L	2.20	3.19
Dissolved nitrate-N	mg/L	1.97	1.30
Total P	mg/L	0.112	0.632
Suspended sediments	mg/L	2.29	5.01

3.1. Enterococci Abundance

Outfalls of WWTP do not have enterococci concentration standards in their National Pollutant Discharge Elimination System (NPDES) permits; however, as a general reference, five out of fourteen sampling events exceeded the Puerto Rico enterococci standards for SD waters of 35 cfu/100 mL [39] (Figure 2). One-, two-, and three-day antecedent rainfall were significantly ($p < 0.05$) positively correlated with both WWTP effluent flow ($r > 0.90$) and with enterococci concentrations in the effluent ($r > 0.54$). In Puerto Rico, sanitary sewer systems are intended to be separated from stormwater inputs; however, runoff and infiltration entering the collection system can increase hydraulic loading, shorten retention time within treatment units, and reduce disinfectant contact time, potentially decreasing the efficiency of bacterial pathogen removal [40].

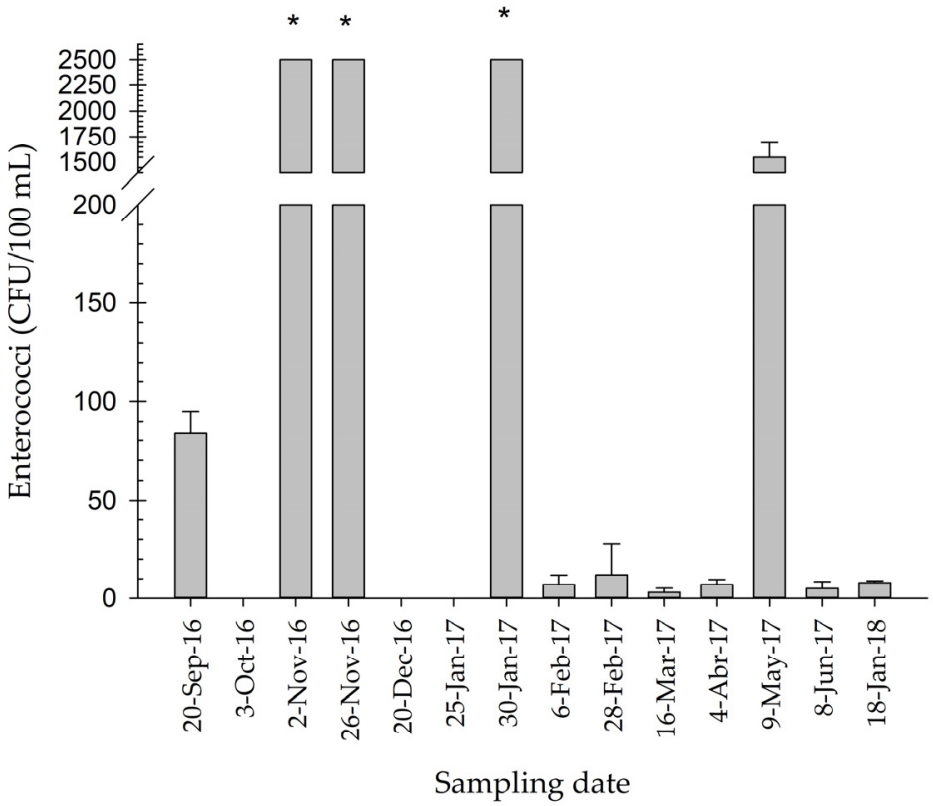


Figure 2. Mean enterococci species abundance at the WWTP effluent; * indicates values above the method detection limit; error bar denotes ±1 standard deviation.

