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Phenotypic Virulence Potential of Bacterial Isolates from Ada River in Okada, Edo State, Nigeria

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ABSTRACT

Ada River is an important freshwater resource whose microbiological quality may be influenced by human activities and environmental contamination. Assessing bacterial loads and the characteristics of resident bacteria is therefore essential for evaluating potential public-health risks. This study assessed bacterial abundance, diversity, and phenotypic virulence characteristics along different sections of the river using samples collected from upstream, midstream, and downstream locations during two sampling visits. Heterotrophic plate counts (HPC) were highest at the midstream section (145.00 ± 13.23 CFU/mL), followed by downstream (95.00 ± 22.64 CFU/mL) and upstream (46.88 ± 16.25 CFU/mL). Sampling location significantly influenced HPC [F (2,6) = 14.01, p = 0.01], whereas visitation and the interaction effect were not significant. Total coliform counts varied between 20.0 and 47.5 CFU/mL, but neither sampling location nor visitation significantly affected the counts. Sixty bacterial isolates representing seven genera were identified, with *Pseudomonas* spp. predominating overall (45.00%), followed by *Bacillus* spp. (28.33%) and *Proteus* spp. (13.33%). Bacterial distribution differed significantly across river sections ($\chi^2 = 24.15$, p = 0.02). Phenotypic screening revealed biofilm formation in 40.00% of isolates, while protease, lipase, and cellulase production occurred in 40.00%, 36.67%, and 28.33%, respectively. β-haemolysis occurred in 26.67%, whereas γ-haemolysis predominated (73.33%). However, none of the virulence-associated characteristics varied significantly by sampling location. These findings indicate that Ada River harbours diverse bacteria with virulence-associated traits, highlighting potential environmental and public-health concerns and the need for regular water-quality monitoring and protection.

Keywords: Okada, Ada River, Water Quality, Bacterial Diversity, Biofilm Formation, Phenotypic Virulence, Public Health.

Introduction

Rivers are important freshwater resources that support domestic activities, agriculture, livestock production and other socioeconomic activities. However, they can also provide suitable environments for the persistence and dissemination of potentially pathogenic microorganisms. In communities where wastewater, agricultural runoff and other wastes enter surface waters with limited treatment, rivers may serve as interfaces connecting environmental, animal and human microbial populations. Franklin *et al*. (2024) emphasized that surface waters are important components of One Health surveillance because water can provide a conduit for the movement and persistence of microorganisms between humans, animals and the environment.

Similarly, Martak *et al*. (2024) reported that anthropogenic effluents can introduce potentially pathogenic and antimicrobial-resistant bacteria into aquatic environments, particularly in settings where sanitation infrastructure is inadequate.

Beyond the simple detection of bacteria in water, their capacity to express virulence-associated characteristics is important for determining their potential public-health significance. Virulence represents the ability of microorganisms to colonize hosts, evade host defenses, obtain nutrients, damage tissues and establish infection (Chakkour et al., 2024). Phenotypic characteristics such as haemolysis, biofilm formation and production of extracellular enzymes can provide useful preliminary evidence of these capabilities.

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Biofilm formation, for example, enables bacteria to attach to surfaces and persist under adverse environmental conditions, while haemolytic activity may indicate the production of factors capable of damaging host cells. Proteolytic, lipolytic and cellulolytic activities may further contribute to environmental persistence, nutrient acquisition and tissue degradation (Hu et al., 2024).

The public-health relevance of environmental bacterial virulence has received increasing attention because aquatic systems may harbour organisms with characteristics associated with human and animal infections. Abeni *et al*. (2024) demonstrated that *Escherichia coli* isolates from human and non-human sources in Rivers State, Nigeria, commonly carried virulence-associated genes, with *fimH* detected in 97% of isolates. Their findings showed that potentially virulent *E. coli* can occur outside conventional clinical settings and highlighted the importance of investigating bacterial virulence across different ecological sources.

Ada River in Okada, Edo State, forms part of the local aquatic environment and may receive microorganisms originating from surrounding human activities, domestic wastes, agricultural practices and animal-associated sources. Consequently, examining the phenotypic virulence characteristics of bacteria recovered from the river can provide useful information about their potential ecological persistence and public-health relevance.

Franklin *et al*. (2024) recommended environmental surveillance as a means of identifying microbial hazards and understanding conditions that facilitate their persistence and dissemination in freshwater systems.

Therefore, this study was undertaken to evaluate the phenotypic virulence potential of bacterial isolates recovered from Ada River in Okada, Edo State, Nigeria, with emphasis on observable characteristics associated with pathogenicity and environmental persistence.

The findings are expected to contribute to understanding the microbiological quality of the river and provide baseline information for environmental health surveillance and One Health risk assessment.

Materials and Methods

Study Area

The study was conducted on bacterial isolates recovered from Ada River in Okada (6º 43'N 5º23'E), Edo State, Nigeria. Okada is located in Ovia North-East Local Government Area of Edo State. The river constitutes an important freshwater resource within the community and is potentially influenced by domestic activities, agricultural practices, animal production and other anthropogenic activities occurring around its drainage basin. The study area was selected because of the importance of the river to surrounding communities and the potential for surface-water systems to harbour bacteria of environmental and public-health significance. Map of the Study Area is shown in Figure 1.

