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Article

A One Health Perspective: Occurrence Study of Carbapenem-Resistant Bacteria and Other Emerging Pathogens from Recycled Wastewater Used in Agriculture

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Abstract

A Recycled wastewater is vital for circular economy, especially on water-scarce islands. This study explored the presence of Carbapenem-Resistant Enterobacterales and other emerging pathogens in irrigation water on four Canarian Islands, applying a One Health perspective. Using membrane filtration and MALDI-TOF mass spectrometry, 69 bacterial isolates were identified. The finding reveals that 50% were gram-negative bacilli like *Pseudomonas aeruginosa* and *Acinetobacter* spp., while 30% were Enterobacteriaceae, including *Klebsiella pneumoniae* and *Escherichia coli*. The main mechanisms of carbapenem resistance in *Pseudomonas* and *Acinetobacter* were oxacillinases, followed by metallo- β -lactamases (MBL). In Enterobacteriaceae, characterization of carbapenemase types was less frequent, with OXA-48 being the most prevalent. The detection of multidrug-resistant organisms in recycled wastewater highlights an urgent need for routine microbiological monitoring in water management protecting public health and agricultural sustainability.

Keywords: recycled wastewater; carbapenems; *Enterococcus* spp.; *Acinetobacter* spp.; *Pseudomonas* spp.; One Health

1. Introduction

Water stress constitutes a critical challenge for many countries, with implications for present and future generations [1,2]. Currently, over 2.4 billion individuals reside in regions experiencing water scarcity. The confluence of high population density, recurrent droughts, and anthropogenic pressures exacerbates environmental vulnerability, particularly in isolated regions such as oceanic islands [3–5].

Waterborne pathogens in aquatic ecosystems are already responsible for hundreds of millions of human illness cases annually [6]. Emerging evidence indicates that climate change is exacerbating this burden by promoting the proliferation and impact of numerous pathogenic microorganisms [7–9]. Monitoring the health effects associated with these hazards remains challenging, as many waterborne diseases are either undetected or underreported [8]. Furthermore, risks are expected to

escalate in response to global warming, intensified precipitation events, and the increasing frequency of floods [9].

One of the primary solutions to the global water crisis lies in improving irrigation systems for agriculture, which accounts for nearly 72% of global freshwater withdrawals [10–13]. One effective approach to mitigating water scarcity is wastewater reuse. Recycled wastewater undergoes physicochemical and biological treatments to comply with regulatory standards for different end-uses [14–17]. Despite undergoing treatment, recycled wastewater may still harbor pathogenic microorganisms, including multidrug-resistant bacteria. The overuse of antibiotics, coupled with insufficient wastewater treatment, has led to the dissemination of antibiotic residues into the environment [18–21]. This disruption of the One Health paradigm poses significant risks to human, animal, and ecosystem health [22,23]. Recycled wastewater, often reused for agricultural purposes, can contain a complex mixture of contaminants, including pathogenic microorganisms, heavy metals, and contaminants of emerging concern (CECs). These substances may pose direct threats to human and animal health and, in the case of CECs, potential long-term risks to ecosystems [24]. Agricultural reuse of recycled wastewater inherently connects multiple environmental compartments—such as wastewater treatment plants, surface water, groundwater, soil, and crops—facilitating the transfer and persistence of these contaminants. Consequently, the consumption of crops irrigated with contaminated water represents a tangible, non-negligible risk to human health [25].

The World Health Organization (WHO) considers bacterial resistance as a major global health threat due to the alarming rise in infections caused by multidrug-resistant bacteria, both in healthcare settings and in the community [26]. Multidrug-resistant bacteria are defined as those exhibiting resistance to one or more antibiotics from at least three distinct classes [27]. Antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are continuously released into aquatic environments, with hospital effluents recognized as important contributors to the spread of antimicrobial resistance beyond clinical settings [28]. Among the most concerning forms of antimicrobial resistance is carbapenem resistance, mediated by carbapenemase production and transmitted via plasmids among bacteria of various species [26]. These plasmids often carry genes conferring resistance to other antibiotics belonging to different antibiotic families [29–31]. The widespread use of carbapenems has driven the emergence of resistance to this critical group of antimicrobial drugs [32].

Wastewater treatment plants (WWTPs) and their associated disinfection process's function are critical barriers to limit the environmental dissemination of ARB [33,34]. Following discharge from the treatment plant into recycled water and storage facilities for reuse, recycled water is generally not subjected to systematic sanitary monitoring. Previous studies have demonstrated that the quality of recycled water exhibits variability throughout the distribution network, and it has been observed that its characteristics may change prior to use at different locations, such as within pipelines, storage tanks, and other points along the system. [35] It has been widely documented that one of the main factors contributing to variability in recycled water quality is the formation of biofilms within distribution systems. Biofilm development is generated by multiple interacting factors and environmental conditions. Furthermore, several studies have highlighted the role of biofilms as potential sinks for ARGs in aquatic ecosystems [36–39].

The variability observed in recycled water quality can be attributed to the multifactorial interactions between treatment performance and post-treatment management practices. Key determinants include storage conditions, which exert a strong influence on microbial persistence, regrowth, and community shifts, as well as geographic and hydrological drivers—such as the high agricultural demand characteristic of certain regions—that impose additional stress on water reuse systems [7,8]. Furthermore, the structural and operational attributes of distribution infrastructures, particularly the reliance on open reservoirs, extended retention times, and long conveyance networks, have been consistently associated with conditions favorable to biofilm development [35]. The subsequent establishment of biofilms not only facilitates the diversification and stabilization of microbial communities but also poses potential risks for water quality deterioration and the

persistence and dissemination of antibiotic resistance genes (ARGs) within aquatic environments [40].