It was hypothesized that Station #1 (downstream of WWTP) would show a substantial increase in enterococci concentrations due to bacterial contributions from the WWTP outfall located upstream of this station. However, this hypothesis was not supported by the observed data, as comparisons between the upstream station (#34, median of 1285 cfu/100 mL) and downstream station (#1, 1467 cfu/100 mL) were non-significant ($p > 0.05$). Nevertheless, the consistently high concentrations of enterococci observed in surface waters such as Quebrada Mongondo (Table 2) should be a cause for water quality concern among sub-basin stakeholders. The mean streamflow was significantly higher downstream at Station #1 (0.10 m^3/s) than upstream at Station #34 (0.04 m^3/s). This difference was likely due to the WWTP effluent, which contributed an estimated mean of 0.03 m^3/s during the sampling events. Any potential dilution of enterococci from the WWTP effluent appears to be offset by other external inputs to the stream after the effluent.

Table 2. Enterococci abundance measured during simultaneous sampling at three stations relative to the WWTP (upstream, outfall, and downstream) in Quebrada Mondongo, Lajas Valley watershed.

Station	30 January 2017	28 February 2017	9 May 2017	8 June 2017	18 January 2018	Median
			CFU/100 mL			
Upstream (#34)	>2500 [1]	>2500 [1]	833	>2500 [1]	207	1708
WWTP	>2500 [1]	12	1550	5	8	76
Downstream (#1)	>2500 [1]	>2500 [1]	1250	1985	213	1689

Note: [1] Values with > represent TNTC samples exceeding 250 colonies per Petri dish.

3.2. *Enterococci spp.* Identification

Of the 360 isolated colonies (24 from each of three sites, collected during five sampling events), 95% (342 colonies) were presumed to be *Enterococcus*, based on the growth on mE selective media and BHI agar, positive esculin hydrolysis, and absence of catalase activity after exposure to hydrogen peroxide (Table 3). These 342 isolates were evaluated using species-specific *soda* primers developed by Jackson et al. [34]. To guide PCR analysis, isolates were grouped based on phenotypic characteristics. Group 1 included non-pigmented, non-motile isolates and were tested with primers targeting *E. faecalis* and *E. faecium*. Group 2 included pigmented, motile isolates and were tested with primers targeting *E. gallinarum* and *E. casseliflavus*. Group 1 accounted for 249 of the 342 presumptive *Enterococcus* colonies. Group 2 accounted for 32 isolates out of 342 (or 8.8% of the isolates), all of which exhibited motility and pigmentation. PCR analysis of Group 1 showed that *E. faecium* was the most abundant species, appearing at twice the frequency of *E. faecalis*. PCR analysis of Group 2, which aimed to differentiate between *E. gallinarum* and *E. casseliflavus*, yielded amplicons only for *E. casseliflavus*; no isolates amplified with the *E. gallinarum*-specific primers.

Table 3. Enterococci species distribution at upstream and downstream stations and the WWTP outfall in Quebrada Mondongo, Lajas Valley watershed, from January 2017 to January 2018.

	E. faecalis	*E. faecium*	*E. casseliflavus*	Non-Amplified with *E.* spp. Species Specific Primer	Amplified with Tuf Gene Primer	False+	Total
Isolates	80	149	52	61	54	25 [2]	360
Relative frequency % [1]	22.2	41.4	14.4	16.9	15.0	6.9	100.0

Notes: [1] The relative frequency was calculated based on the total number of colonies isolated (24 isolates per site, three sites across five sampling events, for a total of 360). [2] This total includes seven isolates that did not amplify with the *tuf* gene primer and 18 isolates that were not selected based on biochemical criteria.

Of the 342 presumed *Enterococcus* isolates, 61 did not amplify with the Jackson multiplex PCR primers employed. These 61 non-amplified isolates were then subjected to *tuf* gene amplification [35] to verify whether these isolates belonged to the *Enterococcus* genus, but not to the four species targeted by the Jackson et al. primers [34]. Of these, 54 isolates did amplify with the *tuf* gene primers, confirming their identity as *Enterococcus*. Therefore, seven of the 342 presumptive isolates (based on biochemical tests) were determined to be false positives and not members of the *Enterococcus* genus.