Study Design

A laboratory-based descriptive study was conducted to determine the phenotypic virulence characteristics of bacterial isolates recovered from Ada River. The study involved the recovery and enumeration of bacterial colonies and identification of bacterial isolates from three sampling locations (upstream, midstream and downstream) followed by phenotypic screening for selected virulence-associated characteristics. The characteristics investigated included haemolytic activity, biofilm formation and extracellular enzyme production, specifically protease, lipase and cellulase activities. These phenotypic characteristics were selected because they provide useful indicators of bacterial capacity for host-cell damage, surface colonization, persistence and extracellular substrate degradation.

Collection of River Water Samples

River-water samples were collected aseptically from upstream, mid-stream and downstream sampling points along Ada River. Sterile, appropriately labelled sampling bottles were used for collection. At each sampling point, the bottle was opened immediately before collection, filled with river water, from a depth of 20 to 30 cm, and securely closed to minimize external contamination. Samples were placed in a clean insulated container and transported to the microbiology laboratory for processing within 4hrs.

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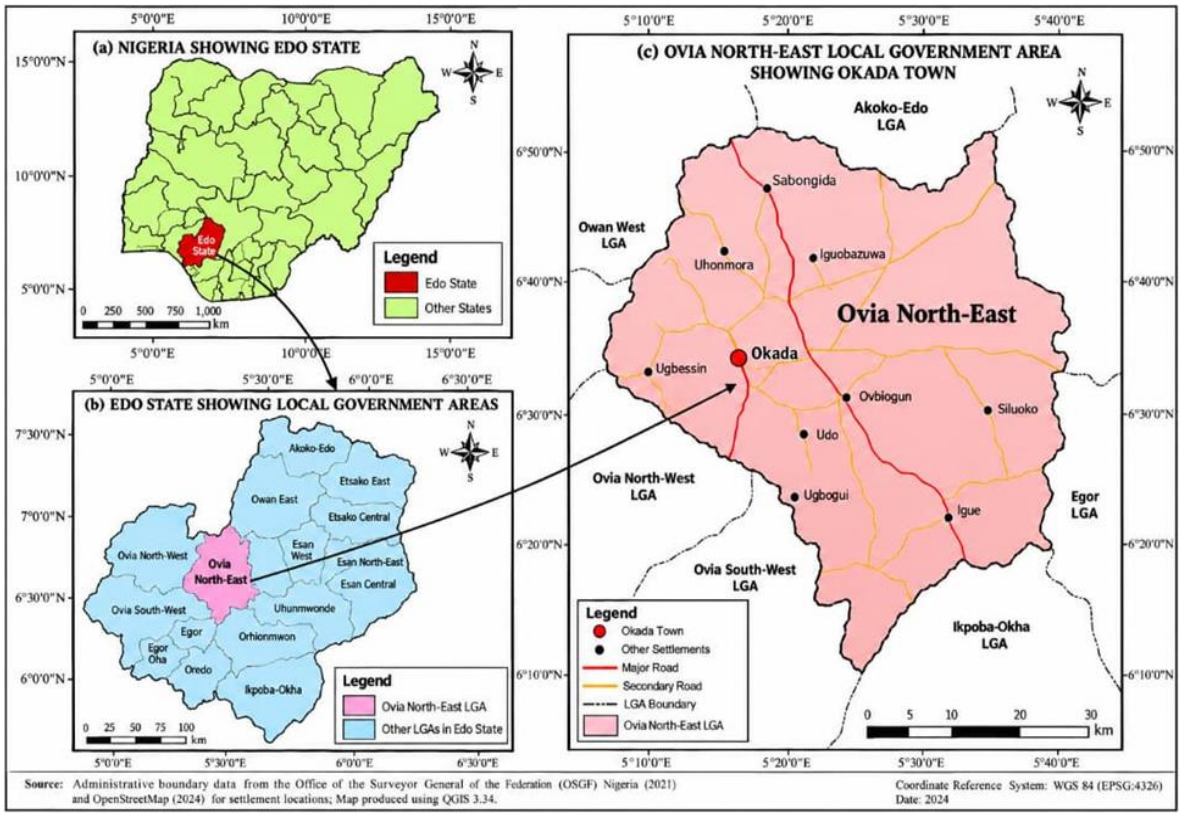


Figure 1: Map of the Study Area

Cultivation, Enumeration and Isolation of Bacteria

Bacterial isolation and enumeration were performed using the pour-plate method following the procedure described by Public Health England (2014). Briefly, 25 mL of each sample was aseptically introduced into a sterile conical flask containing 225 mL of 0.1% (w/v) sterile peptone water to obtain the initial suspension. Serial ten-fold dilutions were subsequently prepared from the suspension up to 10^{-6}. Aliquots of 1 mL from each dilution were aseptically dispensed into duplicate sterile Petri plates.

Approximately 15 mL each of molten Nutrient Agar and MacConkey Agar (HiMedia, India) were added to the appropriate plates and carefully mixed with the inoculum to ensure uniform distribution. Following solidification of the media, the plates were incubated at 35°C for 24 hours. After incubation, visible colonies were enumerated from plates containing countable numbers of colonies, and the results were used to estimate the bacterial loads in the samples.

Colony counts obtained on Nutrient Agar were reported as Total Heterotrophic Plate Counts (HPC), whereas counts obtained on MacConkey Agar were recorded as Total Coliform Counts (TCC).

