UNIVERSIDAD DE COSTA RICA
SISTEMA DE ESTUDIOS DE POSGRADO

CARACTERIZACIÓN MOLECULAR DE LAS COMUNIDADES BACTERIANAS ASOCIADAS A MICROPLÁSTICOS ENCONTRADOS EN EL SISTEMA DIGESTIVO DE LA LANGOSTA DE AGUA DULCE *Procambarus clarkii* (DECAPODA: CAMBARIDAE)

Tesis sometida a la consideración de la Comisión Programa de Estudios de Posgrado en Biología para optar al grado y título de Maestría Académica en Biología

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Ciudad Universitaria Rodrigo Facio, Costa Rica

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DEDICATORIA

A mis papás, a mis hermanos, a Jimmy, a mis amigos y a mi familia. El appoyo incodicional que me birndaron durante este proceso, me ayudó a culminar con éxito uno de mis sueños

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RESUMEN

La contaminación por plásticos está causando daños irreparables en el planeta y se agrava cada día. Los microplásticos (MPs) son un contaminante peligroso de interés mundial que amenaza a los organismos, los ecosistemas acuáticos y la salud pública. En este estudio experimental, elegimos como modelo animal de agua dulce a la langosta de río *Procambarus clarkii*, por ser una especie invasora exitosa, cosmopolita y muy tolerante condiciones extremas de nuevos ambientes, e investigamos la composición del microbioma del tracto intestinal de 12 individuos en tres condiciones: muestreados directamente del medio ambiente y en condiciones de laboratorio, expuestos con o sin MPs en los alimentos, utilizando PCR-DGGE y secuenciación de alto rendimiento del gen 16S rRNA, para determinar su efecto en el microbioma. Determinamos que Firmicutes, Proteobacteria, Bacteroidota y Actinobacteria eran los filos predominantes en la microbiota intestinal y desempeñaban papeles importantes en las funciones intestinales de digestión, absorción e inmunidad de *P. clarkii*. Tras 96 horas de exposición a los MPs, se observó una evidente disbiosis de la microbiota intestinal, como un aumento de las Alfaproteobacterias y las Actinobacterias. Además, se pudo evidenciar un aumento en la abundancia de los géneros *Klebsiella, Acinetobacter, Hydromonas, Pseudomonas, Gemmobacter* y *Enterobacter*. La mayoría de estos géneros se encuentran también en la microbiota intestinal de los humanos y causan infecciones debido a su resistencia a los antibióticos, por lo que podrían considerarse como microorganismos potencialmente perjudiciales para la salud de *P. clarkii*. Además, la abundancia de la clase Clostridia y Bateroides disminuyó en las muestras con MP, siendo microorganismos cruciales para el desarrollo del sistema inmune y para la prevención de patógenos, por lo que el desequilibrio podría ser un indicador de alguna alteración de la salud en *P. clarkii*. En general, estos resultados revelan una diferencia significativa en la riqueza (p=0,028) de las comunidades bacterianas intestinales tras el consumo de MPs en comparación con las muestras sin MPs, lo que favorece la abundancia de bacterias oportunistas y una posible transferencia de bacterias entre MPs a la microbiota intestinal del cangrejo de río, pudiendo afectar también a la salud de otros animales acuáticos y alterar los ecosistemas.

Palabras clave: Microbiota intestinal, cangrejo de río, microplásticos, agua dulce, disbiosis, medio ambiente.

ABSTRACT

Plastic pollution is causing irreparable damage to the planet and it is getting worse every day. Microplastics (MPs) are a hazardous pollutant of global concern that threatens organisms, aquatic ecosystems and public health. In this study, we chose the invasive, cosmopolitan and highly environmentally tolerant the red swamp crayfish *Procambarus clarkii* as a freshwater animal model and studied the composition of the intestinal tract microbiome of 12 individuals under three conditions: individuals sampled directly from the environment, individuals reared under laboratory conditions and exposed with or without MPs in feed, using PCR-DGGE and high-throughput sequencing of the 16S rRNA gene to determine its effect on the microbiome. The results revealed that Firmicutes, Proteobacteria, Bacteroidota and Actinobacteria were the predominant phyla in the gut microbiota, which play important roles in the intestinal functions of digestion, absorption and immunity of *P. clarkii*. After 96 hours of exposure to MPs, an evident dysbiosis of the intestinal microbiota was observed, such as an increase in alphaproteobacteria and actinobacteria. Moreover, results revealed an increase in the abundance of Klebsiella, Acinetobacter, Hydromonas, Pseudomonas, Gemmobacter and Enterobacter genera. Most of these genera are present also in the intestinal microbiota of humans and cause infections due to their resistance to antibiotics, so they could be considered as microorganisms potentially detrimental to the health of *P. clarkii*. In addition, the abundance of the Clostridia and Bateroides class decreased in samples with MPs, being crucial microorganisms for the development of the immune system and the prevention of pathogens. Therefore, the imbalance could be an indicator of some health alteration in *P. clarkii*. Overall, these results revealed a significant difference in the richness ($p=0.028$) of intestinal bacterial communities after consumption of MPs compared to samples without MPs, which favors the abundance of opportunistic bacteria and a possible transfer of bacteria between MPs to the intestinal microbiota of the crayfish, which could also affect the health of other aquatic organisms and alter ecosystems.

Keywords: Gut microbiota, crayfish, microplastics, freshwater, dysbiosis, environment.

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LIST OF ABBREVIATIONS

MPs: Microplastics

rPET: Recycled Polyethylene Terephthalate

DO: Dissolved oxygen

V: Voltios

SEM: Scanning Electron Microscope

DSC: Differencial scanning calorimetry

PCR: Polymerase Chain Reaction

DGGE: Denaturing Gradient Gel Electrophoresis

dNTP: Deoxynucleotide

TAE: Tris-Acetate–EDTA

OTUs: Operational Taxonomical Units

ASV: Amplicon Sequence Variants

NCBI: National Center for Biotechnology Information

UPGMA: Unweighted Pair-Group Method Using an Arithmetic Average

NMDS: Non-metric multidimensional scaling analyses

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INTRODUCTION

Plastic pollution is causing irreparable damage to the planet, and the situation is getting worse every day (Lebreton et al., 2018). It is estimated that by 2050 there will be more plastic than fish in the sea (World Economic Forum and Ellen MacArthur Foundation, 2017). In Costa Rica, about 550 tons of plastic are discarded daily, where 80% (440 tons) is thrown into rivers and seas; 11% (60.5 tons) remains in landfills and the environment; while only 9% (49.5 tons) is recycled (Gutiérrez et al., 2017; Grajales, 2018).

Plastic takes hundreds of years to degrade (Cole et al., 2011). However, by the effect of abrasion and other physicochemical processes, it can fragment into smaller pieces (<5 mm), commonly known as microplastics (MPs) (Cole et al., 2011; Law & Thompson, 2014). Additionally, they can adsorb additives, heavy metals, antibiotics, pesticides, as well as other toxicants and can harbor other types of beneficial or pathogenic microorganisms (plastisphere) (Hirai et al., 2011; Zettler et al., 2013; Jahnke et al., 2017; Harrison et al. 2018; Liu et al., 2021). Because of their ubiquity, they have been found all over the planet and also throughout the water column. They have been encountered in the north (Kanhai et al., 2018) and the south pole (Waller et al., 2017), in surface waters (Kanhai et al., 2017), rivers (Lechner et al., 2014; Klein et al., 2015; Lebreton et al., 2017), lakes (Blettler et al., 2017), estuaries (Peng et al. 2017), sewage treatment plants (McCormick et al., 2014; Mintenig et al., 2017), attach to raindrops (Elizalde-Velázquez et al., 2021), as well as at the bottom of oceans (Woodall et al., 2014).

In Costa Rica, a recent study in the National Park Las Baulas found the presence of microplastics in the digestive system of 89% of fish and 76% of crustaceans, with an average of 3.75 ± 1.70 pieces of MP/fish and 2.64 ± 1.36 pieces of MP/crustacean (Astorga-Pérez, 2020). Other studies (Lundström & Mårtensson, 2015; Bermúdez-Guzmán et al., 2020) found evidence of the presence of MPs in planktivorous fish from the Pacific coast of Central America; all analyzed individuals contained MPs, and from the 1100 pieces, 20.5% were particles and 79.5% fibers.

Setälä et al. (2014) showed for the first time the ingestion of MPs via planktonic organisms from one trophic level (mesozooplankton) to a higher level (macrozooplankton), suggesting

that the impacts of plastic transfer and possible accumulation in the food web require further research. Matsson et al. (2014) demonstrated that nanoparticle uptake occurs along the algal-zooplankton-fish-human food chain, with consequent genetic, morphological, and behavioral alterations. This problem will flow from lower to higher nutrient levels in the food chain, causing human health risks from transferring MPs between mussels and crabs (Miranda & de Carvalho-Souza, 2016).

Studies with decapods showed that wild populations in marine environments are exposed to microplastics, *Crangon crangon* (Devriese et al., 2015), *Nephrops norvegicus* (Murray & Cowie, 2011). In both studies, predominantly plastic fibers were detected in 63% and 83% of the animals examined, respectively. Most studies on the effects of microplastics have focused on marine organisms, while the biological impacts of these types of particles on freshwater organisms remain relatively unknown (Eerkes-Medrano et al., 2015). Previous studies have indicated the presence of microplastics in both freshwater and terrestrial biosphere (Derraik, 2002; Eerkes-Medrano et al., 2015). Therefore, further research focusing on the effects of microplastics on the biology of freshwater organisms is needed (Scherer et al., 2018).

In China, the species *Eriocheir sinensis* is an economically important crab for freshwater fisheries (Yu et al., 2018). In addition, this benthic decapod is typical in the Yangtze River of China, where the accumulation of microplastics in both water and sediments is increasingly serious (Zhao et al., 2014). Yu et al. (2018) demonstrated that microplastics can accumulate in *E. sinensis* tissues and negatively affect growth. Furthermore, they evidenced that microplastic exposure causes damage and induces oxidative stress in the hepatopancreas of *E. sinensis*. These results provided basic biological data for environmental and human risk assessments of microplastics of major global concern, demonstrating the value of conducting such studies (Yu et al. 2018).

When ingested, especially at the micro-and nanoscale, MPs can also have toxic effects by entering the circulatory system of organisms as nanoparticles (Avio et al., 2015; Grigorakis et al., 2017). Microplastics' toxic cellular effects have also been reported in blue mussels (*Mytilus edulis*) and mesenchymal stem cells (Jiang et al., 2011; Von et al., 2016).

However, little is known about the effects of MPs on the gut microbiota of fish (Jin et al., 2018) and freshwater crustaceans (Scherer et al., 2018).

The toxicity of MPs in aquatic organisms does not only cause physical damage in fish and crustaceans (Pedà et al, 2016; Jovanović, 2017). Moreover, several studies revealed the toxic impact of MPs causing internal damage such as blocking the digestive tract, reducing growth rates, blocking enzyme production, inducing oxidative stress, and even affecting reproduction and interaction with microorganisms in the environment (Wright et al, 2013; Jeong et al, 2016; Sussarellu et al, 2016; Rodríguez-Seijo et al, 2017; Caruso, 2015; Grigorakis et al, 2017).

Plastic particles, having a high surface-to-volume ratio, are colonized by microorganisms, including pathogens, which disrupt food webs, nutrient cycles and the balance of aquatic ecosystems (Zettler et al., 2013; Kirstein et al., 2016; Arias-Andres et al., 2018). Considering the widespread presence of microplastics especially in aquatic systems, plastic is frequently found in the digestive tract of zooplankton, invertebrates and fish (Cole et al., 2016; Steer et al., 2017). However, little is known about the effects of microplastics on the gut microbiota of fish (Jin et al., 2018) and freshwater crustaceans (Scherer et al., 2018).

Gut microbiota refers to the community of microorganisms residing in the gastrointestinal tract, is essential for health, and is involved in the regulation of many physiological functions in the host organism (Hsiao et al., 2013; Yano et al., 2015; Jin et al., 2017). Gut microorganisms are crucial for the healthy culture of aquatic animals (Zhou et al., 2014). Studies have shown that environmental stresses, such as changes in the aquaculture environment and contaminant input, can destroy microbial community dynamics in aquatic animals (Huang et al., 2021). This leads to significant changes in the intestinal microflora (Guo et al., 2020; Zhang et al., 2020; Zhang et al., 2021a; Wu et al., 2021; Zhenbing et al., 2021), thus impairing host immunity. Bacterial diversity is essential for ecological function and changes in the gut microbial community of aquatic organisms, such as crustaceans, increase their risk of disease (Guo et al., 2020).

