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Article – One Health

Influence of Urban Wastewater and Pig Farming on Aquatic Microbiomes and Waterborne Pathogens in Southern Brazil

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HIGHLIGHTS

- Predominance of *Proteobacteria* in most samples, with notable differences in microbial diversity and composition between upstream and downstream sites.
- *Pseudomonas aeruginosa* and *Escherichia coli* persisted in the aquatic environment surrounding pig farms and cities
- Detection of pathogenic bacteria such as *Escherichia coli O157:H7* and *Shigella flexneri 2a* in areas influenced by pig farming and urban wastewater discharge.

Abstract: Urbanization and intensive animal farming generate large volumes of waste that introduce microbial contaminants into aquatic ecosystems, increasing the risk of pathogen dissemination. We analyzed 16S rRNA-based microbial community profiles from water samples collected upstream and downstream of pig farms and urban areas in southern Brazil including Itambaracá (P1, P3), Chapecó (P2, P4), Curitiba (P5) and Joinville (P6). Proteobacteria dominated most samples, with *Gammaproteobacteria* enriched near pig farming sites. Urban-impacted waters showed reduced microbial diversity and clear taxonomic shifts consistent with wastewater influence. Notably, pathogenic bacteria of public health concern, including *Escherichia coli O157:H7*, *Shigella flexneri 2a*, and *Pseudomonas aeruginosa*, were detected in areas affected by livestock production and urban effluent discharge. These findings demonstrate that animal production systems and urban wastewater may act as interconnected sources of environmental contamination, reshaping aquatic microbiomes and introducing clinically relevant pathogens into natural waters. This convergence highlights the need for integrated monitoring and management strategies to protect ecosystem integrity and reduce human health risks.

Keywords: microbiome; aquatic; livestock; microbial community

INTRODUCTION

Intensive farming and urban development wastewater management share similarities, using advanced technological integration such as precision agriculture tools and municipal water treatment technologies [1,2]. Although both conditions may have a significant environmental impact (i.e., soil degradation and water pollution), they involve using resources, maximizing crop yields, and water treatment for reuse [3]. Human and animal health protection is increasingly centered on sustainable practices to reduce their ecological footprint. These specific factors are subject to (inter)national regulations to protect the environment [4].

The global demand for protein-rich diets has pushed for intense animal farming, particularly in developing countries [5]. The data reflect the increase in global meat production reaching 357 million tons in 2021, a growth of 53% compared to 2000 [6]. To address the rise in meat (protein-rich food) consumption, it is necessary to strengthen animal production by adopting high animal density, high-protein diets, increasing the frequency of medications, and improving farm infrastructure [7]. Urban lifestyle and intensive animal production, especially pig farming, are widely recognized for their significant environmental impact, stemming from the considerable demand for natural resources and the associated pollution risks [8]. Hospitals and intense animal production have a corresponding waste generation, particularly within the concentrated animal feeding operations, which monitor the discharge of conventionally treated livestock waste to the environment [9].

Wastewater from livestock farms and cities contains antibiotic residues, pathogenic bacteria, and antimicrobial-resistant (AMR) bacteria that are eliminated by feces and urine (i.e., hospitals, industrial sewages, etc.) [10-13]. Moreover, conventional wastewater treatment processes commonly employed on farms and cities can partially remove multidrug-resistant bacteria (MDR) and their genes [14,15]. Consequently, the risk of pathogen transmission persists even after the water filtration/purification process [16]. The ecological impact resulting from the high quantity of waste generated by housing several animals, like pigs, in a single area [17] undermines the sustainability of production [18,19].

The World Health Organization (WHO) has published a list of 12 bacterial families representing the most significant risks to public health. *Pseudomonadaceae* and *Enterobacteriaceae* were classified as critical and the highest level of concern with high AMR, pathogenicity, virulence, and mortality rates in humans and animals [20-23]. Moreover, some species, such as *Pseudomonas aeruginosa* and *Escherichia coli*, are important pathogens in humans and veterinary clinics due to their large number of opportunistic infections and intestinal infections [24,25].