Representative colonies were subsequently subcultured to obtain pure cultures. The purified bacterial isolates were maintained on agar slants, appropriately labeled, and refrigerated 4°C until required for subsequent analyses.

Phenotypic identification of the bacterial isolates

The isolates were initially examined by Gram staining to determine their Gram reaction and cellular morphology, after which appropriate biochemical tests were performed for further identification.

Phenotypic identification of the bacterial isolates was carried out using conventional microbiological identification procedures as described by Krieg and Holt (1984) and Eltai *et al*. (2018).

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The identification scheme comprised a combination of enzymatic, metabolic and carbohydrate utilization tests, including catalase, coagulase, oxidase, citrate utilization, indole, methyl red (MR), and Voges–Proskauer (VP) tests. Carbohydrate fermentation tests involving glucose, lactose and sucrose were also performed, with observations made for acid and gas production.

Hydrogen sulphide (H_2S) production was additionally assessed as part of the biochemical characterization. The resulting biochemical reaction patterns were compared with established bacteriological identification criteria of Krieg and Holt (1984) and Cheesbrough (2006) to obtain the presumptive identities of the isolates.

Determination of Haemolytic activity of Bacterial Isolates

Haemolytic activity was determined by inoculating each bacterial isolate onto blood agar plates. The inoculated plates were incubated under appropriate conditions and subsequently examined for zones of erythrocyte lysis surrounding the bacterial colonies Cheesbrough (2006). Isolates producing a clear zone around the colony were recorded as haemolytic, whereas isolates without visible haemolysis were classified as non-haemolytic.

Where partial greenish discoloration was observed, the isolate was recorded as exhibiting alpha-haemolysis, while complete clearing around the colony was interpreted as beta-haemolysis. Absence of visible haemolysis was recorded as gamma-haemolysis.

Determination of Biofilm Production

Biofilm-forming ability of the bacterial isolates was assessed using the Congo red agar (CRA) method described by Freeman *et al*. (1989). Briefly, each bacterial isolate was inoculated onto sterile CRA plates by streaking and incubated aerobically at 37°C for 24–48 hours.

Following incubation, the colony morphology and colour were examined. Isolates that produced rough, black colonies were regarded as positive for biofilm production, whereas isolates producing smooth, red colonies were classified as non-biofilm producers.

Determination of Proteolytic activity of the bacterial isolates

Proteolytic activity of the bacterial isolates was evaluated according to the method described by Cappuccino and Sherman (2014). Under aseptic conditions, the isolates were inoculated onto the surface of the casein agar medium by either streaking or spot inoculation. The inoculated plates were incubated aerobically at 35–37°C for 24–48 hours. During incubation, protease-producing bacteria hydrolyzed the casein incorporated into the agar through the action of extracellular proteolytic enzymes. Protease production was indicated by the formation of distinct clear zones surrounding the bacterial colonies as a result of casein degradation. Isolates that showed no visible zone of clearance around the growth were considered negative for protease production.

Determination of Lipase-producing ability of the bacterial isolates

Lipase-producing ability of the bacterial isolates was assessed using the plate method described by Sierra (1957). Pure cultures of the isolates were aseptically inoculated onto tributyrin agar plates using sterile inoculating loops. The inoculated plates were incubated aerobically at 35–37°C for 24–72 hours, depending on the growth characteristics of the individual isolates. During incubation, isolates capable of producing extracellular lipase hydrolyzed the tributyrin incorporated into the agar medium. This hydrolytic activity was manifested by the formation of distinct, transparent zones of clearance surrounding the bacterial colonies. The presence of a clear halo around an isolate was therefore recorded as a positive indication of lipase production, whereas isolates showing no zone of clearance were regarded as negative for lipolytic activity.

Determination of Cellulase-producing ability of the bacterial isolates

Cellulase-producing ability of the bacterial isolates was assessed using the CMC agar plate method (Hendricks *et al*., 1995). Pure cultures were aseptically inoculated onto carboxymethyl cellulose (CMC) agar plates by either streaking or spot inoculation with sterile inoculating loops.

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The inoculated plates were incubated aerobically at 35–37°C for 24–48 hours to permit bacterial growth and extracellular enzyme activity. Following incubation, the surface of each plate was flooded with 0.1% Congo red solution and allowed to stand for approximately 15 min. The excess stain was then carefully removed, and the plates were gently rinsed with 1 M sodium chloride solution to enhance the differentiation between hydrolyzed and non-hydrolyzed regions of the CMC-containing medium. Isolates producing extracellular cellulase hydrolyzed the CMC substrate surrounding their colonies, resulting in distinct transparent zones against the red-stained background. The appearance of a visible clearance zone was interpreted as a positive reaction for cellulase production, whereas isolates without a discernible halo were recorded as negative.

Statistical Analysis

Results obtained from the phenotypic virulence assays were recorded and summarized using frequencies and percentages. Virulence profiles were compared according to bacterial genera. Statistical analysis was performed using Number Cruncher Statistical Software (NCSS) version 12. Associations between categorical variables were assessed using the chi-square test or Fisher's exact test. Statistical significance was considered at $p < 0.05$.

