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ORIGINAL RESEARCH ARTICLE

Genetic diversity of pathogenic bacteria and incidence of selected antibiotic resistant genes in water used by eateries and open markets in Juja, Kenya.

Alfrick Makori[1], *Johnstone Neondo*[1], *Ian Mwangi*[1], *Cecilia Mweu*[1]

[1]*Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.*

Corresponding email: *alfrickomune@gmail.com*

ABSTRACT

Water is an important bacterial habitat and a major avenue of dissemination of antibiotic-resistant bacteria between and among different environments. Contamination with antibiotic-resistant bacteria in water used in eateries and open markets poses a direct threat to human health. This is because antimicrobial resistance monitoring is not currently a routine standard for drinking water. This study aimed to investigate the occurrence of ARGs and variation in the composition of bacterial communities in eighty-two water samples collected from eateries and open markets in Juja. The purposeful sampling technique was used to collect water samples used in eateries and open markets in Wakulima, Gachororo, and Mwerevu in Juja. The samples were collected from water containers placed at the entrance of eateries, taps at the market entrance, and jerricans used by traders within the market to wash groceries. Special media of MacConkey, Triple Sugar Iron Medium, Thioglycollate, and Lauryl Tryptose Broth media were used for the isolation of Enterobacteriaceae, the detection and numeration of F. streptococci, the differentiation and enumeration of E. coli, as well as the detection of bacterial coliforms, respectively. Culture-dependent and independent techniques were used to profile the bacteria and detect antibiotic-resistant bacteria (ARBs) and antibiotic-resistant genes (ARGs) present in the samples. Metagenomics sequencing and analysis were applied to investigate the ARB genetic profiles. Data was analysed using R software and Quantitative Insights into Microbial Ecology (QIIME2, version 2021.4) software. Heatmaps showed the morphological and biochemical characterization relationships, antibiotic susceptibility, and detection of ARGs in the culturable bacteria. The DNA extracted from the eighty-two raw water samples was pooled into six tubes according to the site of collection: Wakulima Eateries (WE), Wakulima Open Market (WOM), Gachororo Eateries (GE), Gachororo Open Market (GOM), Mwerevu Eateries (ME), and Mwerevu Open Markets (MOM). There is an abundance of the bacterial phylum Proteobacteria (above 86%) across all the collected water samples from the open markets and eateries. The dominant genera belonging to Proteobacteria were: WE (Acinetobacter 44.180%), WOM (Duganella 28.201%), GE (Acinetobacter 15.189%), GOM (Acinetobacter 30.675%), ME (Acinetobacter 40.823%), and MOM (Curvibacter 48.785%). This study reported the presence of antibiotic-resistant priority pathogens from the WHO list: Priority 1 (critical), where Pseudomonas spp. was detected in samples ME (12.163%) and WOM (0.323%), Acinetobacter baumannii was detected in samples GE (2.975%) and WE (5.495%), and Enterobacteriaceae was detected in samples WE (0.125%), MOM (0.326%), WOM (0.875%), GE (0.452%), and ME

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(0.206%). The genus of Escherichia coli, Klebsiella, Raoultella, and Enterobacter dominate the Enterobacteriaceae. Priority 2 (high) pathogen detected was the genus Staphylococcus in samples MOM (1.826%) and GE (0.962%). The ARGs of qnrD and sul2 were detected in all six samples; int1 and FloR genes were present in five samples, while those of strB, catA, and blaTEM were found in one sample each using qualitative polymerase chain reaction (PCR) assays. The findings of this study form a critical reference point for policy development and rapid mitigation strategies to prevent a possible outbreak of water-borne diseases in the area.

Key words: antibiotic-resistant bacteria (ARBs); antibiotic-resistant genes (ARGs); genetic diversity; pathogenic bacteria

1.0 Introduction

Maintaining hygiene in drinking water is challenging in urban areas with persistent anthropogenic influence, as it efficiently transmits diseases. Despite being indispensable, the purity of drinking water is often compromised in such environments. (Numberger et al., 2019). The availability of biologically safe drinking water is a major public health concern and relies on adequate protection of water sources, effective water treatment, and proper distribution system maintenance (Han et al., 2020). Most developing countries require the persistence of residual disinfectant, which is often ineffective, across all water distribution networks to control microbial waterborne pathogens (Han et al., 2020). The presence and growth of microbes in treated drinking water and at the customers' taps is undesirable both for biosafety reasons and because of process-related microbial problems during distribution such as nitrification, biocorrosion, and the persistence of pathogens (Liu et al., 2018). The majority of eateries in peri-urban settlements in developing countries depend on unsafe groundwater (wells and boreholes) that are potentially contaminated with antibiotics, ARBs, and ARGs for their daily requirements (Sanganyado & Gwenzi, 2019). The quality and organoleptic features, such as flavour, smell, and colour, of groundwater are badly affected by both human activities and natural factors (Singh et al., 2020). The implications of climate change, uncleanness or deleterious use of water reservoirs, population explosion, the mixing of sewage and agricultural runoff with water supply, and the high per capita cost of new hydroelectric infrastructure are a few features that auxiliary to the provision of water (Gwenzi et al., 2020; Singh *et al.,* 2020; Anand et al., 2016). As per the World Health Organization (WHO), the foremost parameters affecting water quality in terms of fit for utility are microbial (the presence of coliform Enterobacteriaceae like *Escherichia coli* or thermo-tolerant coliforms) and heavy metal contaminants (Waqar & Ali, 2020). Considering the shallow groundwater table, permeable aquifers, and the indiscriminate and abusive prophylactic and therapeutic use of antibiotics, the available untreated groundwater poses a huge public health risk (Obayiuwana & Ibekwe, 2020). The contaminated underground water therefore provides a perfect selective and natural medium for interaction between ARB and environmental bacteria for the horizontal shift of ARGs (Anand et al., 2016). ARGs have been detected in various water bodies, including reservoirs (Huerta et al., 2013; Su et al., 2014), sources of drinking water (Jiang *et al.,* 2013), rivers (Ling et al., 2013; Xu et al., 2016), lakes (Devarajan et al., 2015; Huerta et al., 2013), and oceans (Chen et al., 2019; Zhang et al., 2019; Jiang et al., 2018).

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Although the quality and safety of drinking water are monitored by water authorities based on indicator microorganisms in bulk water, surveillance of biofilms or microorganisms during distribution is not part of current regulations (Douterelo et al., 2020). For instance, *Escherichia coli* is a WHO-recommended faecal indicator bacteria for drinking water, while *Enterococci* is the indicator bacteria for bathing water (Acharya et al., 2019). The routine disinfection of all pathogens is impractical, as each requires a unique period, and there is a demand for more rapid and comprehensive screening methods to detect Fecal indicator organisms (FIO) and/or their markers and putative pathogens in water samples (Acharya et al., 2019). These traditional indicators are therefore limited with respect to their sensitivity, robustness, and specificity to detect failures within drinking water distribution systems (DWDS) (Acharya et al., 2019). These indicators are also not necessarily correlated with the occurrence of new emergent pathogens and are only able to indicate high-intensity disturbances (Douterelo et al., 2020). As a consequence, there is a need to develop alternative bioindicators of water quality that can predict, measure, and monitor changes within DWDS (Douterelo et al., 2020). Next-generation sequencing platforms offer high throughput, scalability, and efficiency, generate a large dataset with legitimately high taxonomic precision, and yield statistically robust assessments of community composition and population structural analysis. This culture-independent approach is thus a powerful tool for elucidating the complex microbial diversities and the mechanisms of anti-microbial resistance in drinking water samples (Fang et al., 2019). Quantitative microbial risk assessment (QMRA) is a suitable method for evaluating and quantifying this health risk. However, information about the exposure to ARB and ARGs in drinking water in peri-urban settlement schemes in developing countries is lacking for many scenarios, and dose-response models regarding ARB infections have not been developed yet.