While intensive animal production has enabled efficient meat, egg, and milk production to meet global demand, it poses significant environmental challenges. Addressing the above issues requires a multifaceted approach that includes technological innovation, sustainable living, and agricultural practices. In addition, there is a need for supportive transnational policies to mitigate the adverse effects on natural resources (river beds, etc.) and fragile ecosystems (native grassland and forests). This study aimed to analyze the microbiome of water samples from urban areas and surrounding pig farms in southern Brazil. Given their clinical and epidemiological significance, particular emphasis was placed on the search for the *Pseudomonadaceae* and *Enterobacteriaceae* families.

MATERIAL AND METHODS

Sample areas and rivers

The selection of samples was conducted in Paraná and Santa Catarina states, south of Brazil, known for pig production (Table 1). The sample areas were divided into places near pig farms and two types of urban areas. Itambaracá, in the Paraná state (PR), and Chapecó, in the Santa Catarina state (SC), were selected for water (river samples) near pig farms (P1 and P2) and cities (city samples) near pig farms (P3 and P4). Samples from strictly urban areas were collected from Curitiba (PR) and Joinville (SC), designated P5 and P6, respectively. Moreover, water samples from near the pig farms were classified as Up (upstream) before the presence of the pig farm, and Down (downstream), representing samples after the presence of the pig farm. Samples from strictly urban areas were collected from the source of the rivers (Up) and near the end of the metropolitan perimeter after crossing the city center (Down). Curitiba and Joinville are the largest and most urban-dense municipalities of their respective states, not being influenced by the intense animal wastewater.

Table 1. Sample points, municipalities, rivers, and geographic coordinates from Paraná and Santa Catarina states, southern Brazil.

Sample	Municipality	River	Geographic coordinates
P1-Up	Itambaracá, PR	Das Cinzas	23°01'35.79"S 50°22'33.47"W
P1-Down	Itambaracá, PR	Das Cinzas	23°02'03.49"S 50°20'52.14"W
P2-Up	Chapecó, SC	Bom Retiro	27°06'31.72"S 52°44'13.34"W
P2-Down	Chapecó, SC	Bom retiro	27°06'37.74"S 52°44'02.63"W
P3	Itambaracá, PR	Das Cinzas	23°00′49.63″S 50°24′15.51″W
P4	Chapecó, SC	Bom retiro	27°05′46.11″S 52°39′24.33″W
P5-Up	Curitiba, PR	Belém	25°35’32.50”S 49°26’59.85”W
P5-Down	Curitiba, PR	Belém	25°46’95.46”S 49°24’72.87”W
P6-Up	Joinville, SC	Cachoeirinha	26°15'59.20"S 48°53'44.20"W
P6-Down	Joinville, SC	Cachoeirinha	26°18'49.40"S 48°49'42.20"W

Pre-DNA extraction techniques and DNA extraction

Water samples were processed using a commercial kit for cell growth cultivation for DNA isolation or a vacuum membrane-filtration (VMF) method. All P1, P2, and P4 were subjected to the cell growth Pseudalert and Colilert tests from the IDEXX Laboratories (Westbrook, USA) kit. Water samples were collected in triplicate sterile plastic containers, with approximately 1000 ml/sample point. Subsequently, samples were pooled to approximately 200 ml for each sample point. All samples were transported to the laboratory and refrigerated (< 4°C) within 24 h after collection. For cell concentration, P3, P5, and P6 underwent vacuum membrane filtration (VMF) using the MF-Millipore 0.22 µm membranes (Merck Millipore, Burlington, USA). For VMF, water samples were collected in triplicate in autoclaved 1000 ml glass containers from each sample point. All samples were refrigerated (< 4°C) and transported to the laboratory within 24 h after collection. The water samples were filtered through two membranes using a funnel, a Kitasato apparatus, and a suction pump to create a vacuum. After filtration, the membranes underwent DNA extraction [26] using the Wizard Magnetic DNA Kit (Promega, Madison, USA).