In summary, of the 360 colonies that grew on mE selective media, 342 were presumptive *Enterococcus* isolates based on biochemical testing. Of these, 281 (79%) were confirmed as *Enterococcus* at the species level using the *soda* primers developed by Jackson et al. [34]. Of the 61 isolates that did not amplify with Jackson primers, 25 isolates were identified as false positives, 18 failed to grow in selective media and did not meet the biochemical criteria, and seven did not amplify with either the Jackson primers or the *tuf* gene genus-specific PCR.

The remaining 54 isolates were confirmed as members of the *Enterococcus* genus but did not belong to the four species targeted by the Jackson primers (*E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum*) (Table 3). Among these 54, nine exhibited motility and yellow pigmentation. Since they did not amplify with the primers targeting *E. casseliflavus*, they could potentially be *E. sulfureus*, *E. flavescens*, or *E. mundtii*, as only three *Enterococcus* species have been reported to produce yellow pigmentation [41]. The remaining 45 isolates could not be identified.

3.3. Enterococci Species Frequency and Abundance

The species frequency and abundance data from this study revealed that *E. faecium* was relatively more abundant than *E. faecalis* at both the upstream station (Station #34) and directly at the WWTP outfall (Table 4). However, downstream at Station #1, the pattern shifted: *E. faecalis* increased in both frequency and abundance, while *E. faecium* tended to decline. The abundance of *E. casseliflavus* remained low at the WWTP outfall but was comparable upstream and downstream, suggesting that the WWTP effluent did not significantly contribute to *E. casseliflavus* levels. This species distribution suggests dynamic microbial community shifts influenced by point and nonpoint fecal inputs as well as environmental persistence traits.

Table 4. Median number of isolates and species diversity across sampling sites of Quebrada Mondongo within the Lajas Valley Watershed.

	E. faecalis		***E. faecium***		***E. casseliflavus***		**Non-Amplified [4]**	
	# isolates [1]	% diversity [2]	# isolates	% diversity	# isolates	% diversity	# isolates	% diversity
Upstream (#34)	5b [3]	18.3	11a	44.2	5	23.3	n/a [5]	14.2
WWTP	4b	17.6	12a	57.4	1b	3.7		21.3
Downstream (#1)	8a	32.8	7a	29.3	4ab	18.1		19.8

Notes: [1] Number of isolates within sites is based on a median of 24 isolates for each of the five sampling events. [2] Diversity within sites is based on 24 isolates for each of the five sampling events. [3] Medians with different letters for number of isolates within a species are significantly different at $p < 0.05$, as determined with Fisher's LSD test on $\log_{10}$-transformed data. [4] Other Enterococcus species not amplified for the four primers employed. [5] Not available.

This observed pattern is consistent with global trends but also highlights regional complexity in *Enterococcus* species distribution. In several studies, *E. faecium* has been reported as the dominant species in treated wastewater effluents, especially in developed regions. For instance, Ferguson et al. [16], using comparable methodologies, reported that *E. faecium* was more prevalent than *E. faecalis* (44% vs. 13%) in U.S. treated sewage. Similar trends were observed in Spain [42], Sweden [43], and other U.S. studies [44], where *E. faecium* predominated in treated wastewater.

E. faecalis is typically the most abundant *Enterococcus* species in the human gut microbiota [13,45] and often dominates in municipal wastewater [46]. Hamiwe et al. [47], in South Africa, observed that *E. faecalis* was more prevalent in the wastewater samples as compared to *E. faecium*. The study by Iweriebor [46] and colleagues in Alice, Eastern Cape (South Africa), found *E. faecalis* to be more prevalent than *E. faecium* in WWTP-influenced environments—a result attributed to its higher representation in both human and animal fecal inputs. Interestingly, in Tunisia and the UK, *E. faecium* was found to be more dominant [43,48], showing that species ratios vary geographically depending on population microbiota, land use, and wastewater treatment characteristics.