In response to these challenges, the Methodological Spanish Guide for the Development of Recycled Water Risk Management Plans for Agricultural Use was developed in 2024 [41]. This framework seeks to provide robust technical criteria that enable more reliable, safe, and sustainable management of recycled water resources, while also integrating considerations of microbial ecology, infrastructure resilience, and agricultural water demand. However, although progress has been made with the development of these guidelines, the absence of binding regulatory frameworks mandating their adoption represents a major limitation for their practical implementation and constitutes a significant barrier to their systematic application.

The absence of post-treatment surveillance in recycled water may constitute a potential public health risk, particularly in contexts where recycled water is likely to come into direct contact with human populations [25]. It is important to underscore that climate change is profoundly altering the physical, chemical, and biological characteristics of aquatic systems, including storage tanks, which are subjected to extreme temperature fluctuations. These alterations may adversely affect human health and socioeconomic stability by disrupting aquatic microbial communities [7–9].

Although wastewater and WWTPs have been extensively investigated to assess the prevalence of ARB the contribution of recycled water storage practices to the dissemination of ARB remains comparatively underexplored. This study aims to determine the prevalence of emerging and carbapenemase-producing bacteria in recycled wastewater samples collected from irrigation points for agriculture in water-stressed oceanic islands.

2. Materials and Methods

2.1. Study area and fieldwork

This study was carried out in the Canary Islands Archipelago (Spain), located in the Atlantic Ocean near the African coast. The Canary Islands have different water necessities between islands, with some experiencing water stress. Desertification, high population density (278.77 inhabitants/km²), tourism (17.77 millions of tourists in 2024), insufficient rainfall, water losses in network or inadequate management are among the stressors contributing to a water demand that exceeds supply [42–44].

The easternmost islands from the Canary Islands demonstrate the greatest water stress, demanding the use of recycled wastewater and desalinisation, also in agriculture [42,45]. Currently, agricultural production on these territories relies heavily on non-conventional water resources [46].

In each water stress island, 5 different sampling points were taken from agricultural water storage for irrigation. Samples were assigned numbers according to the following order: Fuerteventura (B1, B2, B3, B4 and B5), Lanzarote (B6, B7, B8, B9 and B10), Gran Canaria (B11, B12, B13, B14 and B15) and Tenerife (B16, B17, B18, B19 and B20) (Fig. 1).

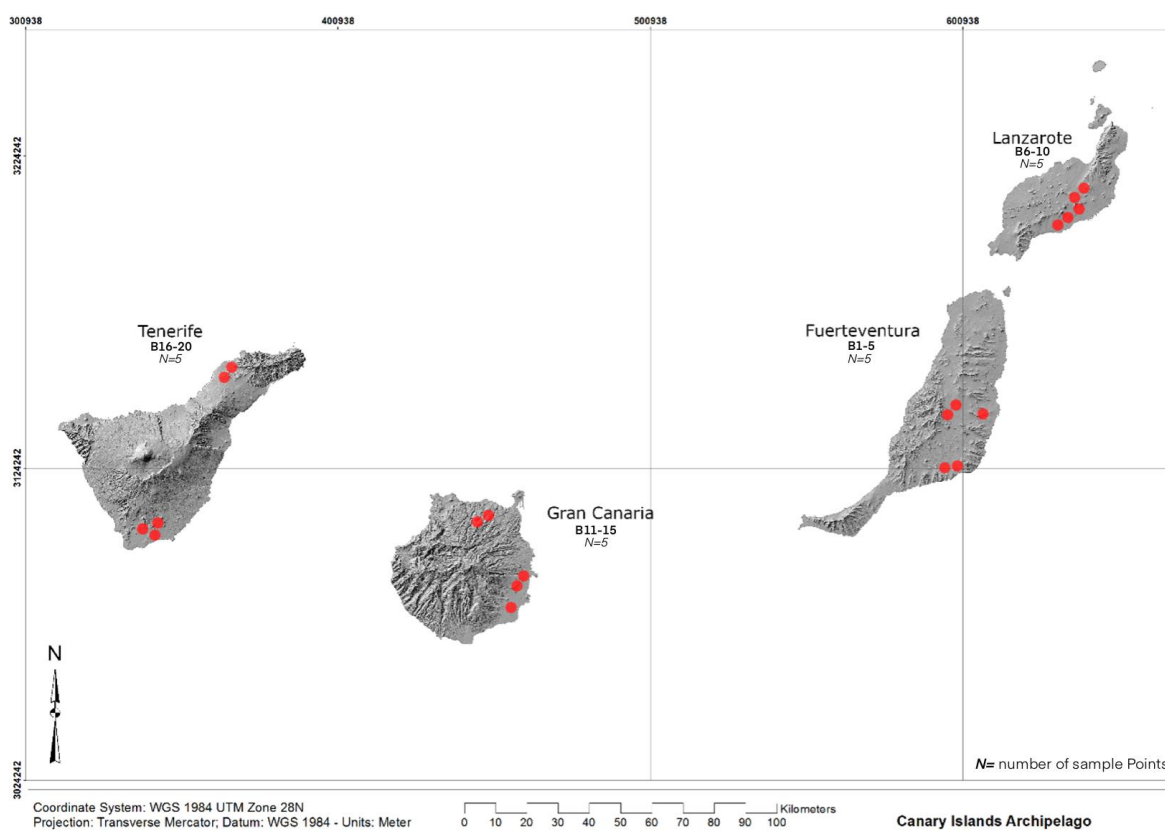


Figure 1. The sampled islands and sampled number that employ recycled wastewater for irrigation due the water stress.

