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Zoonotic Pathogens Isolated from an Introduced Population of Red Swamp Crayfish (*Procambarus clarkii*) in Tenerife (Canary Islands, Spain)

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Abstract: The red swamp crayfish (*Procambarus clarkii*) is a widely distributed invasive species that is listed in the Delivering Alien Invasive Species Inventory for Europe. Native to North America, it has been introduced to numerous regions, such as the Canary Islands, Spain. Previous studies have confirmed the role of this crayfish in the maintenance of several foodborne pathogenic bacteria. Therefore, the aim of this study was to analyze the main zoonotic bacterial and parasitic pathogens present in a *P. clarkii* population introduced to the island of Tenerife, Canary Islands, and to assess the potential risk to public health and native fauna. A total of 22 crayfish from Tenerife were analyzed using Biofire FilmArray Gastrointestinal Panels and culture–PCR methods. The results show the presence of *Plesiomonas shigelloides*, *Shigella*/enteroinvasive *Escherichia coli*, enteropathogenic *Escherichia coli*, *Salmonella* ser. Enteritidis, *Salmonella* ser. Typhimurium, and *Salmonella* ser. Typhi. These results demonstrate the presence of a variety of pathogenic bacteria in the red swamp crayfish in Tenerife that represent a significant concern in terms of public health and conservation. Implementing educational campaigns to inform the community about the risks associated with handling and consuming contaminated crayfish, as well as initiatives for the restoration of the contaminated ecosystem, are necessary to prevent the transmission of the foodborne pathogens.

Keywords: *Procambarus clarkii*; zoonotic bacteria; *Plesiomonas shigelloides*; *Shigella*/enteroinvasive *Escherichia coli*; enteropathogenic *Escherichia coli*; *Salmonella* ser. Enteritidis; *Salmonella* ser. Typhimurium; *Salmonella* ser. Typhi; Canary Islands

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1. Introduction

Invasive exotic river crayfish have expanded beyond their natural distribution areas, causing ecological threats and negative economic impacts. The red swamp crayfish, *Procambarus clarkii* (Girard, 1852), is widely distributed across all continents except Australia and Antarctica [1–3]. Its high biological plasticity and resistance to environmental contamination contribute to its enormous invasive capacity, competitiveness, and aggressiveness toward native species, making it nearly impossible to eradicate once introduced into a territory [4]. It is an omnivorous predator, opportunistic generalist, and extremely active both day and night, causing significant impact on habitats and altering interspecific relationships, modifying water transparency, eutrophying it, and thus reducing plant diversity [5]. Additionally, *P. clarkii* is a potential asymptomatic carrier of the crayfish plague (*Aphanomyces astaci*) and an importer of pathogenic fungal species such as *Phoma glomerata*

(Coelomycetes), which are potentially harmful to human health, flora, and fauna [6,7]. *Procambarus clarkii* is listed in the Delivering Alien Invasive Species Inventory for Europe (DAISIE) as one of the 100 worst invasive exotic species and is on the list of species of interest to the European Union linked to EU Regulation 1143/2014 on invasive exotic species. The red swamp crayfish exerts undesirable impacts on natural water resources, resulting in a loss of ecosystem services.

Native to North America, this crayfish has been introduced to numerous countries for commercial farming [8,9], becoming one of the most important crustacean crops in countries such as China [10,11]. *Procambarus clarkii* was first introduced to Spain in 1973 [12,13] as an economic resource to alleviate the low availability of the native crayfish population *Austropotamobius pallipes* (Lereboullet 1858), which had been decimated by the crayfish plague [14]. After being introduced to the Guadalquivir Marshes, it spread throughout the Iberian Peninsula, the Balearic Islands [2,15], reaching the Azores islands (Portugal) [16], and finally, the Canary Islands. In 1997, the presence of the red swamp crayfish was detected for the first time on the island of Tenerife (Canary Islands), introduced for human consumption in El Cercado ravine, located in the northwest part of the island, and two years later, it was found on the island of Gran Canaria in a reservoir located in the northwest part of this island [17].

With the introduction of these crayfish into El Cercado ravine in Tenerife, there have been no problems so far with them spreading through irrigation systems, as has happened in other places. Additionally, since there are no native freshwater crayfish species, there has been no impact on any. On the other hand, *P. clarkii* has no natural predators in Tenerife, so the number of individuals can increase excessively, although the ravine topography seems to act as a natural barrier [18]. Regarding its impact on the ravine native fauna, these crayfish appear to be related to the disappearance or drastic reduction in populations of some endemic aquatic arthropods, such as the beetle *Graptodytes delectus* [19].

El Cercado ravine is located on the southern slope of the Anaga massif on the island of Tenerife. It originates at an altitude of 789 m above sea level and flows into the sea after joining with Las Huertas ravine. The coastal section preserves typical Canary coastal vegetation, mainly consisting of "tabaibal-cardonal" (*Euphorbia canariensis*, *Euphorbia balsamifera*, *Euphorbia obtusifolia*, and *Euphorbia aphylla*), as well as ruderal communities. In the middle section, there is a palm grove primarily formed by *Phoenix canariensis* species, located along the watercourse and slopes. The summits are mostly covered with fayal–brezal forest (*Morella faya* and *Erica canariensis*), with some remnants of laurel forest. Numerous rupicolous species are present in the area, particularly those belonging to the genera *Aeonium* and *Sonchus*. El Cercado ravine constitutes a true stream, thus supporting abundant hygrophilous vegetation. Notable among these are the populations of the Canary willow *Salix canariensis* arranged in gallery forests, and *Cyperus* sp., which are a regular part of the red swamp crayfish diet [18]. Throughout the entire course of the ravine, one can observe hamlets, as well as numerous orchards where various types of crops are cultivated.