Results

The heterotrophic plate count (HPC) of Ada River water samples collected from the upstream, mid-stream and downstream sections of Ada River at Okada, Edo State, is presented in Table 1. The heterotrophic plate count (HPC) data obtained from Ada River were analyzed using both the original CFU/mL scale and a $\log_{10}$-transformed scale. The transformation was performed on each individual biological replicate before calculation of the logarithmic mean and standard deviation.

A two-way factorial analysis of variance (ANOVA) was subsequently performed using visitation (period of sample collection) and sampling point as fixed factors. The analysis was based on 12 independent biological observations, with two observations for each visitation-by-location combination.

The descriptive results showed a consistent spatial pattern in bacterial abundance. The mid-stream location had the highest overall HPC (145.00 ± 13.23 CFU/mL), followed by the downstream location (95.00 ± 22.64 CFU/mL), whereas the upstream station recorded the lowest value (46.88 ± 16.25 CFU/mL). The same ranking was observed on the logarithmic scale, with mean values of 2.16 ± 0.04, 1.98 ± 0.11 and 1.67 ± 0.18 $\log_{10}$ CFU/mL for mid-stream, downstream and upstream, respectively. During the first visitation, the mid-stream HPC was approximately 3.26 times the upstream concentration. During the second visitation, it was approximately 2.95 times the upstream concentration. The downstream concentration was also higher than the upstream concentration during both visits. The two-way ANOVA demonstrated a statistically significant effect of sampling point on bacterial abundance, $F(2,6) = 14.01$, $p = 0.01$, partial $\eta^2 = 0.82$. This indicates that approximately 82.4% of the explainable variance associated with the model's effects and error was attributable to sampling location after accounting for the other factor. In contrast, the effect of visitation was not statistically significant, $F(1,6) = 0.69$, $p = 0.44$. The visitation × sampling-point interaction was also not significant, $F(2,6) = 0.11$, $p = 0.90$. Therefore, there was no statistical evidence that the effect of sampling location differed between the two visits. Because sampling point had a significant effect, Tukey's honestly significant difference (HSD) procedure was used to determine which locations differed from one another. The Tukey HSD analysis showed that the upstream station had significantly lower bacterial abundance than both the mid-stream and downstream stations. The difference between mid-stream and upstream was 0.49 $\log_{10}$ CFU/mL ($p = 0.001$), while the difference between downstream and upstream was 0.31 $\log_{10}$ CFU/mL (adjusted $p = 0.01$).

In contrast, the difference between the mid-stream and downstream stations was not statistically significant (mean difference = 0.18 $\log_{10}$ CFU/mL, $p = 0.12$). Thus, the statistically supported grouping is: Mid-stream = a; Downstream = a; Upstream = b. This means that both mid-stream and downstream had significantly greater HPC than upstream, whereas mid-stream and downstream were statistically comparable. The analysis was conducted on $\log_{10}$-transformed HPC values to reduce the influence of the magnitude differences inherent in bacterial-count data.

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Table 1: Heterotrophic Plate Count of Bacteria in Ada River

Sampling of River Water	Heterotrophic Plate Count (HPC) of Bacteria in Ada River					
	Upstream		Mid-stream		Downstream	
	Mean HPC ± SD		Mean HPC ± SD		Mean HPC ± SD	
	CFU/mL	Log_{10} CFU/mL	CFU/mL	Log_{10} CFU/mL	CFU/mL	Log_{10} CFU/mL
1st visit (28th April 2026)	43.75 ± 26.52	1.64 ± 0.28[b]	142.50 ± 7.07	2.15 ± 0.02[a]	86.25 ± 26.52	1.94 ± 0.14[a]
2nd visit (9th June 2026)	50.00 ± 7.07	1.70 ± 0.04[b]	147.50 ± 21.21	2.17 ± 0.01[a]	103.75 ± 22.98	2.02 ± 0.06[a]
All visitations	46.88 ± 16.25	1.67 ± 0.18[b]	145.00 ± 13.23	2.16 ± 0.04[a]	95.00 ± 22.64	1.98 ± 0.11[a]

Key: SD = standard deviation; superscript letter a and b: Tukey grouping. Means sharing the same letter are not significantly different at $p > 0.05$ while means with different letters differ significantly at $p < 0.05$; ANOVA was performed on Log_{10}-transformed HPC values

Table 2: Total Coliform Count of Bacteria in Ada River

Sampling of River Water	Total Coliform Count (TCC) of Bacteria in Ada River					
	Upstream		Mid-stream		Downstream	
	Mean TCC ± SD		Mean TCC ± SD		Mean TCC ± SD	
	CFU/mL	Log_{10} CFU/mL	CFU/mL	Log_{10} CFU/mL	CFU/mL	Log_{10} CFU/mL
1st visit (28th April 2026)	23.75 ± 1.77	1.38 ± 0.03[a]	27.50 ± 7.07	1.44 ± 0.11[a]	22.50 ± 3.54	1.35 ± 0.07[a]
2nd visit (9th June 2026)	30 ± 7.07	1.48 ± 0.10[a]	37.5 ± 14.14	1.57 ± 0.17[a]	21.25 ± 1.77	1.33 ± 0.04[a]
All visitations	26.88 ± 5.54	1.43 ± 0.08[a]	32.5 ± 10.80	1.52 ± 0.14[a]	21.88 ± 2.39	1.34 ± 0.05[a]