Samples were collected between 13th October and 29th of November 2023 from the irrigation outlet of each water storage tank. Samples were collected in sterilized 500 mL bottles, ensuring the exclusion of air contact, and transported to the laboratory of Preventive Medicine and Public Health of University of La Laguna, Tenerife, for analysis within 24 hours in accordance with the guidelines outlined in Regulation (EU) 2020/741 for bacteriological analysis.

2.2. Microbial Culturing

From the initial sample volume, only 10 mL were used to prepare four serial tenfold dilutions for each sampling point, yielding a final dilution of 10^{-4} . Owing to the anticipated high bacterial growth and to prevent filter biofouling a 10 mL aliquot from each dilution was passed through a 0.45 μm membrane filter, using the membrane filtration method. The filters were then incubated on selective and differential media: Tergitol® 7 Agar OXOID at 44°C and Slanetz-Bartley Agar Base (Scharlau, Sentmenat, Spain), thiosulfate-citrate-bile-sucrose (TCBS) Agar OXOID®, and MacConkey M3 OXOID® at 37°C for 24 hours.

These media were used to isolate *E. coli*, *Enterococcus* spp., *Vibrio* spp., and Gram-negative bacilli, respectively. Filters yielding 20-80 colony-forming-units (CFU) were selected for repeat the process in triplicate further incubation over 24 hours. The most probable number (MPN) per 100 mL was determined for each microorganism.

2.3. Identification and Antimicrobial Susceptibility Testing

Once pure isolates were obtained, they were subsequently were performed to the species or genus level using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry MALDI-TOF MS. (VITEK-MS, bioMerieux, France) automated system as previous described by Ashfaq et al. (2022) and Puljko et al. (2024) [47,48]. Enterobacteriaceae and non-fermentative Gram-negative bacilli (NFGNB) were screened for carbapenemase production using

CHROMID CARBA SMART agar as previous other studied was carried [49,50]. Colonies exhibiting typical growth were then confirmed and characterized using the ROSCO Diagnostica KPC, MBL and Oxacillinase detection kits [51]. *Pseudomonas aeruginosa* and *Acinetobacter* spp. were also tested for these carbapenemase types (Sun et al., 2017) [52]. Vancomycin susceptibility of *Enterococcus* was determined using the disk diffusion method using vancomycin disc and Muller-Hinton agar, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [53]. Diameters of inhibition halos surrounding the disc were measured and expressed in millimeters. Results were interpreted as sensitive, intermediate or resistant, following the EUCAST criteria [53].

3. Results and Discussion

Focusing on the analysis of bacterial isolates obtained from the irrigation outlets sampled on each island, the results of the microbial diversity observed in each recycled wastewater irrigation outlet are represented in Fig. 2. The figure displays the microbial counts (CFU/mL) for each irrigation outlet grouped by island. Each bar represents a different outlet, allowing for a visual comparison of microbial load across sampling sites. The colors represented in the bars of the bar chart correspond to the different species isolated, with the bar height indicating the number of CFU per 100 ml. The graph also includes a line that indicates the number of isolations. Also, information on each bacterial isolation and the microbiological counts (CFU/100 ml) are extensively presented in Supplementary Materials (tables 1, 2, 3, and 4).

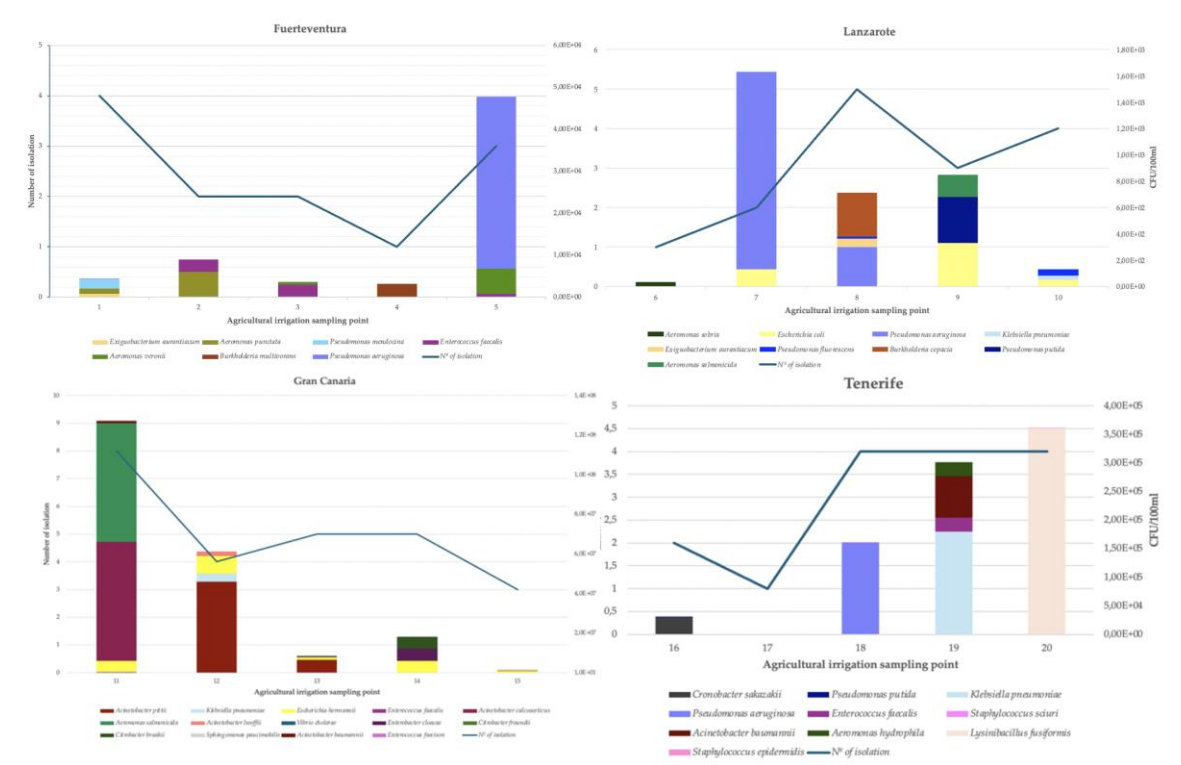


Figure 2. Bacterial isolates from island irrigation outlets. Y-axis: Number of isolates. X-axis: Irrigation outlet sampling points. Colors indicate different bacterial species (see legend). Blue line: Total bacterial isolates per sampling point.