There are numerous studies demonstrating that the red swamp crayfish can carry foodborne pathogenic bacteria such as *Vibrio* spp. [20,21], *Salmonella* spp. [22–24], or *E. coli* [21,22]. While these bacterial pathogens can infect people through the ingestion of improperly cooked contaminated animals, there is also a risk of infection due to the improper handling of these crustaceans. In fact, several outbreaks of tularemia, a disease caused by the pathogenic bacterium *Francisella tularensis*, have been reported in Spain, associated with crayfish fishing [25] or improper handling [26].

Crustaceans like crabs and shrimp can acquire potentially harmful pathogens for human health by feeding on contaminated mollusks and filtering large volumes of water through their gills to breathe, particularly if the crustaceans are eaten raw [27–29]. Shellfish, in general, can actively become infected with a wide variety of pathogens when they develop in waters contaminated by sewage discharged into the bodies of water where these invertebrates live [30,31]. Pathogens can also be found in the intestine and can access the muscles through wounds or bruised areas [32,33].

Based on these data and the absence of previous studies on these crayfish, the objectives of this work were to evaluate the main zoonotic bacterial and parasitic pathogens present in the *P. clarkii* population introduced into El Cercado ravine on the island of Tenerife and to assess the potential risk to public health and native fauna.

For this purpose, the Biofire FilmArray™ System by BioMérieux was employed due to its numerous advantages. This rapid molecular technique enables the detection of multiple pathogens in a single assay, while also minimizing contamination risks associated with handling. In addition, PCR–culture techniques were used to study pathogens not included in this system.

2. Materials and Methods

A total of 22 crayfish were collected in El Cercado ravine (Tenerife, Canary Islands, Spain) (Figure 1) between March and April 2021 by the staff of the "Red de Alerta Temprana de Canarias para la Detección e Intervención de Especies Exóticas Invasoras" (REDEXOS) during a control action, with the authorization of the "Dirección General de Lucha Contra el Cambio Climático y Medio Ambiente" (Gobierno de Canarias, Expte. 1-2024-0131100523) and donated to be analyzed.

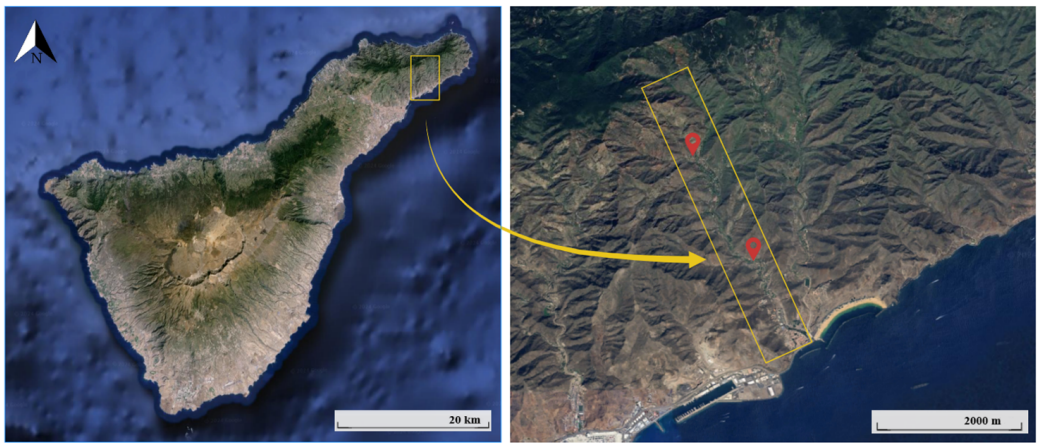

Figure 1. Sampling locations (in red) for *Procambarus clarkii* in El Cercado ravine (yellow), Tenerife (Canary Islands, Spain). Images captured from Google Earth Pro and edited with BioRender.com (consulted on 27 May 2024).

2.1. Bacterial Strains

All bacterial strains used as positive controls were obtained from the American Type Culture Collection (ATCC) (Table 1). All bacteria were stored at −70 °C and were grown on Trytic Soy Broth (TSB, Labkem, Barcelona, Spain) at 37 °C for 18 to 24 h under aerobic conditions.

Table 1. Bacterial strains from American Type Culture Collection (ATCC) used as control in PCR assays.

Bacterial Strain
Salmonella enterica serovar Typhimurium ATCC® 14028
Salmonella enterica serovar Enteritidis ATCC® 13076
Salmonella enterica serovar Typhi strain ATCC® 19430
Staphylococcus aureus ATCC® 653
Staphylococcus epidermidis ATCC® 12228
Staphylococcus saprophyticus ATCC® 15305
Staphylococcus aureus derived from ATCC® BAA-1708™ (Methicillin and Mupirocin resistant)
Staphylococcus haemolyticus ATCC® 29970
Staphylococcus lugdunensis ATCC® 49576
Staphylococcus hominis ATCC® 19536

2.2. Sample Preparation

Every sacrificed crayfish was aseptically and individually cut, and 10 g of material (muscle and internal organs) was taken and placed into a stomacher bag. Then, 90 mL of buffered peptone water (BPW) was added. Using a stomacher, the tissues were homogenized and analyzed.