Crustaceans are the second-largest subphylum on Earth and consist of crabs, crayfish, lobsters and shrimps (Vogt, 2021). The digestive system of crustaceans consists of

the digestive tract and digestive gland (Zhenbing et al., 2021a). In research with economically important shrimp, the involvement of the microbiome in the regulation of health and disease in shrimp was studied, it was also possible to verify how the intestinal microbiota changes with the introduction of various pathogens in the decapod (Chae et al., 2019; Foysal et al., 2021; Holt et al., 2021). Likewise, several characterization studies of the gut microbiota in another decapod of importance for aquaculture, *P. clarkii* have been carried out to evidence the importance of the interaction of bacterial communities and how dysbiosis can affect the health of the organisms, altering their ecosystem and, therefore, their productive efficiency in aquaculture (Guo et al., 2020; Zhang et al., 2020; Zhang et al., 2021b).

In Costa Rica, the taxonomic composition of the freshwater decapod fauna is composed of three families: Palaemonidae and Atyidae (Caridea), and Pseudothelphusidae (Brachyura) (Magalhães & Türkay 1996). *Procambarus clarkii* (Girard, 1852) is a decapod of the family Cambaridae, has a natural geographic distribution in northeastern Mexico and south-central United States of America (USA) and has been extensively cultivated in the USA (Hobbs, 1972). Due to its importance for aquaculture, *P. clarkii* has been introduced in numerous countries around the world and is currently considered the most cosmopolitan crayfish ("acosil" in Mexico), present in natural environments in all continents except Australia and Antarctica (Viccon-Pale et al., 2016). It is considered a successful or model invasive species, as it possesses characteristics such as early sexual maturity, rapid growth, high fecundity, direct development, a relatively short life cycle (Hobbs, 1972; Gherardi, 2006). In addition, this organism can adapt to new environments, being tolerant to low oxygen levels, resistant to high temperatures, flexible to prolonged periods of drought, and able to survive in saltwater, for all these characteristics it has been recognized as the species with the greatest ecological plasticity of all Decapods (Rodríguez et al. 2015).

According to Huner (1977), *P. clarkii* was introduced in Costa Rica around 1966. It is an omnivorous species, but can also be saprophagous, although it only consumes prey that is not decomposing (Hobbs, 1972). Among its favorite foods are worms (annelids), insects, insect larvae, fish, frog eggs and salamander (Campos, 2005). Their feeding behavior is changeable to survive since they are attracted to animal or artificial foods

through remote receptors (telereceptors) and when food becomes scarce, these organisms become vegetarian, changing their bacterial structure (Gutiérrez-Yurrita et al., 1998).

The identification of the digestive bacterial genetic structure associated with microplastic ingestion in *P. clarkii* can be considered a substantial avenue to obtain information on additive exposure to this pollutant (Capanni et al., 2021; Xavier et al., 2021). Above all, to fully understand the impact of absorbed and plastic-associated contaminants on aquatic organisms (Capanni et al., 2021). So far, no similar studies have been conducted in the country to assess the transfer of (environmentally relevant) concentrations of chemicals, both contaminants, and additives, like MPs, in host organisms, clearly indicating that this is an area in need of further research (Li et al., 2018).

Therefore, obtaining information on the impact of MPs on this species will be very important and innovative, especially for its effects on ecosystems and public health since this crustacean is widely consumed due to its abundance in one place (Heindler et al., 2019; Zhang et al., 2020; Huang et al., 2021; Wu et al., 2021). The gut microbiome and its bacteria-host interactions are a complex network of associations and to explore that complexity, molecular tools are the most suitable options for such studies, as more robust and meaningful results are obtained (Pompanon et al., 2012; Sun et al., 2018). Therefore, high-throughput sequencing technology provides high-definition tools to investigate complex taxonomic assemblages of bacterial communities in depth (Ding et al., 2017).

Molecular methods based on the recovery of microbial diversity by 16S RNA gene sequence analysis are the most suitable options for this type of study to obtain robust and meaningful results on the structure (i.e. species composition and abundance) of very diverse bacterial communities (Sun et al., 2018) such as lake sediment samples, soils (Li et al., 2012), gut microbial diversity of terrestrial and aquatic animals (Huber et al., 2004; Janczyk et al., 2007; Liu et al., 2011).

The gut microbiome and its bacteria-host interactions are a complex network of associations and to explore that complexity, experimental molecular methods with high resolution are needed (Pompanon et al., 2012). Despite their usefulness, denaturing gradient gel electrophoresis (DGGE), cloning or culture-based methods have shown limitations in the accurate characterization of complex bacterial communities (Boutin et al., 2013). On

the other hand, high-throughput sequencing technology provides high-definition tools to deeply investigate complex taxonomic assemblages of bacterial communities (Ding et al., 2017). These techniques allow characterizing the species composition and abundance of highly diverse bacterial communities (Sun et al., 2018). Therefore, this study aimed to characterize the structure of bacterial communities associated with the presence of MP in the digestive system of the red swamp crayfish *Procambarus clarkii* in Costa Rica, using the molecular technique of high-throughput sequencing by analyzing the 16S RNA gene.

RESEARCH QUESTION

How do MPs affect the structure of bacterial communities associated with the intestinal tract of the the red swamp crayfish *Procambarus clarkii*?

OBJECTIVES

2.1. General Objective

Analyze the structure of the bacterial communities associated with the presence of MPs in the digestive system of the red swamp crayfish *Procambarus clarkii* from the Cachí reservoir of Costa Rica, using high-throughput sequencing of the 16S rRNA gene, to determine its effect on the microbiome.

2.2. Specifics objectives

- Identify the bacterial composition associated in the intestinal tract of the freshwater the red swamp crayfish *P. clarkii*.

- Determine microplastics-associated bacterial communities in the intestinal tract of *P. clarkii* through sequencing of the 16S rRNA gene.

- Analyze bioinformatically and statistically the differences in the taxonomic composition of the bacterial communities of the intestinal tract of *P. clarkii* and microplastics-associated bacteria to demonstrate the dysbiosis effect of the contaminant on the freshwater the red swamp crayfish intestinal microbiome.

MATERIALS AND METHODS

3.1 Sample collection

Individuals of *P. clarkii* were collected at the Cachí reservoir, the sampling site was chosen where the species was known to be introduced, access to the site was easy to obtain specimens of this decapod, and where plastic contamination was visible. A total of 30 red swamp crayfish were collected, 10 individuals (seven females and three males of *P. Clarkii*) were specifically sampled at the points with the following coordinates 9°49'42.8"N 83°48'45.3"W and 9°49'42.0"N 83°48'44.0"W (**Figure 1**), these samples were analyzed immediately after sampling (Control Cachí reservoir)**,** see **Appendix 1**. On the other hand, for the experiment in laboratory conditions (fed pellets without and with MPs), 20 male individuals of *P.clarkii* of similar sizes were purchased from local fishermen in the Cachí reservoir. These samples had their acclimatization and processing period, a procedure that will be detailed later in section 3.2. To continue with a homogeneous study, we focused only on males of *P. clarkii* since they were the individuals that were collected the most in this research and, therefore, could be compared with the other organisms selected for the laboratory conditions. In addition, males are the most consumed by the people for the size and the abundance because the monthly growth rate is higher in males than in females due to the incubation and juvenile care periods (Hazlett & Rittschof, 1985; Hamasaki et al., 2020) (**Figure 2**).

To avoid possible contamination of the samples with external plastic material, the individuals were captured manually, using nitrile gloves and clean cloth bags, and subsequently stored in ice coolers until further processing. Water temperature and pH were measured at the sampling site with an alcohol thermometer and pH test strips (Merck Millipore, Darmstadt, Germany). The identification of the red swamp crayfish was carried out following the morphometric and morphological characteristics described by Campos (2005).

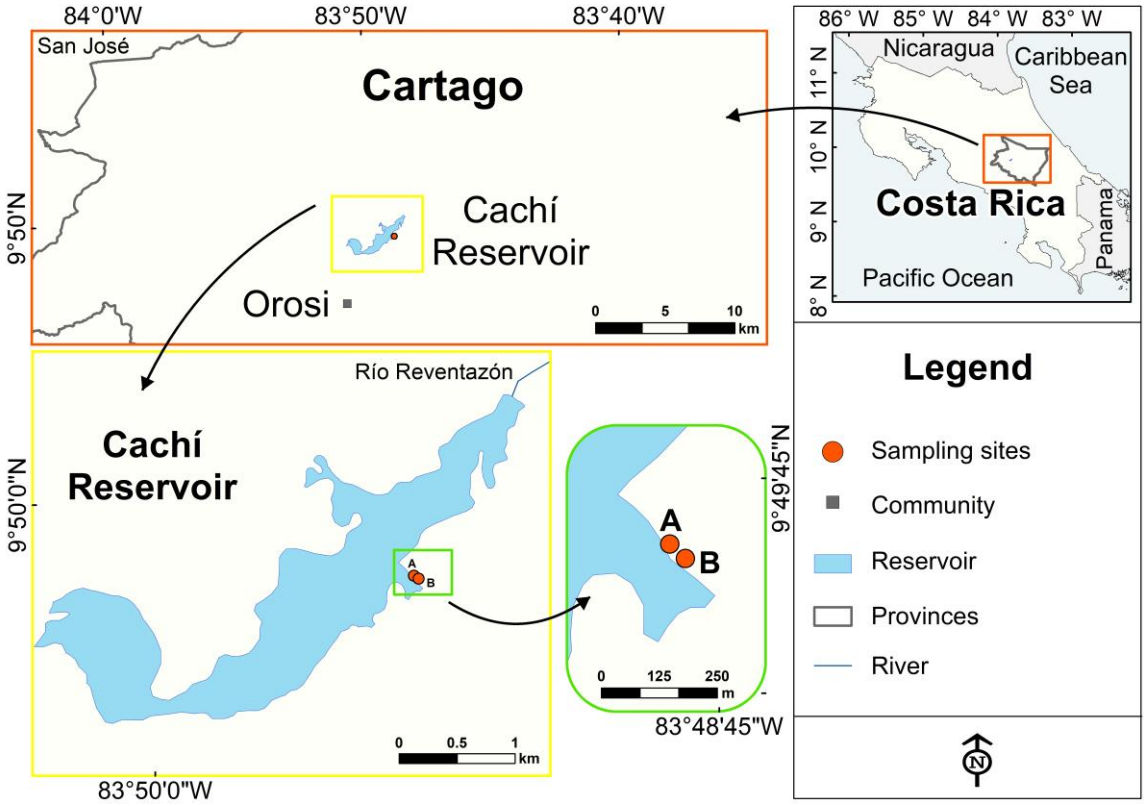

Figure 1. Map of the sampling site at the Cachí reservoir, Cartago, Costa Rica. A) and B) represent the sampling sites. This map was created with ArcMap 10.8.1 (https://desktop.arcgis.com/es/arcmap/).

After collection, the individuals were weighed, measured, and dissected at the laboratory of the Research Unit for Fishery and Aquaculture (UNIP) of the Research Center of Marine Science and Limnology (CIMAR), located at the University of Costa Rica, San José. The intestinal contents were washed three times with sterile water and were placed in sterile glass tubes until genomic DNA extraction.