Notably, outlet 11 from Gran Canaria Island exhibited the highest density and diversity of microorganisms, with a total of eight distinct species identified (*Acinetobacter pitii*, *Klebsiella pneumoniae*, *Escherichia hermannii*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *Enterococcus faecium*, *Acinetobacter calcoaceticus*, and *Aeromonas salmonicida*). The high prevalence of microorganisms and the elevated microbiological load observed on the island may be associated with multiple factors, the influence of which has not yet been fully elucidated. One potential explanation

In addition, the intensive livestock activity characteristic of Gran Canaria is a relevant factor to consider. According to data from the Canary Islands Institute of Statistics (ISTAC, 2024), this island, with a surface area of 156,151.87 hectares, reported 87,523 heads of livestock. This figure is particularly noteworthy when compared with Tenerife, the largest island in the archipelago (203,352 hectares), where 75,367 livestock animals were reported in the same year [54]. The high livestock density in Gran Canaria, relative to its available area, may be associated with an increased propagation and persistence of microorganisms in the environment.

To enhance the visualization of microbial isolates in the irrigation outlets, Fig. 3 depicts the outlets where microorganisms were detected, with each outlet represented by a distinct color.

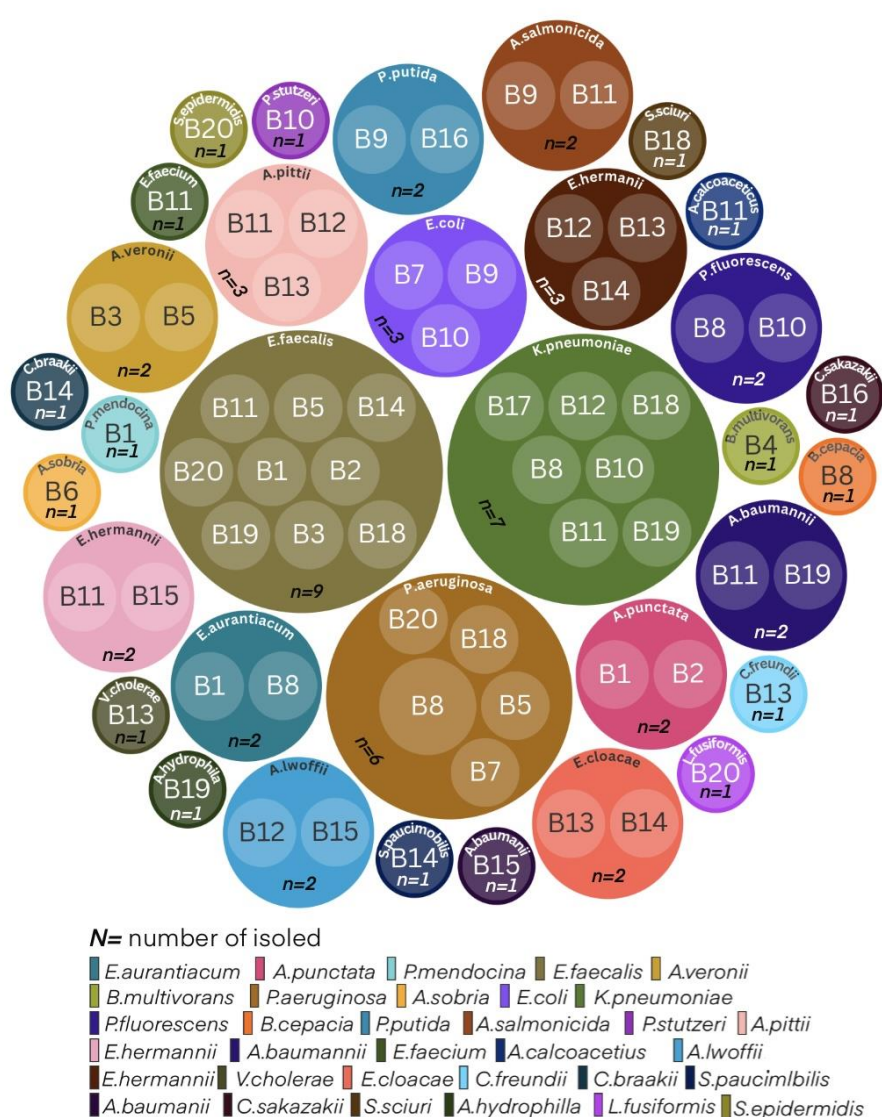


Figure 3. Details the recurring microbial species isolated from various irrigation outlets.

Dominant among these were *E. faecalis*, followed by *K. pneumoniae*. These species are frequently encountered in treated wastewater due to their common origin in the human gut microbiome. While recycled wastewater regulations often prioritize *E. coli* as an indicator organism, this does not preclude the presence of other potential pathogens [55–57]. The presence of

Enterococcus spp. especially *E. faecalis*, and *Enterobacteriaceae*, which are commonly found in the intestinal microbiota of mammals, indicated fecal contamination in the water bodies [56–59]. In many countries, the detection of *E. coli* serves as a specific marker for fecal contamination from human, animal, or untreated wastewater sources. According to Regulation EU 2020/741 on minimum requirements for water reuse, recycled wastewater for agricultural use is restricted to a maximum of 100 CFU/100 ml of *E. coli*, while the WHO has established guidelines primarily focused on *E. coli* within the *Enterobacteriaceae* family [60,61].