The Pseudalert test employs an enzymatic detection technology for *P. aeruginosa* through substrate hydrolysis. Bacterial cells grow, and reproduction is fast using the nutrient-rich supply. Substrate cleavage in the reagent produces a blue fluorescence when exposed to ultraviolet light. The Colilert test uses two nutrient indicators, ortho-nitrophenyl-β-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-β-D-glucuronide (MUG). The coliform enzyme, β-galactosidase, and the *E. coli* enzyme, β-glucuronidase, can metabolize the nutrient indicators. The samples were then incubated for 24 h, as indicated by the manufacturer, at 38°C for Pseudalert and 35°C for Colilert. After this period, the tests were interpreted to confirm the absence or presence of the respective bacterial species. As coliforms grow, they metabolize the indicators, causing the colorless medium to turn yellow (first result). The substrate cleavage by *E. coli's* β-glucuronidase produces a green fluorescence under ultraviolet light (second result). Subsequently, a 50 ml pool of each processed sample was centrifuged at 10,000 rpm for 10 minutes. The resulting pellets were transferred to 2 ml tubes for DNA extraction (Wizard Magnetic DNA Kit by Promega), following the manufacturer's instructions.

Metagenomic analysis of bacterial community diversity

DNA was quantified using the NanoDrop One/Onec UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Metagenomic libraries for each sample were constructed following the manufacturer's protocol using the Illumina DNA Prep kit (San Diego, USA). The libraries were individually quantified on the Qubit 2.0 (Thermo Scientific, Waltham, USA) with the Qubit dsDNA Quantification Assay Kits. They were assessed for size using the Agilent 2100 Bioanalyzer with the Agilent High Sensitivity DNA kit (Agilent Technologies, Santa Clara, USA). The concentration of each library was determined using the following method: the libraries were diluted to 2nM and combined into a pool, which was then diluted again to 750 pM. From this final dilution, 20 µl were loaded into the NextSeq 1000/2000 P1-600 cartridge, and microbiome sequencing was performed on the NextSeq 1000 equipment (Illumina, San Diego, USA). The results have been deposited in the NCBI (National Center for Biotechnology Information, Bethesda, USA) BioSample database under the ID number PRJNA1241837.

Raw sequencing data were processed using the QIAGEN CLC Microbial Genomics Module (version 24.0) within the QIAGEN CLC Genomics Workbench 24.0 (QIAGEN, Aarhus, Denmark). This specialized module was selected for its comprehensive pipeline, which enables the identification and quantification of

microbial taxa at single-nucleotide resolution through Amplicon Sequence Variants (ASVs) determination. This provides higher-resolution taxonomic profiling than traditional Operational Taxonomic Unit (OTU) clustering approaches.

The bioinformatics workflow consisted of quality control, where raw paired-end reads were subjected to quality filtering to remove low-quality sequences (Q-score < 20), adapters, and reads shorter than 250 bp. The ASV determination was used to detect amplicon sequence variants, implementing an error profiling methodology similar to the DADA2 algorithm [27] to distinguish authentic biological variation from sequencing errors. A taxonomic assignment consisting of ASVs was taxonomically classified using the SILVA 16S rRNA database (version 138.1) and Genome Taxonomy Database [28] with a minimum similarity threshold of 97%. The taxonomic assignment was performed using the QIAGEN CLC's implementation of the RDP Naive Bayesian Classifier algorithm with a confidence threshold of 0.8. A diversity analysis was performed with QIAGEN CLC Microbial Genomics Module's built-in algorithms, which calculated alpha diversity metrics (Shannon diversity index, observed ASVs, and Faith's Phylogenetic Diversity) and beta diversity measures (UniFrac and Bray-Curtis dissimilarity). All parameters for quality filtering, ASV determination, and taxonomic assignment were kept at the QIAGEN CLC Microbial Genomics Module's recommended settings, as these have been optimized for accurate microbial community profiling with 16S rRNA gene amplicon data.