The present study used Next Generation Sequencing (NGS), PCR-based molecular markers for screening antibiotic resistance genes, and biochemical approaches to catalogue the molecular origin of known and emerging antibiotic resistance in water used by eateries and open markets in Wakulima, Gachororo, and Mwerevu markets in Juja town.

2.0 Materials and methods

2.1 Study area and sampling

The study was done in Juja town (1.1018° S, 37.0144° E) and Kiambu County (1.0314° S, 36.8681° E) in Kenya. The purposeful sampling technique was used to collect water samples used in eateries and open markets in Wakulima, Gachororo, and Mwerevu in Juja (Figure 2). The samples were collected during the outbreak of the COVID-19 pandemic, when hand washing was encouraged, and the study was seeking to know if Juja residents were using clean water. The sampling also considered the nearness of dumping sites for waste from these markets, which are likely sources of contamination of the water. A total of 82 water samples—Wakulima Eateries (WE)—17, Wakulima Open Market (WOM)—12, Gachororo Eateries (GE)—21, Gachororo Open Market (GOM)—12, Mwerevu Eateries (ME)—9, and Mwerevu Open Markets (MOM)—11—were collected. Sterile small plastic water containers were used to collect 250 ml of water from water containers placed at the entrance of eateries, taps at the market entrance, and jerricans used by traders within the market to wash groceries, which included two (2)

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samples from two commercial water vendors in Wakulima and Mwerevu markets who sell purified water from boreholes. Juja town relies on water supply from wastewater treatment plants and boreholes. The samples were labelled properly and kept on ice, then transported to the Molecular Biology Laboratory at the Institute of Biotechnology Research at Jomo Kenyatta University of Agriculture and Technology (JKUAT) for immediate use and stored at-20 °C for subsequent experiments.

2.2 Characterization of bacteria in water

2.2.1 Isolation of bacteria for morphological and biochemical characterization.

Isolation was done using MacConkey media (MaC 51.53 g/l), Triple Sugar Iron medium (TSIA, 64.5 g/l), Thioglycollate medium (brewer) (TM) (26 g/l), Lauryl Tryptose Broth (LTB, 35.60 g/l), and Nutrient Agar (NA, 28 g/l). From each water sample, 50 µl of water was pipetted and placed on each of the five different types of media in petri dishes and then spread using a sterile glass spreader till dry (Mbithi, 2013). The petri dishes were kept in the incubator for 24 hours at 37°C. Discrete colonies were isolated and sub-cultured on nutrient agar using the streak plating method to obtain pure cultures (Temitope et al., 2013). A total of 42 pure cultures were obtained and preserved using a sterile loop to pick a colony from the plate and place it in a 20% (v/v) glycerol solution in cyro vials, then vortexed and stored at-80°C (Akinbankole et al., 2015), which were morphologically and biochemically characterised (Marzan et al., 2017).

2.2.2 Morphological characterization

Gramme staining was done on all the isolates, while the cell shape and colony morphology (form, elevation, margin, size, colour, surface, opacity, and cell shape) were viewed and recorded as pictures using a computerised microscope fitted with the Scope Image 9.0 application.

2.2.3 Biochemical characterization

2.2.3.1 Citrate utilization test

This was done using Simmons's citrate test protocol (Akinbankole et al., 2015). *Klebsiella pneumoniae* culture was used as a positive control, while *Escherichia coli* culture was used as a negative control. The positive reaction showed a green colour that changed to an intense blue colour, while the negative reaction showed no growth and no colour change along the slants.

2.2.3.2 Methyl red test and voges proskauer test

The tubes containing sterile Methyl Red-Voges Proskauer Broth were inoculated with freshly prepared cell colonies of the isolates (24 hours old) on nutrient agar in petri dishes. The tubes were incubated at 37°C for 24 hours, after which aliquots of 1 ml of the broth were transferred to clean test tubes. A volume of 0.6 mL of 5% α-naphthol was added, followed by 0.2 mL of 40% dipotassium phosphate to the broth. The tubes were shaken gently to expose the medium to atmospheric oxygen and allowed to remain undisturbed for 30 minutes. *Klebsiella aerogenes* culture was used as a positive control, while *Escherichia coli* culture was used as a negative control. The positive test showed the development of a red colour after 15 minutes, while the negative result indicated a yellow-brown colour.

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2.2.3.3 Motility indole urease test

Motility Indole and Urea tests were done using Motility Indole agar, according to Arya et al. (2020). The tubes were observed for the urease test through a change of colour from yellow-orange to pink-red for a positive reaction and no colour change for a negative reaction upon the addition of Kovac's reagent. *Escherichia coli* culture was used as a positive control, while *Klebsiella pneumonia* was used as a negative control for the indole test.

2.2.3.4 Catalase test

A sterile wooden stick was used to transfer a small amount of freshly grown colony to the surface of a clean, dry glass slide. A drop of 3% hydrogen peroxide was placed in the colony on the glass slide. The slides were observed for the evolution of bubbles. A positive reaction showed active bubbling, while a negative reaction showed no or few bubbles. The positive control used was *Salmonella,* while the negative control was *Enterococcus faecalis.*

2.2.3.5 Triple iron sugar (TSI) agar test

This was used to test for lactose, sucrose, and glucose sugar fermentation and the production of gases such as hydrogen, carbon IV oxide, and hydrogen sulphide. The TSI agar was prepared by dissolving 65.524 g of this agar in 1000 ml of distilled water. It was distributed into test tubes, sterilised in an autoclave at 121°C for 15 minutes, and left to solidify in a slanted position to give a 2.5 cm butt and a 3.8 cm slant in the safety cabinet. A sterile wire loop was used to touch the top of a colony (24 hours old), followed by stabbing the butt through the middle of the agar to the bottom of the tube and then streaking of the slant. The test tubes were loosely closed with sterile cotton wool and incubated at 37°C for 24 hours. A yellow colour in both the butt and the slant indicated lactose and sucrose fermentation. A red slant and a yellow butt indicated glucose fermentation. Both the slant and butt remained red in some tests, which indicated lactose, sucrose, and glucose non-fermenters. A black precipitate in the butt showed the production of hydrogen sulphide gas. Production of hydrogen and carbon IV gases was indicated by the presence of bubbles, cracks, or lifting of the media.