Vancomycin-resistant *Enterococcus faecalis* and *E. faecium* represent a significant clinical challenge in hospital settings, as they are capable of rapidly spreading within healthcare environments and causing infections that are notoriously difficult to treat. Data from the 2023 European Antimicrobial Resistance Surveillance Network (EARS-Net) indicate that 19.8% of *E. faecium* isolates and 24.3% of *E. faecalis* isolates were resistant to vancomycin [62]. These findings underscore the importance of implementing environmental surveillance strategies to detect potential reservoirs of resistance, thereby contributing to a more comprehensive understanding of its dissemination pathways.

From a total of 69 isolates, 32 corresponded to different microorganisms. The predominant groups identified were Gram-negative bacilli (34 isolates, 50%) *Enterobacteriaceae* (20 isolates, 30%), and Gram-positive bacteria (15 isolates, 22%). Within the Gram-negative bacilli group, non-fermenting Gram-negative bacilli accounted for 25 isolates (75%), the most relevant being *Pseudomonas aeruginosa* (6 isolates, 18%), *Acinetobacter pittii* (3 isolates, 9%), and *Acinetobacter baumannii* (3 isolates, 9%). The counts of the isolates mentioned are presented in Tables 1, 2, and 3.

Table 1. Counts of the most relevant non-fermenting Gram-negative (NFGN) species isolated at each sampling point.

Irrigation sampling point	Microorganism	CFU/100mL
B5	<i>Pseudomonas aeruginosa</i>	4,1 x104
B7	<i>Pseudomonas aeruginosa</i>	1,5x104
B8	<i>Pseudomonas aeruginosa</i>	2,9x102
B8	<i>Pseudomonas aeruginosa</i>	6,7x100
B18	<i>Pseudomonas aeruginosa</i>	1,6x105
B20	<i>Pseudomonas aeruginosa</i>	1,6x102
B11	<i>Acinetobacter baumannii</i>	1,2x106
B15	<i>Acinetobacter baumannii</i>	6,0x104
B19	<i>Acinetobacter baumannii</i>	7,3x104
B11	<i>Acinetobacter pittii</i>	1,9x105
B12	<i>Acinetobacter pittii</i>	4,6x107
B13	<i>Acinetobacter pittii</i>	6,5x106

Among the *Enterobacteriaceae*, table 2 shows that *Klebsiella pneumoniae* was the most prevalent species, representing 35% of the isolates (7/20). This was followed by *Escherichia hermannii* (25%, 5 isolates), *Escherichia coli* (15%, 3 isolates), and *Enterobacter cloacae* (10%, 2 isolates), highlighting a predominance of clinically relevant opportunistic pathogens in the irrigation outlets.

Table 2. Counts of the most relevant *Enterobacteriaceae* species isolated at each sampling point.

Irrigation sampling point	Microorganism	CFU/100mL
B8	<i>Klebsiella pneumoniae</i>	6,7x100
B10	<i>Klebsiella pneumoniae</i>	3x101
B11	<i>Klebsiella pneumoniae</i>	1,5x105
B12	<i>Klebsiella pneumoniae</i>	4,1x107
B17	<i>Klebsiella pneumoniae</i>	5x100
B18	<i>Klebsiella pneumoniae</i>	6x102

B19	Klebsiella pneumoniae	1,8x105
B7	Escherichia coli	1,3x100
B9	Escherichia coli	3,3x100
B10	Escherichia coli	5x101
B11	Escherichia hermannii	5,7x106
B12	Escherichia hermannii	8,8x106
B13	Escherichia hermannii	1,3x106
B14	Escherichia hermannii	6,0x107
B15	Escherichia hermannii	8,3x105
B13	Enterobacter cloacae	3,0x106
B14	Enterobacter cloacae	6,0x107

Among the Gram-positive bacteria, table 3 shows that *Enterococcus* spp. represented the most relevant genus, with 10 isolates (66%). Notably, *Enterococcus faecalis* accounted for the majority of these (9 isolates, 90%), positioning it as the most frequently recovered species in this study and underscoring its clinical relevance as a well-recognized opportunistic pathogen.

Table 3. Counts of the most relevant gram-positive bacteria species isolated at each sampling point.

Irrigation sampling point	Microorganism	CFU/100mL
B11	Enterococcus faecium	9,3x100
B1	Enterococcus faecalis	5,2x101
B2	Enterococcus faecalis	3x103
B3	Enterococcus faecalis	3x103
B5	Enterococcus faecalis	7,7x102
B11	Enterococcus faecalis	1,3x102
B14	Enterococcus faecalis	2,8x103
B18	Enterococcus faecalis	7,3x101
B19	Enterococcus faecalis	2,4x104
B20	Enterococcus faecalis	2,3x102

The ESKAPE group—an acronym proposed by Rice in 2008 to designate *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.—encompasses pathogens of critical clinical relevance. These microorganisms account for a significant global burden of morbidity and mortality, particularly within healthcare settings, where they are leading causes of severe and frequently fatal nosocomial infections. Beyond their clinical impact, the ability of ESKAPE pathogens to persist in environmental reservoirs and disseminate across human, animal, and ecological domains highlights their importance within the One Health framework and emphasizes the urgent need for integrated strategies aimed at surveillance, prevention, and control. [63].

Analysis of bacterial isolation from recycled wastewater intended for agricultural use revealed significant heterogeneity in bacterial composition among different islands, and even within individual islands, depending on the specific water outlet. Pathogens variability may be attributed to water storage and exposure to open storage tanks accessible to wild animals, domestic animals, or farm animals that may inhabit the vicinity of the storage area. Given the predominantly open-air water storage conditions, it is imperative to establish regulatory limits not only at the treatment plant discharge point but also to implement comprehensive hygiene and sanitation guidelines for water storage practices by farmers.