We hypothesized that there would be a greater human fecal signal (a greater predominance of *E. faecium*) at Station #34 and WWTP outfall, which would be reduced due to dilution or non-survival downstream. Upstream of Station #34 there were residential buildings suspected of discharging graywater (from laundry, sinks, showers and not from toilets),

many of which relied on septic systems. Numerous open-pipe outfalls were observed in the northern and southern parts of this basin, suggesting the presence of wastewater and potential septic-point source discharges (as described below). Dense algae growth was observed both upstream and downstream of the WWTP during all sampling events. The ratio of *E. faecium* to *E. faecalis* was 2.3 and 3.2 in Station #34 and WWTP effluent, respectively, while 0.89 in Station #1. In our study, the shift from upstream *E. faecium* dominance to downstream *E. faecalis* prevalence could indicate secondary non-traditional or anthropogenic fecal input, or reflect the greater environmental persistence of *E. faecalis*, which is known to form biofilms and survive better under environmental stressors [49]. Thus, species-level profiling can help infer the nature and source of fecal pollution more accurately than genus-level indicators alone.

The WWTP is an important discharge source to Quebrada Mondongo. Based on published daily discharge data during 2016 [50] it is estimated that the WWTP contributed between 42% and 57% of the Mondongo stream's total discharge. Sotomayor-Ramírez et al. [29] reported that the WWTP contributed substantial mass loads of nitrogen and phosphorus to Quebrada Mondongo where total N concentrations immediately downstream of the WWTP discharge point increased sixfold compared to upstream and reference stations, respectively, while total P concentrations increased twofold and 6.6-fold, respectively, compared to upstream and reference stations. The downstream increase in *E. faecalis* may reflect nutrient-driven selection, as this species is more environmentally persistent, metabolically versatile, and capable of biofilm formation than the more host-associated *E. faecium*.

Also, though the diversity and abundance of *E. faecalis* concentration at the WWTP were relatively low, the large discharge volume of the WWTP, combined with enhanced persistence due to surface adhesion, may explain the increase in *E. faecalis* downstream. *E. faecalis* possesses the ability to adhere to surfaces and colonize by expressing the *gelE* and *esp* virulence genes, which aid in surface adhesion and biofilm formation [51–53]. Furthermore, these genes were observed in 75% of all *E. faecalis* isolates obtained from the three sampling sites, as well as in biofilms extracted on an exploratory basis from cobbles and rocks directly beneath the WWTP outfall, which may support the genomic potential for the persistence of this species downstream

Beyond persistence, these traits also raise the possibility of naturalization, meaning that *E. faecalis* may establish a stable, self-sustaining population within the creek, independent of continuous fecal inputs. Such naturalized populations could confound water quality assessments by masking signals of recent fecal contamination and reducing the reliability of *E. faecalis* as a fecal indicator. Studies have shown that *E. faecalis* can enter the viable but nonculturable state, adhere to abiotic and biotic surfaces (e.g., chitin, plankton), and persist in aquatic environments over long periods [10,54,55]. These findings provide empirical support for the possibility that *E. faecalis* may become naturalized in stream systems, maintaining populations even in the absence of recent fecal contamination. This highlights the importance of incorporating molecular species identification and virulence profiling when interpreting enterococci data in tropical and subtropical environments, where favorable conditions for persistence and naturalization may be common. In addition to serving as a fecal indicator, *E. faecalis* is often part of microbial communities within wastewater environments, where some strains may persist without representing immediate health concerns. However, the species is also recognized for carrying virulence traits that enhance survival and attachment. Evaluating its role in treatment systems therefore requires consideration of both ecological function and pathogenic potential.

The occurrence of virulence-associated genes in environmental enterococci is particularly relevant in the context of a growing global population and the increasing demands placed on wastewater infrastructure. The detection of *esp* and *gelE* in a high proportion

of *E. faecalis* isolates suggests that receiving waters may function not only as indicators of fecal contamination, but also as environments where bacteria with enhanced survival and attachment capabilities can persist. These findings reinforce the importance of integrating species-level and genetic information into water quality management strategies.

