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Thriving Digestive Systems: How Isopods Are Conquering Extreme Geothermal Environments

VOLUME 1

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RESUMO

Os isópodes terrestres são invertebrados bem-adaptados a praticamente todo o tipo de ambientes, encontrando-se mesmo em locais com atividade vulcânica como os campos geotermais, onde fatores de stress como temperaturas elevadas, hipoxia, pH ácido e existência de metais pesados desencorajam a presença de organismos. Enquanto decompositores, os isópodes desempenham um papel essencial nas funções do ecossistema do solo, fragmentando a vegetação em decomposição e aumentando a área de superfície para a colonização e atividade microbiana. Estes processos resultam na mineralização de nutrientes essenciais para o ciclo de nutrientes do solo e para o crescimento das plantas. Este estudo teve como objetivo avaliar as adaptações no sistema digestivo dos isópodes devido à atividade vulcânica secundária de um campo geotérmico. Foram realizados procedimentos histológicos para determinar diferenças morfológicas no intestino e hepatopâncreas dos isópodes. Assim como análises metagenómicas no gene 16S rRNA para avaliar a diversidade microbiana no trato gastrointestinal da espécie *Porcellionides sexfasciatus*, encontrada no campo geotermal das Furnas (Ilha de São Miguel, Portugal). Os resultados mostraram alterações morfológicas na espessura do epitélio na câmara anterior do intestino do isópode. As células epiteliais da câmara anterior dos isópodes das Furnas não são tão altas como dos isópodes do local de referência (Fajã de Baixo). A microbiota do intestino e do hepatopâncreas diferiu significativamente em termos de riqueza e composição. As Furnas apresentaram níveis mais elevados de diversidade no intestino quando comparadas com os locais de referência (Fajã de Baixo, Ribeirinha e Lagoa). Em contrapartida, a riqueza bacteriana do hepatopâncreas foi inferior à dos locais de referência. *Bacillus*, *Candidatus Hepatoplasma*, *Staphylococcus*, *Rickettsiella*, *Vibrio* e *Candidatus Rhabdochlamydia* foram os principais géneros encontrados em ambos os órgãos. *Sphingobium* e *Ralstonia* estavam entre os 10 géneros mais encontrados no hepatopâncreas, enquanto *Streptomyces*, *Actinomadura*, *Streptococcus* e *Paenibacillus* estavam entre os 10 géneros mais encontrados no intestino. Porém, não se verificaram diferenças na microbiota das Furnas para os locais de referência. Este trabalho pretende adicionar informação quanto às adaptações morfológicas e das comunidades bacterianas no sistema digestivo de *P. sexfasciatus*, fornecendo assim informações valiosas sobre a biologia dos isópodes terrestres e a sua capacidade de se adaptarem e colonizarem estes ambientes extremos.

ABSTRACT

Terrestrial isopods are widely distributed and can be found in reducing environments such as volcanic geothermal fields, where stress factors such as elevated temperature, hypoxia, acidic pH and heavy metals discourage biota from living in these environments. As decomposers, woodlice play essential roles in the soil ecosystem's functions by fragmenting leaf litter and increasing the surface area for microbial processing. These processes result in nutrient mineralization, which is essential for further nutrient cycling in soil and plant growth. This study aimed to evaluate adaptations within isopods' digestive system due to the secondary volcanic activity of a geothermal field. Histological techniques were performed to determine the cellular structure and overall tissue morphology in the hindgut and hepatopancreas of the species *Porcellionides sexfasciatus*, found in the geothermal field of Furnas (São Miguel Island, Portugal). Using a metabarcoding approach of the 16S rRNA gene, the microbial composition associated with the isopods' digestive system was also analyzed. Results showed morphological differences in epithelium height in the anterior chamber of the isopods' hindgut. The epithelial cells of the anterior chamber of the isopods living in the geothermal site (Furnas) are smaller when compared to isopods from the reference site (Fajã de Baixo). Gut and hepatopancreas microbiota significantly differed in diversity and composition across the analyzed sites. Furnas showed the highest levels of diversity in the hindgut compared to the reference sites (Fajã de Baixo, Ribeirinha and Lagoa). In contrast, the hepatopancreas' bacterial diversity was lower than the reference. Bacillus, Candidatus Hepatoplasma, Staphylococcus, Rickettsiella, Vibrio and Candidatus Rhabdochlamydia were the main genera found in Top 10 of both organs. Sphingobium and Ralstonia were in the top 10 genera found in hepatopancreas, while Streptomyces, Actinomadura, Streptococcus and Paenibacillus were in the top 10 genera for hindgut. The microbiota core was similar between reference sites and Furnas. This study sheds light on the adaptations in the digestive system of *P. sexfasciatus* reflected by altered tissue and different bacterial communities in the animals inhabiting a geothermal field, thus providing valuable insight into the biology of terrestrial isopods and their capability to adapt and colonize these extreme environments.

Table of Contents

List of Figures

List of Tables

1.Introduction

Volcanic Islands as Natural Laboratories for Evolution

Island`s ecosystems are excellent for evolutionary studies due to their discrete geographical nature and diversity of species and habitats (Amaral et al., 2006; Cunha et al., 2011; Rodrigues et al., 2008). New species on islands occur predominantly because of a reliable time frame for geological events superimposed on molecular and/or morphological variation patterns, allowing inferences about allopatric differentiation (Emerson, 2002). Adaptation is a term that describes an evolutionary process in which an organism's characteristics change over time in response to selective pressures from the environment.

The Azores archipelago near the Mid-Atlantic Ridge is composed of nine volcanic islands divided into three groups (Western, Central and Eastern) near the Mid-Atlantic Ridge, spanning an area of 530 (latitudes 36°55'–39°43' N) by 320 km (longitudes 25°–31°17'). São Miguel stands out among these volcanic islands as the largest. It is home to three active volcanoes: Sete Cidades, Fogo, and Furnas (Figure 1.1). Furnas is particularly noteworthy for its intense secondary volcanic activity, emitting approximately 1000 tons of CO_2 per day. Within Furnas, the valley boasts an array of mesmerizing hydrothermal manifestations, making it a focal point of scientific interest (Viveiros et al., 2010).

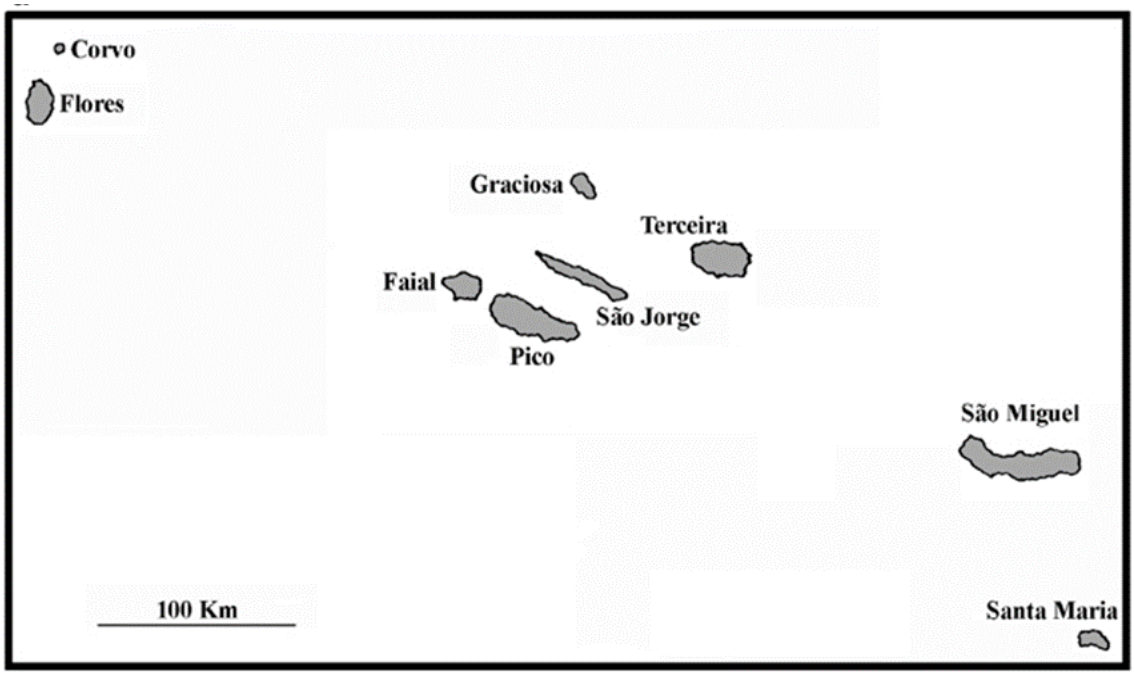


Figure 1. 1 The Azores archipelago (Forjaz 1997).

Terrestrial geothermal fields are reducing environments characterized by elevated soil, water and atmospheric elemental composition, constant diffuse degassing and high temperatures. Volcanic gases typically comprise water vapor, carbon dioxide, sulfur dioxide, hydrogen sulfide and hydrogen chloride with lesser amounts of hydrogen fluoride. Rocks and volatiles of volcanic origin are enriched with metals/metalloids, such as aluminum (Al), arsenic (As), copper (Cu), mercury (Hg), plum (Pb) and zinc (Zn), with diffusion of acidic volcanic gases through the rocks mobilizing the metals in associated soils and water bodies (Cunha et al., 2011). These environments provide an unusual opportunity to research the dynamics driving population structure and genetic diversity in these communities since specialization and adaption are required for population persistence in the habitat (Cunha et al., 2014). For species persistence on this environment, it is require the mechanisms to face this changes by increasing its physiological tolerance, plasticity (acclimation capacity) and evolutionary adaptation (adaptive capacity) of physiological tolerance (Morley et al., 2019). The environmental stress experienced by parents lead to plasticity (acclimation capacity) in offspring by changing its phenotype through non-genetic or epigenetic processes this is the key for evolutionary adaptations though multigeneral life cycles (Lee et al., 2020).

Ecosystems showing extreme environmental conditions support original biological communities, especially bacteria, bacteria-like organisms, protozoans, viruses, and fungi. However, species from distinct invertebrate groups (i.e., ice worms, rotifers, nematodes, oligochaetes, and crustaceans) (Dattagupta et al., 2009; Flot et al., 2014; Howarth & Moldovan, 2018; Sarbu et al., 1994) have adapted physiologically, metabolically, and behavioral to live in environments which are unlivable for most life forms. The geothermal field of Furnas has species from distinct invertebrate groups (e.g. rotifers, nematodes, oligochaetes) (Zaldibar et al., 2006; Esin et al., 2020) that have shown acclimatization strategies. For example, *Amynthas gracilis,* an earthworm inhabiting volcanic soils, demonstrated physiological adaptations to Furnas`s hypoxic, elevated temperatures and metallic soils. The continuous exposure showed that the epidermis thickness decreased by approximately 30% - a vital aspect necessary to reduce the oxygen (O_2) diffusion distance across its dermal respiratory surface. Since high temperatures increase their metabolic oxygen (O_2) and due to a hypoxic native soil, the O_2 presence in the environment is low. If on one hand, the reduced thickness improves the trans-epidermal diffusion efficiency of the earthworm, on the other hand, it increases metals exposure. As a result, *A. gracilis* adapted by having significantly higher goblet cell counts (expressed as numbers per unit epidermis area) responsible for mucus production with mucopolysaccharides to trap heavy metals on the dermal surface (Cunha et al., 2011). In the case of *Pseudaletia uninpuncta* larvae, the hydrothermal vents and soil degassing affected the digestive epithelium by increasing its thickness and apoptotic nuclei. High levels of Cu explained these responses found in larvae from the active volcanic environment that induced the midgut cells' apoptotic activity (Rodrigues et al., 2008). Digestive epithelium and apoptosis under metallic exposure, as a result of secondary volcanic activity, have been reported in other invertebrates such as earthworms (Amaral et al., 2006) , snails (Zaldibar et al., 2006) or limpets (Cunha et al., 2008).

While in physiology the focus is in how organisms evolve acclimatize strategies to confront the unique challenges presented by stressful environments (Morgan et al.,

2007). In ecotoxicology, researchers aim to establish clear and easily understandable connections between environmental exposure and observed phenotypic responses, much like traditional toxicology (Dahms et al., 2011). However, a significant complication in field-based ecotoxicology arises from the possibility that locally adapted populations may exhibit responses (e.g., accumulation of metals in their tissues or variations in functional parameters) that deviate substantially from predictions based on combining environmental measurements with response patterns observed in laboratory tests on naïve organisms lacking multi-generational exposure to the stressor(s) (Morgan et al., 2007, Zhang et al., 2022). Ecotox tests can provide information on temperature and metals influence on invertebrates physiology and acclimatization to geothermal field environments, for instance temperature can effect metals toxicity to soil organisms (Morgado et al., 2022).