To elucidate the carbapenem-resistance mechanisms identified in this study, Table 4 presents the distribution of carbapenem-resistant Gram-negative bacilli isolates and their associated resistance mechanisms (KPC, MBL, OXA-48, ESBL with porin loss, and AmpC with porin loss) by island and

irrigation point. A total of 34 microorganisms has been found in 14 of the 20 irrigation outlets sampling points.

Table 4. Multidrug-resistant microorganisms and the resistance mechanisms detected in samples from each sampling point.

Island	Irrigation sampling point	Microorganism	Resistant mechanism	Number of isolates
Fuerteventura	B1	Aeromonas punctata	Intrinsic/other resistance mechanism	2
Fuerteventura	B1	Pseudomonas mendocina	Intrinsic/other resistance mechanism	1
Fuerteventura	B2	Burkholderia multivorans	Intrinsic/other resistance mechanism	1
Lanzarote	B6	Aeromonas sobria	Intrinsic/other resistance mechanism	1
Lanzarote	B8	Pseudomonas aeruginosa	MBL	1
Lanzarote	B8	Pseudomonas aeruginosa	KPC	1
Lanzarote	B8	Klebsiella pneumoniae	ESBL + Loss of porin	1
Lanzarote	B8	Burkholderia cepacia	Intrinsic/other resistance mechanism	1
Lanzarote	B9	Pseudomonas putida	Intrinsic/other resistance mechanism	1
Lanzarote	B9	Aeromonas salmonicida	Intrinsic/other resistance mechanism	1
Lanzarote	B10	Pseudomonas stutzeri	Intrinsic/other resistance mechanism	1
Lanzarote	B10	Pseudomonas fluorescens	Intrinsic/other resistance mechanism	1
Gran Canaria	B11	Acinetobacter pittii	Oxacilinas	2
Gran Canaria	B11	Klebsiella pneumoniae	OXA-48	1
Gran Canaria	B11	Acinetobacter baumannii	Oxacilinas	1
Gran Canaria	B11	Acinetobacter calcoaceticus	MBL	1
Gran Canaria	B11	Aeromonas salmonicida	Intrinsic/other resistance mechanism	1
Gran Canaria	B12	Acinetobacter pittii	Intrinsic/other resistance mechanism	1
Gran Canaria	B12	Klebsiella pneumoniae	Intrinsic/other resistance mechanism	1
Gran Canaria	B12	Acinetobacter lwoffii	Oxacilinas	1
Gran Canaria	B14	Escherichia hermannii	OXA48 + ESBL	2
Gran Canaria	B14	Enterobacter cloacae	AmpC+ loss of porin	1
Gran Canaria	B14	Sphingomonas paucimobilis	Intrinsic/other resistance mechanism	1
Gran Canaria	B14	Enterobacter cloacae	KPC	2
Gran Canaria	B15	Escherichia hermannii	ESBL+loss of porin	2
Gran Canaria	B15	Acinetobacter baumannii	Oxacilinas	1
Gran Canaria	B15	Acinetobacter calcoaceticus	MBL	1

Tenerife	B16	<i>Pseudomonas putida</i>	Intrinsic/other resistance mechanism	1
Tenerife	B18	<i>Pseudomonas aeruginosa</i>	KPC	1
Tenerife	B18	<i>Pseudomonas aeruginosa</i>	Intrinsic/other resistance mechanism	1
Tenerife	B19	<i>Klebsiella pneumoniae</i>	Intrinsic/other resistance mechanism	1
Tenerife	B19	<i>Acinetobacter baumannii</i>	Oxacilinas	1
Tenerife	B19	<i>Aeromonas hydrophila</i>	Intrinsic/other resistance mechanism	1
Tenerife	B20	<i>Pseudomonas aeruginosa</i>	MBL	1

Regarding antibiotic resistance, all enterococcal and staphylococcal isolated were susceptible to vancomycin, which aligns with the low prevalence of this resistance reported in Canary Islands hospitals [64]. Among Gram-negative bacilli (n=53), 64% demonstrated non-intrinsic carbapenem resistance. Enterobacteriaceae accounted for 24% of these resistant isolates and were most prevalent in Gran Canaria. According to the Global Antimicrobial Resistance and Use Surveillance System (GLASS), *Escherichia coli* represents one of the principal pathogens associated with antibiotic resistance. Data from 76 countries in 2022 indicated that 42% of *Escherichia coli* isolates exhibited resistance to third-generation cephalosporins, while 35% demonstrated resistance to methicillin. *Klebsiella pneumoniae* ranked second in prevalence, with both species belonging to the family Enterobacteriaceae. These bacteria, along with other members of the same family, are major contributors to a broad spectrum of intestinal infections [65]. Carbapenems are frequently employed as the last line of therapy against multidrug-resistant bacterial infections. However, over the past decade, numerous studies have reported the emergence of carbapenem-resistant Enterobacteriaceae, mediated by diverse resistance mechanisms [48].

The role of the environment as a reservoir and vehicle for the dissemination of resistance genes has been increasingly investigated in recent years. Hospital wastewater has been identified as a major hotspot, facilitating the horizontal transfer of resistance genes. For instance, a study conducted in India reported the isolation of an *Escherichia coli* strain producing NDM-4, harboring the bleomycin resistance gene (*bleMBL*) on a plasmid associated with the complete IS *Aba125* sequence, suggesting that hospital effluents represent an important reservoir of resistance determinants. Similarly, the release of antibiotics into municipal wastewater, together with human excretions, contributes to the selection of such bacteria in surface and/or groundwater. Supporting evidence includes the detection of *Klebsiella pneumoniae* strains producing VIM-1 in rivers in Spain [66].