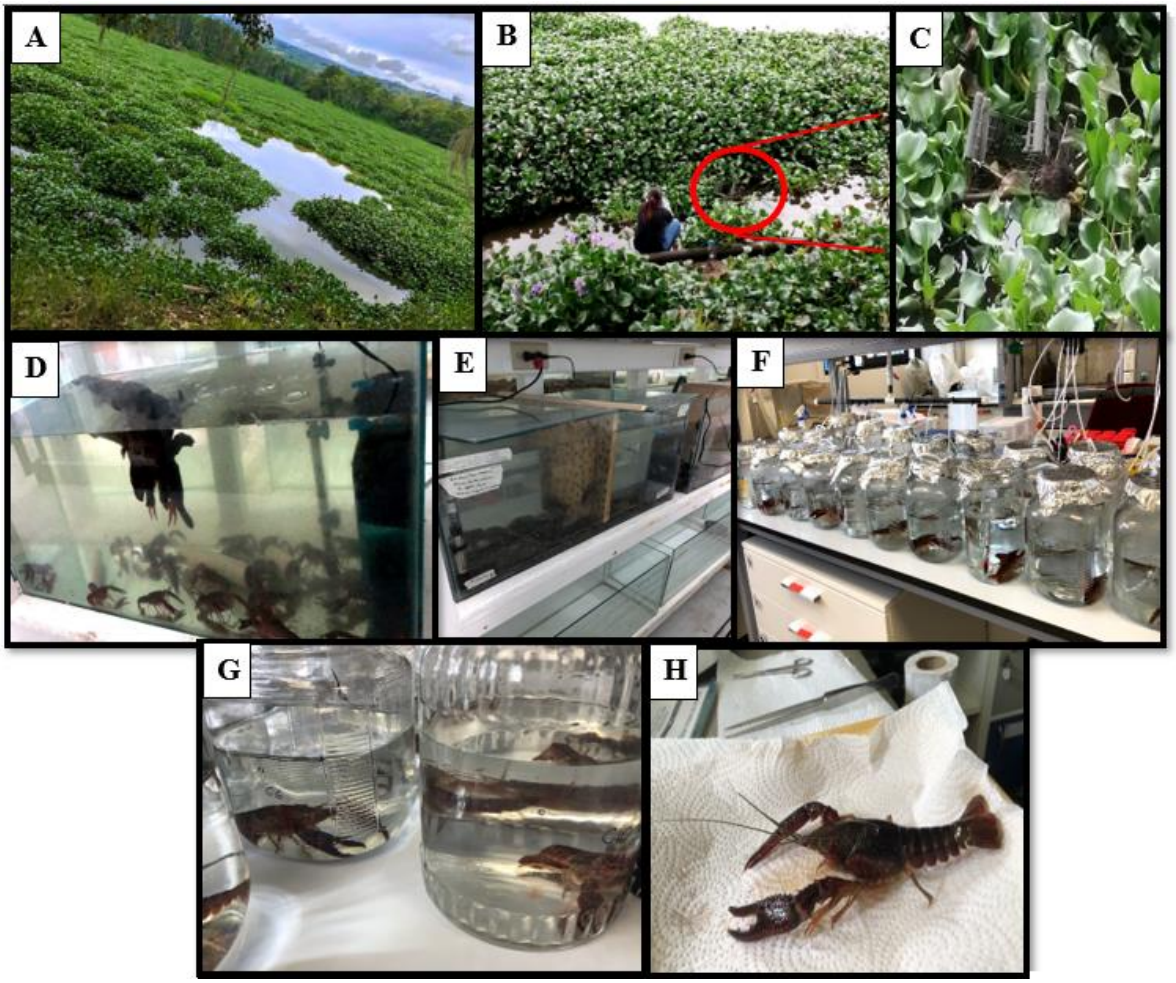

Figure 2. Images of sampling site and experimental essay with *P. clarkii*. A) and B) Sampling site. C) Plastic contamination at the sampling site. D) and E) Acclimatization conditions at CIMAR Laboratory. F) Bioassay at Ecotoxicological Studies Laboratory (ECOTOX). G) 2L container for the bioassay with red crayfish inside. H) *P. Clarkii*.

3.2 Laboratory conditions to evaluate the effect of microplastics on bacterial communities in the gut of *P. clarkii.*

In the laboratory, specimens of *P. clarkii* were acclimatized following the procedure proposed by Yu et al. (2018) with the following modifications. The laboratory conditions were maintained for 12 days for acclimatization at 26-28 °C ± 2 ° C in fresh culture water (drinking water, filtered and sterilized with UV and well aerated; pH 7.6 ± 0.5) (Jin et al., 2019). During this period, they were fed once a day every 48 h for five days with

commercial feed (Nutrafin commercial brand). Feed, fecal pellets, and water change were done every 48 h. Before starting the laboratory experiments, 20 males of the red swamp crayfish *P. clarkii* were weighed and measured. We ensured that the individuals selected for the experiment were of similar size. They were not fed for 48 h to empty their intestine tract.

A bioassay was conducted at the Regional Institute for Studies on Toxic Substances of the National University (IRET-UNA), specifically at the Ecotoxicological Studies Laboratory (ECOTOX), to observe the effect of MPs on bacterial communities. The feed without microplastics (powdered fish food with 38% protein) and with MPs made from recycled polyethylene terephthalate (30% rPET 500 µm -1 mm particle size+70% feed) was provided by IRET-UNA collaborators. 20 males of *P. clarkii* were cultured in 20 sterile glass flasks with a capacity of approximately 3000 ml. Each flask contained a volume of 2000 ml of UV-sterilized and filtered water as well as one of the red swamp crayfish. ten specimens were exposed to a daily dose of 0.69 g of feed with MPs and the other red swamp crayfish individuals were exposed to feed without MPs for 96 h. Before changing the water, the following parameters were measured daily with a portable multi-parameter: pH, temperature (°C), dissolved oxygen (mg/L), and conductivity (µS/cm), see this result in **Appendix 2**. Two specimens died before the end of the bioassay (one without MPs and one with MPs), so they were eliminated from the study. After completion of 96 h, 18 red swamp crayfish the individuals were measured and weighed before the dissection of the digestive system. Intestinal tracts dissections were performed with sterile surgical forceps and scissors. After dissection, the crayfish were measured and weighed before being divided into two portions, one for extraction of genomic DNA and the other for the extraction of MPs with 10% KOH digestion, following the methodology described below, see the this results in **Appendix 3 and 4**.

3.3 Microplastic extraction in the intestinal tract of the red swamp crayfish *P. clarkii*.

Each sample of the intestine tract was divided into two portions: one was used to determine the presence of plastids following protocols established by Dehaut et al. (2016). The other part of the sample was used for genomic DNA extraction.

For the determination of the presence of plastids, tissues were transferred and maintained for three weeks in an alkaline medium with 20ml of 10% m/v KOH (Sigma Aldrich, St. Louis, Missouri, USA) (Dehaut et al., 2016; Kühn et al., 2017). After digestion, the remaining solution was vacuum filtered through 0.45 μm μm microfiber filter papers (47 mm diameter; Sartorius Stedim Biotech, Goettingen, Germany). Subsequently, these filters were oven-dried for 48 h at 60 °C.

3.3.1 Observation and identification of MPs

A stereomicroscope (Leica Microsystem, Wetzlar, Germany) was used to visually inspect the MP particles collected from the intestinal tracts; MPs were photographed and subsequently analyzed for color and shape. According to their shape, the particles were classified into fibers (elongated) or fragments (angular and irregular pieces). In addition, to determine the presence of microplastics in the study samples, particles were separated by size, with a length of less than 2.5 mm, and stored in aluminum foil and glass Petri dishes.

A Hitachi High-Technologies TM3000 tabletop scanning electron microscope (SEM) with an accelerating voltage of 15 kV was used to observe the microstructure of both the intestinal tissue of *P. clarkii* and MPs. A cross-sections of the samples were placed on aluminum holders attached to a carbon sheet. Subsequently, we metalized the samples with gold-palladium to increase electrical conductivity using an EMS 150R ES ionic cover instrument (EMS, Hatfield, PA) (Samal, 2020; Chae et al., 2019).

We performed DSC using an SDT-Q600 thermal analyzer (TA Instruments, New Castle, DE) to detect the characteristic endothermic reactions of the mixture of pellets with pure MPs and MPs used for the bioassay. To perform the dynamic and isothermal analyses, 10 mg of pellet samples with pure MPs and rPET were used. All DSC experiments were performed in a nitrogen atmosphere with a determined purge flow rate of 100 mL/min. The dynamic DSC was heated from room temperature to 800 °C at a heating rate of 10 °C/min (flow rate: 30 cm3 /min). The temperature was monitored using a thermocouple inserted into the reactor to provide a graphical representation of the changes in sample mass as the temperature increased. Finally, the rate of change of sample mass as a function of temperature was plotted to simplify the weight reading as a function of the temperature thermogram peaks, which occur close together (Majewsky et al., 2016).

3.4 DNA extraction from bacterial communities present in intestinal tract the red swamp crayfish of *P. clarkii*.

The dorsal surface of each of the red swamp crayfish from the experiments was washed with sterile water and disinfected for five minutes with 70% v/v ethanol. The intestine was removed by applying mechanical force (Meziti et al., 2010; Zhang et al., 2016). Approximately 250 mg of the tissue were grounded and extracted using the protocol of the commercial DNeasy PowerSoil Pro Kit (QIAGEN, Germantown, MD, USA). Subsequently, DNA quality was measured on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) with an approximate yield of 1.7 to 1.8 purity at a wavelength ratio of 260:280 nm and a concentration of >70 ng/µl.

3.5 PCR-DGGE

For cost reasons, it should be mentioned that the composition of bacterial communities in the intestinal tract had to be analyzed using 12 similarly sized male individuals of *Procambarus clarkii*, divided into three conditions: direct samples from the environment, control Cachí reservoir (three specimens), as well as reared in laboratory conditions, exposed to feed with (five specimens) or without MPs (five specimens). The selection criteria for choosing *P. clarkii* and continuing with the study were based on the quality and high concentration of DNA obtained in the extraction.

3.5.1. PCR Amplification

PCR was performed in an *AB applied biosystems* thermocycler (Thermo Fisher Scientific, New York, United States) in 50 µl reaction volumes containing 0.3 mM of each primer, 0.2 mM of each dNTP (Thermo Fisher Scientific, New York, United States), and 0.03 U/µ Dream Taq DNA Polymerase (Thermo Fisher Scientific, New York, United States) as well as 1X of the Dream Taq Buffer, which contains KCl, $(NH_4)_2SO_4$ and 20 Mm $MgCl_2$ (Thermo Fisher Scientific, New York, United States). The primers used for the PCR were 341F-GC (with GC clamp) (CCTACGGGAGGCAGCAGCGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG) and 534R (ATTACCGCGGCTGCTGG) as a universal bacterial 16S rDNA reverse primer (Schäfer & Muyzer, 2001). The thermal cycling was as follows: initial denaturation at 94°C for 1 min, followed by 20 cycles of 94°C for desnaturation 45 min,

65°C for annealing45 s (temperature decreases by 0.5 °C with each new cycle, touchdown PCR-DGGE on the AB applied biosystems thermal cycler) and 72°C for extension 2 min. After that, another 20 cycles of denaturation at 94°C for 30 s were conducted, followed by annealing of 55°C for 30 s, extension at 72°C for 2 min, one cycle for a final extensión at 72°C for 10 min. The PCR products (expected sizes about 200 bp) were analyzed by running 5 µ aliquots of the reaction mixtures in 2% agarose (Merck, Darmstadt, Alemania) gels.

3.5.2. DGGE

The DGGE technique was performed using The Dcode Universal Mutation Detection System (BIO-RAD, California, United States). We used 8% polyacrylamide gels (ratio of acrylamide and bisacrylamide 37:1) with a gradient of 45% to 65% denaturants (100% denaturant was defined as 7 M urea plus 40% formamide). The gels were run at 60°C (65 V) for 15 h in a 1X TAE buffer (40mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) and visualized with 1X GelRed® Nucleic Acid Gel Stain (Biotium, California, United States). Then the gel staining was observed using a UVISAVE HD2 transilluminator (Thermo Fisher, Diepoldsau, Switzerland). The bands obtained by DGGE were analyzed by clustering using the Unweighted Pair-Group Method Using an Arithmetic Average (UPGMA) method to construct molecular phylogenetic trees using the program Minitab® 19.1.1 (Minitab, LLC, United States) (https://www.minitab.com/en-us/products/minitab/free-trial/). The generated dendrogram was elaborated using Pearson's correlation coefficient using the program Minitab® 19.1.1 (Minitab, LLC, United States).

3.6. Amplification and sequencing with Illumina on Miseq 300bp PE, 100K reads of the 16S DNA gene

The DNA samples from the intestine of *P. clarkii* were sent to Macrogen Corp (Beotkkot-ro Geumcheon-g, Seoul, Rep. South Korea) for 16S rRNA gene sequencing, targeting the V3-V4 region using the universal primers Bakt_341F: 5'- CCTACGGGGNGGCWGCAG-3' and Bakt_805R: 5'- GACTACHVGGTATCTAATCC -3', following the procedure of Klindworth et al. (2013). Sequencing was performed with the MiSeq sequencing platform (Illumina, San Diego, CA, USA) (https://dna.macrogen.com/). Libraries were prepared on

a paired-end Illumina platform using the Nextera XT Index Kit V2 to generate 300bp paired-end raw reads.

3.7. Bioinformatics and statistical analyses

3.7.1 Taxonomic assignation

The DADA2 version 1.18 was used to process the Illumina-sequenced paired-end fastq files and to generate a table of amplicon sequence variants (ASVs), which are higher-resolution analogs of the traditional OTUs (Callahan et al., 2016). Briefly, we removed primers, inspected the quality profiles of the reads, filtered and trimmed sequences with a quality score < 30, estimated error rates, modeled and corrected amplicon errors, and inferred the sequence variants (Xavier et al., 2021). Then, the forward and reverse reads were merged to obtain the full denoised sequences, removed chimeras, and constructed the ASV table. Taxonomic inferences were made to the ASVs with the function *assignTaxonomy*, of DADA2, which uses as input the set of sequences to be classified and a training set of reference sequences with known taxonomy, which in this case was the SILVA database version 138 (Quast et al., 2013). An additional taxonomic assignment of the ASVs was carried out using the tool IDTAXA of DECIPHER (Murali et al., 2018) with the same version of SILVA and using the RDP database version 18 (http://rdp.cme.msu.edu/). The consistency between the taxonomic assignments of the different programs and databases was verified followed by performing a manual curation. In case of discrepancies in taxonomic inferences, a comparison was made with BLAST tool of the NCBI Genbank. All sequences assigned to Eukaryota or Chloroplast were removed. The sequence data were deposited in the NCBI Sequence Read Archive under. This process generated 671.092 sequences from the 12 samples (mean length = 409 nt). The average number of sequences per sample was 55.924 (ranging from 49.089 to 64.961).