In certain terrestrial areas on São Miguel, one of the nine islands forming the Azores archipelago, the presence of ongoing volcanic activity offers unique "field laboratories" to explore the capacity of soil macroinvertebrates to thrive in natural habitats continuously subjected to potentially toxic chemical inputs and bioavailability (Cunha et al., 2011; Ferreira et al., 2021; Taheri et al., 2018). Also, the organisms living in extreme environments are good models to understand the evolutionary processes and historical factors involved in speciation and adaptation to severe environmental conditions (Pop et al., 2023).

The Ecological Role of Terrestrial Isopods

Woodlice (also called sow bugs, pill bugs and slaters) are terrestrial isopods (class of Crustacea, sub-order Isopoda) of the family Oniscidea. It is estimated that the total number is larger than the 3,710 valid isopod species belonging to 527 genera and 37 families (Sfenthourakis & Taiti, 2015).

Isopods are cosmopolitan, having an ecological distribution that ranges from supralittoral zones far into forests, rangelands, agroecosystems, mountains and subterranean caves, absent only in polar regions and at very high altitudes (RAND, 1986). The transition of isopods from water to land possibly happened during the Paleozoic (Sfenthourakis & Hornung, 2018). For the evolutional process, *Oniscideans* had to develop morphological, ecological and behavioral solutions to the terrestrial ways of reproduction, respiration, excretion and protection against desiccation. In the study of Hornung (2011), it is mentioned that the main adaptations to the terrestrial environment are the size reduction, water-resistant cuticle, diverse surface morphology (e-g. increase in the number of surface structures), pleopodal lungs, a water-conducting system and closed brood pouch. All these aspects were fundamental for isopods to become abundant. The size reduction was essential to escape from predators, and the existence of a cuticle allow them to become relatively permeable to water and protected them from desiccation. The ornamentation variety of cuticular surfaces has sensory structures that mediate sensory information for behavioral responses. Additionally, a water-conducting system was an adaptive solution for thermoregulation, excretion,

osmo- and ion-regulation under terrestrial conditions, and a marsupium brood care evolved for the mechanical protection of eggs and developing embryos, providing parental care, protection and nutrition for the developing progeny (Sfenthourakis & Hornung, 2018).

Terrestrial isopods are described as being small to medium-sized organisms (1.2–30 mm), with several life forms: (1) runners, which have large eyes, long legs, and sometimes mimetic colors; (2) rollers, capable of rolling into a tight ball when disturbed; (3) clingers, less mobile than the preceding forms and with depressed margins of the body which they press down on flat surfaces, and (4) creepers, which have developed tergal ribs and live in narrow interstices, caves, etc. (Paoletti & Hassall, 1999). Their diet consists mostly of decaying organic materials such as leaf litter, decayed wood, fungi and bacterial mats. Isopods can also predate insect larvae and be cannibalistic (Paoletti & Hassall, 1999).

These invertebrates play an important role in the functioning of soil ecosystems and restoration (Van Gestel et al., 2018). They act on the decomposition process by fragmenting leaf litter, which increases the surface area available for microbial processing. This subsequent microbial processing of organic matter is a critical component of soil development, resulting in the mineralization of nutrients necessary for further nutrient cycling in soil for plant growth. Even the isopods` faecal pellets contribute to soil structure. These are hot spots for microbial activity when fresh (Snyder & Hendrix, 2008).

These organisms have the capability to inhabit heavy metal-contaminated areas, since they ingest metals from the environment and achieve tolerance/resistance by accumulative immobilization. Isopods have different strategies to handle with the exposure of different metals, they can modify their behavior and assimilated these in specific cell types and intra-cellular compartments (Pope et al., 2023). When long-term exposed with nickel, isopods mortality increased, and oxidative stress was induce. However, the organism had the effective strategies to handle with nickel exposure by modifying their behavior, accumulating energy reserves, and demonstrating enzymatic processes to counteract oxidative stress induced. In the end, they had a positive response to nickels exposure. It is important to note that the effects of metals on isopods is not linear, while some metals induce hermetic responses at low concentrations, nickel's toxicity appeared to increase in higher concentrations (N. G. C. Ferreira et al., 2015; N. G. C. Ferreira et al., 2016).

Besides its profound ecological impact, isopods research has been neglected, reflecting the relatively little funding for research (Cameron et al., 2019). Vittori & Dominko (2022) points out that during the last two decades, the rate of publishing paper (isopod related) has increased, being the major research topics on: (1) ecotoxicology, (2) systematics, (3) microbe-host interactions, (4) ecology, with a significant focus on population ecology and life histories, and (5) physiology. However, they should be given more opportunities for invertebrates' research, such as in terrestrial isopods, once these marginal studies can lead to flourishing applicative research on future issues.

The Digestive System of Terrestrial Isopods

The woodlice role in soil ecosystem functioning is highly related to its diet and digestive process that indirectly promotes microbial distribution in terrestrial ecosystems by its ingestion and dispersal by faeces (Kostanjšek et al., 2006).

The terrestrial isopods` digestive system comprises the foregut, hindgut and hepatopancreas (midgut glands). The foregut and hindgut have ectodermal origin lined with cuticle and form the entire alimentary canal. In comparison, the midgut (hepatopancreas) has an endodermal origin (Hames & Hopkin, 1989).

The foregut is composed of an oesophagus and a proventriculus (proventriculus), this last structure sorts, masticates and filters food material. The hindgut is divided into an anterior chamber, papillate region and short rectum (separated from the rest of the structure by a muscular sphincter). In the anterior chamber, a folded dorsal hindgut wall forms a dorsal longitudinal fold termed typhlosole, with two typhlosole channels. In the papillate region, the dome-shaped basal parts of epithelial cells bulge into the haemocoel between the longitudinal and circular muscles surrounding the hindgut (Figure 1.2) (Bogataj et al., 2018).

The midgut consists of two pairs of bilobed structures connected to the digestive system tract at the junction of the foregut and hindgut by a common hepatopancreatic duct. It has two cell types: the larger *B*-cells and the smaller *S*-cells. The *B*-cells are responsible for the absorption of food and secretion of digestive enzymes. This cells usually contain lipid droplets, glycogen and metals stored in granules. *S*-cells are storage cells of contaminants, such as metals, calcium and urate (Figure 1.2) (Lešer et al., 2008; Odendaal & Reinecke, 2007).

According to Hames & Hopkin (1989), the anterior chamber is where digestion of food mixed with digestive enzymes takes place, while the papillate region is involved in the compaction of faecal pellets and water removal. The hepatopancreas is an important organ in the digestive system. It secreted digestive enzymes to help digest food, and products of digestion are transported into the organ for absorption. In the end, the hepatopancreas has intestinal, hepatic and pancreatic functions.

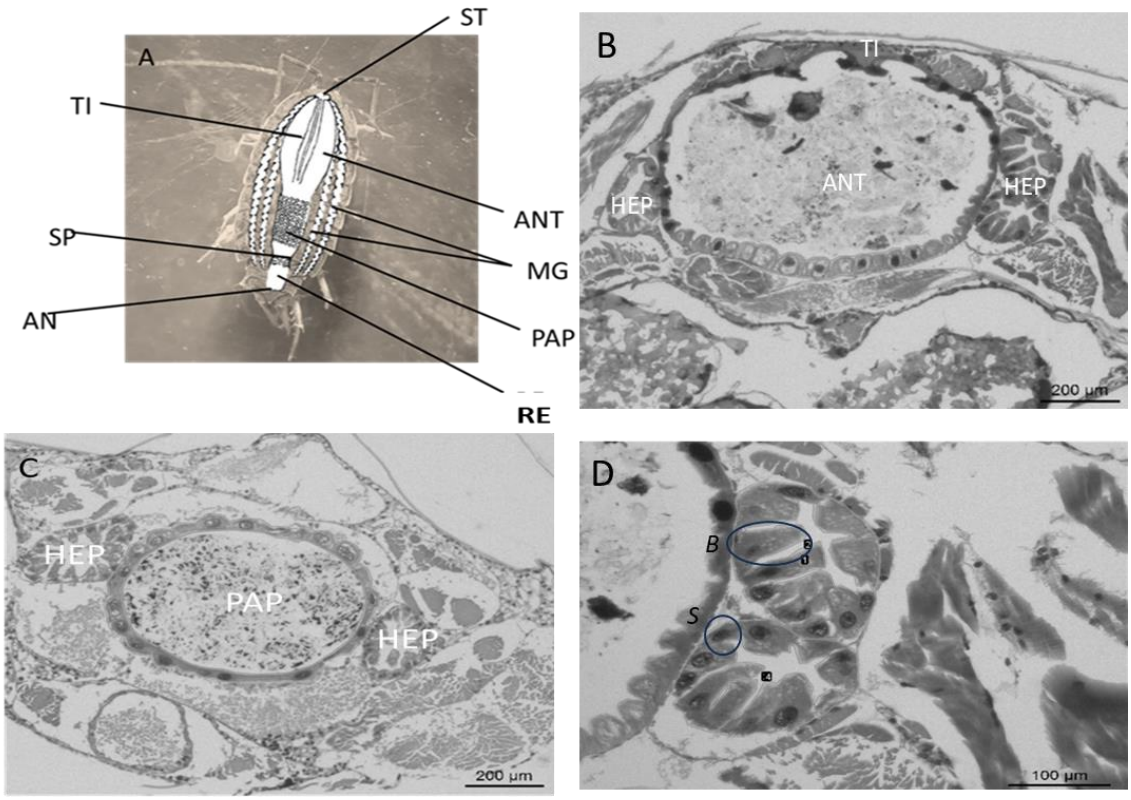


Figure 1.2 A) Schematic diagram of the digestive system of the isopod *Porcellionides sexfasciatus*. ST- Stomach; HEP- Hepatopancreas (Midgut glands); ANT- Anterior Region of the hindgut (Anterior Chamber); TI- Typhlosole; PAP- Papillate Region of the hindgut; SP- Sphincter; RE- Rectum; AN- Anus. **B)** Histological structure of anterior chamber in cross section (hindgut). TI- Typhlosole; HEP- Hepatopancreas (Midgut glands); ANT- Anterior Region of the hindgut (Anterior Chamber). **C)** Histological structure of the papillate region in cross section (hindgut). MG- Midgut glands (hepatopancreas); PAP- Papillate Region of the hindgut. **D)** Cross section of a hepatopancreas caeca. B- *B*-cell, S- *S*-cell.

Physicochemical conditions in the gut of woodlice, such as pH levels vary from acidic in the midgut glands and anterior chamber to slightly acidic in the papillate region, determine enzymatic conditions for litter degradation in the anterior chamber and bacterial proliferation in the papillate region (Zimmer, 2002). Microbial activity can change physiological gut conditions such as pH level, redox potential and oxygen concentration differing with microbial density (Zimmer & Brune, 2005). According to Zimmer & Brune (2005), pH homeostasis is maintained by a counter-balancing respiratory CO_2 production, resulting from microorganisms' proliferation in the posterior hindgut and formation of acidic fermentation products by anaerobic bacteria. Most of aerobic bacteria are placed in the anterior chamber, and because of their activity micro-oxic or even anoxic conditions can be create at the center of the anterior

chamber. Since the gut periphery experiences a continuous influx of oxygen from the surrounding hemolymph via the gut epithelium, oxygen-dependent processes are thought to occur in this region. In the anoxic center of the hindgut fermentation prevails, affecting the pH regime of the central hindgut lumen.

Some enzymes used to degrade lignocellulose, are assumed to be ingested with food or to be produced by endosymbiotic bacteria in the midgut glands. Isopods, as macro decomposers degrade dead plant biomass, mostly composed of lignocellulose that can be divided into cellulose, hemicellulose and lignin. The degradation of these polymers requires the synergistic action of multiple Carbohydrate-Active enzymes (CAZymes) (Bredon et al., 2018). According to Bredon et al. (2020), host and microbiota complement CAZomes (CAZyme repertoire) to achieve effective lignocellulose deconstruction and a great diversity of CAZymes of potential isopods halobionts were found on its digestive tract.

The Isopods` Digestive System Microbiome

Symbiosis is described as an association (temporal or spatial) between individuals who do not belong to the same species. Symbiotic relations between host and symbiont can be negative (parasitism), neutral (commensalism) or positive to the host (mutualism) (De Bary, 1879). In animals, host-symbiont associations include transient gut passengers that pass through the digestive tract with ingested food or permanent gut residents. Symbiosis plays an important role in adaptation by allowing the host to explore new ecological niches (Horváthová et al., 2015). Isopods are constantly in contact with environmental microbes directly from soil, by ingesting organic matter rich in bacteria/fungi or via feeding on their congeners´ faeces (coprophagy). These symbiotic microbes located in the digestive tissues may benefit the host fitness by providing them enzymes, vitamins and fatty acids. The acquisition of these symbiont bacteria might have been beneficial for isopods to colonize land (Bouchon et al., 2016).