Key: SD = standard deviation; superscript letter a: Tukey grouping. Means sharing the same letter are not significantly different at $p > 0.05$ while means with different letters differ significantly at $p < 0.05$; ANOVA was performed on Log_{10}-transformed HPC values

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The Shapiro–Wilk test of the ANOVA residuals gave W = 0.99, p = 1.00. Since p > 0.05, there was no evidence of a significant departure from normality of the residuals. Bartlett's test gave $\chi^2(5) = 4.51$, p = 0.48. Thus, there was no statistical evidence of heterogeneity of variance among the six visitation × sampling-point groups. Independence was considered satisfied because each pair of observations represented independently collected biological water samples, rather than duplicate measurements of the same sample. Therefore, the basic assumptions required for the selected factorial ANOVA were considered sufficiently supported for this dataset.

Table 2 presents the evaluations of total coliform counts (TCC) of water samples collected from the upstream, mid-stream and downstream sections of Ada River during two sampling visits. Total coliform counts ranged between 20.0 and 47.5 CFU/mL during the first visit, the upstream section recorded a mean TCC of 23.75 ± 1.77 CFU/mL, corresponding to 1.38 ± 0.03 $\log_{10}$ CFU/mL. The mid-stream section had a slightly higher mean count of 27.50 ± 7.07 CFU/mL (1.44 ± 0.11 $\log_{10}$ CFU/mL), whereas the downstream section recorded 22.50 ± 3.54 CFU/mL (1.35 ± 0.07 $\log_{10}$ CFU/mL). Thus, during the first visit, the mid-stream section had the highest TCC, while the downstream section had the lowest. During the second visit, TCC increased at the upstream and mid-stream sections but declined slightly downstream. The upstream section recorded 30.00 ± 7.07 CFU/mL (1.48 ± 0.10 $\log_{10}$ CFU/mL), while the mid-stream section recorded the highest value of 37.50 ± 14.14 CFU/mL (1.57 ± 0.17 $\log_{10}$ CFU/mL). The downstream section recorded 21.25 ± 1.77 CFU/mL (1.33 ± 0.04 $\log_{10}$ CFU/mL). When both visits were considered together, the mean TCC was highest at the mid-stream section (32.50 ± 10.80 CFU/mL; 1.52 ± 0.14 $\log_{10}$ CFU/mL), followed by upstream (26.88 ± 5.54 CFU/mL; 1.43 ± 0.08 $\log_{10}$ CFU/mL), while downstream had the lowest overall count (21.88 ± 2.39 CFU/mL; 1.34 ± 0.05 $\log_{10}$ CFU/mL). The Tukey grouping showed that all three sampling locations were assigned the same group (**a**), indicating that the observed differences were not statistically significant. A two-way factorial ANOVA conducted on the $\log_{10}$-transformed TCC data showed that sampling location did not have a statistically significant effect on TCC, $F(2,6) = 2.80$, $p = 0.14$. Visitation also had no significant effect, $F(1,6) = 1.51$, $p = 0.27$, while the interaction between visitation and sampling location was not significant, $F(2,6) = 0.65$, $p = 0.56$. The corresponding partial eta-squared values were approximately 0.48 for sampling location, 0.20 for visitation and 0.18 for the interaction, suggesting that location accounted for a relatively larger proportion of the observed variation than the other factors, despite not reaching statistical significance.

A total of 60 bacterial isolates were isolated and were phenotypically examined. The bacterial comprised 20 isolates each from the upstream, midstream and downstream sections of Ada River. Seven bacterial genera were presumptively identified across the three sampling sections: *Bacillus* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Enterococcus* spp., *Proteus* spp., *Micrococcus* spp. and *Proteus* spp**.**

The percentage frequency of distribution of the bacterial isolates along the Ada River is as presented in Table 3. The distribution differed between the three sections, indicating spatial variation in the cultivable bacterial community.

Table 3: Frequency of Occurrence of Bacterial Isolate along Ada River in Okada

Bacteria Isolate	**Frequency of Occurrence (n = 24 /%) in Location of MSW**		
	Upstream	**Midstream**	**Downstream**
Bacillus spp.	8 (40.00)	4 (20.00)	5 (25.00)
Enterococcus spp.	2 (10.00)	-	-
Micrococcus spp.	-	-	1 (5.00)
Proteus spp.	-	5 (25.00)	3 (15.00)
Pseudomonas spp.	8 (40.00)	10 (50.000	9 (45.00)
Staphylococcus sp.	2 (10.00)	1 (5.00)	2 (10.00)