2.3. Detection and Identification of Pathogenic Bacteria

2.3.1. Detection of Pathogenic Bacteria Using the Biofire FilmArray™ System (BioMérieux)

The Biofire FilmArray™ System (BioMérieux, Craponne, France) is a multiple-PCR system certified by the FDA (Food and Drug Administration), CE-IVD (European in vitro diagnostic devices), and TGA (Therapeutic Goods Administration) that integrates sample preparation, amplification, detection, and analysis. It is a simple system that can simultaneously detect multiple species of pathogens in approximately one hour of testing, with just 2 min of handling time. This system was used to investigate the presence of 16 common gastrointestinal pathogens, including bacteria, (*Campylobacter* (*C. jejuni, C. coli,* and *C. upsaliensis*), *Clostridium difficile* toxin A/B, *Plesiomonas shigelloides*, *Salmonella*, *Vibrio* (*V. parahaemolyticus, V. vulnificus,* and *V. cholerae*), *Yersinia entercolitica*, Enteraggregative *E. coli* (EAEC), Enteropathogenic *E. coli* (EPEC), Entertoxigenic *E. coli* (ETEC) *lt*/*st*, Shiga-like toxin-producing *E. coli* (STEC) *stx1*/*stx2*, *E. coli* O157, *Shigella*/Enteroinvasive *E. coli* (EIEC), and the parasites *Cryptosporidium* spp. and *Giardia lamblia*, causing gastroenteritis (Biofire FilmArray™ GI Panel).

Homogenized tissues from each individual were analyzed using a Biofire FilmArray™ GI Panel and the Biofire FilmArray™ System following the manufacturer instructions. Each kit includes a FilmArray pouch and buffers. For each assay, a FilmArray pouch was hydrated using a hydration injection vial containing 1.5 mL of hydration solution. The sample mixture was then prepared by adding 1 mL of sample buffer to the sample injection vial and 1 mL of the homogenized stool sample. The sample injection vial was then mixed and inserted into the sample port of the pouch, allowing the sample to mix in the corresponding well inside the pouch. After this final step, the analysis was performed using the FilmArray instrument following the manufacturer instructions (https://www.biomerieux.ca/sites/subsidiary_ca/files/biofire_v2.0_operator_manual_en.pdf; accessed on 22 March 2021).

2.3.2. Detection of Pathogenic Bacteria Using Culture–PCR

For the identification of bacterial pathogens not included in the Biofire FilmArray™ GI Panel and to specifically identify some pathogens identified only at the generic level, such as *Salmonella* spp., the enriched liquid cultures of 13 crayfish underwent culture on selective media and subsequent confirmation by PCR of suspicious colonies.

For this purpose, 100 µL of the enriched culture was spread on the surface of Baird Parker agar plates (Labkem, Barcelona, Spain) for the isolation of *Staphylococcus* spp. For the isolation of *Salmonella* spp., 0.5 mL of each BPW culture was transferred to 4.5 mL of Rappaport Vassiliadis Broth (VWR International, Leuven, Belgium) and incubated at 42 °C for 20 h. Subsequently, these samples were plated on XLD agar selective medium (Merck, Darmstadt, Germany) and incubated at 37 °C.

2.3.3. Molecular Identification of Isolates

DNA Extraction

Five or six colonies were randomly selected from those that displayed size and morphology characteristics compatible with *Salmonella* and *Staphylococcus*, grown on the culture media used for isolation (XLD agar and Baird Parker agar, respectively). These colonies were resuspended in 1 mL of PBS and centrifuged at 12,000× *g*. The supernatant was discarded, and the resulting pellet was resuspended again in 1 mL of PBS and centrifuged under the same conditions. The resulting pellet underwent DNA extraction following the instructions of López et al. [34].

PCR Assays

For the identification of the most prevalent zoonotic serovars of *Salmonella* spp., two m-PCR assays were used according to Guimarães de Freitas et al. [35].

The first PCR includes primers for *Salmonella* spp., *S. enterica* ser. Enteritidis, and *S. enterica* ser. Typhi, and the second PCR includes primers for *Salmonella* spp. and *S. enterica* ser. Typhimurium (Table 2).

Table 2. Primers used in the PCR amplifications for *Salmonella* spp., *S. enterica* ser. Enteritidis, *S. enterica* ser. Typhi, and *S. enterica* ser. Typhimurium.

Bacteria	Target Gene	Primers	Sequence (5′-3′)	Size (bp)
Salmonella spp.	ompC	OMPCF OMPCR	ATC GCT GAC TTA TGC AAT CG CGG GTT GCG TTA TAG GTC TG	204
S. enterica ser. Enteritidis	Sdf1	ENTF ENTR	TGT GTT TTA TCT GAT GCA AGA GG TGA ACT ACG TTC GTT CTTCTG G	304
S. enterica ser. Typhi	ViaB	ViaBF ViaBR	CAC GCA CCA TCA TTT CAC CG AAC AGG CTG TAG CGA TTT AGG	738
S. enterica ser. Typhimurium	Spy	TyphF TyphR	TTG TTC ACT TTT TAC CCC TGA A CCC TGA CAG CCG TTA GAT ATT	401

ser. = serovar.