The symbiont-host relationship has three transmission mechanisms: the vertically transmitted symbionts are passed from mother to progeny by infecting milk glands, eggs or embryos; the horizontally transmitted symbionts may be acquired through feeding on infected corpses, faeces, exuviae or through physical contact between conspecifics and the environmentally transmitted, symbionts are acquired through contact with or uptake of any matter (Bright & Bulgheresi, 2010; Gros et al., 2012; Kikuchi et al., 2007). Results from Bouchon et al. (2016) suggested that isopods gut symbionts are mediated through horizontal and environmental transfer. The vertical transfer may exist during the phase when the marsupium is filled with liquid.

Molecular studies of isopod microbiota, based on 16S rRNA gene clone libraries, have been focused on digestive tissues due to the interest in bacterial components potentially involved in the nutritional process (Bouchon et al., 2016). Research hinted already a high bacterial diversity in isopod tissues such as the gut, midgut caeca, gonads, nerve cord and hemolymph (Braquart-Varnier et al., 2015). It is registered a total of 208 bacterial genera from 31 classes, 19 phyla and additional 28 genera were detected in

faeces and absent on tissues. Alphaproteobacteria, Gammaproteobacteria, and Mollicutes together represented 92% of all sequences due to highly predominant taxa (i.e., Wolbachia, Halomonas, Pseudomonas, Rickettsiella, Shewanella, and Hepatoplasma). In the other hand Firmicutes, Actinobacteria, and Bacteroidetes represented 2%, 1.8%, and 1% of all sequences. Actinobacteria was the second most represented phylum in terms of taxonomic richness, with 54 observed genera, after Proteobacteria with 88 genera (Dittmer et al., 2016).

Most of the bacterial community is likely acquired from environmental sources from ingested bacteria in different food sources. About 70% of all taxa of isopods' microbiome were also detected in faeces and/or soil. The ingestion of microbes in leaf litter improves survival and growth rates of juveniles and adults and female reproductive success (Oliveira et al., 2021). The role of bacteria causing this positive effect on woodlice is still unprecise, but there are some hypotheses to explain it: (I) Environmental microbes may be themselves a digestible supplementary food source (Ihnen & Zimmer, 2008); (II) microbial enzymes are used for digestion (Muscorum et al., 1975) and (III) microbial colonization is an indication of high-quality food (Zimmer et al., 2003).

There are few studies on how environmental stress factors influence the microbiota. As an example, the earthworm *Lumbricus rubellus* obtained from a mine site contaminated with arsenic, the earworms` host community did not demonstrate significantly changes in diversity and richness (Pass et al., 2015). However, arsenic exposure changes organisms' guts microbiota composition due to different levels of arsenic uptake in body tissues (H. T. Wang et al., 2021). In Lafuente et al. (2023) the aquatic isopod *Asellus aquaticus,* nonfiltered wastewater affected the host performance and gut microbiota composition but not in diversity.

Study Subject and Experimental Scope

The aim of this study was to determine morphological and microbial alterations in the digestive system, specifically in the hindgut and hepatopancreas of animals chronically exposed to secondary volcanism activity, using the terrestrial isopod species *Porcellionides sexfasciatus* as a model.

P. sexfasciatus (Oniscidea; Family: *Porcellionidae;* Genus: *Porcellionides)* has a characteristic grey bloom, the body has a stepped outline and it is capable of very rapid movement, the antennal flagella comprise two segments and two pairs of pleopodal lungs (Gregory et al., n.d.-a). It exhibits sexual dimorphism, being the male more heavily pigmented than females, their length ranges between 6-12mm depending on the collection site. Males have a characteristic exopod 1, it is elongated into a gradually tapering point and lacking a cleft on the outer margin of the pseudotracheae. This species is distributed across the western Mediterranean region, which includes Spain, France, Italy, Malta, Morocco, Algeria, Tunisia, some Atlantic islands (Azores) and England. This specimen can be found under loose stones in dry situations, with no other species of woodlouse present (Gregory et al., 2021). For this study *P. sexfasciatus* were selected for being the most abundant isopod species close to the geothermal field of Furnas and dwell in other parts of the island without geothermal activity, making the comparisons possible between sites.

In this study, two different digestive system organs were targeted, namely the hindgut and the hepatopancreas. Histological techniques and 16S rRNA gene metabarcoding analyses were made to understand the influences of the geothermal field on morphology and in the microbiome of these organs, following two different scientific hypotheses:

(1) Animals chronically exposed to the volcanic environment will show morphological alterations in their digestive system when compared to conspecifics living in reference sites, confirming previous studies in other taxa (Amaral et al., 2006; Cunha et al., 2011; Navarro-Sempere et al., 2021; Rodrigues et al., 2008; Zaldibar et al., 2006). Previous studies have shown that invertebrates dwelling in volcanic soils change their morphology as a response to these environments. In the case of *P. sexfasciatus* the adaptative abilities will also depend on the capability of hindgut and hepatopancreas cells to deal with the environmental stress.

(2) Dissimilarities in microbiome composition and richness of gut and hepatopancreas between animals from Furnas compared to animals from reference sites. Since the geothermal stress factors are going to affect the composition of microbial communities, some bacterial communities can have a reduction in the number of members, while sensitive species can get extinct and microorganisms tolerant to high temperatures, acidic and rich metallic soils can become dominant, common in geothermal areas. In the isopods' digestive system can be found symbiont bacteria or passenger microorganisms that come from the ingestion of organic matter and return to the environment at the end of the digestion process in faeces (Dittmer et al., 2016; Zimmer, 2002). If bacterial communities in soil are adapted to environmental pressures, microbes in the isopods digestive system must come from the same bacterial communities.

The extreme environmental conditions presented by this geothermal field create a challenging habitat for various organisms, including isopods. Understanding how isopods thrive and adapt to such harsh conditions within the Azores archipelago holds scientific significance, and evaluating the digestive systems of isopods living in this geothermal environment can provide valuable insights into their remarkable resilience and survival strategies. Such research can shed light on the interactions between these fascinating creatures and their volcanic surroundings, enriching our knowledge of the delicate balance between life and the ecosystem.

2.Material and Methods

Sampling Sites and Experimental Design

Terrestrial isopods belonging to the species *Porcellionides sexfasciatus*, were collected from four sites in São Miguel (Azores, Portugal).

A group was collected in Furnas Village (37˚46´23´´N, 25˚18´15´´W) close to the geothermal field. This site has the volcanic characteristics relevant to study the isopod's adaptations (table 1.1). Other three groups were collected in sites without any secondary volcanic activity: (1) Lagoa (37˚45´01´´N, 25˚34´09´´W), (2) Fajã de Baixo (37˚45´37´´N, 25˚38´49´´W), (3) Ribeirinha (37˚47´58´´N, 25˚29´14´´W-Figure 2.1). Lagoa and Fajã de Baixo are characterized as being gardens with agriculture practices. In Ribeirinha isopods were collected in a garden without agriculture practice in a soil full of leaves from many different tree's species. In Furnas isopods were also collected is a garden with agriculture practices in the middle of the village close to the geothermal field.

P. sexfasciatus specimens were captured by hand and preserved in 96% ethanol until further downstream analysis.

Table 1.1 *Physical properties and concentrations of metals (µg g-1 dry weight) in soils of Furnas. Source:* (Amaral et al., 2006)

Physical properties	Soil CO_2 (% vol.)	54.45
	Soil O_2 (% vol.)	9.7
	CO (ppm)	0.5
	Soil Temp. (˚C)	37
	Surface Temp (˚C)	17
	Moisture (%)	25
	pH (H_2O)	5.8
	Clay-silt (%)	76
	Organic Matter (%)	34
Metal Content (µg g-1 dry weight)	Cu	42
	Pb	81
	Zn	225
	K	32909
	Ca	9149

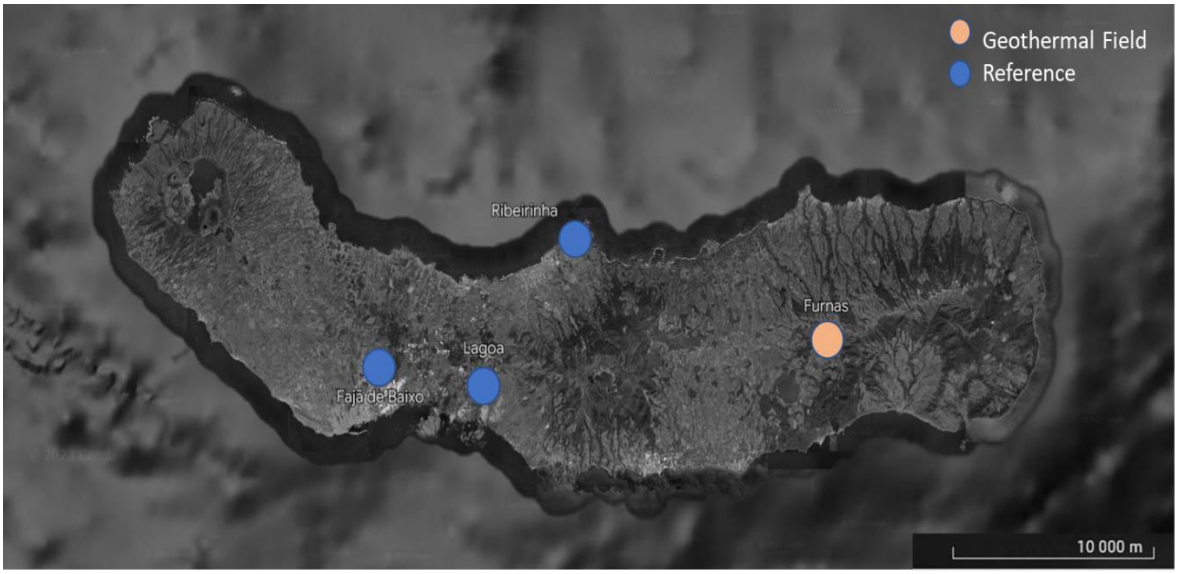


Figure 2.1 Sampling sites in São Miguel Island (Azores, Portugal) Source: Google Earth.

Scanning Electron Microscopy Samples Processing

Five individuals of *P. sexfasciatus* were collected and stored in ethanol 96% in two locations – Fajã de Baixo and Furnas. Each isopod was placed in small glass recipients. The initial fixative was replaced with 0.1M HEPES, after 60min it was replaced with EDTA, for 24h at room temperature. Then the EDTA was cleaned using 0.1M HEPES for 30min and replaced for plus 30 min. The samples were then placed in Osmium Tetroxide 2%, during 2h. In the end, it was washed with deionized water twice for 30min and dehydrated (50%-70%-90% and 100%). The absolute ethanol was replaced with xylene (30min). The isopods were placed in paraffin to facilitate the cut of isopods surface to expose the hindgut and hepatopancreas. The samples stayed overnight in xylene. Then it was replaced with ethanol during 1h to remove paraffin. The ethanol were replaced with HMDS (1h). The samples were attached to a strobe using silver paste and stayed in the fume cabinet for 12h. Scanning electron micrographs were recorded with a JEOL JSM-7500F field-emission scanning electron microscope.

Histological Sample Processing

Seven individuals of *P. sexfasciatus* were collected and stored in ethanol 96% in two locations – Fajã de Baixo and Furnas. Each isopod was placed in small glass recipients. The samples were submitted to hydration, followed by decalcification in 10% ethylenediaminetetraacetic (EDTA), for 48h, at room temperature. Samples were then dehydrated in an ethanol series (50%, 70% and 96%) (1min in each), cleared in xylene (1min) and placed in paraffin overnight. Transversal cuts of the tissues were sectioned (7 µm) on a rotary microtome, collected on glass slides, deparaffinized with xylene and rehydrated in an ethanol series (1 min each). A set of 120 sections were stained with

hematoxylin and eosin for general histological observations. After staining, sections were dehydrated in an ethanol series, washed in xylene and mounted in Pertex. All light micrographs were recorded with an Axiolmanager Z.1 microscope (Zeiss).

Morphological Analysis of Hindgut and Hepatopancreas

The thickness of gut and hepatopancreas cells were measured with Fiji/ImageJ software *Version* 1.53. Seven individuals were dissected per group (a) Furnas and (b) Fajã de Baixo (reference).

To compare cell's thickness between treatments, cell's height needed to be calculated. The cell´s height was obtained indirectly by measuring the entire area occupied by cells in each section using Fiji software. The cell area measuring was applied in all hepatopancreas and papillate region sections.

In the hindgut, measurements were only made in ventral cells due to heterogeneity in the anterior chamber.