2.3 Antimicrobial susceptibility/resistant testing

All the pure culture isolates obtained during isolation were subjected to an antimicrobial susceptibility test to determine the sensitivity or resistance of the bacteria using commercially available standard impregnated antibiotic discs (Biyela et al., 2018) containing Ampicillin (AMP) 25 mcg, Tetracycline (TE) 25 mcg, Co-Trimoxazole (COT) 25 mcg, Streptomycin (S) 10 mcg, Kanamycin (K) 30 mcg, Gentamycin (GEN) 10 mcg, Sulphamethoxazole (SX) 200 mcg, and Chloramphenicol (C) 30 mcg antibiotics. The isolates were spread on nutrient agar in petri dishes using the spread plate method, and then, using sterile forceps, the discs were laid on the spread bacterial isolates and incubated at 370 °C for 24 hours. Antibiotic susceptibility was determined by measuring the diameters of zones of inhibition using a ruler. The isolates were classified as resistant by indicating zero as there was no zone of inhibition or susceptible to a particular antibiotic (Bhargav et al., 2016).

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2.4 Molecular characterization.

2.4.1 DNA extraction

DNA extraction was done from the 82 raw water samples collected from eateries and open markets in Wakulima, Gachororo Centre, and Mwerevu-Juja. The extraction was done using the Isolate II Genomic DNA Kit from Meridian Bioscience Company. The extracted DNA was stored in the freezer at-200 °C in PCR tubes for further analysis.

2.4.2 Polymerase chain reaction for amplification of 16S rRNA gene

Amplification of the 16S rRNA gene of genomic DNA obtained from all the 82 raw water samples was performed using the 16S universal bacterial primers 515F GTGCCAGCCGCCGCGGTAA and 806R GGACTACTCGGGTTTCTAAT to amplify approximately 300 bp of the 16S rRNA gene (Figure 1). PCR was prepared in a 40-µl reaction mixture per tube containing 0.5 µl of 0.25 µmol/L of each primer, 18 µl of sterile ddH_2O, and 20 µl of 5X MyTaq Reaction Buffer, to which 1 µl of template DNA was added. The amplification of the template DNA was done in a programmed thermocycler. The thermal cycling protocol involved 5 minutes of initial denaturation at 95°C, followed by 30 cycles of denaturation for 10 seconds at 98°C, 30 seconds of annealing at 55°C, and 45 seconds of extension at 72°C. The final extension was performed for 1 minute at 72°C. The resultant amplicons were stored in PCR tubes at-20°C.

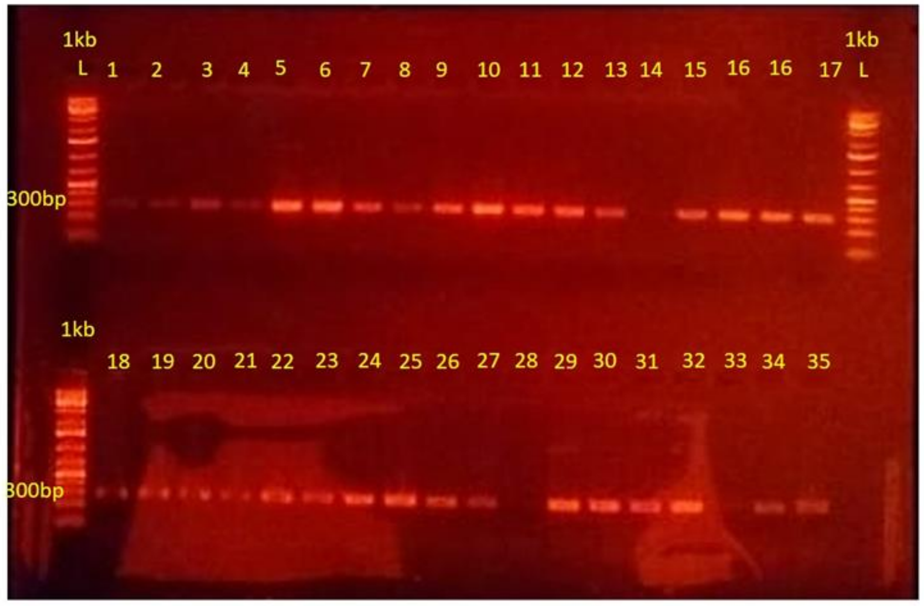

Figure 1: Gel photograph of bacterial 16S rRNA gene PCR products; L) Molecular marker (1kb ladder), 1) WE1, 2) WE2, 3) WE3, 4) WE4, 5) WE5, 6) WE6, 7) WE7, 8) WE8, 9) WE9, 10) WOM10, 11) WOM11, 12) WOM12, 13) WOM13, 14) WOM14, 15) WOM15, 16) WOM16, 17) WOM17, 18) WOM18, 19) WOM19, 20) WOM20, 21) ME26, 22) ME22, 23) ME23, 24) ME24, 25) ME25, 26) ME26, 27) MOM27, 28) MOM28, 29) MOM29, 30) MOM30, 31) MOM31, 32) MOM32, 33) MOM33, 34) GE34 and 35) GE35.

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2.4.3 DNA sequencing and phylogenetic analysis

A total of six samples containing pooled PCR products were sent for sequencing at Molecular Research LP (MR DNA) in the United States of America. The amplified DNA was pooled into six tubes according to the sites of collection: Wakulima Eateries (WE), Wakulima Open Market (WOM), Gachororo Eateries (GE), Gachororo Open Market (GOM), Mwerevu Eateries (ME), and Mwerevu Open Markets (MOM). Sequencing was done on the Illumina MiSeq platform.

Phylogenetic analysis was done using Quantitative Insights into Microbial Ecology (QIIME2 version 2021.4) software to determine the genetic diversity of bacteria present in the untreated water. The Next Generation Sequencing (NGS) raw data (already demultiplexed and in CASSAVA 1.8 format) was received, and the quality of the reads was confirmed (Phred score > 25) in the QIIME pipeline by obtaining the interactive quality plots. DADA2 was used for denoising and merging of the sequences to achieve the Operational Taxonomic Units (OTUs) for downstream processing of alpha and beta analyses. The samples had different numbers of sequences, so they had to be standardised by sub-sampling without replacement through rarefaction. A sampling depth of 105230 that considered the lowest number of sequences among the samples was chosen to avoid losing any of the samples.

Alpha analysis was done utilising Faith Phylogenetic Diversity (a measure of community richness) and evenness metrics (a measure of the distribution of the number of features per taxa in a sample). This was followed by beta diversity analysis utilising Emperor plots that allowed the principal coordinate analysis (PCoA) of the data. It generated Emperor plots for weighted and unweighted Unifrac with PCoA and showed the distances of the Operational Taxonomic Units (OTUs) in terms of diversity between the three markets. The last step was taxonomic classification, which utilised a Naive Bayes classifier to assign a class label to the samples. The classifier generated interactive bar plots, which were visualised at the phylum level to determine the dominant phyla in each sample.