RESULTS

Descriptive taxonomic profile of water samples from cities near pig farms

Overall, the taxonomic profile of the samples was dominated by the *Bacteria* kingdom. Exceptionally, P5-Up (99%) and P6-Up (99%) were the only points that did not show 100% dominance of *Bacteria*. *Proteobacteria* was the predominant phylum in 90% of samples (9/10). Only P6-Down had *Firmicutes* as the predominant phylum. *Gammaproteobacteria* were the predominant class (100%) in all samples from rivers near pig farms (P1-Up, P1-Down, P2-Up, and P2-Down). *Gammaproteobacteria* were also the predominant class (66% in P3 and 99% in P4) in samples from cities near pig farms. P3 was composed predominantly of *Burkholderiales* order (43%) and *Burkholderiaceae* family (24%). P4 was composed predominantly of *Enterobacterales* order (98%) and *Enterobacteriaceae* family (93%). The difference in composition between P3 and P4 can be explained by the results of the different pre-DNA extraction methods used. In P3, it was used the VMF, and P4 used a commercial kit, which favored the growth of *Enterobacteria* and *P. aeruginosa* (Figure 1).

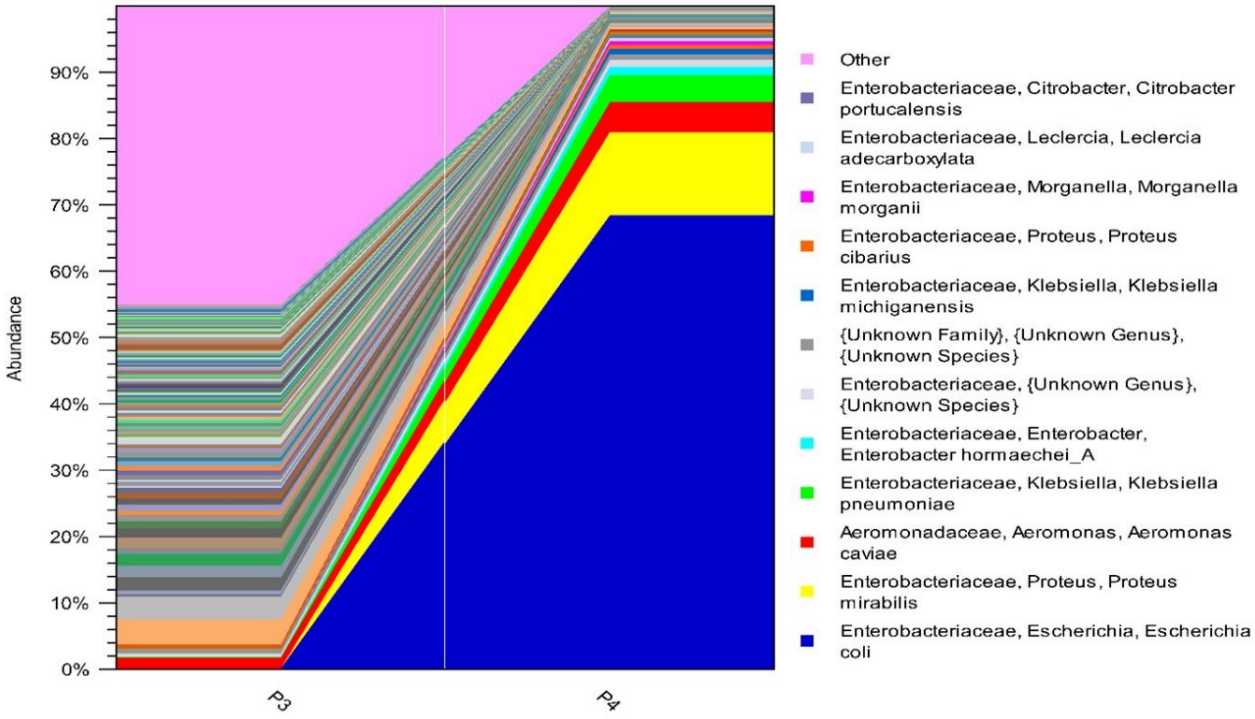

Figure 1. Area chart indicating the microbial composition in genus and species from water samples collected from cities near pig farms (P3 and P4). *Enterobacteria* area increases as it reaches the graphs' right side from sample P4, indicating the favored growth promoted by commercial kit cultivation.