Manual contouring of the inner (A_{int}) and outer (A_{total}) epithelial surfaces was performed (Figure 2.4B) to know the area of the cell (A_{cells}), calculated as $A_{cells} = A_{total} - A_{int}$. To estimate the average epithelial thickness or height (h), the internal perimeter (P_{int}) was calculated by estimating first the circle radius out, $A_{int}=r^2 \times \pi$ and $P_{int}=2 \times \pi \times r$. Then, with the perimeter of the internal area, it was possible to calculate the cell's estimated height (h): $h=A_{cells}/P_{int}$ (Figure 2.2).

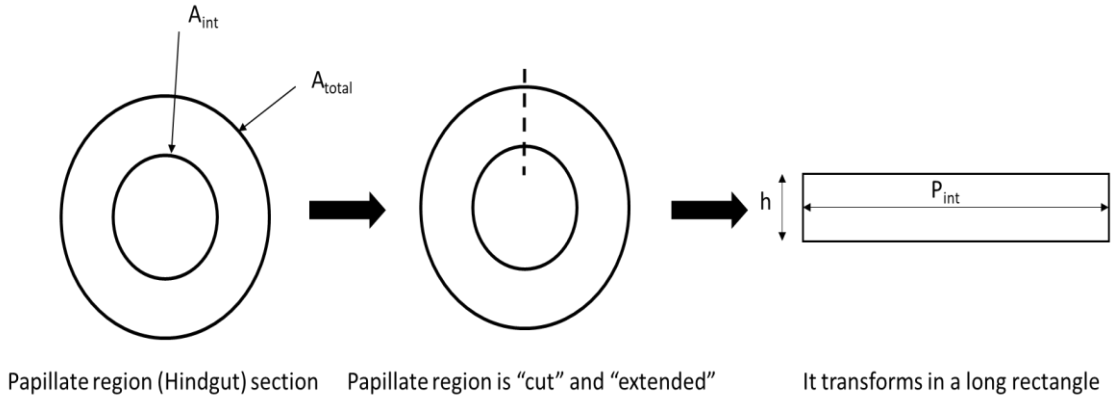


Figure 2.2 Estimation of papillate region (hindgut) epithelial thickness from cross sections. Four measured parameters were used to construct this model. The internal perimeter and cells area were measured values used to calculate epithelial thickness. The calculated epithelial value is an estimate of the average thickness of the papillate region epithelium.

In the hepatopancreas, at each section, the perimeter (HP), external (A_{ext}) and internal (A_{int}) areas were measured based on recorded contours. The Epithelial cross-section Area (EA) started to be calculated as $EA=A_{ext}-A_{int}$. Then for estimation of the average epithelial thickness (ET), the hepatopancreas was modelled as a circle with perimeter HP, from which the hepatopancreas radius (HR) was calculated as $HR = HP/2\pi$ and the hepatopancreas cross-section area (HA) as $HA = \pi HR^2$. The lumen cross-section area (LA) was calculated as LA = HA–EA, and the lumen radius (LR) as $LR = (LA/\pi)^{1/2}$. The epithelial thickness was determined as ET = HR – LR (Figure 2.3 and Figure 2.4A) (Lešer et al., 2008).

For the anterior chamber, in each section the length (L) and area (A) of the ventral cells were measured directly on Fiji software and with this the thickness was calculated as h=A/L (Figure 2.4C).

Epidermal thickness measurements were analyzed by a t-Test Two-Sample assuming unequal variances, with $p \leq 0.05$ considered as the significance level (R package "stats" version 3.6.2), before a Shapiro and Levene test were done to confirm data normality and homoscedasticity.

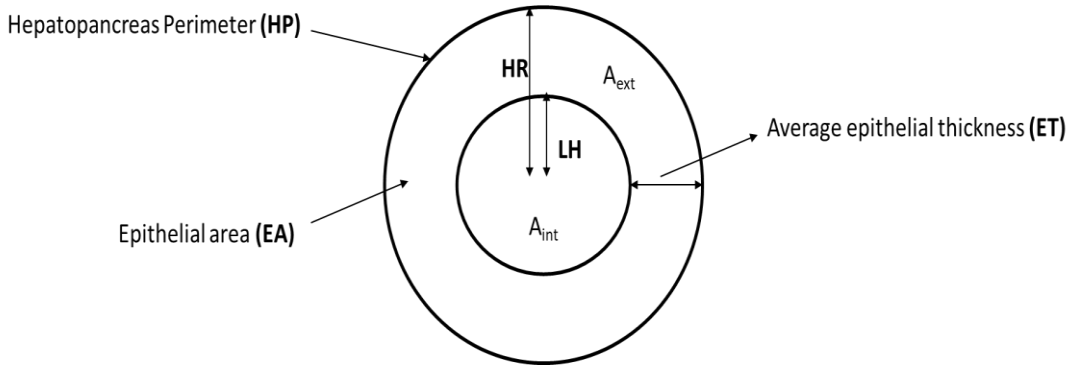


Figure 2.3 Estimation of the hepatopancreas epithelial thickness from cross-sections. Two measured parameters, the Hepatopancreas Perimeter (HP) and the Epithelial Area (EA), were used to calculate the epithelial thickness. This calculated epithelial value is an estimate of the average thickness of the hepatopancreas epithelium.

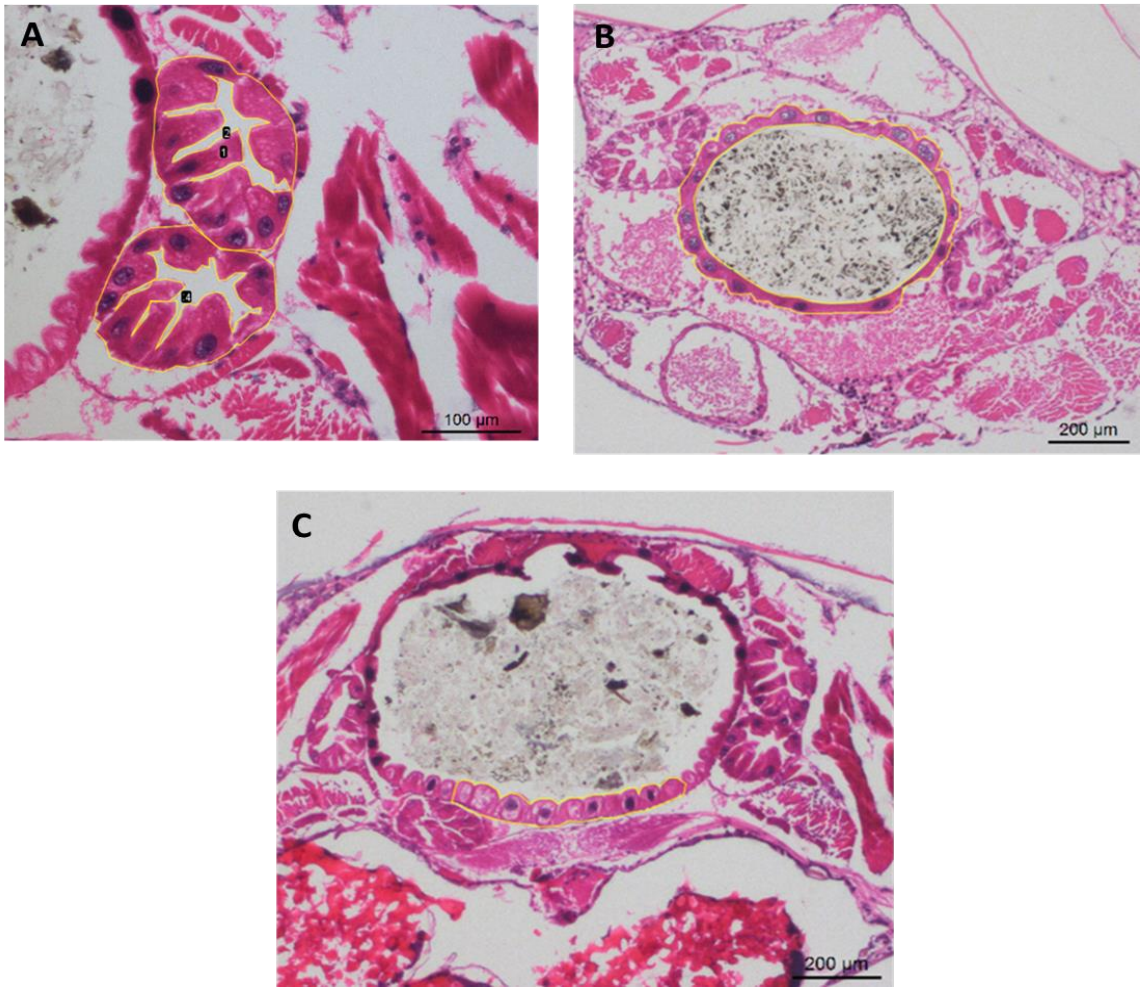


Figure 2.4 Using FIJI software to measure areas, perimeters and length in sections of hepatopancreas **(A)** and hingut; **(B)** Papillate Region; **(C)** Anterior Chamber

DNA extractions from digestive tissues

The hepatopancreas and hindgut of five animals per site were removed from each individual and preserved in ethanol 96% until the DNA extraction. DNA extractions were made using NYZ Soil gDNA isolation kit following the manufacturer protocol with some alterations as follows. Briefly, tissues were transferred to a microtube with 180µl Buffer NSL1 and 20 µl proteinase K and kept at 60 ℃, for 2h for tissue digestion. Then 520µl Buffer NSL1 was added to the sample, and the full volume was transferred to a NZYSpin Soil Bead Tube for additional tissue disruption. Before adding 150 µl of NS Enhancer, samples were subjected to three cycles of tissue homogenisation using a FastPrep Tissue Homogenizer (with 30s at 6m/s, and 5 min pause on ice between cycles). Afterwards, samples were centrifuged for 2min at 11000 x g. Then, 150 µl of Buffer NSL3 was added to precipitate any contaminants and vortex for 5s. Proceed by incubating for 5min at 0-4 ℃ and centrifugation for 1min at 11000 x g. For lysate

filtration, the supernatant from the previous step was loaded into a filter of NZYSpin soil Inhibitor Removal Column placed in a collection tube (up to 700 µl), then centrifuged and NZYSpin soil Inhibitor Removal Column discarded. To adjust DNA binding conditions 250 µl of Buffer NSB was added and vortexed for 5s. The sample (550 µl) was loaded into a NZYSpin Soil Column placed in a collection tube, to be centrifuged at 11000 x g for 1 min. The flow-through was discarded and the column placed in the same collection tube was load with more 550ul of the sample. To wash and dry the silica membrane, 500 µl of Buffer NSB was added to the Spin column and centrifuged for 50s at 11000 x g with flow-through discard. This was repeated with 550 µl of Buffer NSW1 and 700 µl of Buffer NSW2. However, for NSW2 the process was repeated twice. In the end the DNA elution obtained by placing the NZYSpin Soil column into a clean microcentrifuge tube correctly label, and 40 µl of NSE was added. The sample was incubated at room temperature with open lid and was centrifuged at 11000 x g for 30s. Then the genomic DNA was stored at -20℃. DNA quality and quantity was verified using a Thermo Scientific NanoDrop 2000 spectrophotometer.

Metabarcode sequencing of the 16S rRNA

Good quality DNA samples (with A260/A280 > 1.8) were then subjected to 16s rRNA gene amplification and amplicon mtebarcoding sequencing using universal primers for the region V4 in 16s rRNA gene with primers 515F and 806R (Caporaso et al., 2011) at Novogene facilities (Cambridge, UK). Amplicons were sequenced on an Illumina NovaSeq platform (Novogene, UK). Sequencing reads were processed and analysed using DADA2 version 1.16 (Callahan et al., 2016), with default parameters applied for filtering, denoising, merging of paired reads, chimera identification and removal, resulting in the generation of amplicon sequence variants (ASVs).

ASVs (Amplicon Sequence Variant) are used to define units of microbial diversity, defined by exact sequence variants, this means each unique sequence is considered as a separate ASV and the full resolution of the data is preserved. ASVs differentiate closely related organisms and detect more subtle changes in microbial communities. In essence, ASVs allow higher resolution and potentially more accurate representation of microbial diversity (Callahan et al., 2017).

Then both forward and reverse primer sequences, and low-quality fragment ends, were trimmed to attain final read lengths of 160 bp and 200 bp for forward and reverse reads, respectively. The chimera proportion was approximately 0.98%, and taxonomic assignments were performed using IDTAXA (Murali et al., 2018), implemented in the R package DECIPHER (Wright,2016), with a classifier trained on the SILVA v138 database (December 2019 release).

For further analysis in Alpha diversity, taxonomic filters were applied in order to keep only the Domain Bacteria and delete all ASVs appearing a single time in the reads. Alpha diversity was represent by Chao1 index, Shannon index and Simpson index of the bacterial microbiome, was compared among treatments using a One-Way ANOVA test (R package "stats" version 3.6.2), with statistical significance set at $p<0.05$, before a Shapiro and Levene test to confirm data normality and homoscedasticity. Pairwise

comparisons between the four treatments were conducted using the Tukey test, considering $p < 0.05$ as statistically significant.