2.5 Detection of antibiotic resistance genes

The DNA from raw water samples was pooled into six tubes according to the site of collection, the same as the DNA amplicons that were sent for sequencing. Polymerase chain reaction (PCR) was performed using sixteen ARG primers on all extracted genomic DNA. t A PCR reaction volume of 20 µl of a of a mixture containing 0.4 µl of 0.2 µmol/L of each primer, 8.2 µl of sterile ddH_2O, 10 µl of One Taq® quick-load 2X master mix, and 1 µl of template DNA was used. The thermal cycling protocol involved 3 minutes of initial denaturation at 94 °C, followed by 30 cycles of denaturation for 30 seconds at 94 °C, 40 seconds of annealing set at a gradient PCR temperature of between 47 and 64°C to determine the optimum annealing temperature of each primer, and 1 minute of extension at 68°C. The final extension was performed for 5 minutes at 68°C. Upon optimization, PCR was done using all primers on all the extracted DNA. PCR products were then separated by electrophoresis on 1.5% agarose run at 150 A and 160 V for 45 minutes. Amplicons were visualised under UV light and recorded.

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3.0 Results

3.1 Isolation of bacteria

A total of 42 bacterial isolates were obtained from the water samples collected from the eateries (E) and open markets (OM) of Wakulima (W) 24 isolates, Gachororo (G) 9 isolates and Mwerevu (M) 9 isolates. The isolates were coded as follows; Wakulima market (WE1, WE2, WE3, WE4, WE5, WE6, WE7, WE8, WE9, WOM13, WOM14, WOM15, WOM16, WOM17, WOM18, WOM19, WOM20, WOM21, WOM22, WOM23, WOM25, WOM26, WOM27, WOM28), Gachororo market (GE10, GE11, GE12, GE24, GE29, GE30, GE37, GE38, GE40) and Mwerevu market (MWV31, MWV32, MOM33, MOM 34, MOM35, MOM36, ME39, ME41, MOM42). The Gram staining technique was used to profile the morphology of bacterial isolates (Figure 3).

Figure 2: Sampling sites of eateries and open markets in Wakulima, Gachororo and Mwerevu sites.

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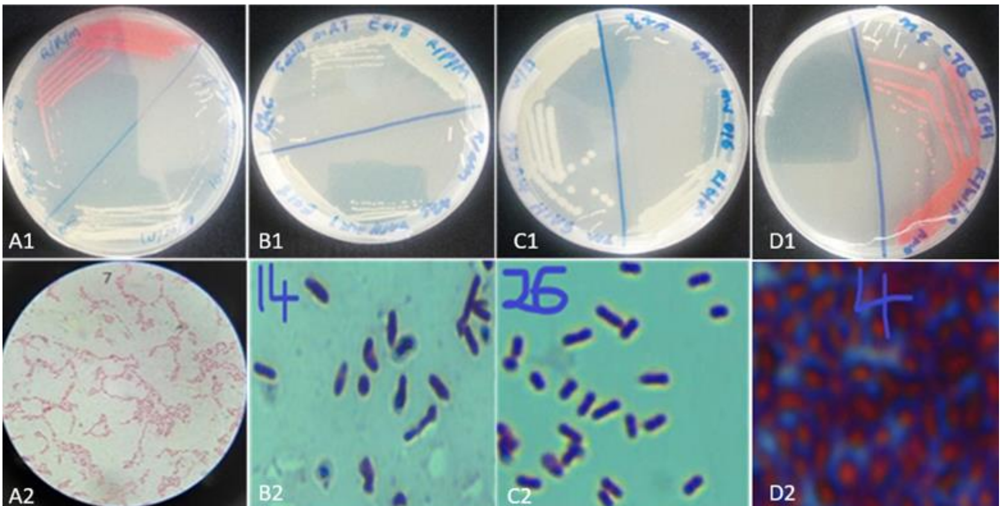

Figure 3: Plate bacterial isolates on culture media and their respective microscopic images. (A) WE7, (B) WOM14, (C) WOM26, and (D) WE4. The roman numbers 1 and 2 refer to macroscopic and microscopic images, respectively.

3.2 Morphological characterization

The correlation between morphological descriptors and their contribution to the phylogeny of the isolates is clearly profiled in Figure 5. Twenty-four out of the 42 bacterial isolates were gram negative bacteria, while 18 were gram positive. Thirty-three of the bacterial isolates were rod-shaped, while nine were cocci-shaped.

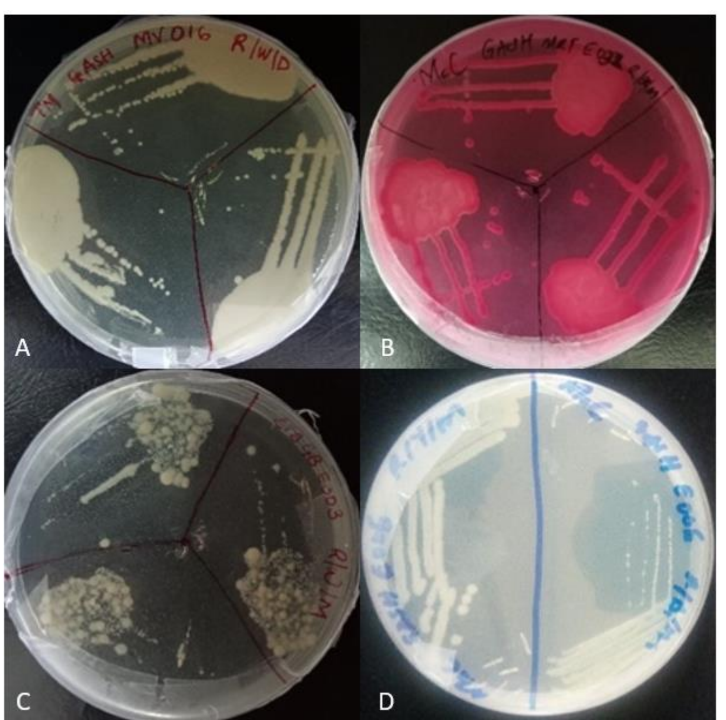

Figure 4: Plate Pure bacterial isolates. (A) MOM33, (B) GE34, (C) WE3, (D) GE37.

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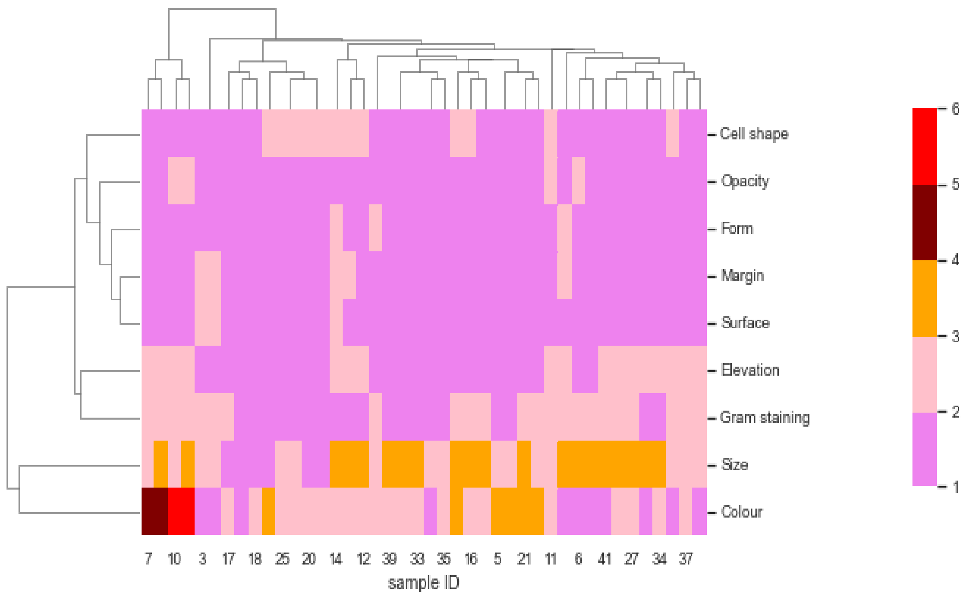

Figure 5: A heatmap showing the morphological relationship among the bacterial isolates.