The PCR amplifications contained 1X Buffer (Bioline, London, UK), 0.2 mM of dinuclueotide (dNTP Bioline), 10 pmol of forward and reverse primers for *Salmonella* genus and those for the serotypes analyzed, 2U of Taq DNA polymerase (Bioline, London, UK), 3.0 mM $MgCl_2$ (Bioline, London, UK), and 5 ng of DNA as a template and MilliQ sterile water for completing 25 µL of reaction.

Amplification was conducted with an XP Cycler (Bioer Technology) using the following parameters: 3 min at 94 °C followed by 29 cycles of denaturation at 95 °C for 2 min, annealing at 57 °C (55 °C for the Typhimurium serotype) for 30 s, and extension at 72 °C for 2.5 min, with a final extra extension step at 72 °C for 5 min.

The detection of the *Staphylococcus* species of major health interest (*S. aureus*, *S. lugdunensis*, *S. saprophyticus*, *S. haemolyticus*, *S. epidermidis* and *S. hominis*) and relevant antibiotic resistance genes present in *S. aureus* (methillicin and mupirocin resistance) employed an m-PCR assay as per the specifications described by Pérez-Roth et al. [36] (Table 3).

For *Staphylococcus* species, the PCR amplifications contained 1X Buffer (Bioline, London, UK), 0.2 mM of dinuclueotide (dNTP, Bioline), 1 µM nucA primer pair, 0.5 µM mvaA primer pair, 0.5 µM sep primer pair, 0.5 µM fbl primer pair, 0.5 µM sap primer pair, 0.5 µM mecA primer pair, 0.5 µM ileS2 primer pair, 0.5 µM hom primer pair, 2.5U of Taq DNA polymerase (Bioline, London, UK), 2.4 mM $MgCl_2$ (Bioline, London, UK), and 5 ng of DNA as a template and MilliQ sterile water for completing 25 µL of reaction. Amplification was conducted with an XP Cycler (Bioer Technology, Hangzhou, China) using the following parameters: 5 min at 94 °C followed by 45 cycles of (i) 10 cycles of denaturization at 94 °C for 30 s, annealing at 64 °C for 45 s, and extension at 72 °C for 45 s; (ii) 10 cycles of denaturization at 94 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min; and (iii) 25 cycles of denaturization at 94 °C for 45 s, annealing at 52 °C for 45 s, and extension at 72 °C for 60 s, ending with a final extension step at 72 °C for 10 min.

After PCR amplification, 5 µL of the product was analyzed on an 1.2% agarose gel (Fisher Bioreagents, Madrid, Spain) to estimate product sizes by comparison with a molecular size standard ladder (GeneRuler 50 bp DNA Ladder, Thermo Scientific, Vilnius, Lithuania, and HyperLadder 50 bp, Bioline, London, UK). The gel was stained with Real-Safe (Durviz SL, Valencia, Spain), and the amplicons were visualized using the ChemiDoc™ XRS+ (Bio-Rad, Hercules, CA, USA) system.

Table 3. Primers used in the PCR amplifications for the *Staphylococcus* species of major health interest and relevant antibiotic resistance genes present in *S. aureus*.

Specie (Locus)	Primer	Sequence (5′-3′)	Size (pb)
S. lugdunensis (fbI)	fbIF fbIR	AAA TCT CCA AGT TGA CCA AAC ATA C GAT TGC GCT GAA AGA ATT GC	550
Mupirocin resistance (ileS2)	ileS2F ileS2R	TAT ATT ATG CGA TGG AAG GTT GG AAT AAA ATC AGC TGG AAA GTG TTG	456
S. saprophyticus (sap)	sapF sapR	AAC GGG CGT CTC GAT AGA AAA AAC GGG CGT CCA CAA AAT CA	380
S. aureus (nuc)	nucF nucR	TCG CTT GCT ATG ATT GTG G GCC AAT GTT CTA CCA TAG C	359
Methicillin resistance (mecA)	mecA1 mecA2	GTA GAA ATG ACT GAA CGT CCG ATA A CCA ATT CCA CAT TGT TTC GGT CTA A	310
S. haemolyticus (mvaA)	mvaA1 mvaA2	GGT CGC TTA GTC GGA ACA AT CAC GAG CAA TCT CAT CAC CT	271
S. epidermidis (sep)	sepF sepR	CAG TTA TAC GGT ATG AGA GC CTG TAG AGT GAC AGT TTG GT	219
S. hominis (hom)	homF homR	TAC AGG GCC ATT TAA AGA CG GTT TCT GGT GTA TCA ACA CC	177

2.4. Statistical Analysis

Results are presented as proportions (prevalence), and 95% confidence intervals are included using the Clopper–Pearson exact method. A chi-square test was performed, with a *p*-value set at 0.05, to compare prevalence between age and sex using the SPSS for Windows statistical software v. 25 (IBM Corporation, Armonk, NY, USA).

3. Results

Two assays were carried out for the detection and identification of the analyzed pathogens. One assay was conducted with the Biofire FilmArray™ System, and the other assay was based on the isolation through culturing on selective media followed by identification by PCR.

3.1. Assays Conducted with the Biofire FilmArray™ System

Of the 16 pathogens investigated using this multiplex PCR system, in the 22 analyzed crayfish, *P. shigelloides*, enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and *Salmonella* spp. were detected (Table 4).