The comparison between Up and Down samples showed that In Up, there was a predominance of *P. aeruginosa* (P1- 51%) and *E. coli* (P2 - 36%), and in Down, there was a predominance of *Plesiomonas shigelloides* (P1 - 27%) and *Morganella morganii* (P2 - 53%). The presence of the pig farm did not result in a noticeable correlation between the influence of pig farming and the alteration of microbial composition in the samples in Up and Down samples (Figure 2).

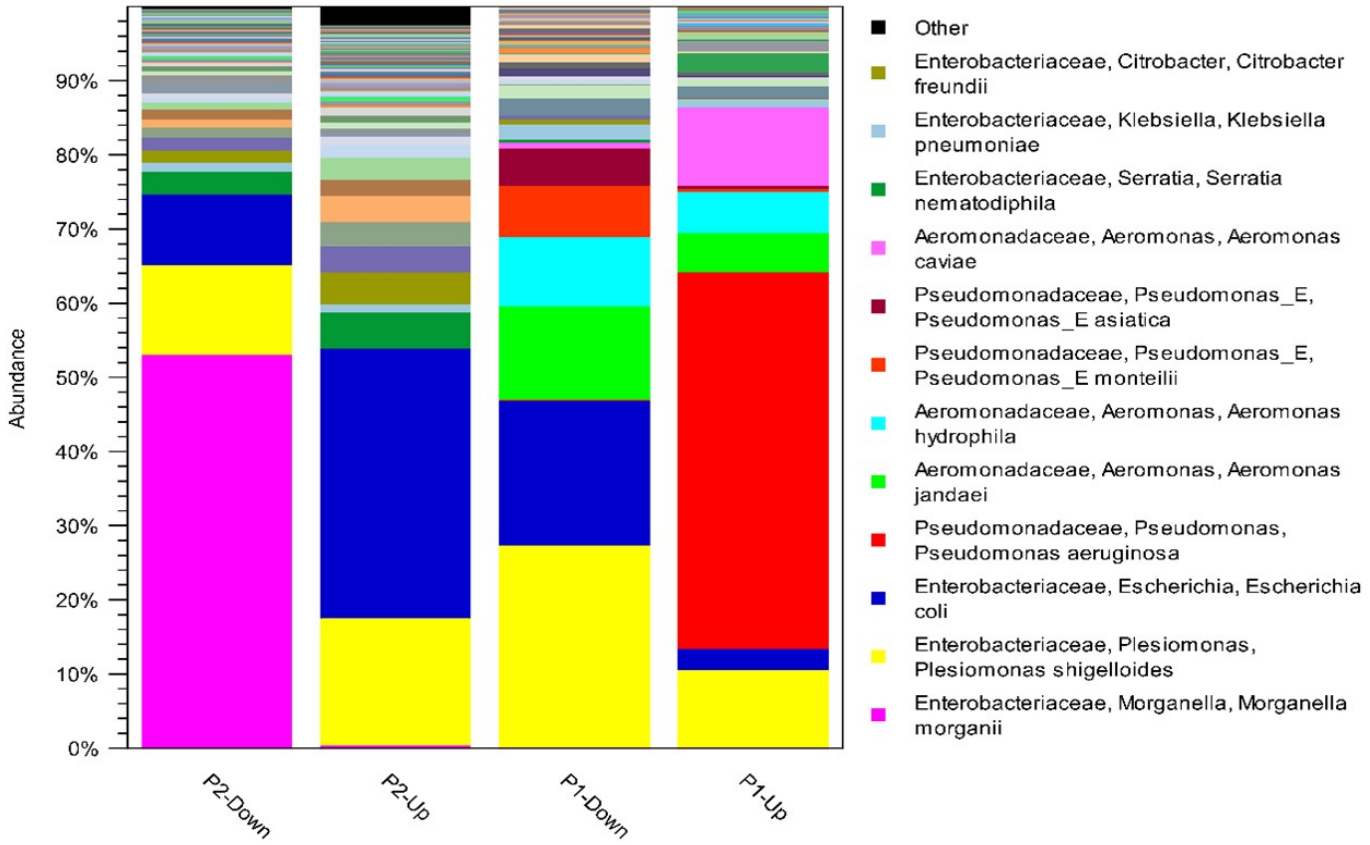

Figure 2. Microbiome compositions at family, genus, and species levels of samples P1-Up, P1-Down, P2-Up, and P2-Down in water samples from southern Brazil.