A heatmap was generated using the various phenotypic characteristics obtained from the colony morphology of the bacterial isolates. The heatmap shows the relationship between the isolates and the observed characteristics. The coloured scale bar indicates the quantified significance of the observed phenotypic characteristics. The numbers 1–6 represent scales of values, with lower and higher numbers representing less and more significant characteristics of identifying the isolates. Red and purple colours in the heatmap indicate the highest and least recorded significant mean values, respectively, at P≤0.05 for the observed characteristics.

3.3 Biochemical characterization

The ability of isolates to utilise different substrates was equally useful in studying relationships between isolates, as shown in Figure 6. The differential strength in utilising the substrates significantly clustered isolates, as shown in Figure 7.

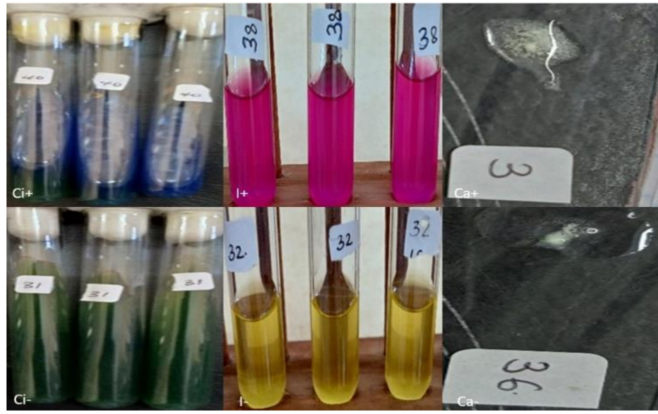

Figure 6: Plate Biochemical tests. Citrate positive (Ci+), Citrate negative (Ci-), Indole positive (I+), Indole negative (I+), Catalase+ (Ca+) and Catalase negative (Ca-).

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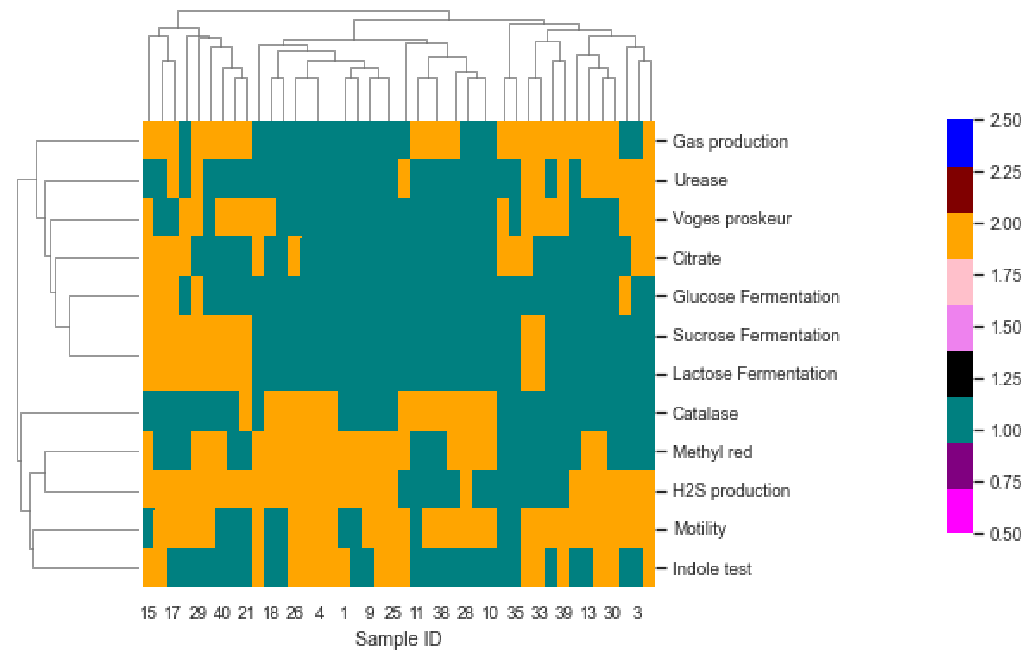

Figure 7: A heat map showing the biochemical characterization relationship among the bacterial isolates.

The heatmap was generated using various products from biochemical characterization by the bacterial isolates. The heatmap shows the relationship between the bacterial isolates and the measured biochemical descriptor. The coloured scale bar indicates the quantified significant strength of the assayed biochemical descriptor. Blue and purple colours in the heatmap indicate the highest and the least recorded significant mean values, respectively, at $P \leq 0.05$ for the assayed treatments.

3.4 Antimicrobial susceptibility testing

The antibiograms of the tested isolates showed susceptibility or resistance to the eight antibiotics used, as shown in Figure 8. Out of the 42 isolates, 12 (28.6%) were resistant to ampicillin, 27 (64.3%) were resistant to co-trimoxazole and sulphamethoxazole each, 8 (19.1%) were resistant to streptomycin and kanamycin each, 3 (7.1%) were resistant to gentamycin, and 9 (21.4%) were resistant to chloramphenicol. On the other hand, 30 (71.5%) were susceptible to ampicillin, 15 (35.7%) were susceptible to co-trimoxazole and sulphamethoxazole each, 34 (81.0%) were susceptible to streptomycin and kanamycin each, 39 (92.9%) were susceptible to gentamycin, and 33 (78.6%) were susceptible to chloramphenicol. All 42 isolates were susceptible to tetracycline, as shown in Figure 9.

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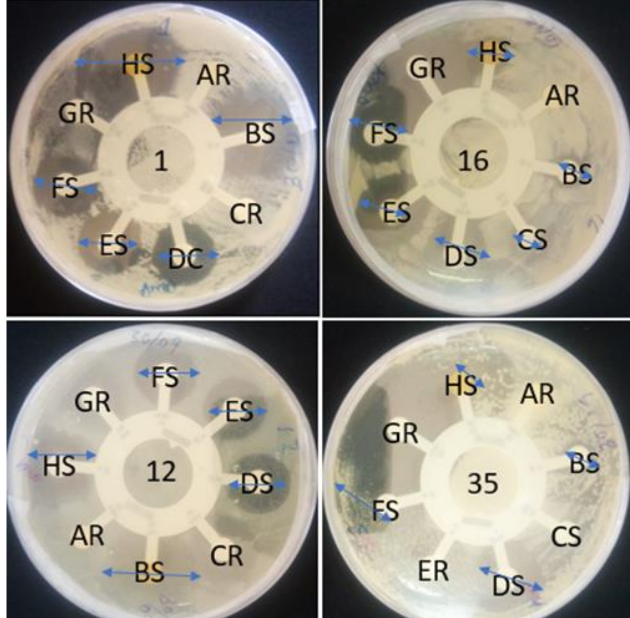

Figure 8: Plate antimicrobial susceptibility of microbial isolates against standard impregnated antibiotic disks 1) Disc inoculum of WE1, 16) Disc inoculum of MOM16, 12) Disc inoculum of GE12, 35) Disc inoculum of MOM35. Letters A–H represent the eight antibiotics used.