There is also evidence of the transmission of resistance genes among Enterobacteriaceae strains in agriculture–aquaculture interface systems, as reflected in a recent study in Egypt on the presence of β -lactamases and CRE, where several resistant Enterobacteriaceae strains were isolated, predominantly carrying the carbapenem-resistance gene *blaKPC*, either alone or in combination with β -lactamase genes (*blaCTX-M-15*, *blaSHV*, *blaTEM*, and *blaPER-1*) [67].

In this study *P.aeruginosa*, *A.baumannii*, and Enterobacteriaceae, pathogens classified as critical priority by the WHO, were detected. Additionally, it is important to highlight the presence of primary pathogens such as *Vibrio cholerae* (Gran Canaria), diverse *Aeromonas* spp., and *Burkholderia cepacia* complex (Lanzarote and Fuerteventura). This bacterium is widely distributed across diverse environmental settings and is characterized by remarkable metabolic flexibility, together with a high mutation rate that facilitates adaptation. These traits enable it to persist under nutrient-limited conditions and even to exploit certain antimicrobials as a carbon source. From an epidemiological perspective, it is mainly associated with opportunistic infections in immunocompromised individuals, particularly in patients with cystic fibrosis [68].

Expanding the scope of investigation to a broader spectrum of pathogens reveals the significant public health threat posed by multidrug-resistant microorganisms, particularly those exhibiting carbapenem resistance. The facile transfer of resistance genes [22] among these pathogens underscores the importance of adopting a One Health perspective to mitigate the escalating global crisis of antibiotic resistance [22]. Indeed, authors such as Peter *et al.* 2017 have reported the transmission of blaVIM resistance genes from *Pseudomonas putida* isolates to *Pseudomonas aeruginosa*, the former being primarily associated with environmental niches and the latter representing a pathogen of major clinical importance [69].

The carbapenem resistance profile is quite alarming since we have detected that over 64% of the isolates presented resistance, with 24% of them being Enterobacteriaceae and 38% of these being produced by the presence of OXA-48 and KPC carbapenemases, which coincides.

Most of isolates microorganisms were identified as NFGNB consistent with their prevalence in environmental niches. However, *P. aeruginosa* and *A. baumannii*, which are more commonly associated with clinical settings, were also detected. Highlight that *P. aeruginosa* is one of the main pathogens that cause nosocomial infections, primarily affecting immunocompromised patients, those with invasive devices, severe burns, and post-surgical patients [70,71]. The main resistance mechanisms identified in *Pseudomonas aeruginosa* in our study were two isolates producing metallo- β -lactamases (MBL) and two isolates producing KPC-type carbapenemases. These findings are of particular clinical relevance due to their significant impact on therapeutic options. In recent years, Spain has reported an increasing number of MBL-producing strains, particularly of the VIM and IMP types [72]. This trend has been associated with the growing detection of resistance to novel agents used in the management of multidrug-resistant *P. aeruginosa*, such as ceftolozane-tazobactam and ceftazidime-avibactam (CZA) [73,74].

Similarly, the use of CZA has led to the emergence of *Pseudomonas aeruginosa* strains resistant to this antibiotic, a phenomenon in which several resistance mechanisms have been described that directly compromise its efficacy. Among these, the emergence of KPC-type carbapenemase variants is particularly noteworthy [75]. In 2017, Shields *et al.* reported for the first time the development of resistance to CZA in patients infected with *Klebsiella pneumoniae*, attributed to mutations in the blaKPC-3 gene [76]. In the case of *P. aeruginosa*, reports are relatively scarce; however, several KPC variants capable of conferring resistance to this antibiotic have been identified, such as blaKPC-31 [77].

Pseudomonas aeruginosa strains possess multiple intrinsic resistance mechanisms against carbapenems, the most frequent being the alteration of outer membrane permeability through modifications or loss of the OprD porin. Additional mechanisms include the overexpression of efflux systems, the synthesis of carbapenemase enzymes capable of inactivating these compounds, and the hyperproduction of chromosomal AmpC β -lactamases [78–80]. Moreover, this species exhibits a remarkable ability to acquire additional resistance via horizontal transfer of antimicrobial resistance genes, which are often located within integrons and mobile genetic elements such as plasmids and transposons [80]. On the other hand *A. baumannii* is also associated with nosocomial infections, including bacteremia, pneumonia, and infections of the skin and urinary tract [81], mainly in critical care units as an opportunistic pathogen [82,83]. *Acinetobacter baumannii* can be able to withstand desiccation, exhibit tolerance to biocides, and remain viable even under conditions of limited nutrient availability [84,85]. *Acinetobacter* strains were isolated in Gran Canaria from four out of five irrigation outlets and in one outlet in Tenerife. Among these isolates, the primary resistance mechanism identified is the production of oxacilinases, enzymes that belong to the class D β -lactamases. In *A. baumannii*, six oxacilinase groups have been described to date: OXA-51, OXA-23, OXA-40/24, OXA-58, OXA-143, and OXA-48 [51,86,87]. Recent reports indicate that these strains have disseminated globally [87], causing hospital outbreaks in countries such as United States, Canada, Spain, among others [88]. Moreover, multidrug resistance is a common feature of this microorganism due to its extraordinary adaptive capacity. *Acinetobacter baumannii* isolates can acquire resistance through several mechanisms, including horizontal gene transfer, natural transformation, the

emergence of mutations, and the mobilization of genetic elements that modulate the expression of intrinsic genes, such as *blaOXA-51-like* gene conferring carbapenem resistance, as well as through the acquisition of additional resistance genes [82,84,85,88].