3.7.2 Statistical analyses

Statistical analyses and the visualization of results were performed with the R statistical program (R-Core-Team, 2019) and the Rstudio interface. Package Vegan v2.5-6 (Oksanen et al., 2020) was used to calculate alpha diversity estimators and non-metric multidimensional scaling analyses (NMDS). Data tables with the amplicon sequence variants (ASV) abundances were normalized into relative abundances and then converted

into a Bray–Curtis similarity matrix. To determine if there were significant differences between the bacterial community composition according to samples factors direct individuals from the environment, as well as reared in laboratory conditions, exposed to feed with or without MPs, we used the non-parametric multivariate analysis of variance (PERMANOVA) and pairwise PERMANOVA (adonis2 function with 999 permutations). In addition, we performed Indicator Species Analysis to identify ASVs associated with a specific treatment, using the Package Indicspecies version 1.7.9 with 999 permutations (De Cáceres et al., 2011).

For a better understanding of the methodology used, a summary has been provided (**Figure 3**).

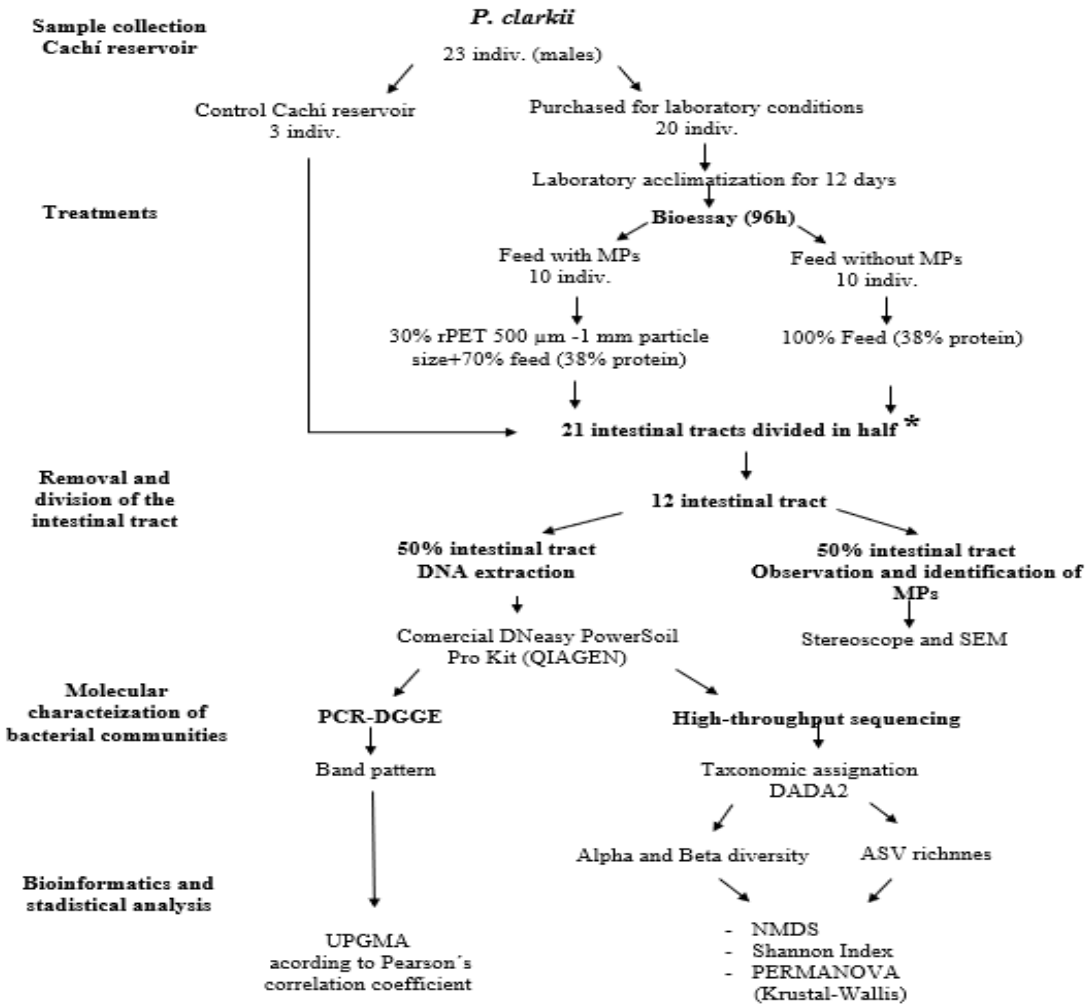

Figure 3. Summary of the methodology used.* One specimen died after 48 h and 72 h.

RESULTS

4.1. Sampling and bioassay with red swamp crayfish

The males of *P. clarkii* control collected from the Cachí reservoir had a smaller mean length of 4.36 ± 0.19 cm and mean weight of 19.56 ± 2.12 g compared to the 20 males used for laboratory conditions, these were of similar size with a mean initial length of 6.19 ± 0.60 cm and mean initial weight of 29.11 ± 6.69 g (Appendix 1, 2 and 3). In addition, during sampling, the water temperature was maintained at 21°C and a neutral pH.

Under laboratory conditions, males red swamp crayfish fed pellets without MPs showed a slight increase in final weight relative to *P. clarkii* fed pellets with MPs. In terms of size, growth was not affected in either treatment (**Table 1**).

Table 1. Measurements of individuals under laboratory conditions.

Description	Minimum	Maximum	Average	Standard deviation
Size (cm)				
Inicial treatment with pellets without MPs	5.61	7.45	6.28	0.62
Final treatment with pellets without MPs	5.81	7.56	6.39	0.61
Inicial treatment with pellets with MPs	5.31	7.30	6.09	0.60
Final treatment with pellets with MPs	5.86	7.14	6.58	0.46
Weight (g)				
Inicial treatment with pellets without MPs	18.87	39.16	28.59	6.69
Final treatment with pellets without MPs	22.82	39.33	30.27	6.10
Inicial treatment with pellets with MPs	25.21	34.84	29.62	3.09
Final treatment with pellets with MPs	19.07	39.33	29.99	5.39

The pattern of the averages of the water parameters measured under experimental conditions was very similar in the two treatments (in pellet-fed individuals without MPs and with MPs). In both cases, pH and DO decreased after 96h, while temperature and conductivity increased (**Table 2**). Despite all those slight changes among parameters, all water characteristics were suitable for red swamp growth.

Table 2. Measured water parameters of individuals under experimental conditions for 96 h.

Description	pH		Temperature (°C)		DO (mg/L)		Conductivity (µS/cm)	
Treatment with pellets without MPs	**Inicial**	**Final**	**Inicial**	**Final**	**Inicial**	**Final**	**Inicial**	**Final**
Minimum	7.17	6.30	20.70	20.90	7.96	6.51	253	275
Maximum	7.17	7.15	20.70	21.40	7.96	7.87	253	282
Average	7.17	6.58	20.70	21.18	7.96	7.43	253	279.50
Standard deviation	0.00	0.39	0.00	0.22	0.00	0.63	0.00	3.11
Treatment with pellets with MPs								
Minimum	7.17	6.28	20.70	21.20	7.96	7.38	253	270
Maximum	7.17	6.45	20.70	21.80	7.96	7.86	253	280
Average	7.17	6.35	20.70	21.52	7.96	7.59	253	275.60
Standard deviation	0.00	0.06	0.00	0.26	0.00	0.18	0.00	4.16

4.2 Observation of microplastics

To verify the presence of possible MPs in the samples, half of the intestinal tracts of the 12 *P. clarkii* males maintained in the laboratory were analyzed. In this study, the presence of MPs particles and fibers was observed in 60% of the intestinal tracts of the individuals in the treatment fed pellets with MPs (**Table 3, Figures 4** and **5**). No MPs residues were found in the control samples from the Cachí reservoir or in the intestinal tract of the individuals fed pellets without MPs.

Table 3. Microplastics (MPs) identified in the samples of intestinal tracts of *Procambarus clarkii* maintained in the laboratory and fed with pellets with MPs.

Sample	MPs types		Color
	Fibres	**No. of fragments**	
T1	1	6	Transparent
T3	2	1	Transparent
T7	2	0	Black

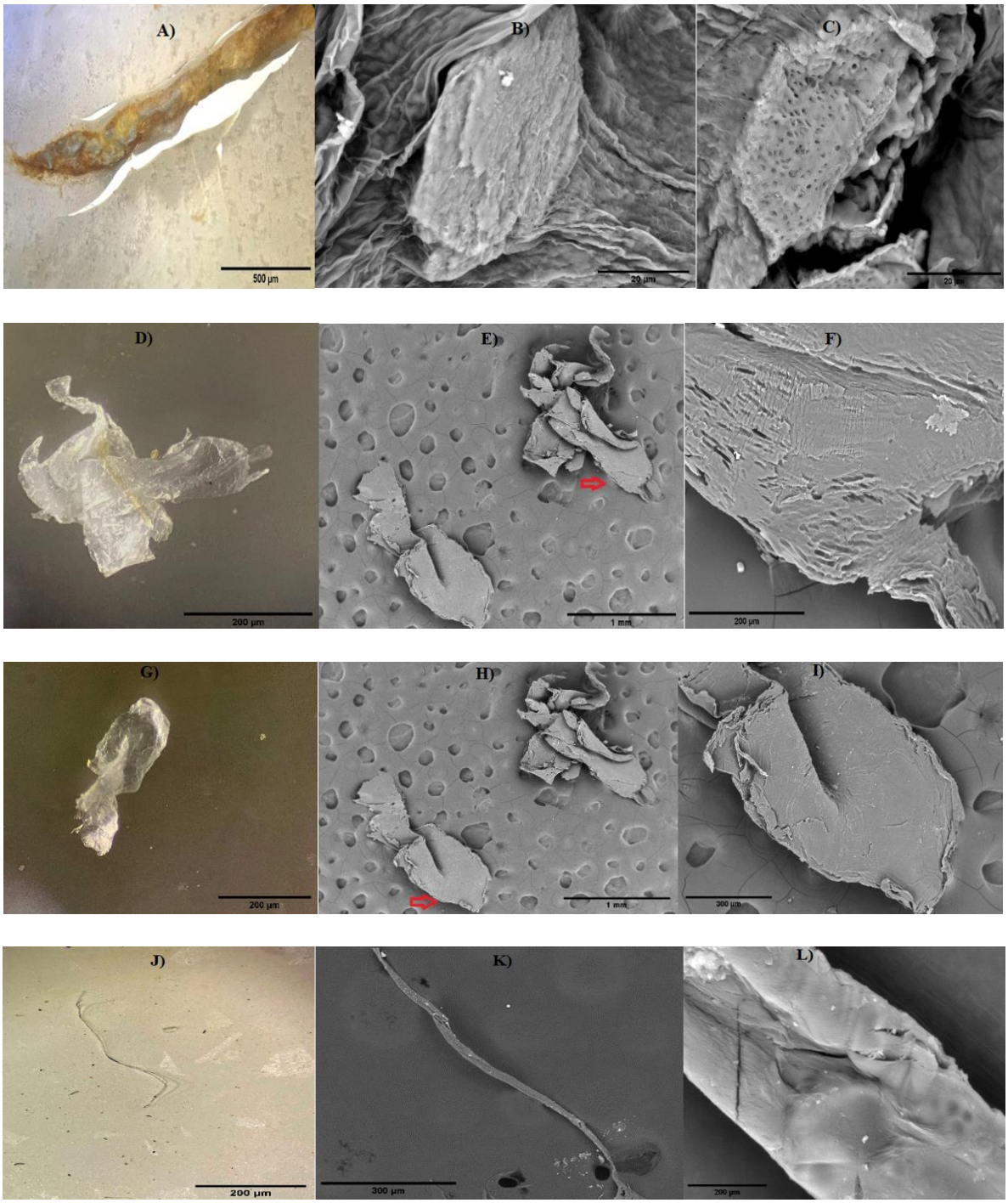

Figure 4. Morphotypes of microplastics were found in gut samples of *Procambarus clarkii* fed with MP (T1, T3 and T7). Microplastics were found in the intestinal tract (A, B and C), pellets or fragments (D, E, F, G, H and I), fibers (J, K and L).