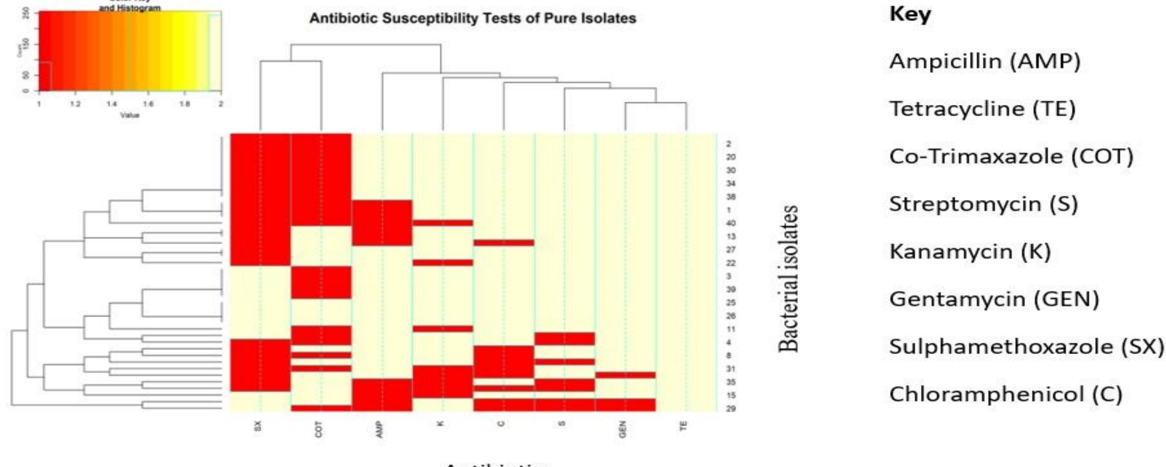

Figure 9: A heat map showing antibiotic susceptibility tests of pure cultures isolated from the water collected from Wakulima, Gachororo, and Mwerevu markets.

The heatmap was generated by means of zones of inhibition caused by antibiotics against the bacterial isolates. The heatmap shows the relationship between the isolates and the measured morphometric descriptor, as shown in Figure 9. The coloured scale bar indicates the quantified significant strength of the assayed morphometric descriptor. The red and yellow colours in the heatmap indicate the highest and the least recorded significant mean values, respectively, at P≤0.05 for the assayed treatments.

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The results of the antimicrobial resistance pattern of the isolates showed that 16 (38.1%) were resistant to 2 antibiotics, 7 (16.7%) were resistant to 3 antibiotics, 3 (7.1%) were resistant to 4 antibiotics, and 2 (4.8%) were resistant to 5 and 6 antibiotics each. From this analysis, it was evident that 30 (71.4%) isolates were multidrug resistant when considered resistant to two or more drugs.

3.5 Molecular characterization

3.5.1 Assessment of bacterial genetic diversity in water samples

Quality filtering of the sequence data from the water samples yielded 1,591,729 high-quality reads with a per-sample read mean of 265,288. A total of 2,589 features were generated based on 97% similarity. The Faiths phylogenetic alpha diversity metric boxplots showed a p-value of 0.651, indicating that there was no statistically significant difference in terms of bacterial diversity in samples collected within the same market. Pielou's evenness alpha diversity boxplots also showed a p-value of 0.180, indicating that there was no statistically significant difference in terms of the distribution of bacterial species in samples collected within the same market as compared to another market. Beta diversity analysis using PCoA plots of Bray-Curtis distances showed no significant distinction between the water samples.

Water from all three markets was dominated by the bacterial phylum Protobacteria, with WE (96.057%), WOM (94.763%), GE (91.851%), GOM (88.955%), ME (95.285%), and MOM (86.063%) (Figure 10). Other phyla that were found in the samples and highest in their respective sites are Bacteroidota (MOM = 9.967%), Verrucomicrobiota (GOM = 1.646%), Deinococcota (GOM = 4.200%), and Firmicutes (WOM = 1.229%), as shown in Figure 10.

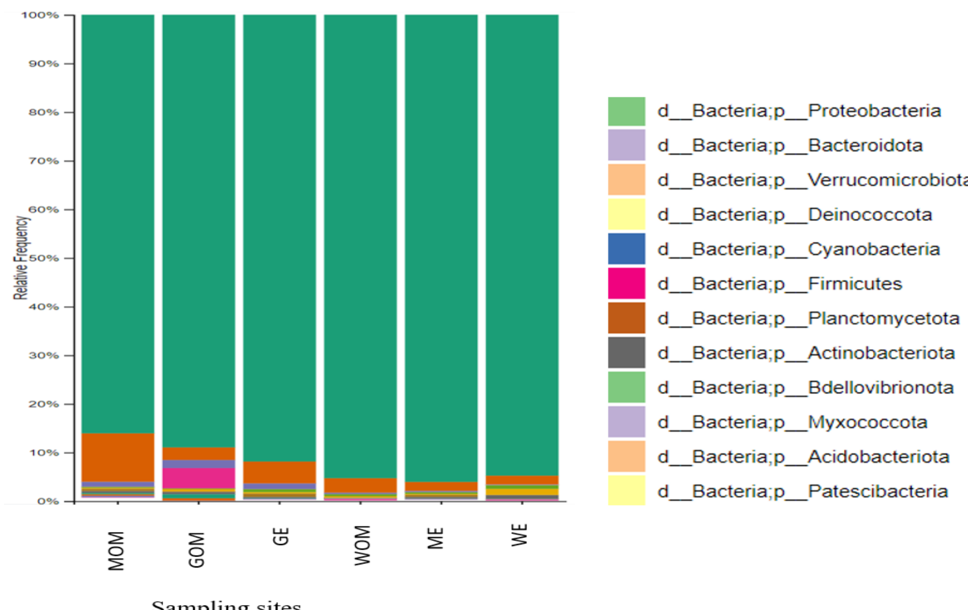

Figure 10: Bacterial relative abundance of phylum-level taxonomic classification of water microbiota collected from Wakulima, Gachororo, and Mwerevu markets.

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The genera belonging to Proteobacteria dominant in the six sites were: WE (*Acinetobacter* 44.180%), WOM (*Duganella* 28.201%), GE (*Acinetobacter* 15.189%), GOM (*Acinetobacter* 30.675%), ME (*Acinetobacter* 40.823%), and MOM (*Curvibacter* 48.785%). The genus *Acinetobacter* was the most abundant across 4 out of the 6 samples as shown in Figure 11. The species of *Acinetobacter baumannii* were detected in samples GE (2.975%) and WE (5.495%), while those of *Acinetobacter iwoffi* were detected in samples GOM (0.093%) and ME (5.296%).