Descriptive taxonomic profile of water samples from urban areas

Samples from the urban perimeter were used to determine the general presence of bacteria and to compare the differences between large cities and rural/farming environments. P5-Up (73%), P5-Down (66%), and P6-Up (70%) showed a predominance of *Proteobacteria*. Differently, P6-Down showed a predominance of *Firmicutes* (89%). The water source points (P5-Up and P6-Up) were dominated by *Alphaproteobacteria*. They had many different genera and species (615 genera and 736 species in P5-Up and 640 genera and 802 species in P6-Up), representing high alfa diversity (Figure 3). Samples from P5-Down and P6-Down showed major reduction of (50,1% and 96% genera decrease; 47,5% and 97% species decrease, respectively) bacterial alfa diversity compared to the water source points, with a predominance of *Gammaproteobacteria* (270 genera and 386 species in P5-Down and 25 genera and 24 species in P6-Down) (Figure 4).

Figure 3. Sunburst chart indicating the percentage of each taxonomic category from the water source (P5-Up and P6-Up) (A) of the Belém river from Curitiba, PR, and the Cachoeirinha river from Joinville, SC, respectively. Kingdom: *Bacteria* (99%), Phylum: *Proteobacteria* (72%), Class: *Alphaproteobacteria* (52%), Order: *Rhizobiales* (71%), Family: *Xanthobacteraceae* (65%), Genus: *Bradyrhizobium* (75%), Species: *Bradyrhizobium stylosanthi* (2%).

Figure 4. Sunburst chart indicating the percentage of each taxonomic category from urban perimeter (P5-Down and P6-Down) of the Belém river from Curitiba, PR, and the Cachoeirinha river from Joinville, SC, respectively. Kingdom: *Bacteria* (100%), Phylum: *Proteobacteria* (62%), Class: *Gammaproteobacteria* (90%), Order: *Burkholderiales* (60%), Family: *Burkholderiaceae* (90%), Genus: *Acidovorax* (54%), Species: *Acidovorax temperans* (2%).

Within the class *Gammaproteobacteria*, the most predominant order was *Burkholderiales* (P5-Down = 35%, and P6-Up = 76%). Exceptionally, P5-Up showed a predominance of the class *Alphaproteobacteria* (56%), and the most predominant order for this class was *Rhizobiales* (74%). P6-Down had a predominance of bacteria from the phylum *Firmicutes* (89%), and the predominant class was *Bacilli* (100%) and the order *Thermoactinomycetales* (100%). In source water samples, there was a predominance (75%) of bacteria from the genus *Bradyrhizobium*. In contrast, the samples taken after passing through the urban perimeter were predominantly composed of bacteria from the genus *Acidovorax* (54%). The Principal Coordinate Scatter plot (PCoA) indicates that samples from upstream (P5-Up and P6-Up) had related (beta diversity) microbial composition as well as the downstream samples (P5-Down and P6-Down). However, the comparison from upstream to downstream indicated dissimilar (beta diversity) microbial composition, as indicated by the large PCoA distance. Our results indicate that the diversity of bacteria decreased in the samples after passing through the urban perimeter of the cities (from P5-Up to P5-Down and P6-Up to P6-Down).

Commercial kits cultivation

The cultivation of water samples surrounding pig farms revealed that all five (100%) sample points (P1-Up, P1-Down, P2-Up, P2-Down, and P4) tested positive for *E. coli*, and 3 (60%) tested positive for *P. aeruginosa* (Table 2). The 16S taxonomic profile from water samples in Parana and Santa Catarina revealed the presence of *E. coli O157:H7* and the *2a str. 301 Shigella flexneri* serotypes in all five samples from the commercial cultivation kit and only one sample from the vacuum membrane (P5-Down) (Table 3).