Table 4. Pathogenic bacteria detected by the the Biofire FilmArray™ System in *Procambarus clarkii* from Tenerife, Canary Islands (Spain).

Bacteria	+ (Prevalence) [95% CI] (n = 22)
Plesiomonas shigelloides	9 (40.90%) [20.71, 63.64]
Enteroinvasive *E. coli*	7 (31.81%) [13.86, 54.87]
Enteropathogenic *E. coli*	2 (9.09%) [1.12, 29.16]
Salmonella spp.	4 (18.18%) [5.19, 40.28]

+ = positive crayfish; n = number of crayfish analyzed; CI: confidence interval.

The analyses of these 22 crayfish captured in El Cercado ravine revealed that pathogenic bacteria *P. shigelloides* and EIEC were detected in 5 individuals (PCB11, PCB12, PCB13, PCB15, PCB16). Two crayfish (PCA22, PCA24) were positive for *P. shigelloides* and *Salmonella* spp. And, in another two individuals (PCB17, PCB21), *P. shigelloides*, *Salmonella* spp., EPEC, and EIEC were detected.

These data indicate that, according to this analysis system, the prevalence of *P. shigelloides* in crayfish was 40.9% [95% CI: 20.71, 63.64], being 31.81% [95% CI: 13.86, 54.87] for EIEC, 9.09% [95% CI: 1.12, 29.16] for EPEC, and 18.18% [95% CI: 5.19, 40.28] for *Salmonella* spp. (Table 4).

3.2. Culturing-PCR Assays

A total of 13 individuals were analyzed for the presence of *Staphylococcus* spp. While presumptive colonies (black colonies surrounded or not by clear zones) of the genus *Staphylococcus* were isolated from all individuals, none of the six species investigated in this study wer identified.

The search for the three most prevalent zoonotic serovars of *Salmonella* (*Salmonella* ser. Enteritidis, *Salmonella* ser. Typhimurium, and *Salmonella* ser. Typhi, was conducted for the same 13 individuals analyzed for *Staphylococcus* spp. All individuals tested positive for some species of this genus (100%) (13/13) [95% CI: 75.29, 100]. In the case of individual PCB5, both *Salmonella* ser. Enteritidis and *Salmonella* ser. Typhimurium were isolated, while in specimen PCB9, all three investigated serotypes were identified. These data indicate that the prevalence of the Enteritidis and Typhimurium serovars in individuals from the ravine was 15.38% (2/13) [95% CI: 1.92, 45.45], while the prevalence of the Typhi serovar was 7.69% (1/13) [0.19, 36.03] (Table 5).

Table 5. Pathogenic serovars of *Salmonella* detected by culture–PCR assays in *Procambarus clarkii* from Tenerife, Canary Islands (Spain).

Bacteria	+ (Prevalence) [95% CI] (n = 13)
Salmonella ser. Enteritidis	2 (15.38%) [1.92, 45.45]
Salmonella ser. Typhimurium	2 (15.38%) [1.92, 45.45]
Salmonella ser. Typhi	1 (7.69%) [0.19, 36.03]

+ = positive crayfish; n = number of crayfish analyzed; CI: confidence interval; ser. = serovar.

4. Discussion

In this study, two techniques were employed, the Biofire FilmArray™ System and culture–PCR assay. The use of the Biofire FilmArray™ System enabled the analyses of multiple pathogens in a single assay for each sample. Using this method, bacteria previously detected in *P. clarkii*, such as *P. shigelloides*, *Salmonella* spp., and *E. coli*, were identified. Furthermore, this system facilitated, for the first time for crayfish, the identification of *E. coli* pathotypes. Additionally, the culture–PCR technique provided the first data on *Salmonella* serotypes in crayfish.

The presence of zoonotic bacteria in the introduced American red swamp crayfish in Tenerife represents a significant concern in terms of public health and conservation. The analysis of individuals from El Cercado ravine in Tenerife revealed the presence of bacterial species known for their ability to cause diseases in humans.

One of the detected enterobacteria, *P. shigelloides*, is common in aquatic environments [37], both freshwater and marine estuaries in tropical and temperate climates [38], and it can be isolated in both clean and stagnant waters [39,40]. This would explain the presence of *P. shigelloides* in the analyzed animals, as they live in small pools along the ravine where the water is more stagnant, providing a suitable habitat for the bacteria's survival. Additionally, the main reservoirs of this bacterium include fish, shellfish, birds, reptiles, and mammals such as dogs, cats, cows, goats, pigs, and monkeys [41,42], and it has been isolated in crabs such as the blue crab (*Callinectes sapidus*) [43] and in *P. clarkii* itself [44,45]. Therefore, the isolation of this bacterium in the American red swamp crayfish from El Cercado ravine was expected. *Plesiomonas shigelloides* has been associated with sporadic cases and outbreaks of diarrhea in different parts of the world, as well as with traveler's diarrhea [46–48]. It can cause extraintestinal infections, cholecystitis, splenic abscess, meningoencephalitis, etc. [48–50]. Therefore, the presence of this microorganism in the studied crayfish poses an infection risk to the human population. Although these

animals are not usually consumed by local inhabitants, there are cases of infections from simple contact with the animals [51] or from the accidental ingestion of the bacteria after handling the animals [52–54].