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Twenty bacterial isolates were recovered from the upstream section. *Bacillus* spp. and *Pseudomonas* spp. were the predominant isolates, each accounting for 8 (40.00%) of the isolates. *Staphylococcus* spp. and *Enterococcus* spp. occurred at lower frequencies of 2 (10.00%) isolates each. The presumptive *Bacillus* spp**.** produced mucoid to dry colonies, were Gram-positive rods, motile and endospore-forming. The midstream samples yielded a different distribution of bacterial groups. *Pseudomonas* spp. constituted the largest proportion, with 10 (50.00%) isolates, followed by *Proteus* spp. with 5 (25.00%) and *Bacillus* spp. with 4 (20.00%). *Staphylococcus* spp. accounted for only 1 (5.00%) isolate. Five bacterial groups were recovered from the downstream section. *Pseudomonas* spp. remained the most frequently recovered organism, occurring in 9 (45.00%) isolates. This was followed by *Bacillus* spp. at 5 (25.00%), Proteus spp. at 3 (15.00%), *Staphylococcus* spp. at 2 (10.00%) and *Micrococcus* spp. at 1 (5.00%). The presumptive *Micrococcus* isolate was a non-motile, non-spore-forming Gram-positive coccus producing yellow colonies. When all 60 isolates were considered together, *Pseudomonas* spp. were the most frequently represented group, with 27 isolates (45.00%), followed by *Bacillus* spp. with 17 (28.33%). *Proteus* spp. accounted for 8 (13.33%), *Staphylococcus* spp. for 5 (8.33%), *Enterococcus* spp. for 2 (3.33%) and *Micrococcus* spp. for 1 (1.67%). Thus, the overall bacterial profile was dominated by Gram-negative organisms, particularly *Pseudomonas* and *Proteus*, although spore-forming Gram-positive *Bacillus* spp. were also prominent. A Pearson chi-square test of independence was used to determine whether the distribution of phenotypically identified bacterial groups was independent of sampling position along Ada River. The analysis produced a χ^2 value of 24.15 with 12 degrees of freedom ($p = 0.02$)**.** The result indicates a statistically significant association between sampling section and bacterial-group distribution ($p < 0.05$). Therefore, the null hypothesis that bacterial-group distribution was independent of sampling location was rejected. Additional exploratory comparisons showed that the distribution of *Pseudomonas* spp**.** did not differ significantly among the three sections ($\chi^2 = 0.40$, df = 2, $p = 0.82$). Similarly, *Bacillus* spp. showed no statistically significant spatial difference ($\chi^2 = 2.13$, df = 2, $p = 0.34$), nor did *Staphylococcus* spp. ($\chi^2 = 0.44$, df = 2, $p = 0.80$). *Proteus* spp. approached but did not reach conventional statistical significance ($\chi^2 = 5.48$, df = 2, $p = 0.07$).The phenotypic virulence characteristics of bacterial isolates recovered from the upstream, midstream and downstream sections of Ada River are presented in Table 4.

Sixty bacterial isolates were examined, comprising 20 isolates from each sampling location. The isolates demonstrated considerable variation in biofilm formation, extracellular enzyme production and haemolytic activity. Biofilm formation was detected in 24 of the 60 isolates examined, giving an overall prevalence of 40.00%**.** The proportion of biofilm-positive isolates was highest among the midstream isolates, where 9 of 20 isolates (45.00%) produced biofilm. This was followed by the upstream isolates, with 8 of 20 (40.00%), while the downstream isolates had the lowest proportion, with 7 of 20 (35.00%). Among the bacterial groups, biofilm formation was particularly evident among *Staphylococcus* spp. and *Enterococcus* spp. in the upstream samples. Biofilm production was also observed among *Pseudomonas*, *Proteus* and *Bacillus* isolates, although the frequency varied between sampling locations. The findings indicate that a substantial proportion of the bacterial population recovered from Ada River possessed the phenotypic capacity to attach to surfaces and develop structured microbial communities. The results demonstrated variation in extracellular enzyme production among the bacterial isolates. Cellulase production was recorded in 17 (28.33%) of the 60 isolates. The highest proportion occurred in the midstream samples, where 9 isolates (45.00%) were positive. In contrast, only 4 isolates (20.00%) from the upstream and 4 isolates (20.00%) from the downstream were cellulase-positive. Protease production was detected in 24 isolates (40.00%) overall. Midstream isolates recorded the highest prevalence, with 12 positive isolates (60.00%), whereas both upstream and downstream locations had 6 positive isolates (30.00%) each. Lipase production was observed in 22 isolates (36.67%). The prevalence was relatively similar between the three locations, ranging from 35.00% upstream to 40.00% midstream and 35.00% downstream. Thus, among the extracellular enzymes examined, protease production was the most frequently observed enzyme-associated phenotype, followed by lipase and cellulase production.

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Table 4: Phenotypic virulence characteristics of bacteria isolated from Ada River