Table 2. The origin of the sample points, the name of the river, and the results of two tests, from Itambaracá, PR, and Chapecó, SC, southern Brazil.

Sample point	Origin	Municipality	River	Colilert	Pseudalert
P1-Up	Near pig farm	Itambaracá	Das Cinzas	Positive	Positive
P1-Down	Near pig farm	Itambaracá	Das Cinzas	Positive	Negative
P2-Up	Near pig farm	Chapecó	Bom Retiro	Positive	Negative
P2-Down	Near pig farm	Chapecó	Bom retiro	Positive	Positive
P4	City near pig farm	Chapecó	Bom retiro	Positive	Positive

Table 3. Sample points with *Escherichia coli* serotypes and their respective percentages (%) of composition from water samples in southern Brazil.

Serotype	P1-Up	P1-Down	P2-Up	P2-Down	P4	P5-Down
Shigella flexneri 2a str. 301	15	19	13	15	15	12
Escherichia coli str. K-12 substr. MG1655	39	32	42	37	39	40
Escherichia coli O157:H7 str. Sakai	16	16	13	15	14	17
Escherichia coli (Unknown)	30	33	32	33	32	31

DISCUSSION

This study provides an exploratory assessment of how anthropogenic pressures associated with pig farming and urbanization may influence aquatic microbial communities in southern Brazil. Water samples were collected from sites influenced by intensive animal production in Itambaracá and Chapecó, as well as from urban centers without direct pig farming activity, Curitiba and Joinville. Given the limited number of sampling points, the absence of temporal replication, and the use of cultivation-based enrichment methods, our findings should not be interpreted as representative of regional environmental or epidemiological patterns. Rather, they offer descriptive preliminary insights into how localized agricultural and urban inputs may converge as sources of microbial contamination in aquatic ecosystems.

The predominant *Proteobacteria* found in the taxonomic profile of water samples in southern Brazil have also been reported to be one of the most prevalent phyla in humans [29] and domestic animals' gut microbiota [30]. Members from this phylum, such as *E. coli* and *Salmonella*, are well-known pathogens causing diseases in humans and animals [31]. Analyses of samples from swine wastewater treatment plants (WWTPs) and the swine's pen floor, from the same area used in this study, were primarily composed of members from the phyla *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* [32]. *Proteobacteria* (from 30.2 to 45.3%) was also the most predominant phylum in samples from soil contaminated with swine waste in China [33]. However, because this phylum is ubiquitous across ecological contexts, its dominance alone does not allow source attribution. More informative patterns emerged at finer taxonomic resolution, where opportunistic and clinically relevant taxa were detected in waters influenced by both pig farming areas (Itambaracá and Chapecó) and urban centers (Curitiba and Joinville). This overlap reinforces the concept of rivers as integrative systems receiving microbial inputs from multiple anthropogenic sources rather than from a single sector.

Contrary to expectations, consistent upstream–downstream shifts were not observed near pig farms in Itambaracá and Chapecó. This likely reflects the complex and multifactorial nature of microbial dispersion in fluvial systems, shaped by hydrological dynamics, sediment resuspension, wildlife activity, and mixing with diffuse sources of contamination. These results highlight a key limitation of snapshot-based sampling and

suggest that short-term or low-frequency sampling may be insufficient to detect persistent impacts of livestock operations. Longitudinal designs and source-tracking approaches would be necessary to clarify these dynamics. The two different pre-DNA extraction methods used resulted in a higher abundance of *Enterobacteria* and *P. aeruginosa* in samples from commercial kit cultivation, as this method enriches the number of these microorganisms. Therefore, the heterogeneous pre-processing methods also contributed to making it harder to observe upstream–downstream shifts, besides favoring the detection of important waterborne pathogens.