To evaluate differences in bacterial community composition between the treatments, non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity was employed, using phyloseq (v1.16.2-McMurdie & Holmes, 2013). These analyses provided valuable insights into the variations and associations within the bacterial populations across the different treatment conditions.

For the analysis of the bacterial composition, relative abundances of observed bacterial taxa at the phylum and family level were compared between different treatment groups using bar plot charts created with the microbiome R package (v1.18.0). Differential abundance analyses were performed by DESeq2 (Love et al., 2014). Changes with an adjusted p-value lower than 0.05 were considered significant.

3.Results

Gut Structure and Bacterial Presence

Bacteria were found in the papillate region of isopods from Furnas and Fajã de Baixo. Those bacteria were detected abundantly in hindgut regions with fragmented food. However, a particular bacteria attached to papillate region hindgut walls were found. A similar bacterium was associated to the hindgut walls of *Porcellio scaber,* designed as "*Candidatus* Bacilloplasma" (a Novel Lineage of *Mollicutes*) (Kostanjšek et al., 2007). This demonstrate this specific bacterium can dwell in this organ, it is not a passenger bacteria. Unfortunately, bacteria were not easily found in the anterior chamber of the hindgut and was not possible to explore the hepatopancreas due to its danification during isopods cut in paraffin (Figure 3.1).

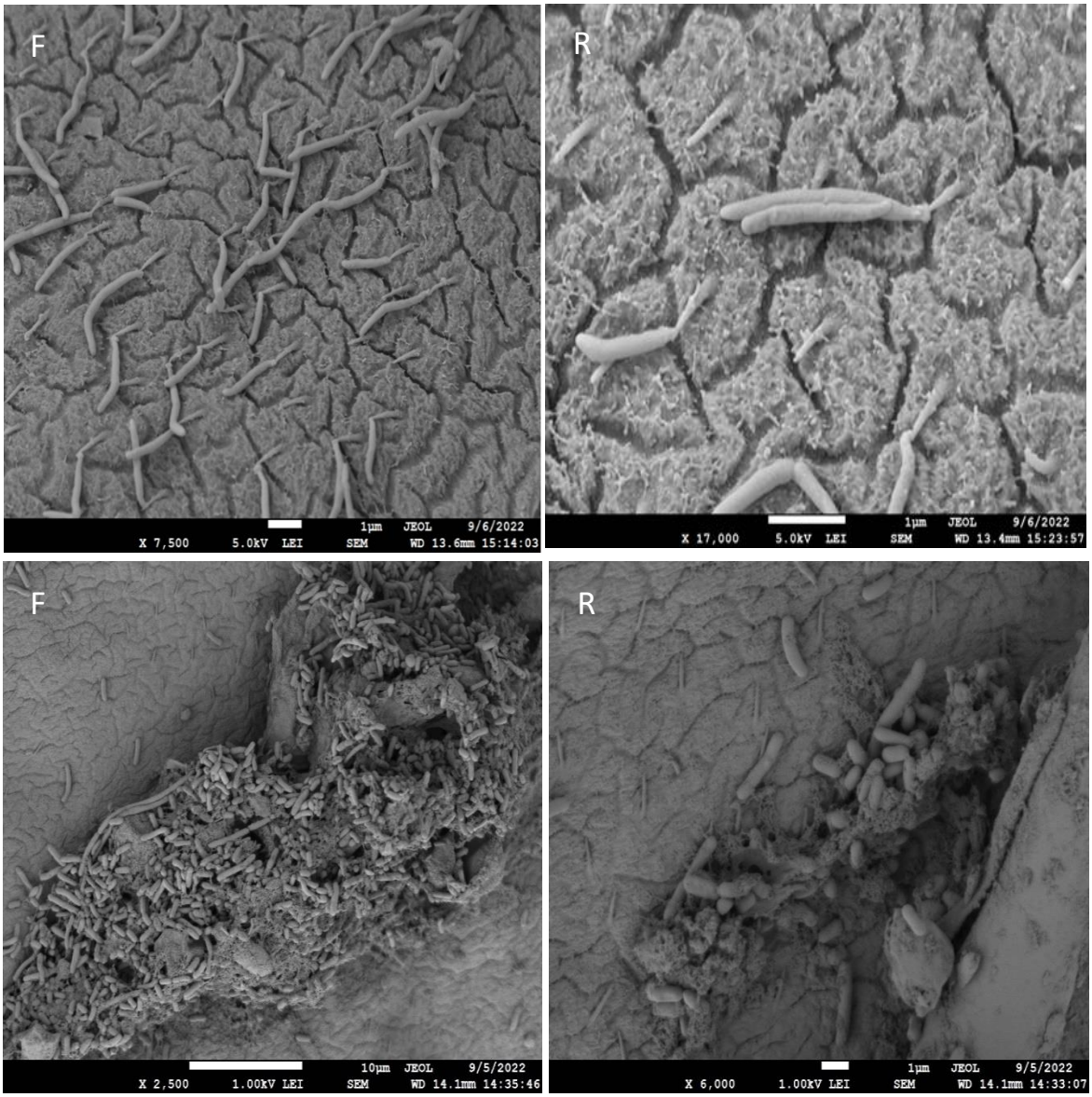
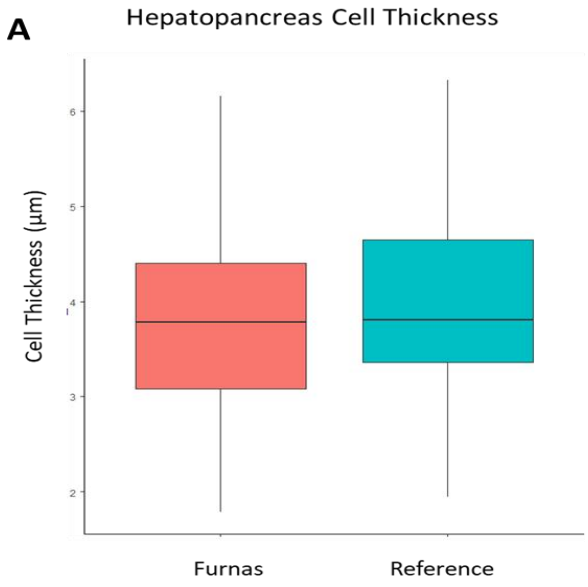
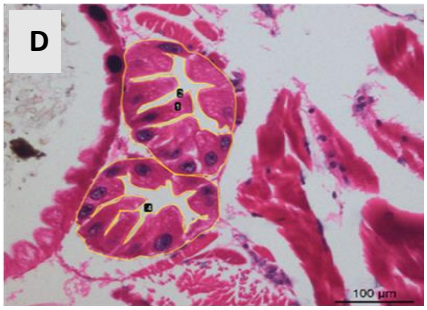


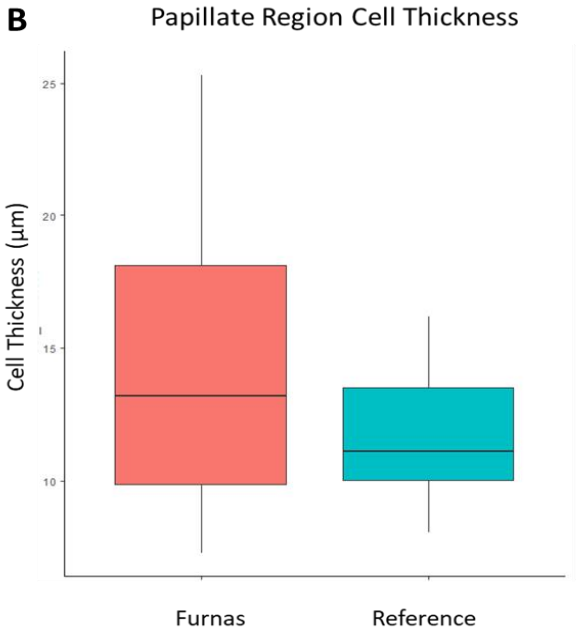
Figure 3. 1 Detected bacteria in papillate region of hindgut isopods from (F) Furnas and (R) Reference - Fajã de Baixo (SEM images).

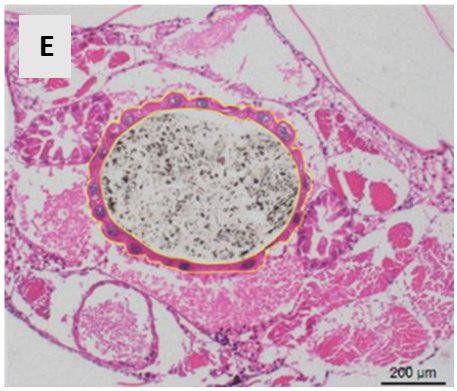
Morphological Analysis of Hindgut and Hepatopancreas

No significant differences were observed between populations of the species of *P. sexfasciatus* from Fajã de Baixo (reference) and at Furnas for what concerns the hepatopancreas (t(207)=-1.1573; p=0.2485 – Figure 3.2 A) and the papillate region of the hindgut t(33)=2.0134; p=0.0523 - Figure 3.2 B). Nonetheless, the thickness in the anterior chamber of the reference site was significantly higher than the exposed site - Furnas (t(38)=-2.2751;*p*=0.0286; n=20 – Figure 3.2 C).









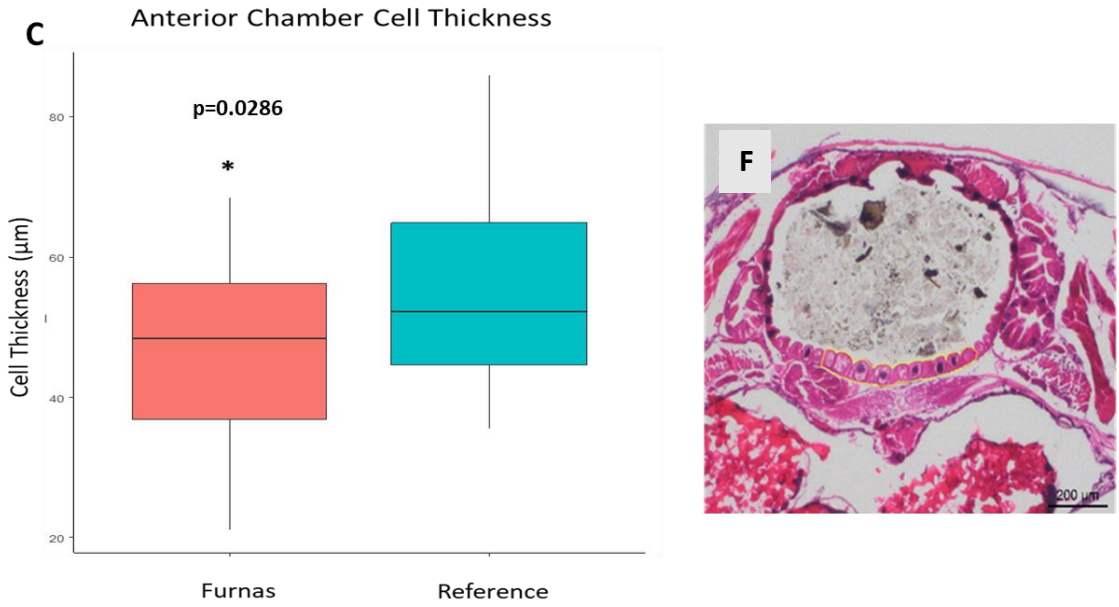


Figure 3. 2 The epidermal thickness (µm) variation in Furnas (with volcanic activity) and Fajã de Baixo (refence), corresponding to: (A) cell thickness in hepatopancreas, (B) the papillate region and (C) the anterior chamber of the hindgut. * - significant differences ($p \leq 0.05$) between groups. Histological cuts of hepatopancreas (D), papillate region of the hindgut (E) and anterior chamber of the hindgut (F).

Bacterial diversity

In the hindgut, from a total of 2712366 reads with ~250 bp pair-end, 2192107 high-quality reads between 252:254bp were kept after denoising, merging and chimaera removal, resulting in 11791 ASVs. For hepatopancreas, from a total of 2685212 reads with ~250 bp, 2184067 high-quality reads between 252:254bp were kept, resulting in 11052 ASVs. For normalisation, the datasets were rarefied in a total of 1707920 reads for the hindgut resulting in 9841 ASVs. Hepatopancreas datasets were rarefied in a total of 1622524 reads, resulting in 8922 ASVs after filtration.