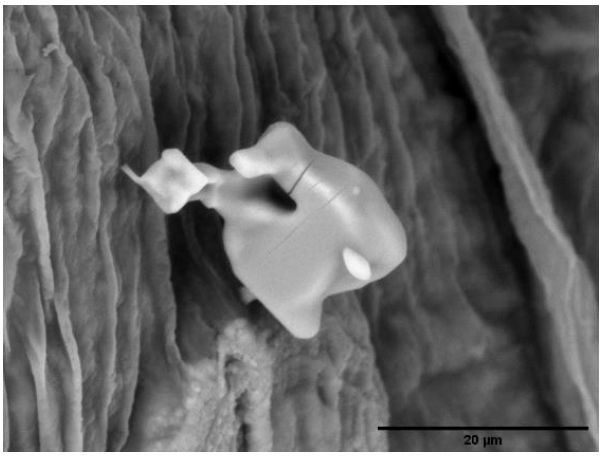

Figure 5. Microplastic fragments encountered in intestinal tissue of *Procambarus clarkii* in the samples used for the laboratory experiment (T1).

DSC curves of pure rPET (red line) and pellet mixture with MPs (black line) were measured to detect the characteristic endothermic reactions of the feed mixture with MPs and pure MPs used for the bioassay. The results of the DSC thermal analysis showed two marked peaks in the mixture of pellets with MPs and pure rPET, the first peak was at a melting temperature between 50 - 100 °C and the second peak at a melting temperature of 250 °C. (**Figure 6**).

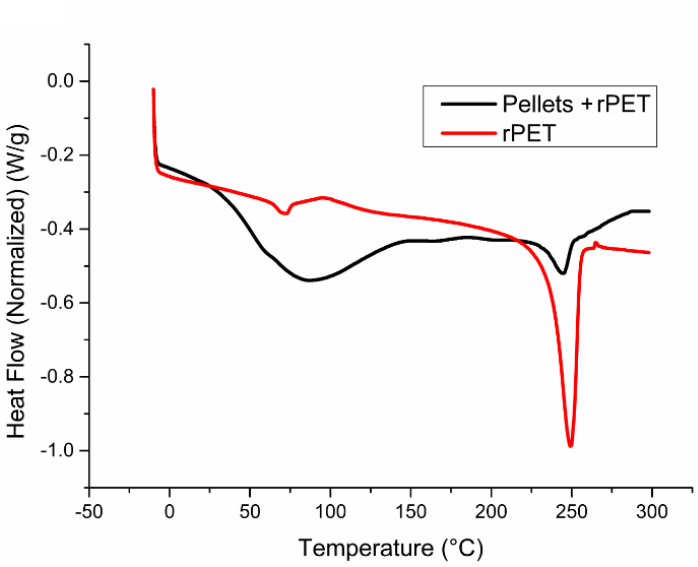

Figure 6. DSC signals from mixtures of pellets (30% protein) with MPs (30% rPET), black line, and only MPs (pure rPET), red line.

4.3. Molecular analyses and taxonomic assignation

4.3.1 PCR-DGGE

The PCR was completely satisfactory. DNA banding patterns amplified satisfactorily between 190 and 200 bp, and the water control was not contaminated (**Figure 7**).

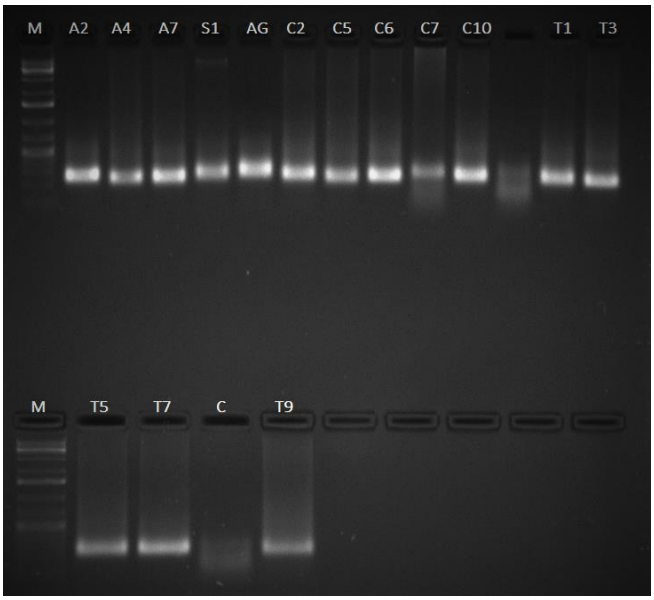

Figure 7. 16S rRNA PCR amplification of red swamp crayfish *Procambarus clarkii* intestinal tract. C= Control Cachí reservoir, S= Soil, AG= Water, C= fed pellets without MPs and T= fed pellets with MPs.

The results of PCR-DGGE were about 25 discernable bands for each sample. Some common bands for all samples collected (control samples from the Cachí reservoir and samples from the experimental conditions) and other unique bands only in the samples under experimental conditions. Also, the patterns of dominant 16S rDNA bands (most intense bands) and those that were well-separated bands (**Figure 8**). The samples fed pellets with MPs showed a decrease in the intensity of the common bands 5, 6, 9, 11, and 12 concerning the control samples from the Cachí reservoir and fed pellets without MPs. Four unique bands could be identified in individuals fed with pellets with MPs (18, 19, 20, and 21). In contrast, in samples from red swamp crayfish fed with pellets without MPs only one band was identified (24). In the control samples from the Cachí reservoir, no unique bands could be detected. On the other hand, the common bands of the samples obtained from the

individuals fed pellets with and without MPs were 10 and 22. The common bands of gut samples of specimens fed without MPs and those collected in Cachí reservoir were 8 and 25, and the only common was band 7 in red swamp crayfish fed with pellets with MPs and those from the Cachí reservoir (**Figure 8**).

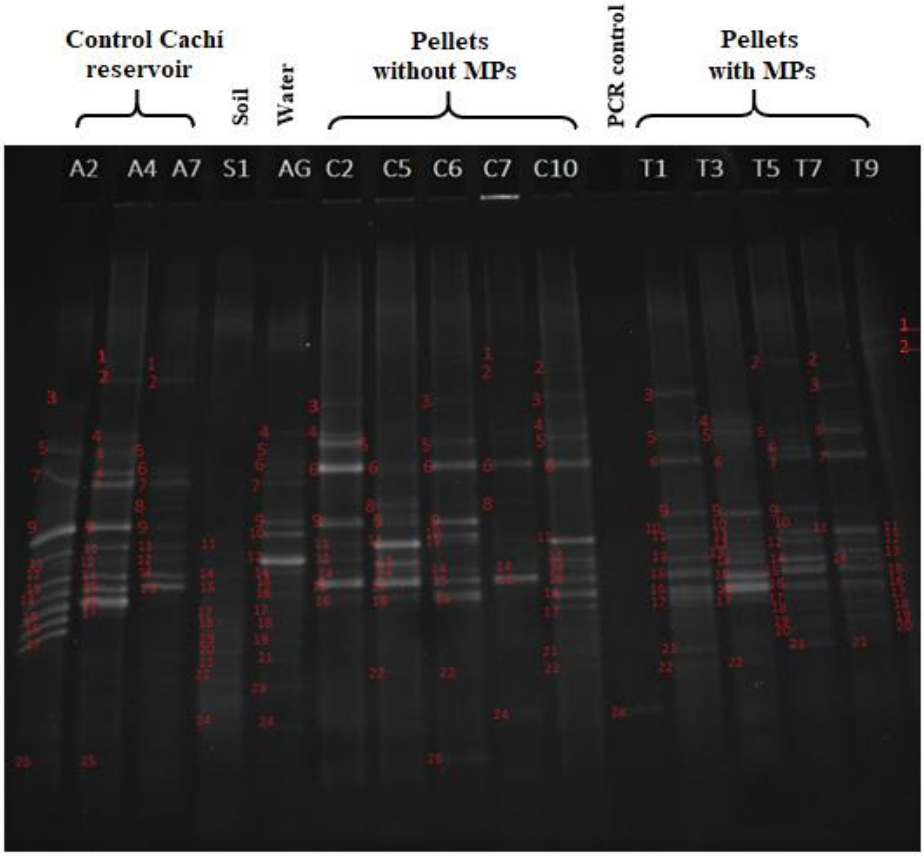

Figure 8. Results of PCR-DGGE from the red swamp crayfish *Procambarus clarkia* guts. A= Control Cachí reservoir, S= Soil, AG= Water, C= fed pellets without microplastic and T= fed pellets with microplastic. A total of 25 different bands were counted in all samples analyzed.

Table 4 shows a band profile matrix based on the presence and absence of important bands identified in the DGGE. Table 4 shows a band profile matrix based on the presence and absence of important bands identified in the DGGE. Bands 7, 8, 14, 15, 15, 18, 19, 20, 21, 23, 24 and 25 (highlighted in red, Table 4), show a possible change in the structure of the bacterial communities as they are absent in all the intestinal tracts of a sample and present

in another, depending on the type of sample. For example, band 7 was absent in red swamp crayfish fed with pellets without microplastics (C) but present in individuals fed with pellets with microplastics (T). The opposite case occurred with band 8 where intestinal tract content of specimens fed pellets without MPs (C) presented the band, which was absent in samples from red swamp crayfish fed pellets with MPs (T). Bands 18 to 21 were not found in individuals collected from Lake Chachí (A and C) but were encountered in individuals from treatment (T).

Table 4. Profile matrix of 16S RNA banding of 13 *Procambarus clarkii* intestinal tract samples for banding pattern analysis, based on the presence and absence of bands.

	Samples														
Bands	**A2**	**A4**	**A7**	**S1**	**AG**	**C2**	**C5**	**C6**	**C7**	**C10**	**T1**	**T3**	**T5**	**T7**	**T9**
1	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1
2	0	1	1	0	0	0	0	0	1	1	0	0	1	1	1
3	1	0	0	0	0	1	0	1	0	1	1	0	0	1	0
4	0	1	0	0	1	1	0	0	0	1	0	1	0	0	0
5	1	1	1	0	1	1	1	1	0	1	1	1	1	1	0
6	0	1	1	0	1	1	1	1	1	1	1	1	1	0	0
7	1	1	1	0	1	0	0	0	0	0	0	0	1	1	0
8	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0
9	1	1	1	0	1	1	1	1	0	0	1	1	1	0	0
10	0	0	0	0	1	0	0	1	0	0	1	1	1	0	0
11	1	1	1	1	0	1	1	1	0	1	1	1	1	1	0
12	1	1	1	0	1	1	0	0	0	0	0	1	1	1	0
13	1	0	0	0	0	0	1	0	0	1	1	1	0	1	0
14	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0
15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16	1	1	0	0	1	1	1	1	0	1	1	1	1	1	0
17	1	1	0	1	1	0	0	0	0	1	1	1	1	1	0
18	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0
19	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0
20	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1
21	0	0	0	1	1	0	0	0	0	1	1	0	1	1	1
22	0	0	0	1	0	0	1	0	0	1	1	1	0	0	0
23	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
24	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0
25	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0

* 1 means presence of the band and 0 is absence of the band. Red highlighting qualitatively shows a possible change in bacterial community structure when one or more of the bands are absent in one sample but present in another type of sample.

Figure 9 shows the banding patterns obtained by DGGE grouped by the unweighted pair group method using an arithmetic mean (UPGMA) for the construction of molecular phylogenetic trees. The dendrogram highlights the division of two groups correlated with each other according to the Pearson's Correlation Coefficient and reveals the presence of a distance between 5.23 and 7.84 in the samples of Group 1 (A2, C6, C2, T3, C5, C10, T1, A4, T5, T7, AG) and Group 2 (S1, C7 and T9). What separates group 1 from group 2 is the higher number of bands in Group 1, i.e., they are samples with a higher diversity of bacteria. In contrast, Group 2 contains samples with fewer bands, i.e. they are less genetically diverse.