Source of bacteria	Isolated bacteria	N	Phenotypic virulence characteristics						
			Biofilm	Enzyme production			Haemolytic activity		
			formation n (%)	Cellulase n (%)	Protease n (%)	Lipase n (%)	β-haemolysis n (%)	α-haemolysis n (%)	γ-haemolysis n (%)
Upstream	*Bacillus* spp.	8	0 (0.00)	0 (0.00)	0 (0.00)	1 (12.50)	1 (12.50)	0 (0.00)	7 (87.50)
	Pseudomonas spp.	8	4 (50.00)	2 (50.00)	4 (50.00)	4 (100.00)	1 (12.50)	0 (0.00)	7 (87.50)
	Staphylococcus spp.	2	2 (100.00)	1 (50.00)	1 (50.00)	1 (50.00)	1 (50.00)	0 (0.00)	1 (50.00)
	Enterococcus spp.	2	2 (100.00)	1 (50.00)	1 (50.00)	1 (50.00)	0 (0.00)	0 (0.00)	2 (100.00)
Midstream	*Staphylococcus* spp.	1	1 (100.00)	0 (0.00)	1 (100.00)	1 (100.00)	1 (100.00)	0 (0.00)	0 (0.00)
	Proteus spp.	5	3 (60.00)	2 (40.00)	3 (60.00)	2 (40.00)	2 (40.00)	0 (0.00)	3 (60.00)
	Bacillus spp.	4	1 (25.00)	2 (50.00)	2 (50.00)	0 (0.00)	2 (50.00)	0 (0.00)	2 (50.00)
	Pseudomonas spp.	10	4 (40.00)	5 (50.00)	6 (60.00)	5 (50.00)	3 (30.00)	0 (0.00)	7 (70.00)
Downstream	*Staphylococcus* spp.	2	1 (50.00)	1 (50.00)	1 (50.00)	1 (50.00)	1 (50.00)	0 (0.00)	1 (50.00)
	Proteus spp.	3	2 (66.67)	1 (33.33)	1 (33.33)	1 (33.33)	1 (33.33)	0 (0.00)	2 (66.67)
	Micrococcus spp.	1	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (100.00)
	Bacillus spp.	5	2 (40.00)	0 (0.00)	2 (40.00)	2 (40.00)	1 (20.00)	0 (0.00)	4 (80.00)
	Pseudomonas spp.	9	2 (22.22)	2 (22.22)	2 (22.22)	3 (33.33)	2 (22.22)	0 (0.00)	7 (77.78)

N: number of bacterial isolates tested; n: number of isolates that tested positive for a virulence characteristic; %: percentage virulence characteristic

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Haemolytic testing showed a clear predominance of γ-haemolytic isolates. Overall, 44 isolates (73.33%) demonstrated γ-haemolysis, whereas β-haemolysis was observed in 16 isolates (26.67%). No α-haemolytic isolate was recorded in any of the three sampling locations. The prevalence of β-haemolysis was highest among midstream isolates, where 8 (40.00%) were positive. This was followed by downstream isolates at 5 (25.00%) and upstream isolates at 3 (15.00%). γ-haemolysis was observed in 17 (85.00%) upstream isolates, 12 (60.00%) midstream isolates and 15 (75.00%) downstream isolates.

The predominance of γ-haemolysis indicates that most isolates did not produce visible erythrocyte destruction under the test conditions. The overall results demonstrate that the midstream bacterial population generally exhibited higher frequencies of several virulence-associated characteristics. Midstream isolates recorded the highest prevalence of cellulase production (45.00%), protease production (60.00%) and β-haemolysis (40.00%). Biofilm formation was also slightly more frequent at midstream (45.00%) than upstream (40.00%) or downstream (35.00%). The downstream isolates generally showed intermediate phenotypic activity, while upstream isolates displayed comparatively lower frequencies for most extracellular enzyme and β-haemolytic characteristics. However, the differences were not necessarily statistically significant, as demonstrated by the inferential analysis below. Pearson's chi-square test was used to determine whether the distribution of each phenotypic virulence characteristic differed significantly among upstream, midstream and downstream isolates. Cramér's V was calculated as a measure of effect size. The statistical analysis showed that none of the investigated virulence characteristics differed significantly among the three sampling locations ($p > 0.05$). Biofilm formation showed virtually no association with sampling location ($\chi^2 = 0.42$, $p = 0.81$; Cramér's V = 0.08). Although cellulase production was more common among midstream isolates, the difference did not reach statistical significance ($\chi^2 = 4.104$, $p = 0.13$). Protease production produced the largest chi-square statistic among the enzyme-associated characteristics ($\chi^2 = 5.00$, $p = 0.08$; Cramér's V = 0.29). This represents a small-to-moderate association, but it remained above the conventional 0.05 significance threshold. Lipase production showed almost no relationship with sampling location ($\chi^2 = 0.14$, $p = 0.93$).

Similarly, β-haemolysis was not significantly associated with sampling location ($\chi^2 = 3.239$, $p = 0.198$). The same result was obtained for γ-haemolysis ($\chi^2 = 3.24$, $p = 0.20$). α-haemolysis was absent from all 60 isolates and therefore could not be subjected to a meaningful chi-square comparison because there was no variation in the outcome. Therefore, the null hypothesis that the distribution of the measured phenotypic virulence characteristics was independent of sampling location was retained at the 5% significance level.

Discussion

The present study showed that the microbiological quality of Ada River varied across sampling sections, with the clearest spatial effect observed for heterotrophic bacterial counts (HPC). Mean HPC was lowest upstream (46.88 ± 16.25 CFU/mL; 1.65 ± 0.18 $\log_{10}$ CFU/mL), increased at midstream (145.00 ± 13.23 CFU/mL; 2.16 ± 0.04 $\log_{10}$ CFU/mL), and remained elevated downstream (95.00 ± 22.64 CFU/mL; 1.97 ± 0.11 $\log_{10}$ CFU/mL). The two-way ANOVA confirmed a significant effect of sampling section, $F(2,6) = 14.01$, $p = 0.01$, with a large partial eta-squared ($\eta^2p = 0.82$). Tukey HSD showed that upstream differed significantly from midstream and downstream, whereas midstream and downstream were statistically comparable. The spatial pattern agrees with Tenebe *et al*. (2024), Okanlawon *et al*. (2024), and Bisi-Johnson *et al*. (2023), who reported substantial variation in bacterial loads among Nigerian water sources. WHO (2022) emphasizes that HPC is a general microbiological indicator rather than a specific measure of faecal contamination. Edosomwan *et al*. (2024) similarly demonstrated that different microbiological indicators can provide complementary information about water quality. Temporal effects were less pronounced. Although mean $\log_{10}$ HPC increased from about 1.89 during the first visit to 1.96 during the second, visitation was not significant, $F(1,6) = 0.68$, $p = 0.44$. The visitation-by-section interaction was also non-significant, $F(2,6) = 0.11$, $p = 0.90$, indicating that the spatial pattern remained broadly consistent. Tenebe *et al*. (2024) stressed the importance of spatial and temporal monitoring, while Hu *et al*. (2024) demonstrated the usefulness of logarithmic transformation for microbial-count data.