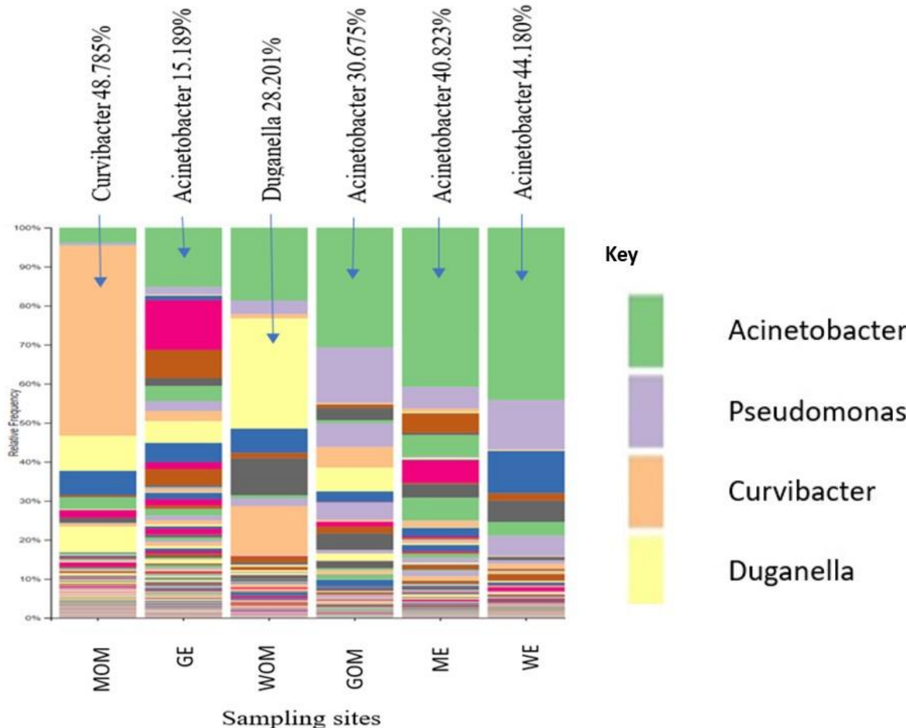

Figure 11: Relative abundance of Curvibacter, Actinobacter, and Duganella in water collected from Wakulima, Gachororo, and Mwerevu eateries and open markets.

This study reported the presence of antibiotic-resistant priority pathogens from the WHO list. The most abundant were pathogens belonging to priority 1 (critical). The dominant group of bacteria from the WHO priority pathogens list was *Pseudomonas spp.* detected in samples WE (12.163%) and WOM (0.323%). The second most frequently isolated pathogen was *Acinetobacter baumannii,* detected in samples GE (2.975%) and WE (5.495%)**.** The third most frequently isolated pathogen was *Enterobacteriaceae,* detected in samples WE (0.125%), MOM (0.326%), WOM (0.875%), GE (0.452%) and ME (0.206%). The genus of *Escherichia coli*, *Klebsiella*, *Raoultella,* and *Enterobacter* dominate the *Enterobacteriaceae*. The pathogen belonging to priority 2 (high) detected was the genus *Staphylococcus* in samples MOM (1.826%) and GE (0.962%).

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3.5.2 Detection of antibiotic resistance genes

The ARGs of *sul2* and *qnrD* were detected in all six pooled samples. The *FloR* and *Int1* ARGs were present in five samples except in MOM and GOM samples, respectively. Genes of *catA* and *strB* were detected in sample MOM, while those of *blaTEM* were detected in sample GOM. The genes of *erm*X, *Amp, aac6, qnrC, qnrS, blaCTMX, aac3, NleA, and TetA* were not detected across all six pooled samples, as shown in Figure 12. This is a clear indication that ARGs have been prevalent in the water used in the three markets.

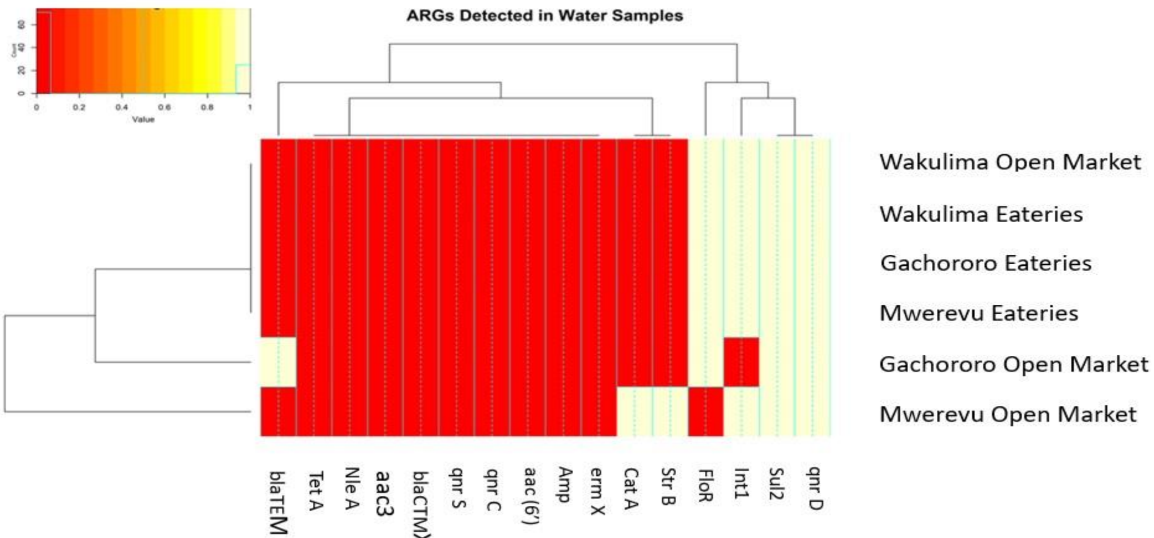

Figure 12: A heat map showing the detection of antibiotic resistance genes in the water samples collected from Wakulima, Gachororo, and Mwerevu markets.

The heatmap was generated by means of the detection of various ARGs in the water samples. The heatmap shows the relationship between the water samples and the detection of the ARGs. The coloured scale bar indicates the quantified significant strength of the assayed resistance. Yellow and red colours in the heatmap indicate the highest and least recorded significant mean values, respectively, at P≤0.05 for the assayed treatments.

4.0 Discussion

4.1 Morphological and biochemical characterization

The aim of this study was to assess the bacterial quality of water samples collected from Wakulima, Gachororo, and Mwerevu markets. Culture-dependent methods have been used in the characterization of environmental samples such as water by identifying microbial characteristics such as appearance and antibiotic resistance (Vaz-Moreira et al., 2017). All the bacterial isolates from the collected water were identified through morphological and biochemical characterization. Antibiotic-resistant bacteria were detected because some of the bacteria could still grow on the media even in the presence of antibiotics (Mian et al., 2020). This means they had acquired antibiotic resistance genes, which conferred resistance to these antibiotics (Szekeres et al., 2018). A total of 30 (71.43%) isolates were multidrug-resistant. These similar findings were reported by Hassan et al. (2018). This means that these antibiotic-resistant

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bacteria are likely to have a genetic recombination of two or more genes that confer resistance to the antibiotics. The antibiogram results formed the basis for screening the antibiotic-resistant genes using primer-specific PCR assays (Zou et al., 2019; Akpan et al., 2020).