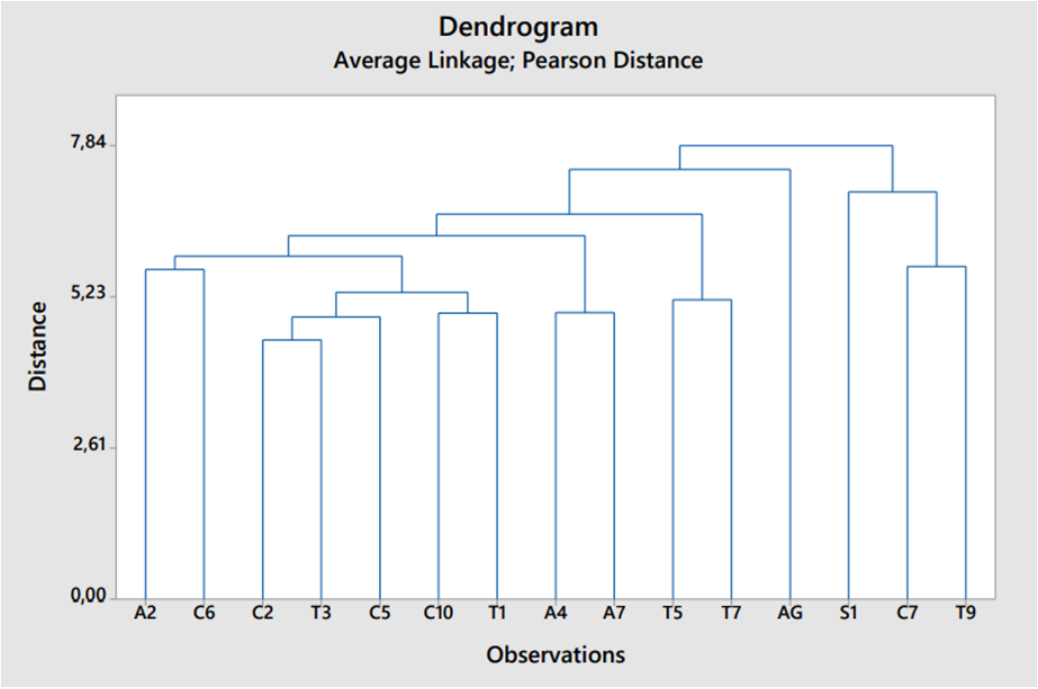

Figure 9. 16S rRNA dendrogram of the intestinal tracts of the red swamp crayfish *Procambarus clarkii* and water and soil samples from the collection site. * A= Control Cachí reservoir, S= Soil, AG= Water, C= fed pellets without microplastic and T= fed pellets with microplastic. Data generated from Pearson's correlation coefficient, performed with Minitab® 19.1.1 statistical software (Minitab, LLC, United States), see **Appendix 4**.

Several samples (C7, water and soil samples) did not show any significant banding patterns and therefore were not subjected to high-throughput sequencing analysis.

4.3.2 High-throughput sequencing of the 16S rRNA

The intestinal tract microbiota of the 12 samples of *Procambarus clarkii* was composed by 1.053 amplicon sequence variants (ASVs), according to the analysis of sequences of the V3-V4 region of the 16S rRNA gene. All the bacterial sequences were assigned to 19 phyla and 37 classes. Firmicutes was the most abundant group of phyla representing 45% of the sequences and 12% of the ASVs, whereas Proteobacteria represented 40 % of the sequences and 46% of the ASVs, Bacteroidota 13% of the sequences and 23% of the ASVs and Actinobacteria represented 0.7% of the sequences and 6.7% of the ASVs. Within Firmicutes, the most abundant genera were *Candidatus*_Bacilloplasma, *Candidatus*_Hepatoplasma, *Erysipelothrix*. The Proteobacteria group was dominated by the genera *Citrobacter*, *Hafnia* and *Shewanella*. *Bacteroides* were the most abundant genus within Bacteroidota and *Leucobacter* within Actinobacteroidota. No Archaea was detected in the intestinal tract of *P. clarkiii* (**Figure 10**).

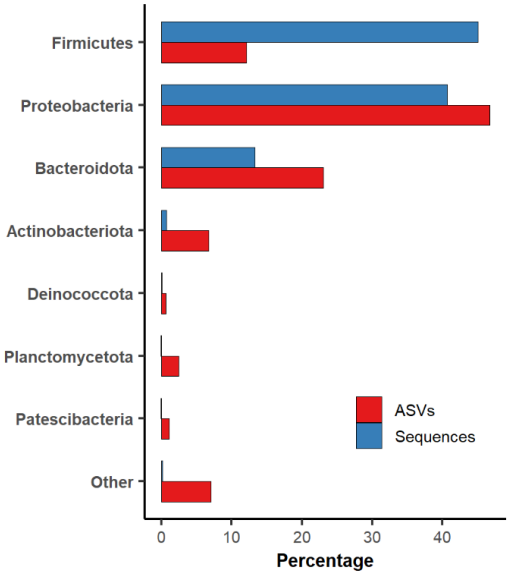

Figure 10. Percentages of microbial taxa detected in individuals of *Procambarus clarkii* in Cachí reservoir and those maintained under laboratory conditions and fed pellets with and without MPs.

Some differences between the treatments analyzed were encountered at the class level (**Figure 11**). Guts analyzed from individuals from Cachí reservoir presented generally a higher abundance of Gammaproteobacteria, Clostridia and Bacteroidia compared to individuals maintained in the laboratory. According to the indicator species analysis, particularly the genus *Tyzzerella* (Clostridia) represented an indicator species of the digestive tract of the guts from the control Cachí reservoir specimens. Samples from the red swamp crayfish of the laboratory experiment had a lower abundance of Clostridia. The intestinal tracts from crayfish fed pellets with MPs contained a higher proportion of Alphaproteobacteria and Actinobacteria compared to control Cachí reservoir and fed pellets without MPs samples According to the indicator species analysis, the predominant genera of this treatment were *Klebsiella*, *Acinetobacter*, *Hydromonas*, *Pseudomonas*, *Gemmobacter* and *Enterobacter*. Also, a significant decrease of bacteria belonging to the Bateroidia class was observed in gut samples of specimens fed pellets with MPs.

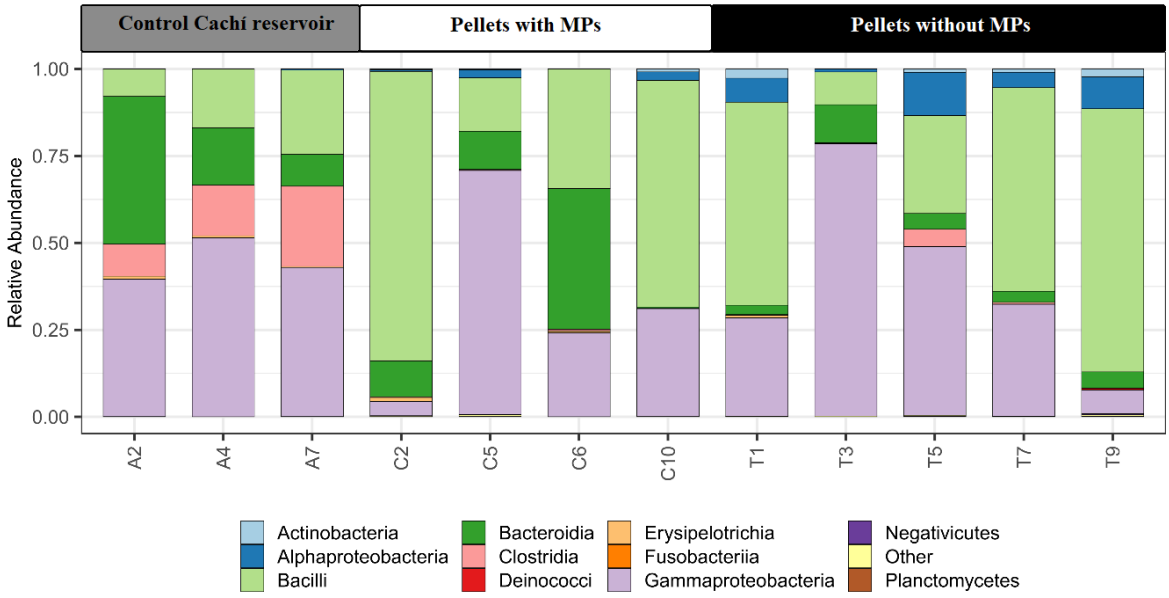

Figure 11. Relative abundance at the class level of predominant bacteria in intestinal tract samples of *Procambarus clarkii*. In Control Cachí reservoir samples (A2, A4 and A7), treatment samples fed pellets without microplastic (C2, C5, C6 and C10) and treatment samples fed pellets with MPs (T1, T3, T5, T7 and T9).

4.4. Bioinformatics and statistical analysis

The NMDS analysis of the bacterial community composition showed that samples from the different treatments were separate from each other (**Figure 12**). The structure of the bacterial communities of the intestinal tract from specimens taken directly from Cachí reservoir separated clearly from the communities of the intestinal tracts of the red swamp crayfish from a laboratory experiments. The samples from *P. clarkii* fed with pellets with and without MPs are generally separated except for some samples that overlap. However, according to the statistical analysis, these genetic differences between the structure of the communities per treatment were significant (Permanova, p=0.034).

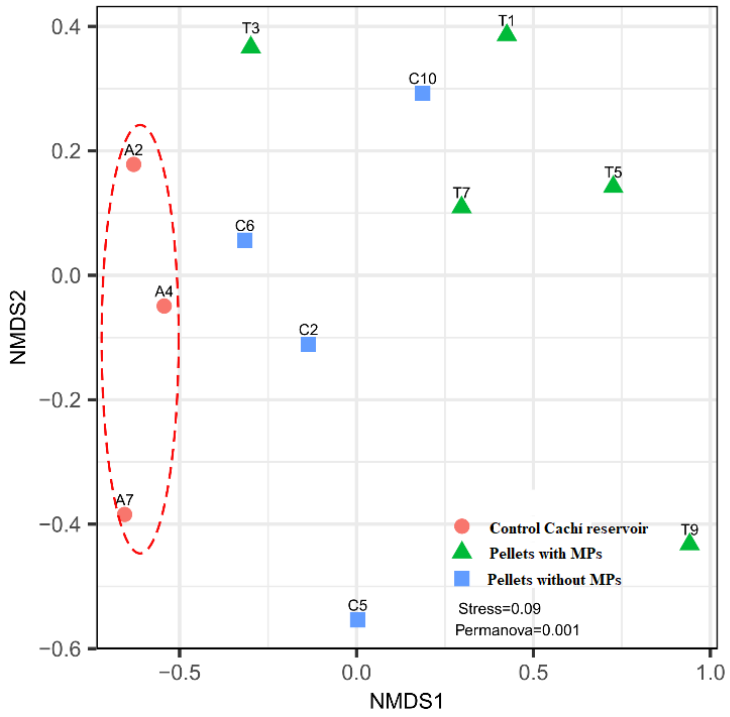

Figure 12. Non-metric multidimensional scale (NMDS) reflecting the degree of variation in intestinal bacterial communities from *Procambarus clarkii*. Different symbols denote especimens from control (Cachí reservoir) and laboratory treatments (fed pellets with MPs and without MPs).

When analyzing the alpha diversity estimators, individuals fed pellets with feed containing MPs presented an average richness of 238 ASVs in their microbiota compared to 156 ASVs in the individuals fed pellets without microplastics and 81 ASVs in the gut samples of *P. clark*ii from Cachí reservoir **(Figure 13A)**. These differences were statistically significant

according to the Kruskal-Wallis test (p = 0.028). The Shannon diversity index values of the gut samples of individuals fed pellets with MPs were slightly higher (average of 3.01) compared to 2.4 and 2.7 of the intestinal samples from red swamp crayfish fed pellets without MPs and specimens from the Cachí reservoir, respectively. These differences, however, were not significantly different (Kruskal-Wallis test: p> 0.5) **(Figure 13B),** see **Appendix 5**.

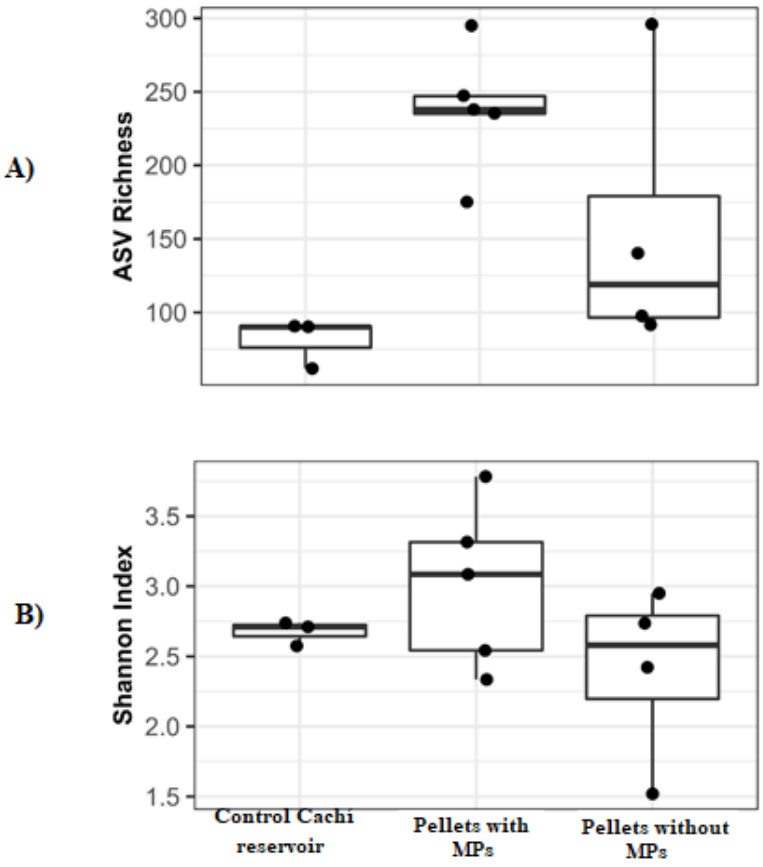

Figure 13. Alpha- and Beta-diversity of the intestinal microbial communities of *Procambarus clarkii* among specimens from Cachí reservoir and individuals maintained in the laboratory and fed pellets with and without MPs. (A) Amplicon sequence variants (ASV) for species richness; (B) Shannon index for species diversity.