ASV richness was determined by the species richness estimator Chao 1. The diversity by the Shannon Index and Simpson Index of alfa-diversity (Chao 1 index, Shannon index and Simpson index: One way-ANOVA, $p \leq 0.05$; (Supplementary Material-Table 1.2 and 1.3). In the hindgut, Chao 1 index had a p=1.4e-05 ***; F= 19.42; df=3; n=5, richness was significantly different between sites. According to Tukey test richness was significantly different between Furnas and Ribeirinha (Chao 1: p=0.00001), Ribeirinha-Fajã de Baixo (Chao 1: p=0.0002) and Ribeirinha-Lagoa (Chao 1: p=0.002). Richness was higher in Furnas. For diversity Shannon index presented a p=2.09e-05 ***; F=18.18; df=3; n=5, while the Simpson index presented a p=0.0252 *; F= 4.068; df=3; n=5. ASVs diversity was higher also in Furnas comparing to the other three reference sites but differences in diversity were significantly higher between Ribeirinha-Furnas (Shannon: p=0.00002; Simpson: p=0.03), Ribeirinha-Lagoa (Shannon: p=0.0002; Simpson: p=0.042) and Ribeirinha-Fajã de Baixo (Shannon: p=0.002) (Figure 3.2A).

In hepatopancreas, Chao 1 index had a p= 0.000301 ***; F= 11.9; df=3; n=5. Differences in richness were significante between Ribeirinha-Fajã de Baixo (Chao 1: p=0.00047), Ribeirinha-Lagoa (Chao 1: p=0.0019) and Furnas-Fajã de Baixo (Chao 1: p=0.024). For diversity, Shannon index presented a p= 0.000226 ***; F=12.57; df=3; n=5, while the Simpson index presented a p= 0.00641 **; F= 6.086; df=3; n=5. For diversity differences were between Ribeirinha-Fajã de Baixo (Shannon: p=0.003; Simpson: p=0.003), Ribeirinha-Furnas (Shannon: p=0.013; Simpson: p=0.013) and Ribeirinha-Lagoa (Shannon: p=0.0002; Simpson: p= 0.0002). Richness was higher in Fajã de Baixo, while diversity in Lagoa in both Shannon and Simpson index (Figure 3.2B).

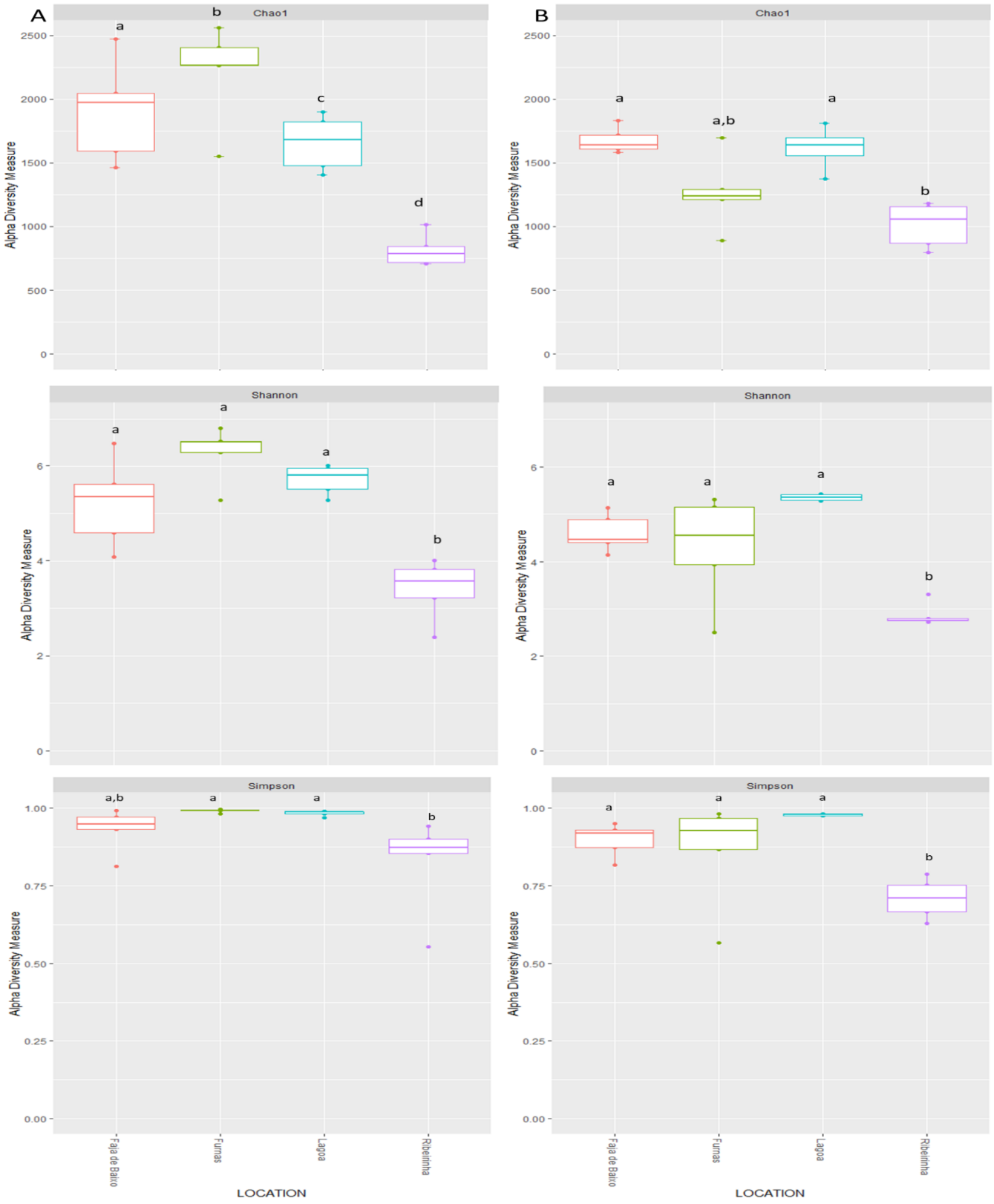


Figure 3.3 Alpha-diversity of bacterial communities in *P. sexfasciatus* hindgut (A) and hepatopancreas (B), Chao 1 index for richness; Shannon index and Simpson index for diversity.

A Non-metric Multidimensional Scaling (NMDS) plot shows the correlation between bacterial ASVs (amplicon sequence variants) based on their Bray-Curtis dissimilarity distance. In gut samples Ribeirinha cluster has a high distance comparing to the other groups, indicating the community of this group has a high level of dissimilarity between the other groups of ASVs. Lagoa samples formed a tighter cluster meaning this group has high similarity among corresponding ASVs, the other three groups have a higher level of dissimilarity (Figure 3.4, A).

In hepatopancreas samples the Ribeirinha cluster distance from the other groups decreases, indicating the community dissimilarity between the other groups of ASVs got lower. Lagoa and Fajã de Baixo samples formed a tighter cluster meaning these two groups have high similarity among corresponding ASVs, Ribeirinha and Furnas have a higher level of dissimilarity (Figure 3.4, B).

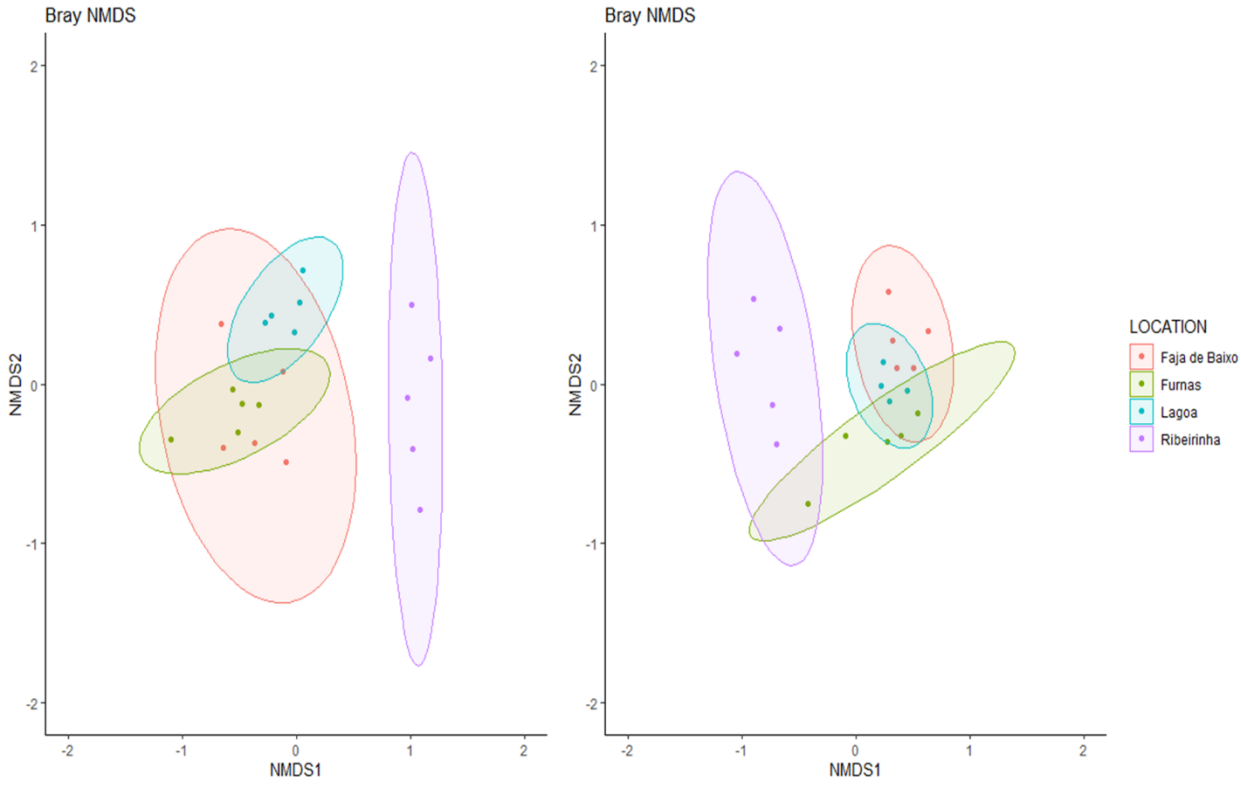
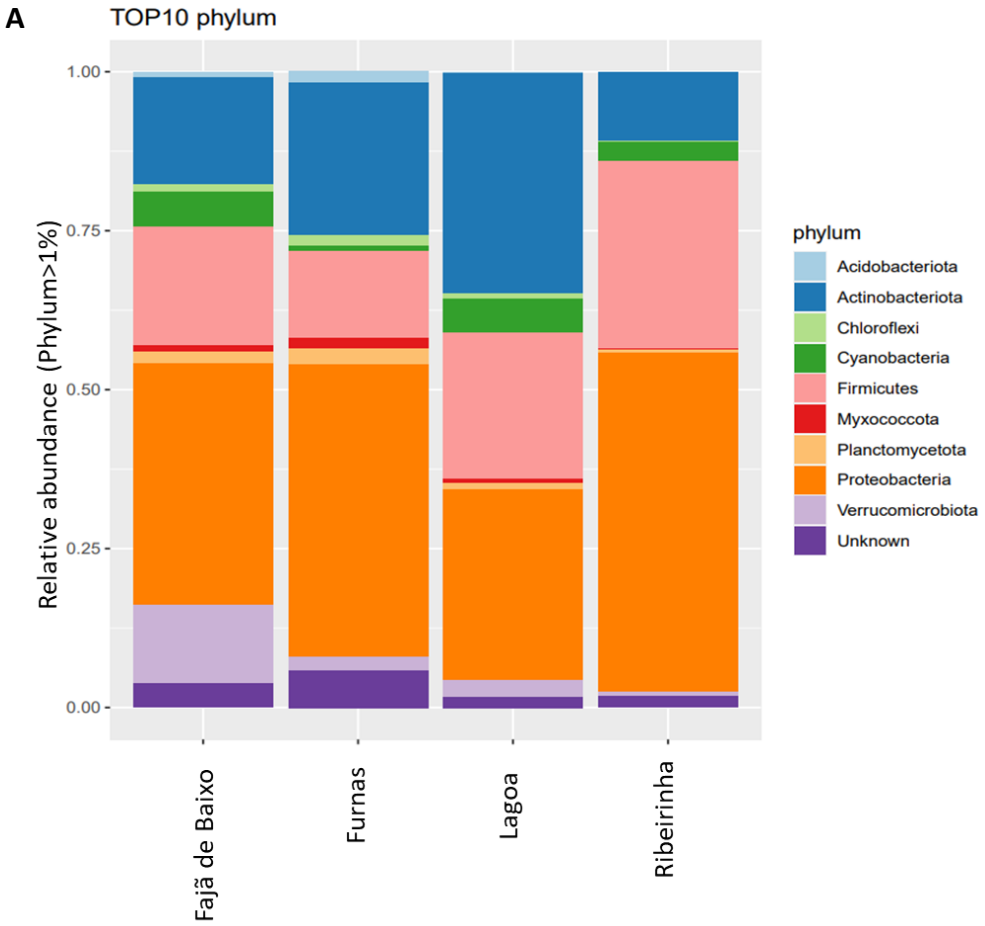


Figure 3. 4 NMDS plot showing the correlation between bacterial ASVs of different locations (Furnas, Fajã de Baixo and Ribeirinha) for gut samples (A) and hepatopancreas samples (B), described by Bray–Curtis dissimilarity distance.