There are few studies on the presence of *Salmonella* spp. in wild populations of *P. clarkii*. Most data pertain to crayfish intended for consumption, either from farms [23] or even dried specimens used for consumption in Nigeria [24]. The results on the prevalence of this enterobacterium in these crayfish vary greatly. Barkate in 1967 [55] detected this bacterium in 2.99% of crayfish obtained from natural habitats in Louisiana (USA). However, Saad El-Deen in 2009 [23] in Egypt detected this bacterium in 44% of the crayfish analyzed from farms, whereas another study in Egypt, in the Nile River, found no presence of *Salmonella* spp. [22], also in farmed crayfish. In the present study, the prevalence detected in crayfish captured in the ravine (18.18%) was significantly higher than those found in Louisiana [55].

The genus *Salmonella* is widely distributed in nature, found in the gastrointestinal tract of domestic and wild mammals, birds, insects, and reptiles [56]. Although there are no data on the presence of *Salmonella* in the birds inhabiting the ravine, some of the present species have been described as carriers of these enterobacteria, such as canaries (*Serinus canaria*) [57,58], Eurasian blue tits (*Cyanistes caeruleus*) [59], long-eared owls (*Asio otus*) [60], common barn-owls (*Tyto alba*) [61], and feral pigeons (*Columba livia*) [62], as well as rats (*Rattus rattus*) [63,64] and mice (*Mus musculus*) [65]. Reptiles in general [66,67], and in particular, endemic lizards (*Gallotia galloti*), which are very common in the area, are also carriers of these bacteria [68]. Therefore, the presence of *Salmonella* spp. in the analyzed crayfish could originate from the fecal matter contributed by this surrounding fauna to the water pools in the ravine where they live.

The multiple-culture–PCR technique used in this study enabled the detection of *Salmonella* species in all analyzed individuals (100%). The difference observed between the prevalences obtained with the Biofire Film Array™ System and the culture–PCR method may be due to the increased sensitivity obtained from the enrichment and selective culture steps employed before the multiple PCRs. The direct detection of this bacterium from crayfish tissues may be limited due to endogenous inhibitors of the PCR reaction and mechanical issues in extracting small quantities of cells or DNA from the tissues. Through using an enrichment phase and selective culture, these potential inhibitors are reduced, ensuring that there are enough organisms present to produce an adequate amount of template DNA, in addition to counteracting any inhibitory effects that might be present in the samples. The risk of false positives is also reduced, as the DNA from viable organisms is the main source of template available for amplification [69].

On the other hand, this culture–PCR technique enabled the detection of the Enteritidis and Typhimurium serotypes in two of the analyzed individuals, while the Typhi serotype was detected in one individual in which the first two were also found. Based on their ability to develop specific pathologies in humans, all known *Salmonella* serotypes are classified as typhoidal and non-typhoidal. The typhoidal *Salmonella* serotypes, including Typhi, Sendai, and Paratyphi, are highly adapted to humans, such that animals are not carriers [70]. On the other hand, non-typhoidal serotypes can be found in a wide variety of animals and are involved in zoonotic salmonellosis. Salmonellosis is perhaps the most widespread zoonosis in the world, with the Enteritidis serotype being the most prevalent globally, followed by Typhimurium, although the predominance of one or the other can vary over time [71]. While the presence of the latter two serotypes in the crayfish may be due to fecal matter from the surrounding fauna, the presence of the Typhi serotype can only be due to fecal material of human origin, likely from wastewater from the hamlets located along the edges of the ravine, a very common issue in the ravines of Tenerife [19].

The hypothesis of the human origin of part of the *Salmonella* contamination is further supported by the fact that, although the incidence of the Enteritidis and Typhimurium serotypes, which are the most prevalent in the Canary population, is low [72], these two serotypes are frequently found in asymptomatic individuals [73], who are not considered carriers or sources of these bacteria. In the case of the Typhi serotype, a similar

situation occurs, as there are chronic carriers who intermittently shed the bacteria over a prolonged and indefinite period in the local environment. Therefore, they can spread the disease in the community and maintain a reservoir of infection [74].

The presence of *E. coli* in *P. clarkii* has been well documented in various parts of the world [9,22,75], with prevalence rates of up to 40% [22]. However, there are no studies on the presence of different pathotypes of this bacterium, such as enteroinvasive (EIEC) or enteropathogenic (EPEC). In other crab species, the search for *E. coli* pathotypes has been conducted, with the enterotoxigenic (ETEC) pathotype being detected [76].

EPEC primarily affects children under the age of two, causing diarrhea of varying degrees [77]. It is particularly important because it is a bacterial etiological agent in a pathology dominated by viruses, which leads to it being underestimated, especially in cases of childhood diarrhea [78]. In recent decades, the incidence of EPEC strains in infectious diarrheal disease has decreased, especially in developed countries. However, these strains are still responsible for numerous cases of watery diarrhea in children, causing sporadic cases as well as outbreaks with significant rates of morbidity and mortality [79–81].