DISCUSSION

The results of the present study revealed, for the first, time that ingestion of rPET caused alterations in the structure of bacterial communities in the intestinal tracts of *Procambarus clarkii* after 96h under experimental conditions. This finding is important since the gut microbiota promotes nutrient absorption and stimulates immune response as well as disease resistance in hosts, thus affecting the health status of individuals (Zhang et al., 2020). It is also recommended to increase the number of individuals to determine whether the results in environmental samples are consistent. Further experimental trials involving both sexes could also be conducted to analyze the behavior of males and females in the presence and absence of MPs.

5.1 Parameters and experimental trial in *P. clarkii*

Water parameters such as dissolved oxygen (DO), temperature, pH and conductivity are monitored periodically because they are important factors affecting aquaculture aquatic environments (Gutiérrez-Yurrita and Montes, 1998; Feng et al., 2021). In our results, these parameters remained constant and within the acceptable range for the growth of *P. clarkii*. This decapod is of global economic importance and a model animal, because it is considered a dominant species in natural environments due to its varied diet, rapid growth and strong adaptability (Gherardi, 2006; Shu, 2014), therefore slight changes in physicochemical parameters do not affect the growth, weight or feeding of the red swamp crayfish, as shown in this study.

The microplastic particles in the pellets used in the laboratory experiments of this study were fiber-shaped, an amorphous form, transparent colors, size less than 2 mm, and polymer of rPET. To test the purity of synthetic materials, examine thermal degradation or phase transitions in environmental and microplastic contaminated samples, thermal analysis such as Differential Scanning Calorimetry (DSC), where a sample is heated using a controlled temperature gradient with a defined heating rate, has been used (Dümichen et al., 2015). To verify that PET was being used in the pellet mixture with MPs, a DSC thermal analysis was performed (**Figure 6**). The DSC results showed very similar behavior between the pellets with MPs and rPET, both showing the presence of two marked peaks. The first marked peak in the pellets with MPs could be due to protein denaturation since proteins

decompose at a temperature between 50-60 °C (Ahmed et al., 2021) and the pellets contained 30% proteins in their composition. The pure polymer shows a characteristic endothermic peak that coincides with the maximum melting temperatures measured and reported in the literature for PET (Majewsky et al., 2016), indicating the possible presence of PET in the pellets.

A total of 60% of MPs treatment samples contained MPs, which may be attributed to the fact that only one-half of the gut was analyzed for the presence of MPs, while the other part was reserved for DNA extraction. Therefore, the part analyzed cannot be overestimated, and in fact, it is quite possible that the reserved part of the gut also contains MPs. These results were similar to those reported by Zhang et al. (2021a) and Capanni et al. (2021), who showed that MPs in the environment could accumulate in the internal tissues of crayfish. They concluded that this finding may pose a potential health risk not only to aquatic animals but also to humans consuming crayfish with MPs accumulated in different tissues.

Although in this study no MPs were found in the control samples from the Cachí reservoir, residual microplastics have been found in the stomach of *P. clarkii* distributed in different areas of Cartago, evidencing the consumption of MPs by this organism in its ecosystem (Vannucchi et al., *in litt*).

5.2 Molecular characterization of intestinal tract bacteria communities

5.2.1. PCR-DGGE

The combination of PCR-DGGE and statistical analysis has already proven useful in determining the effect on aquatic animal health when an external agent affects the structure of the gut microbiome (Steinum et al 2009; Essahale et al., 2010; Cheung et al., 2015). In addition, this was also demonstrated by PCR-DGGE patterns in this study, where it was observed that samples fed with food containing MPs had a higher number of different bands compared to samples fed without MPs and to environmental samples, suggesting that there is a higher richness and possible dysbiosis in the intestinal bacterial communities of *P. clarkii* fed with MPs (**Figure 7, 8 and 9**), these results were similar to our high-throughput sequencing results. These results also agree with those of the study by Liu et al. (2011), who compared the gut microbiota of Chinese shrimp *Fenneropenaeus chinensis*

that we used, were able to demonstrate that both techniques were able to reflect the gut microbial diversity of Chinese shrimp. However, the clone library analysis was more representative of the community. Although similar results were obtained in our research, more quantitative analyses, such as FISH or real-time PCR, should be performed in the future to compare much better with 16S RNA sequencing.

5.2.2 High-throughput sequencing of the 16S rRNA

High-throughput sequencing analysis of 16S rRNA from the 12 intestinal tracts showed that Firmicutes, Proteobacteria, Bacteroidota, and Actinobacteria were the predominant phyla in the intestinal microbiota of *P. clarkii* (**Figure 10**). This finding is in agreement with similar results of other studies on *P. clarkii* (Guo et al., 2019; Zhang et al., 2020; Zhang et al., 2021a; Wu et al., 2021). These studies concluded that these four phyla prevailed in the intestine of aquatic decapods and indicated that they played principal roles in the intestinal functions of digestion, absorption, and immunity of *P. clarki.* In the present study, the Firmicutes were the most abundant group, which include many beneficial bacteria, especially butyrate-producing bacteria (Li et al., 2020; Duan et al., 2021).

At the class level, the gut content of the *P. clarkii* specimens collected in the Cachí reservoir was predominated by Gammaproteobacteria, Bacteroidia, and Clostridia. Most of the dominant microorganisms in environments such as dams and wetlands are related to C and N transformation (Di Pippo et al., 2020). In wetlands the denitrification process depends on the presence of heterotrophic and autotrophic bacteria, generally, the abundance of Gammaproteobacteria and Bacteroides is high in this type of environment (Zhang et al., 2021b). Bacteroides can produce carbohydrate metabolism-related enzymes, which promote food digestion (Karlsson et. al., 2011). Species of the class Clostridia constitute a substantial part of the total bacteria of the intestinal microbiota and their participation is crucial in the modulation of physiological, metabolic, and immunological processes in the gut by interacting with the other resident microbial populations (Setälä et al., 2014). Moreover, they provide specific and essential functions modulating normal intestinal homeostasis and may play an important role in dysbiosis when gastrointestinal disorders occur (Lopetuso et al., 2013). Likewise, its occurrence is associated with the presence of certain stress levels (Li et al., 2020). *Procambarus clarkii* is tolerant to highly contaminated freshwater habitats (Shui, 2020) such as the Cachí reservoir (R. Guillén, comm. pers.).

Therefore, the predominance of species of *Clostridia* in *P. clarkii* from the Cachí reservoir may be related also to the stress caused by contamination.

The most abundant genera of Firmicutes in our research were *Candidatus_Bacilloplasma, Candidatus_Hepatoplasma, Erysipelothrix* and *Candidatus spp*. These genera constituted the core microbiota of another crayfish species, *Cherax cainii,* and are known to play an important role in digestion and immunity (Foysal et al., 2021). Wai et al. (2019) reported that isopods with a *Candidatus_Hepatoplasma* based intestinal tract showed higher survival rates when food was deficient, relative to the other genera of symbiont bacteria they studied. *Candidatus_Bacilloplasma* was another abundant genus found in this study, these results agree with Shui et al., (2020), that although there are not many studies on its effect on the gut of *P. clarkii,* the literature suggests that it is a highly diversified population and played essential roles in the gut microbiota of crustaceans, as it has been grouped into several genetic subgroups of bacteria obtained from various marine animals, mainly in crustaceans such as *Litopenaeus vannamei, Eriocheir sinensis, Pelteobagrus fulvidraco* and *Nephrops norvegicus.* On the other hand, *Erysipelothrix* is a facultatively anaerobic, gram-positive, rod-shaped bacterium and some species have been elucidated as pathogenic (Opriessnig et al, 2020), some have been isolated from erysipelas of many other animals sporadically, and also from healthy cattle, sheep, chickens, surface fish and crustaceans (Imada, 2013). Therefore, the abundance of this genus in this study suggests the presence of possible pathogens in the intestinal tract of *P. clarkii* from this investigation.

Among Proteobacteria, the most abundant genera were *Citrobacter*, *Hafnia,* and *Shewanella*, coinciding with the results of Wu et al. (2021), who studied the gut microbiota of *P. clarkii*. These genera include opportunistic pathogenic bacteria in some freshwater decapods (Zhang et al., 2019; Wu et al. 2021; Zhang et al., 2021a). In addition, the abundance of these genera may be attributed to the presence of foreign compounds in the diet or external environment that may cause an alteration in the stability of the gut microbiota of *P. clarkii* and thus may facilitate or hinder infection by pathogenic bacteria that influence the overall health of *P. clarkii* (Zhang et al., 2019; Foysal et al., 2021).

Bacteroides was the most abundant genus within Bacteroidota encountered in the guts of the red swamp crayfish and *Leucobacter* within Actinobacteroidota (Figure 11).

These results coincided with those reported by Wu et al., (2021); they concluded that the abundance a predominance of *Leucobacter* might be an indicator of possible dysbiosis because these authors detected more opportunistic pathogens, which might be a specific microbial indication of the presence of diseases in *P. clarkii*.

Our analyses of the gut content revealed the presence of the genus *Tyzzerella* exclusively in the specimens collected from the Cachí reservoir while it was absent in the individuals reared under laboratory conditions. This finding is in agreement with the results of Shui et al. (2020), who reported this genus as abundant in *P. clarkii* specimens reared in rice fields. They concluded that the presence of this genus is due to their capacity to metabolize plant polysaccharides and mentioned also that *P. clarkii* as a predominantly carnivorous freshwater decapod has a broad feeding spectrum, including herbivorous items. This result is evidence of the ecological plasticity of this red swamp crayfish to adapt to different environments by changing its diet.

The intestinal tract of the specimens cultivated in the laboratory and fed with pellets that contained MPs showed a high proportion of Alphaproteobacteria and Actinobacteria (**Figure 10**). The Alphaproteobacteria can have genetic activity and are capable of degrade aromatic hydrocarbons (Ghosal et al., 2016) and the Actinobacteria produced various metabolites with antimicrobial activity and degrade recalcitrant compounds (Wang et al. 2021). The Actinobacteria are usually the most abundant bacteria in *P. clarkii* intestine (Zhang et al., 2021a). These bacteria participate in the decomposition of organic matter present in soil and sediments, and studies in mammals have shown a positive correlation with fiber intake (Shui et al., 2020). Moreover, they play a crucial role in maintaining intestinal homeostasis (Glenny et al., 2016; Wang et al., 2021). Therefore, when these classes predominate in the gut content, it is probable that the normal intestinal functions of *P. clarkii* are affected by external or toxic compounds such as diclofenac (Zhang et al., 2021a) or by the presence of MPs as shown by our results (Figure.10). Similar results were obtained Wang et al., (2021) studying the white-leg shrimp *Litopenaeus vannamei*.

The most abundant genera in the samples fed with pellets containing MPs were *Klebsiella, Acinetobacter, Hydromonas, Pseudomonas, Gemmobacter,* and *Enterobacter*. *Klebsiella, Pseudomon*as and *Enterobacter* have been reported as part of the intestinal microbiota of healthy people (Vogel et al., 2012; Hadder et al., 2018). These genera rarely

cause infections, but when the patient is immunosuppressed, these bacteria can cause infections of the urinary or respiratory tract;, they are also of clinical importance due to their resistance to antibiotics (Hadder et al., 2018). Therefore, these microorganisms could be considered potential pathogens, which may affect the health of *P. clarkii*. Moreover, species of *Klebsiella, Acinetobacter, Pseudomonas,* and *Enterobacter* (KAPE) are of particular concern due to their ongoing acquisition of genetic traits, which are associated with the intraoperative spread for their antibiotic resistance, virulence, treatment failure, and increased patient morbidity and mortality (Boucher et al., 2009). Therefore, our results might suggest a possible transfer of human pathogenic bacteria to animals through MPs. Eckert et al. (2018) showed that microplastic particles indeed promote the persistence of typical indicators of microbial anthropogenic pollution in natural waters. However, additional research is needed to demonstrate this type of microbial exchange.