Community composition

The microbiota associated with hindgut and hepatopancreas from different locations, not only differed in terms of bacterial richness and diversity, but also concerning bacterial community composition.

Analyzing in detail the hindguts microbial composition, it's possible to determine Proteobacteria as the dominant phylum in Furnas (45.98%), Faja de Baixo (37.98%) and Ribeirinha (53.30%). The dominant phylum in Lagoa is Actinobacteriota with 34.59%, Proteobacteria occupies the second place with 30.06%. In the other hand, 24% of Actinobacteriota is present in Furnas, being the second dominant group. In Fajã de Baixo (18.66%) and Ribeirinha (29.45%) it belongs to Firmicutes. The prevailing families change between sites: Furnas has very close percentages of four families Comamonadaceae (9.91%), Bacillaceae (9.45%), Pseudomonadaceae (9.14%) and Moraxellaceae (9.11%); Lagoa has Streptomycetaceae (20.64%) and Bacillaceae (15.82%); for Fajã de Baixo it is Simkaniaceae (16.77%), Diplorickettsiaceae (13.15%) and Paenibacillaceae (13.13%); Ribeirinha is predominately dominated by Diplorickettsiaceae with 61.80%. Except for Ribeirinha, all other locations have higher percentages of unknown families (Furnas-34.91%; Lagoa-28.98% and Fajã de Baixo-26.78%) (Figure 3.5A and Figure 3.5B - Supplementary Material Table 1.4).



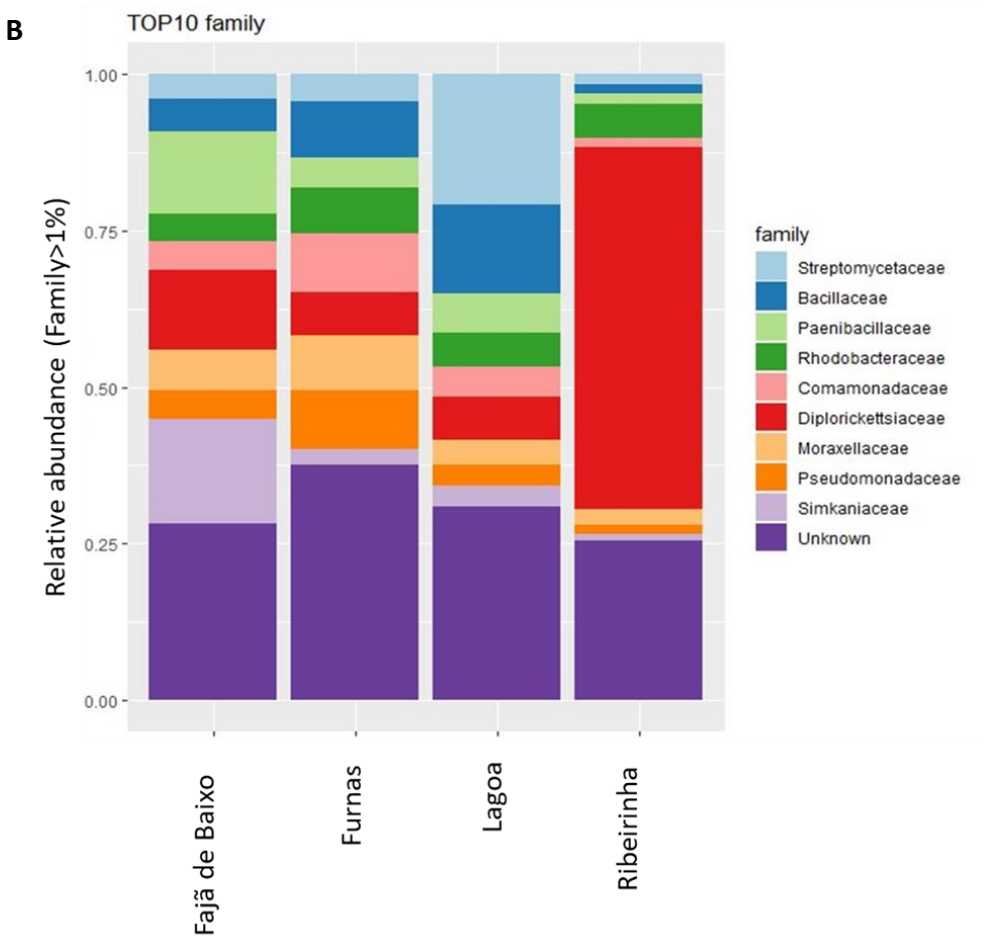
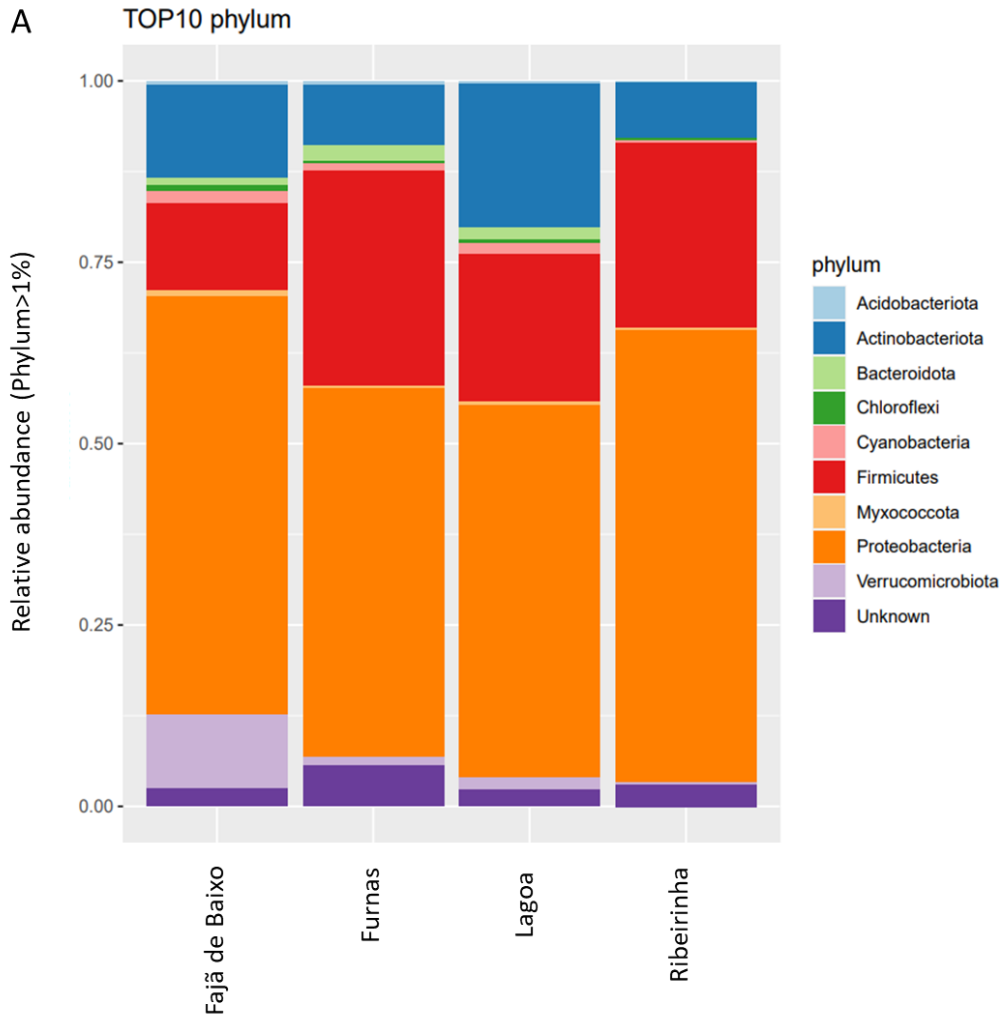


Figure 3. 5 Bacterial community composition (%) of bacteria phylum (A) and bacteria family (B) of the hindgut.

In hepatopancreas Proteobacteria is the dominant phylum in all sites (Furnas-50.40%; Lagoa-51.90%; Fajã de Baixo-57.55%; Ribeirinha-61.63%). Firmicutes is the second dominant phylum in Furnas (30.14%) and Ribeirinha (26.24%), it is the third in Fajã de Baixo (12.10%) and Lagoa (19.77%). Actinobacteriota is the second dominant phylum in Lagoa (20.04%) and Fajã de Baixo (12.71%). In Furnas the predominant family is *Entomoplasmatales Incertae Sedis* (29.26%), followed by *Moraxellaceae* (12.25%). For Lagoa it is "unknown" family (44.65%), *Moraxellaceae* (15.59%) and *Pseudomonadaceae* (11.71%). In Fajã de Baixo it is *Moraxellaceae* (26.52%), *Comamonadaceae* (14.37%) and *Simkaniaceae* (14.31%). For Ribeirinha *Diplorickettsiaceae* is for sure the dominant family with 44.04%, followed by *Entomoplasmatales Incertae Sedis* (22.67%) and *Candidatus Hepatincola* (16.82%) (Figure 3.6Aand 3.6B -Supplementary Material Table 1.5)



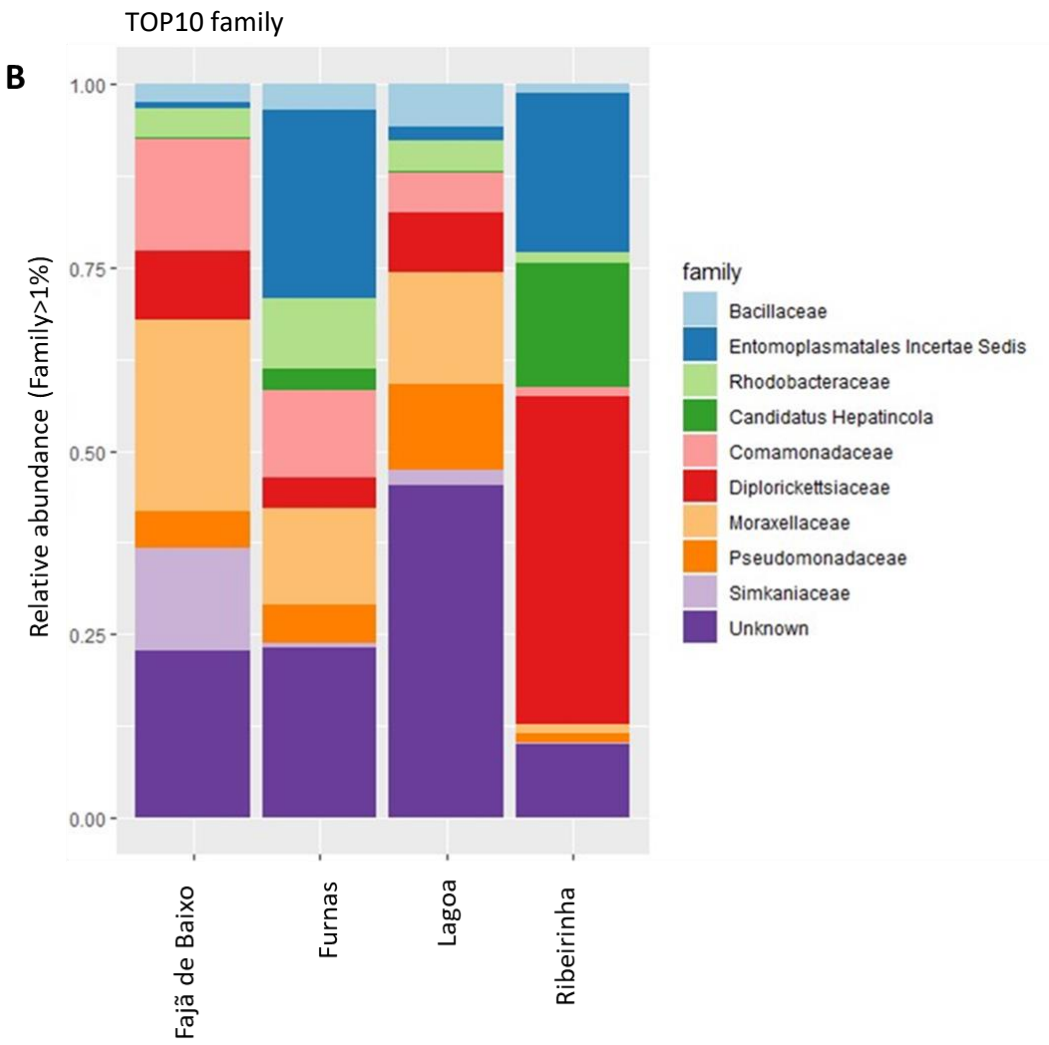


Figure 3. 6 Bacterial community composition (%) of bacteria phylum (A) and bacteria family (B) of the hepatopancreas.