Carbapenemase dissemination, predominantly nosocomial, has prompted a One Health investigation into broader transmission pathways, including hospital wastewater or undercooked food. Consequently, European regulatory frameworks are undergoing revision to encompass agricultural and food sectors in carbapenem resistance surveillance [83]. The detection of carbapenem-resistant environmental *Pseudomonas* species, such as *P. putida* and *P. stutzeri*, underscores their potential role as reservoirs for the horizontal transfer of carbapenemase genes to clinically significant pathogens like *P. aeruginosa*.

A One Health approach highlights the significance of *P. aeruginosa*, *A. baumannii*, and Carbapenem Resistant Enterobacteriaceae as primary pathogens in human health. These microorganisms, classified as critical priority pathogens by the WHO [89], along with *Vibrio cholerae* non-O1/O139 strain, have been detected in recycled wastewater used for irrigation in Gran Canaria. This bacterium, has also been found predominantly in Mediterranean coastal lagoons [90]. *Vibrio cholerae* is a halophilic, Gram-negative, facultative anaerobic bacterium widely distributed in aquatic and estuarine environments. [91] Non-O1/non-O139 strains are not responsible for cholera, as they lack the cholera toxin; however, they are capable of colonizing the gastrointestinal tract, causing self-limiting acute gastroenteritis, and have also been linked to extraintestinal infections such as those of the biliary and urinary tracts, soft tissues, and skin, as well as bacteremia, pneumonia, meningitis, and otitis externa [91,92]. Although bacteremia due to these serogroups is relatively rare, studies indicate that it is associated with higher mortality compared to other *V. cholerae* infections, particularly in immunocompromised patients [93]. From a One Health perspective, the persistence of *V. cholerae* in aquatic environments, including irrigation water, raises important concerns regarding its potential role as an environmental reservoir and transmission pathway across human, animal, and ecosystem interfaces.

The detection of *Aeromonas* spp. in Fuerteventura and Tenerife, this genus is among the predominant bacterial groups in treated wastewater [94]. Specifically, the species *Aeromonas hydrophila*, *A. caviae*, and *A. veronii* are considered clinically relevant due to their pathogenic potential in humans. The infections they cause range from self-limiting gastroenteritis to more severe conditions affecting the heart, skin, eyes, and other organs, which can ultimately progress to fatal septicemia [95]. Moreover, multidrug resistance has been reported in *Aeromonas* spp. isolates obtained from fish as well as from various aquatic sources, including rivers and wastewater treatment plant effluents. These bacteria also exhibit intrinsic resistance mechanisms, supported by mutations in specific genes, efflux pump overexpression, and the acquisition of resistance determinants through horizontal gene transfer, all of which enhance their antimicrobial resistance capacity [96]. Aquatic habitats provide favorable conditions for the development of these processes. In addition, biofilm formation on aquatic environments has been documented, offering protection to the bacteria and facilitating the exchange of resistance genes [40]. Due to their characteristics, high prevalence in aquatic environments, and diverse antibiotic resistance mechanisms, this genus can serve as a valuable indicator of contamination and the dissemination of antibiotic resistance within aquatic ecosystems, including effluents from wastewater treatment plants. [97].

Underscores the importance of establishing rigorous standards for the hygienic quality of recycled wastewater used for irrigation, also under storage conditions, particularly when food contamination is at risk.

Limitations of this study

In cases where carbapenem resistance mechanisms could not be identified due to limitations of phenotypic techniques, sequencing methods were required. However, the study detected resistance mechanisms to β -lactam antibiotics, including MBL, KPC, and OXA-48, which significantly reduce therapeutic options for infections caused by these microorganisms. Our findings demonstrates that recycled wastewater used in agriculture can serve as a reservoir for these microorganisms and

promote their dissemination, highlighting the importance of One Health policies and continued environmental surveillance to mitigate and control this public health threat.

4. Conclusions

In this study, *E. coli* levels in irrigation outlets were within the permitted values established by regulations for recycled water reuse in irrigation. However, elevated counts of other Enterobacteriaceae suggest the need to consider additional microbiological indicators to ensure public health. The isolation of *Vibrio cholerae*, *Aeromonas veronii*, and *Aeromonas hydrophila* underscores the importance of post-treatment for food irrigated with recycled wastewater stored in systems without hygienic control, especially when the edible part of the fruit or vegetable comes into direct contact with the irrigation water.

The results indicate that recycled wastewater stored for agricultural use can serve as a reservoir of carbapenem-resistant bacteria, potentially facilitating their dissemination from the environment to living organisms. To mitigate the spread of antimicrobial resistance, effective management and improved hygienic-sanitary conditions for recycled wastewater storage designated for agriculture irrigation are essential. Achieving this goal requires collaboration among various stakeholder's within a One Health framework.

Supplementary Materials: The following supporting information can be downloaded at Preprints.org, Table S1: Bacterial isolations and their corresponding microbiological counts from each irrigation sampling point on the island of Fuerteventura; Table S2: Bacterial isolations and their corresponding microbiological counts from each irrigation sampling point on the island of Lanzarote; Table S3: Bacterial isolations and their corresponding microbiological counts from each irrigation sampling point on the island of Gran Canaria; Table S4. : Bacterial isolations and their corresponding microbiological counts from each irrigation sampling point on the island of Tenerife.

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Abbreviations

The following abbreviations are used in this manuscript:

WHO	World Health Organization
CFU	colony-forming-units
MALDI-TOF MS	Matrix-assisted laser desorption/ionization mass spectrometry.
NFGNB	non-fermentative Gram-negative bacilli
KPC	Carbapenemase Klebsiella pneumoniae
MBL	Metallo-β-lactamase
OXA	Oxacilinasas
WWTP	Waste Water Treatment Plant

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