The low abundance of *E.casseliflavus* at the WWTP outfall effluent suggests that the WWTP discharge did not contribute to nor alter *E. casseliflavus* concentrations downstream. This trend of higher abundance upstream of the WWTP has been observed in other studies where *E.casseliflavus* tends to predominate in urban runoff rather than in sewage water [56]. Upstream of Station #34, we observed many homesteads and buildings with open-pipe conduits that apparently discharged directly into the creek. A major portion of the Lajas municipality is located upstream of the sampling stations. Although the area is sewered, we are not aware of how many of the households are connected. Further, many household facilities keep small domestic livestock and fowl on their properties near the stream bed which may contribute to enterococci loads.

4. Conclusions

Enterococci abundance and species composition were evaluated upstream and downstream of the wastewater treatment plant (WWTP) discharge in Quebrada Mondongo in southwestern Puerto Rico. Overall, concentrations consistently exceeded the regulatory threshold for SD class waters at all stations, indicating widespread fecal contamination. However, despite the occasional increases associated with higher flows, no significant differences in the total enterococci abundance were detected between upstream and downstream sites.

Species-level analysis showed that *E. faecalis* and *E. faecium* dominated throughout the system. A modest but consistent downstream increase in *E. faecalis* relative to *E. faecium* suggests that contributions are likely from secondary nonpoint inputs or the enhanced environmental persistence of *E. faecalis*, a species known for its tolerance to stress and biofilm-forming capacity. These results indicate that factors beyond the WWTP effluent likely play an important role in maintaining bacterial levels within the watershed.

The presence of virulence-associated markers such as *esp* and *gelE* further emphasizes that the evaluation of water quality should extend beyond total counts to include species distribution and genetic traits. Integrating molecular confirmation with culture-based methods improved accuracy, revealing that presumptive identification alone overestimated enterococci occurrence. Such approaches are particularly valuable in tropical environments where environmental persistence may influence indicator behavior.

As wastewater systems evolve toward greater sustainability and resource recovery, maintaining microbiological safeguards will remain essential. Incorporating species- and gene-level surveillance can strengthen risk interpretation while supporting adaptive management strategies aimed at protecting public health.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/w18080904/s1, Figure S1. Preliminary biochemical test identification workflow. Table S1. Land use description in Mondongo sub-basin; Table S2. Primers used for Group 1 *sodA* gene amplification in *Enterococcus* spp., based on Jackson et al. (2004) [34]; Table S3. Primers used for Group 2 *sodA* gene amplification in *Enterococcus* spp., based on Jackson et al. (2004) [34]; Table S4. Primers used for *tuf* gene amplification of *Enterococcus* genus, based on Ke et al. (1999) [35]; Table S5. Virulence factors primers employed for gelatinase production and enterococcal surface protein genes.

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Writing—review and editing, D.S.-R., L.A.R.-H., G.M., L.P.-A. and E.P.-C.; Supervision, D.S.-R.; Funding acquisition, D.S.-R. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript

BEA	Bile Esculin Agar
BHI	Brain Heart Infusion
BOD	Biochemical Oxygen Demand
CFU	Colony-Forming Unit
DNA	Deoxyribonucleic Acid
esp	Enterococcal surface protein gene
gelE	Gelatinase gene
LSD	Least Significant Difference
Mondongo Basin	Basin in the municipality of Lajas, Puerto Rico, characterized by a mix of intense urban and minor agricultural land use
MGD	Million Gallons per Day
mE	mEnterococcus agar
MST	Microbial Source Tracking
NPDES	National Pollutant Discharge Elimination System
PREQB	Puerto Rico Environmental Quality Board
PRASA	Puerto Rico Aqueduct and Sewer Authority
Quebrada Mondongo	A secondary stream in the Lajas Valley of southwestern Puerto Rico
SD	Saline Surface water classification
SIM	Sulfide–Indole–Motility
sodA	Superoxide dismutase gene
TNTC	Too Numerous to Count
TSB	Tryptic Soy Broth
TSS	Total Suspended Solids
tuf	Elongation factor tuf gene
UV	Ultraviolet
WWTP	Wastewater Treatment Plant

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