The transmission of EPEC occurs through the fecal–oral route, contaminated fluids, surfaces, and water, with asymptomatic carriers being a significant source of infection [82]. For typical EPEC strains, humans (both symptomatic and asymptomatic) are the main known reservoir. These strains are rarely isolated from animals, while atypical strains are frequently detected in both domestic and wild animals [77,82–88]. In this study, the Biofire Film Array™ System used for EPEC detection did not allow for the identification of whether the detected strains were typical or atypical, thus the human or animal origin could not be demonstrated. Therefore, the presence of these *E. coli* strains in the crayfish from El Cercado ravine likely has both a human origin, due to the previously mentioned wastewater input into the water pools where these crayfish are found, and an animal origin, due to fecal matter from the fauna present in the ravine. Based on these data, the improper handling of these animals, especially by children, which is a common occurrence (pers. obs.), poses a clear risk for developing cases of watery diarrhea.

EIEC are the etiological agents of bacillary dysentery in humans, particularly in developing countries [82,89]. However, outbreaks in Europe caused by EIEC O96:H19 have led the scientific community to reconsider the role of EIEC infection in industrialized countries [90–92]. Humans infected by EIEC appear to be the primary source of infection, as no animal reservoirs have been identified and the transmission of these bacteria is primarily through the fecal–oral route [93]. These strains are often found in human-origin wastewater or treated water [94], while they are not commonly detected in surface waters [95]. Based on these data, the presence of EIEC in the analyzed crayfish likely originates from wastewater input into the pools where these animals live, coming from the houses adjacent to the ravine [19]. The improper handling of these animals, especially by children, poses an infection risk from the accidental ingestion of these bacteria, potentially causing symptoms such as vomiting, fever, and even watery diarrhea. In severe cases, dysenteric stools may contain blood and mucus [96].

Based on the results obtained, and as previously mentioned, it appears that there is fecal contamination of human origin due to wastewater seepage. However, the other bacteria analyzed using the Biofire Film Array™ System that affect the human population of the islands, such as *Campylobacter* spp. [97], were not detected.

Bacteria of the genus *Campylobacter* can frequently be isolated from wild birds [98], as well as from mice (*Mus musculus*) and rats (*Rattus rattus*) [99], which are prevalent in the study area. In the case of the human population in the Canary Islands, although cases of *Campylobacter* spp. are reported, the incidence of campylobacteriosis is low [97], and asymptomatic infections are uncommon [100]. These two facts may be related to the absence of *Campylobacter* spp. in the analyzed crayfish because the contribution of these bacteria from human sources via wastewater seepage into the water pools where the crayfish live is minimal. Additionally, the surrounding fauna do not appear to be a significant source of contamination.

The third most reported bacterium in the Canary Islands population is *Yersinia enterocolitica* [101], although its incidence is low [102]. This ubiquitous bacterium is frequently isolated from animals, soil, various water sources, and food products [103–105]. Considering that asymptomatic carriers among adults are not uncommon [106], it is surprising that this bacterium was not detected in the analyzed crayfish, despite the clear presence of wastewater seepage into the pools where the crayfish reside. This absence could be due to the very low incidence of this bacterium in the human population adjacent to the ravine, as well as in the fauna inhabiting the ravine. There are no data on the presence of *Y. enterocolitica* in the wildlife of the Canary Islands, but studies from other regions demonstrate that wildlife can be carriers of this bacterium, such as rats (*R. rattus*) [107] and mice (*M. musculus*) [108], species that are abundant in the study area.

Many species of the genus *Vibrio*, including *V. alginolyticus*, *V. harveyi*, *V. cholerae*, *V. mimicus*, *V. parahaemolyticus*, and *V. vulnificus*, can infect aquatic animals. Vibriosis has been confirmed as a disease affecting marine and freshwater fish, mollusks, and crustaceans [109]. In the case of *P. clarkii*, systemic infections with *V. mimicus* and *V. cholerae* have been recorded [110], although *V. parahaemolyticus* is the most commonly isolated bacterium of this genus in these crayfish [20,111]. Within the genus Vibrio, 11 species are considered to be human pathogens of varying importance. Notable for their relevance and severity are *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* [112], which have been implicated in human disease outbreaks or possess the potential for such [113]. The incidence of *V. cholerae* in the human population of the Canary Islands is very low, with no cases of cholera detected in 2022, and as well for the other two significant species [102]. Contaminations by *V. parahaemolyticus* were often overlooked in freshwater animals; however, it has been shown that these animals can also be emerging vehicles for the propagation of this bacterium, consequently posing a public health threat [114], especially when these animals are used for human consumption. Nevertheless, in this study, the analyzed crayfish were also free of these bacteria.

Although water is the primary reservoir for *Vibrio* spp., species of this genus have been isolated from numerous mammal species, both domestic and wild, and in birds [115,116]. In non-endemic cholera areas, cholera and non-cholera *Vibrio* strains (non-cholera producers) have been isolated from domestic animals (goats, cows, dogs, and birds) [117]. There are limited data on the presence of these bacteria in the wildlife of the Canary Islands [116], but it appears that they are not common bacteria. The absence of *Vibrio* bacteria in the analyzed crayfish seems to be due to the lack of incidence in the human population, which could introduce them into the water pools through wastewater seepage, and to their absence in the wildlife of the Canary Islands in general.

Clostridioides difficile is a ubiquitous bacterium in the environment capable of colonizing the intestinal tract of both animals and humans [118]. Given that wild, domestic, and food animals frequently test positive for toxigenic *C. difficile*, even without showing signs of disease, it seems plausible that *C. difficile* could be zoonotic [118]. On the other hand, this bacterium has been detected in aquatic environments [119,120], including in wastewater [121–123]. Zidaric et al. [120] showed that more than 50% of 34 different strains of *C. difficile* isolated from rivers have also been found in humans and animals. This finding demonstrates the association between the environment, humans, and animals.