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Molale-Tom *et al*. (2024) further indicates that environmental heterotrophs may possess resistance and virulence-associated characteristics.

Total coliforms were detected throughout the river, ranging from 20.0 to 47.5 CFU/mL (1.3010–1.6767 $\log_{10}$ CFU/mL). Midstream had the highest mean TCC (1.50 ± 0.14 $\log_{10}$ CFU/mL), followed by upstream (1.42 ± 0.08) and downstream (1.34 ± 0.05), but the section effect was not significant, $F(2,6) = 2.53$, $p = 0.16$. Tukey HSD similarly placed all sections in the same group. Temporal variation in TCC was also non-significant, $F(1,6) = 1.35$, $p = 0.29$, and the interaction was non-significant, $F(2,6) = 0.63$, $p = 0.56$. TCC should therefore be interpreted as evidence of general microbial contamination rather than definitive proof of recent faecal pollution.

Cultivable bacteria represented a diverse community comprising *Bacillus, Pseudomonas, Proteus, Staphylococcus, Enterococcus* and *Micrococcus*. *Pseudomonas* was predominant (45.00%), followed by *Bacillus* (28.33%). *Bacillus* distribution was non-significant ($p = 0.34$), while *Pseudomonas* also showed no significant spatial difference ($\chi^2 = 0.40$, $p = 0.82$). *Proteus* was absent upstream but occurred at 25.00% midstream and 15.00% downstream; the difference remained non-significant ($\chi^2 = 5.48$, $p = 0.07$). *Staphylococcus* occurred across all sections, whereas *Enterococcus* occurred only upstream and *Micrococcus* only downstream. Overall bacterial-group distribution was significant ($\chi^2 = 24.15$, $df = 12$, $p = 0.02$), apparently influenced by less frequent groups. These findings broadly agree with Titilawo *et al*. (2015), Sule *et al*. (2023), Popoola *et al*. (2024), Esumirhewe and Asamaka (2020), and WHO (2021). The recovery of *Enterococcus* is epidemiologically relevant, although quantitative enumeration is required for formal recreational-water assessment.

The isolates also displayed biofilm formation, cellulase, protease and lipase production, and haemolytic activity. Biofilm formation occurred in 40.00% of isolates, cellulase in 28.33%, protease in 40.00%, and lipase in 36.67%. None showed significant association with sampling location: biofilm $\chi^2 = 0.42$, $p = 0.81$; cellulase $\chi^2 = 4.10$, $p = 0.13$; protease $\chi^2 = 5.00$, $p = 0.08$; and lipase $\chi^2 = 0.14$, $p = 0.93$. γ-haemolysis predominated (73.33%), β-haemolysis occurred in 26.67%, and α-haemolysis was absent; β-haemolysis was not significantly associated with location ($\chi^2 = 3.24$, $p = 0.20$). Flemming *et al*. (2025), Nkemngong and Teska (2024) emphasized the ecological importance of biofilm matrices in adhesion, persistence and stress tolerance. Chakkour *et al*. (2024) and Yang *et al*. (2024) linked biofilm, protease and haemolysin production with *Proteus* pathogenicity, while Letizia *et al*. (2025) highlighted interconnected ecology, biofilm formation, virulence and resistance in *Pseudomonas.* Nevertheless, these phenotypes indicate biological capabilities rather than confirmed pathogenicity. Overall, the findings identify Ada River as a dynamic microbial environment requiring continued surveillance. These findings underscore the need for sustained microbiological monitoring of Ada.

Conclusion

The study established that Ada River contains measurable heterotrophic and coliform bacterial contamination, with notable spatial variation in heterotrophic bacterial abundance. Midstream water recorded the highest HPC, followed by downstream and upstream, and sampling location significantly influenced HPC. In contrast, total coliform counts showed no significant differences among sampling sections or sampling visits. Phenotypic characterization demonstrated considerable bacterial diversity, with *Pseudomonas* spp. being the predominant isolate, followed by *Bacillus* spp. and *Proteus* spp., while bacterial composition varied significantly along the river. The isolates also exhibited important phenotypic characteristics, including biofilm formation, extracellular enzyme production and haemolytic activity.

Although these virulence-associated traits were more frequently expressed among midstream isolates, their distributions did not differ significantly by sampling location.

Collectively, the findings indicate that Ada River supports a diverse cultivable bacterial community containing organisms with potentially important environmental and public-health implications. Regular microbiological monitoring, improved protection of the river from contamination sources, and appropriate public-health interventions are therefore recommended to safeguard water quality.

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