4.2 Genetic diversity of bacteria

Water treatment has been used as a method of water purification for commercial and domestic purposes, but access to this water has been a challenge due to its scarcity, leading to the use of alternative sources of untreated water (Guarino, 2017; Tzanakakis et al., 2020). The study revealed that water from all three markets was dominated by the bacterial phylum Protobacteria. Other phyla found were Bacteroidota, Verrucomicrobiota, Deinococcota, and Firmicutes. A similar study on assessing the impact of bacterial communities in tap water from 46 drinking water supply systems in China showed that the phyla of Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Cyanobacteria dominated the water (Han et al., 2020).

Acinetobacter baumannii is commonly found in environmental samples, such as water and soil. There are several species that belong to this genus; however, the species Acinetobacter baumannii accounts for 80% of infections in humans (Vázquez-López et al., 2020). Other studies have shown that Acinetobacter baumannii is highly resistant due to the multiple antibiotic resistance genes that are harbored in this bacterium (Palavecino et al., 2022). For instance, Acinetobacter baumannii has been reported to contain resistant genes against beta-lactam antibiotics and quinolones, and its resistance to aminoglycosides is increasing (Georgina Solano-Gálvez et al., 2021; Karumathil et al., 2018).

Currently, the microbial indicators for surface water contamination used are: total coliforms, enterococci, fecal streptococci, Escherichia coli, and Clostridium perfringens (Jałowiecki et al., 2022). These bacterial indicators have been widely used in monitoring microbiological water quality. Additionally, these microbiological indicators are both bacteria-specific and multi-antibiotic resistant (Jian et al., 2021). Therefore, the pathogens have acquired a new characteristic of multi-antibiotic resistance, which is a threat to public health. The occurrence of these pathogens in the environment has escalated the mortality rate associated with microbial infections, particularly those caused by multi-drug-resistant species such as Escherichia coli, Acinetobacter spp., Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, and Enterococcus spp. (Asokan et al., 2019). The species of Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa harbor antibiotic-resistant genes against penicillins, cephalosporins, carbapenems, monobactams, quinolones, aminoglycosides, tetracyclines, and polymyxins (Jałowiecki et al., 2022).

This study had a shortcoming due to the failure of the NGS analysis to resolve up to level 7, that is, species level. This is because the 16S rRNA gene primer used only targeted the V4 region of the 16S rRNA gene amplified by the Illumina Miseq platform. It is very costly to sequence the

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entire length of the 16S rRNA gene to deduce the strain level, which our limited budget was unable to cover.

4.3 Antibiotic resistant genes

The study area relies heavily on water supplied from wastewater treatment plants and borehole sources. Earlier studies have shown that drinking water and wastewater treatment processes are incapable of completely removing antibiotic resistance genes (ARGs) (Amarasiri et al., 2020). The observed resistance to antibiotics could possibly arise either from point mutations in the bacterial genome (intrinsic resistance) or through the acquisition of genes encoding resistance determinants (acquired resistance) (Das et al., 2020). The antibiogram results formed the basis for screening the antibiotic-resistant genes using primer-specific PCR assays (Zou et al., 2019; Akpan et al., 2020).

Polymerase chain reaction assays showed that the antibiotic resistance gene *qnrD* was detected in all samples. This ARG is a plasmid-mediated quinolone resistance (PMQR) determinant that encodes a pentapeptide repeat protein, responsible for inducing low susceptibility to quinolone by binding to DNA-DNA gyrase complexes (Zeng et al., 2020). Quinolones (ciprofloxacin, norfloxacin, ofloxacin, etc.) are among the most commonly prescribed antibiotics due to their broad-spectrum antibacterial activity, and the frequent use of quinolones has contributed to the emergence of quinolone resistance worldwide, posing a serious threat to public health (Zhang et al., 2019). The plasmid nature of *qnrD* plays a crucial role in the horizontal transfer of quinolone resistance genes due to their good self-replication and transmission characteristics, which explains why it was detected in all samples (Zhang et al., 2019).

Sulfonamide resistance gene *sul2* was detected in all six samples, exhibiting a frequency of 100%, the same as that of *qnrD* genes. This indicates that the water used in these markets is highly contaminated with sulfonamides, and the bacteria in this water have developed resistance to these antibiotics. *Sul2* is a plasmid-borne gene encoding diyhdropteroate synthase that inhibits sulfa-based drugs due to mutations in the *folP* gene encoding this enzyme (Wang et al., 2019).

Present in gram negative bacteria such as *Salmonella enterica typhimurium*. Sulfonamides function as broad-spectrum antibacterial agents, and their prolonged use is likely to favour the selection of resistant bacteria. This similar occurrence has been shown in the detection of *sul2* genes in water from the River Njoro in Nakuru County, Kenya (Itotia et al., 2018). Previous studies reported domestic water from wastewater treatment plants are 'hot spots' of *sul* genes and MGEs and hence serve as reservoirs for the dissemination of the *sul* genes among bacteria (Lin et al., 2021).

Int genes were detected in all the samples except the Gachororo open market, with a frequency of 83.33%. The presence of *Int* genes in the bacteria indicates the possibility of alternate antibiotic resistance among different bacterial strains, as integrons are mobile and capable of integrating and expressing gene cassettes by site-specific recombination (Adesoji et al., 2015).

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This means that one bacterium can possess multiple cassettes that code for different antibiotic resistance, making it multidrug resistant. This will make it very difficult to treat bacterial infections in Juja residents who use this water due to the presence of these integrons, reducingthe potency of antibiotic drugs. These genes have also been detected and reported in wastewater that is likely to mix with underground water used for human consumption (Gwenzi et al., 2020).

The genes of both *strB* and *catA* were detected in only the Mwerevu open market, while those of *blaTEM* were only detected in the Gachororo open market, which is 16.67% frequency each. The genes of *strB* pose aminoglycoside resistance and have been reported as gene cassettes in relation to integron genes (Adesoji et al., 2015). That is why the genes of both *Int* and *strB* were detected in the same sample. The study showed the lowest prevalence of these genes in the water used in this area. An occurrence of these antibiotic residues has been detected in a study conducted on surface water in Nairobi County (Ngigi et al., 2019).

5.0 Conclusion

The bacterial diversity revealed that the phylum Proteobacteria, largely found in groundwater, had the highest relative abundance (more than 85%), with its members having a relative abundance above 40%: *Curvibacter* (48.78%) and *Acinetobacter* (44.180%).

The molecular detection of ARGs in the water samples is a major public health concern and an indicator of poor water hygiene.

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6.2 General acknowledgement

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6.3 Ethical considerations

None

6.4 Conflict of Interest

None

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