In the Canary Islands, there are only data on the presence of this bacterium in North African hedgehogs (*Atelerix algirus*) with relatively high prevalence [116]. Studies in other regions have shown that rodents are carriers of these bacteria [124], so it would not be surprising if these animals were also carriers in the study area. Regarding the human population, *C. difficile* has been involved in several hospital outbreaks on the islands [125,126], but there are no data suggesting that it is a frequent bacterium in the population. These data may explain why *C. difficile* was not detected in the analyzed crayfish, as although the wildlife surrounding the ravine could contribute this bacterium, it seems that the main source of contamination is humans through wastewater, and the incidence of this bacterium in the human population is very low.

The genus *Staphylococcus* is composed of the common commensal bacteria found in both humans and animals and is often present on the skin and mucous membranes. These are ubiquitous microorganisms that can be isolated from animal products as well as environmental sources such as soil and various types of water [127]. *Staphylococcus* spp. has been isolated from *P. clarkii* in different parts of the world [128–130]. However, in this study, none of the six investigated species, which are the most frequently isolated in hospitals in Tenerife [36], were isolated. Therefore, wastewater discharge into the ravine does not seem to be a source of contamination for these bacteria. Although these species were not detected, colonies with characteristics compatible with *Staphylococcus* spp. were obtained from the cultures of the crab samples, which may correspond to other species that have been isolated from animals present in the study area, such as *S. sciuri*, *S. cohnii*, or *S. gallinarum* [131].

Protozoa of the genus *Cryptosporidium* are globally distributed parasites that can be found both in the environment and parasitizing humans and a wide range of domestic and wild animal species [132], such as pets, livestock, and rodents, among others [133–136]. Additionally, they are considered waterborne and foodborne protozoa due to being a common cause of outbreaks transmitted by water and food [137]. In the Canary Islands, the presence of the zoonotic species of this genus has been described in wildlife such as birds, rodents, and small mammals [138–141], as well as in the human population [142], where the incidence is relatively low [143].

There is little information on the contamination of crabs in general by *Cryptosporidium* spp., and specifically in *P. clarkii*. Data only exist regarding the contamination of *P. clarkii* captured in the Nile River [144,145]. Other studies have shown that some species of crabs, such as Atlantic Blue Crabs (*Callinectes sapidus*), can act as mechanical vectors for the transmission of *Cryptosporidium* spp. oocysts [146], suggesting that *P. clarkii* could also serve as a transmission vector for these parasites. The fact that the fecal contamination in the water wells where this crab species lives in Tenerife is primarily of human origin, and the incidence of this protozoan in the human population is low, could explain why *Cryptosporidium* spp. was not detected in the analyzed crabs.

Giardia duodenalis (syn. *Giardia intestinalis* and *Giardia lamblia*) is a parasite that infects the upper intestinal tract of humans and other animals, causing giardiasis worldwide. In addition to this species, seven other valid species have been identified infecting multiple hosts, ranging from mammals to birds and amphibians [147]. Transmission occurs through the fecal–oral route, and sources of *G. duodenalis* infection include direct contact with infected people or animals, or contaminated water and food [148]. Different species of *Giardia* have been detected in domestic animals and wildlife in the Canary Islands [139,149,150], as well as in wastewater [151,152]. The incidence of *G. duodenalis* in the human population in the Canary Islands is moderate [153].

Giardia spp. cysts have been detected in shellfish such as oysters [154] or mussels (*Mytilus galloprovincialis*) [155]. However, similar to the case of *Cryptosporidium* spp., there are few studies that have investigated the presence of *Giardia* spp. in crabs in general. There are only data on the presence of cysts of these protozoa in *P. clarkii* captured in Egypt [145]. In the absence of more information, it seems that these arthropods can be hosts of these protozoa, although they were not detected in the analyzed crabs, either because the contribution from the surrounding fauna is negligible or because the incidence of *G. duodenalis* in the human population of the ravine is low.

In summary, the results of this study suggest the possibility that the population of *P. clarkii* introduced into El Cercado ravine in Tenerife may act as a reservoir for potentially dangerous bacterial pathogens, posing a risk to health for those individuals, especially children who handle them or for potential consumers of these crustaceans, as well as for the aquatic ecosystem where they are found. It appears evident that the presence of these pathogenic microorganisms primarily originates from human sources due to sewage leaks from neighboring households along the ravine. This raises several serious issues for both human health and the aquatic ecosystem. Firstly, the potential illnesses in

the population result from the consumption of these crustaceans or from the accidental ingestion of pathogens after handling them. Secondly, the eutrophication of the ravine due to the excess nutrients present in the sewage reduces dissolved oxygen and affects other forms of aquatic life. Lastly, it may impact the aquatic fauna, such as through the disruption of the food web due to contaminated species competing disadvantageously for resources.

Possible actions to address these issues could include improving sanitation and wastewater treatment in the area, as well as maintaining and modernizing infrastructure to prevent leaks. Implementing educational campaigns to inform the community about the risks associated with handling and consuming contaminated crayfish, as well as initiatives for the restoration of the contaminated ecosystem, would also be beneficial.

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