In contrast, clearer compositional shifts were detected in urban-influenced sites, particularly in Curitiba and Joinville. The transition from *Alphaproteobacteria* in upstream samples to *Gammaproteobacteria* downstream suggests selective pressure associated with organic loading and wastewater inputs [34]. This pattern supports the hypothesis that urban effluents may exert stronger and more consistent pressure on microbial community structure than localized agricultural sources alone. However, without chemical, hydrological, or quantitative pathogen load data, these associations remain correlative. The detection of *Escherichia coli* O157:H7, *Shigella flexneri*, and *Pseudomonas aeruginosa* in samples from both farm-adjacent (Itambaracá and Chapecó) and urbanized (Curitiba and Joinville) areas highlights a critical convergence of environmental contamination, animal production systems, and potential human health risks. These pathogens are widely associated with waterborne disease outbreaks [35-40], but their presence alone does not establish exposure risk. This limitation underscores the need for integrated frameworks that combine taxonomic detection with quantitative risk assessment, viability assays, and epidemiological data.

From a One Health perspective, these results indicate that rivers crossing rural and urban landscapes act as critical interfaces connecting livestock production, human settlements, wildlife, and aquatic ecosystems. This connectivity facilitates microbial circulation across these sectors, creating pathways for pathogen persistence and the potential selection of antimicrobial resistance (AMR). For instance, the intensive use of antibiotics in swine production has already led to human infections that are unresponsive to conventional therapies [41]. Furthermore, the rising prevalence of pathogenic *Gammaproteobacteria* is a major concern for the One Health initiative, as the impacts of animal production extend far beyond the farm boundaries. Indeed, the presence of such bacteria in rivers and their detection in domestic and wild fauna pose a direct risk to local communities. Additionally, land modifications and occupational activities can influence vector-carrying bacteria, further amplifying disease spread [41]. Ultimately, understanding the epidemiology at this human-animal-environment interface requires a deeper grasp of how microorganisms circulate between these niches [42]. However, a significant gap in environmental governance persists, as current Brazilian regulatory frameworks do not yet mandate explicit microbial or AMR surveillance in water bodies near pig farms or urban wastewater discharge points [43–45]

Although this study does not yet propose a validated monitoring tool, it offers conceptual groundwork for applied development. The use of cultivation-based enrichment, as tested here, could be incorporated into surveillance systems for rapid screening in cities such as Curitiba and Joinville and in livestock-dense regions like Chapecó. This strategy could support early detection of sanitary risks and guide targeted molecular analyses. In conclusion, our findings reinforce the need to conceptualize aquatic microbiomes not as isolated ecological entities but as dynamic systems shaped by the interaction of urbanization, animal agriculture, and environmental processes. The One Health approach provides a comprehensive framework for understanding and addressing pathogenic bacteria in aquatic environments surrounding pig farms and cities. We can develop sustainable solutions to protect ecosystem integrity and public health by fostering collaboration across disciplines and sectors.

CONCLUSION

The data demonstrated the persistent presence of clinically relevant pathogens, including *Pseudomonas aeruginosa* and *Escherichia coli*, in aquatic environments influenced by both pig farming and urban activities. These bacteria were detected not only near livestock operations but also in urban waters within the same hydrographic systems, highlighting the connectivity between rural and urban contamination sources. The use of commercial cultivation-based enrichment substantially improved the detection of pathogenic taxa, supporting its potential as a rapid screening approach for environmental surveillance. No consistent association was observed between proximity to pig farms and broad shifts in microbial community structure, emphasizing the multifactorial nature of riverine systems and the limitations of snapshot-based sampling. In contrast, urban environments exhibited clearer ecological signals, including reduced diversity and a shift from *Alphaproteobacteria* to *Gammaproteobacteria* downstream of city perimeters. Overall, these results suggest that aquatic systems act as integrative reservoirs for pathogenic bacteria at the human–animal–environment interface. Although exploratory, this study underscores the need for longitudinal monitoring and One Health–oriented surveillance strategies to better assess and mitigate environmental and public health risks.

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Use of Generative Artificial Intelligence:

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