Species of *Hydromonas,* also found in our gut samples, comprise bacteria that occur in both environmental and clinical settings, including opportunistic human and plant pathogens as well as potential biodegrades of recalcitrant xenobiotics (Vaz-Moreira et al., 2015). The genus *Gemmobacter* has been reported as a facultative anaerobe and may play an important role in the degradation of some refractory contaminants such as cephalexin and toluene (Liu et al., 2020). The abundance of this genus in our samples may be due to presence of some kind of pollutants in its environment, as in our study the presence of MPs. This conclusion, however, requires further studies.

The decrease of Bacteroides levels observed in treatments T1, T5, T7, and T9 (**Figure 11**) implies that MPs exposure may affect metabolite production of putative beneficial bacteria (Duan et al., 2021; Karlsson et al., 2011). This decrease in abundance suggests that MPs exposure could affect the degradation of lignin and environmental pollutants in the gut of *P. clarkii* as shown in the white-leg shrimp *Litopenaeus vannamei* (see Duan et al., 2021). Our results evidence a possible change in the gut bacterial community structure of *P. clarkii* in individuals fed pellets with MPs. Clostridia is another class of bacteria that suffered a considerable decrease in the gut samples of specimens fed with pellets containing MPs. Generally, these microorganisms play a crucial role in the development of the immune system, modulating immune tolerance, and helping to prevent the establishment of potentially harmful and pathogenic organisms (Kelly et al., 2005;

Lopetuso et al., 2013). Furthermore, this decrease could suggest a decrease in resistance to pathogen colonization and thus cause a possible deficiency or disease in *P. clarkii* organisms fed pellets with MPs and a possible dysbiosis effect in the presence of MPs. (Kelly et al., 2005).

2.3 Bioinformatics and statistical analysis

The NMDS of the Bray-Curtis distance confirmed the presence of three differential gut microbiota patterns among the samples analyzed of specimens from Cachí reservoir and those reared in the laboratory (**Figure 12**). This finding might be compared to the study of Duan et al. (2021), who concluded that the gut of the marine penaeid shrimp *L. vannamei* contained a microbial community whose structure and stability was closely related to the health of the analyzed shrimps. When the host becomes sick, dysbiosis occurs in the gut microbiota and with it various physiological processes of the host, such as digestion, immunity and metabolism (Wu et al., 2021). These changes in the gut microbiota are closely related to the severity of the disease (Xiong et al., 2015). In the present case, the presence of MPs may have affected the gut microbiota of *P. clarkii*, considering that the exposure to MPs causes intestinal mucosal damage, microbiota imbalance and inflammation in aquatic animals (Lu et al., 2019).

Individuals fed pellets with MPs presented higher bacterial richness and diversity in their gut microbiota compared to individuals fed pellets without MPs and control samples from the Cachí reservoir (**Figure 13A**). This could be because the host immune system acts as a selective pressure on the symbiotic microbiota and coevolution that normally restricts the excessive growth of internal bacteria and prevents external bacteria from entering the host's internal organs, thus maintaining a balance between the symbiotic microorganisms and the host that promotes continuous coadaptation (Hooper et al., 2012; Groussin et al., 2020; Wu et al., 2021). Diseases and other stress factors damage the host immune system, resulting in reduced selective pressure on symbiotic bacteria and reduced restriction on external bacteria, leading to dysbiosis of the microbiota induced by overgrowth of opportunistic bacterial pathogens and possible loss of bacterial richness (Clerissi et al., 2020; Tan et al., 2020; Wu et al., 2021).

The microbial community of gut samples from reed swamp crayfish fed with pellets containing MPs was significantly different compared to those fed pellets without MPs (**Figure 13B**). This large number of bacterial communities found in samples with MPs could be becuase during transport by water flow, MPs can be colonized by planktonic microorganisms capable of adhering to plastic surfaces with subsequent formation of biofilms, called plastisphere (Eckert et al., 2018; Di Pippo et al., 2020; Amaral-Zettler et al., 2021). Also, plastisphere microorganisms play an important role in the transport of potentially invasive and pathogenic species (Koelmans et al., 2019), as demonstrated in the present study, which detected bacterial genera that have only been reported from human intestinal microbiota and have been considered as human pathogens (Boucher et al., 2009; Eckert et al. 2018). Our results were similar to those of Wu et al. (2021), who found significant differences in the structure of the digestive system microbiome between different health states, including healthy, anorexic, dying and whitish muscular states of *P. clarkii*, also concluded that the dysbiosis of the diagnostic microbiological system associated with disease outbreaks could be potential indicators to assess the health status of the crayfish. Therefore, our results could also suggest a possible exchange of microorganisms internal to the intestinal tract of *P. clarkii* and MPs and a possible dysbiosis.

CONCLUSIONS

Despite the difficulties encountered in this study, we were able to characterize the structure of the bacterial communities of 12 intestinal tracts of the crayfish *P. clarkii* under control conditions in the Cachí reservoir and under experimental conditions, fed pellets with and without MPs, using molecular tools such as PCR-DGGE and 16S rRNA high-throughput sequencing. In addition, this study reports for the first time a possible dysbiosis effect in the intestinal tract of samples fed pellets with MPs concerning samples fed pellets without MPs and control samples from the Cachí reservoir. It was statistically proven that samples in the presence of MPs presented a higher species richness than samples in the absence of MPs. Therefore, further studies are recommended to examine the toxic effects of MPs on the gut of *P. clarkii* by evaluating histological changes, molecular regulations and microbial responses of the hemolymph in the presence of MPs.

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APPENDICES

Appendix 1. Measurements of the 10 direct environmental samples.

Sample	Sex	size (cm)	Total weight (g)	Observations
A1	**F**	3,87	10,56	with eggs
A2*	**M**	4,22	17,108	-
A3	**F**	3,4	7,769	-
A4*	**M**	4,22	20,75	-
A5	**F**	4,65	23,111	with eggs
A6	**F**	4,87	24,521	with eggs
A7*	**M**	4,55	20,825	without cheliped
A8	**F**	3,31	35,28	with a possible nematode
A9	**F**	4,61	21,25	-
A10	**F**	3,89	7,882	-

*Samples chosen to continue with the study. F= Female and M= Male

Appendix 2. Measurements of the 10 samples reared under laboratory conditions, after exposure to acclimatization conditions 12 days.

Sample	Sex	size (cm)	Total weight (g)
C1	**M**	56,09	23,687
C2*	**M**	70,91	35,441
C3	**M**	61,53	24,586
C4	**M**	68,03	36,87
C5*	**M**	59,54	18,874
C6*	**M**	74,53	39,16
C7*	**M**	57,19	22,602
C8	**M**	62,52	29,025
C9	**M**	58,83	26,495
C10*	**M**	58,82	29,187
T1*	**M**	60,21	25,303
T2	**M**	53,12	25,208
T3*	**M**	56,33	27,694

T4	**M**	62,52	31,056
T5*	**M**	63,22	29,972
T6	**M**	63,14	31,782
T7*	**M**	65,71	32,577
T8	**M**	58,21	29,173
T9*	**M**	72,99	34,844
T10	**M**	53,82	28,587

*Samples chosen to continue with the study. F= Female and M= Male

Appendix 3. Measurements of the 10 samples reared under laboratory conditions, before exposed to feed with or without MPs for 96h.

Sample	Sex	size (cm)	Total weight (g)	Observations
C1	**M**	59,55	24,382	-
C2*	**M**	68,28	36,359	-
C3	**M**	-	-	Died 48 h
C4	**M**	67,93	38,062	Intestinal tract full of food
C5*	**M**	58,8	26,308	
C6*	**M**	75,58	39,326	Intestinal tract full of food
C7*	**M**	59,71	22,816	Intestinal tract full of food
C8	**M**	69,25	28,916	
C9	**M**	59,91	27,508	Intestinal tract full of food
C10*	**M**	66,28	28,723	Intestinal tract full of food
T1*	**M**	58,64	19,071	Intestinal tract full of food
T2	**M**	58,71	26,219	
T3*	**M**	66,15	28,889	Intestinal tract full of possible MPs
T4	**M**	-	-	Died 72h
T5*	**M**	68,05	31,209	-
T6	**M**	70,16	32,352	-
T7*	**M**	66,71	32,96	Intestinal tract full of possible MPs
T8	**M**	68,73	31,39	
T9*	**M**	71,42	36,431	Intestinal tract full

				of possible MPs
T10	**M**	63,66	28,954	Intestinal tract full of possible MPs

*Samples chosen to continue with the study. F= Female and M= Male

Appendix 4. Cluster analysis of observations

Cluster Analysis of Observations: 1; 2; 3; 4; 5; 6; 7; 8; 9; 10; 11; 12; 13; 14; 16; 17; 18; 19; 20; 21; 22; 23; 24; 25

Standardized Variables, Pearson Distance, Average Linkage

Amalgamation Steps

Step	Number of clusters	Similarity level	Distance level	Clusters joined		New cluster	Number of obs. in new cluster
1	14	51,5247	4,47958	6	12	6	2
2	13	47,2332	4,87616	6	7	6	3
3	12	46,5168	4,94236	10	11	10	2
4	11	46,4059	4,95260	2	3	2	2
5	10	43,9959	5,17531	13	14	13	2
6	9	42,5992	5,30439	6	10	6	5
7	8	38,3406	5,69792	1	8	1	2
8	7	37,7991	5,74796	9	15	9	2
9	6	35,8569	5,92743	1	6	1	7
10	5	32,0118	6,28276	1	2	1	9
11	4	28,0256	6,65112	1	13	1	11
12	3	23,8369	7,03819	4	9	4	3
13	2	19,6305	7,42691	1	5	1	12
14	1	15,1772	7,83844	1	4	1	15

Final Partition

	Number of observations	Within cluster sum of squares	Average distance from centroid	Maximum distance from centroid
Cluster1	15	336	4,67841	5,91182

Appendice 5. Bioinformatic and statistical analysis

Permanova

Permutation test for adonis under reduced model
Terms added sequentially (first to last)
Permutation: free
Number of permutations: 1000

adonis2(formula = per ~ Treatment, data = env, permutations = 1000)
Df SumOfSqs R2 F Pr(>F)

Treatment 2 0.75282 0.26462 1.6193 0.03497 *
Residual   9 2.09213 0.73538
Total   11 2.84494 1.00000

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Kruskal-Wallis Riqueza
> kruskal.test(Richness ~ Treatment, data = PerFungi)

	Kruskal-Wallis rank sum test

data: Richness by Treatment
Kruskal-Wallis chi-squared = 7.0962, df = 2, p-value = 0.02878

Kruskal-Wallis Shannon

> kruskal.test(Shannon ~ Treatment, data = PerFungi)

	Kruskal-Wallis rank sum test

data: Shannon by Treatment
Kruskal-Wallis chi-squared = 1.3487, df = 2, p-value = 0.5095

Indicator Species Analysis
RESULTADOS INDICATOR SPECIES

Resultado. Especies indicadoras with MP: Kebsiella, Acinetobacter, Hydromonas, Pseudomonas, Gemmobacter, Enterobacter.
Especie indicadora environmental Tizzerella (Clostridia)

 Multilevel pattern analysis

 Association function: IndVal.g
 Significance level (alpha): 0.05

 Total number of species: 1053
 Selected number of species: 35
 Number of species associated to 1 group: 29
 Number of species associated to 2 groups: 6

 List of species associated to each combination:

```
 Group environmental  #sps.  12
        stat p.value
ASV46   1.000   0.006 **
ASV76   1.000   0.006 **
ASV85   1.000   0.006 **
ASV87   1.000   0.006 **
ASV94   1.000   0.006 **
ASV97   1.000   0.006 **
ASV10   1.000   0.004 **
ASV68   0.953   0.009 **
ASV95   0.916   0.011 *
ASV102  0.816   0.033 *
ASV180  0.816   0.033 *
ASV1538 0.816   0.042 *

 Group with MP  #sps.  16
       stat p.value
ASV25  0.993   0.002 **
ASV73  0.992   0.004 **
ASV119 0.986   0.005 **
ASV65  0.984   0.005 **
ASV44  0.984   0.032 *
ASV37  0.984   0.004 **
ASV80  0.982   0.002 **
ASV51  0.975   0.006 **
ASV91  0.950   0.004 **
ASV41  0.888   0.041 *
ASV43  0.886   0.040 *
ASV114 0.880   0.047 *
ASV344 0.877   0.042 *
ASV123 0.860   0.039 *
ASV99  0.855   0.043 *
ASV70  0.854   0.039 *

 Group without MP  #sps.  1
       stat p.value
ASV131 0.866   0.017 *


---
    Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```