Comparison of bacterial communities

Comparing the microbial composition of hindgut and hepatopancreas samples from the geothermal field in Furnas and the reference sites combined (Fajã de Baixo, Lagoa and Ribeirinha), the results were five bacterial families are more abundant in the reference sites, with significant values. These are different between organs, in the hindgut (Figure 3.7A) those belong to phylum Proteobacteria belonging to family *Xanthobacteraceae* (genus N/A) ($Log_2Fold=8.51$) and *Hyphomicrobiaceae* (genus *Hyphomicrobium*) *(*$Log_2Fold= 8.58$). In hepatopancreas (Figure 3.7B) it also pertains to phylum Proteobacteria but to family *Pseudomonadaceae (*genus *Pseudomonas*) ($Log_2Fold = 3.81$), *Shewanellaceae (*genus *Shewanella*) ($Log_2Fold = 4.98$) and *Legionellaceae* (genus *Legionella*) ($Log_2Fold = 8.72$) (Supplementary Material - Table 1.6 and 1.7).

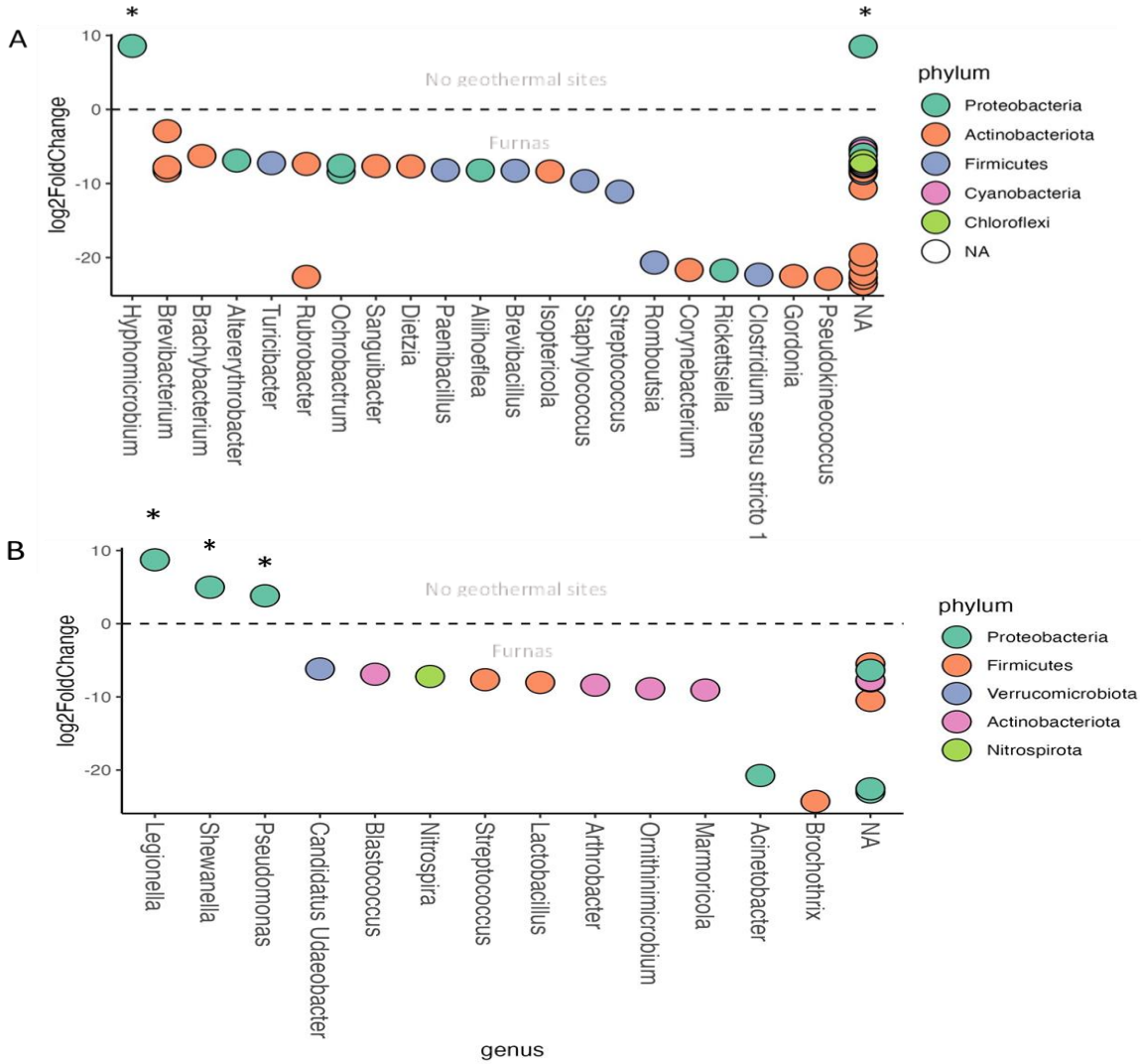


Figure 3. 7 DESeq2 plots comparing microbial composition of hindgut (A) and hepatopancreas (B) samples from the geothermal field in Furnas and the reference sites combined (Fajã de Baixo, Lagoa and Ribeirinha) two bacterial families are contributing to differentiate these sites, with significant differences.

This DESeq2 plots demonstrate a significant bacteria association to Furnas hindgut and hepatopancreas. Some genus are associated to the decomposition of organic matter (*Brevibacterium*, *Brevibacillus*, *Isoptericola*, *Clostridium sensus stricto 1*, *Gordonia*, *Pseudokineococcus*, *Arthrobacter*, *Marmorocola*, *Acinetobacter*, *Brochthrix*), nutrient cycle (*Altererythrobacter*, *Ochrobacttrum*, *Paenibacillus*); skin and mucous membranes of animals (*Corynebacterium, Streptococcus, Staphylococcus, Turicibacter, Brachybacterium, Blastococcus*); nitrogen cycle (*Candidatus Udaeobacter*), gastrointestinal tracts of animals (*Clostridium sensu stricto, Sanguibacter, Lactobacillus*), are intracellular parasites (*Rickettisia*), fermentation of complex carbohydrates and production of short-chain fatty acids (*Romboutsia*). Also, have been isolated from extreme environments *Dietzia* from high-salinity soils and *Rubrobacter* from hot, arid and acidic habitats. In the reference sites the significant associations were related to *Legionella* normally associated to contaminated water droplets, *Hyphomicrobium* and *Shewanella*) to nutrient cycle and *Pseudomonas* to the decomposition of organic matter.

4.Discussion

The present study marks a pioneering endeavour to elucidate the impact of secondary volcanic activity on isopods thriving in the extreme environment of volcanic origin. This study sheds light on the variations in the thickness of various digestive structures and explores the microbial communities present. Our findings reveal intriguing morphological distinctions, particularly within the anterior chamber region and hindgut. Surprisingly, the microbiota did not exhibit the anticipated characteristics of a bacterial community with reduced species richness and evenness in a geothermal field setting. These observations add a new dimension to our understanding of the intricate interactions between isopods and their volcanic habitat.

Tissue Alterations in the Gut of Volcanic Isopods

Each digestive system structure has its own function during digestion. It was reported that the anterior chamber is the site where the food is mixed and digestive enzymes take place, while in the papillate region and rectum is where removal of water and compaction of faecal pellets occur. Thus, papillate region´ epithelial cells are responsible for ion transportation and water re-absorption. Where catalysis acts, the absorption of digestive products up to 1.9 nm passes the organ cuticle (Zimmer, 2002). In comparison to the anterior chamber, nutrients assimilation occurs in majority on hepatopancreas, it is also the main site of synthesis and secretion of digestive enzymes, storage of metabolic reserves (lipids and glycogen) and secretion of waste products, such as degenerated cellular material. Waste products can leave the hepatopancreas by going directly to the anterior chamber, passing throw the hindgut and be voided as faeces. This organ can also accumulate metals in the cells, the concentration of these metals can differ from isopods of different species collected in the same site, it appears to be related to differences in the 'availability' of the metals in the digestive fluids and the efficiencies of uptake and excretory mechanisms (Hames & Hopkin, 1989).

The ventral part of anterior chamber is less thick in individuals belonging to Furnas manifesting metals influence on the digestive track of the organisms. As happened with *Pseudaletia uninpuncta* (Rodrigues et al., 2008), the reduction of cells height can be related to metals presence on digest food, these metals can be toxic to cells, reducing its cellular cycle by inducing apoptosis. In the other hand, the thickness of papillate region of *P. sexfasciatus* in Furnas has a higher range of values than in Fajã de Baixo. Apical and basal surfaces of epithelial cells in this hindgut region are associated with mitochondria, indicating intensive material exchange between hindgut lumen and haemocoel (G. M. Vernon et al., 1974, Laura Coruzzi et al., 1982, Palackal T et al., 1984). This transepithelial transport of ions and water can drive to osmoregulatory processes in the papillate region, such as ion sequestration in the hindgut during dehydration (J. C. Wright et al., 1997). Metals are responsible for morphological damage, it can modify organs structure by inducing disorganization on epithelial structure and critical cellular lesions such as hyperplasia, hypertrophy and necrosis (review in Lignot et al., 2000).

In Issartel et al., 2010, amphipod (*Gammarus fossarum)* exposed to cadmium its haemolymph osmolality (HO) decreased and an inter-individual variation on HO values was only observed on exposed organisms, highlighting a distinct sensitivity to this metal toxic effect. Furthermore, histological changes were noticed, such as hyperplasia (abnormal cell proliferation) on gills of organisms exposed to cadmium. In *P. sexfasciatus* from Furnas this inter-individual variation is observable on epithelium cells height so in this case metals can induce cells hypertrophy in the papillate region which probably would influence osmolality. However, Furnas is a location rich in metals that can interact in cells differently by inducing or restricting fundamental reactions and there are also acidic, high temperature soils. The interaction metal-cell is much more complex in this case.

Hepatopancreas has intestinal, hepatic and pancreatic functions. It is defined by two cell types: secretory and absorptive cells as *B*-cells, containing lipid droplets, glycogen and store ions in granules. *S*-cells, that accumulate metal ions, calcium, and urate. Morphological changes during 24h of digestive cycle were described in *B*-cells, these changed in size from dome-shaped to flat, the accumulation and extrusion of lipids droplets can also be observed. Ultrastructural changes also occur in *B*-cells during fasting, molting and feeding with metal-contaminated food. In the other hand, *S*-cells ultrastructural appearance is similar at all stages of daily and molt cycle, with only some oscillations in calcium content during molting (Lešer et al., 2008). In our study, significant changes in hepatopancreas epithelium between sites were not detected. The epithelium in both sites is very thin (between 3 and 5µm) and the average close. Ecotoxicological studies of metals exposure effectiveness in isopods, demonstrated organisms exposed to elevated metals concentrations have a thinner epithelium comparing to control. Hepatopancreatic *B*-cells when exposed to zinc reduce in size due to its effect on the apical membrane, resulting in less capacity of nutrients absorption (Odendaal & Reinecke, 2007). There are some known specific proteins in both cells responsible for taking, trafficking and distributing metals for storage, nickel (Ni) is an example. For instance, Zinc (Zn) trafficking is done by lysosomes and performed by a pH gradient (N. G. C. Ferreira et al., 2019).

It was expected to find morphological differences on hepatopancreas of organisms from Furnas since it is environmentally rich in metals. No differences probably mean adaptation to the environment, cells can have specific molecular paths to take, traffic and distribute the different metals present in volcanic environments.

The Surprisingly Higher Richness in Microbiota of Furnas Isopods

Symbioses were essential for the origin and diversification of eukaryotes because they cause physiological, morphological, and developmental changes in the species involved (e.g., metabolism, pathogen defense, feeding, reproduction, ecology; (Sapp et al., 2004; Shin et al., 2011). Symbiotic associations are common in environments where there is not enough organic matter to support a heterotrophic lifestyle. Bacteria can access energy resources such as sulfur and methane and benefic eukaryotes with it (Sogin et al., 2021).

Bacterial communities' structure had been shown to be affected by metal contamination, resulting in a reduction in members community, the extinction of more sensitive species and appearance of metal-tolerant microorganisms (Drobne et al., 2002). As soil metal richness is associated to volcanism, it was expected to find less diversity in microbiota´s Furnas samples. However, it was Ribeirinha that most differ from the other three sites. It shows lower bacterial diversity and evenness, indeed bacterial ASVs in both organs have a higher level of dissimilarity compared to the other locations.

The bacterial communities were analysed for both organs and the dominant phyla are the same for all sites. In the gut it is possible to notice the two most dominant phylum appear in higher percentages than in Furnas, Fajã de Baixo and Lagoa, this helps to explain the divergency of ASVs found in Ribeirinha cluster and the low diversity values. In the hepatopancreas the phylum dominant percentages are in majority equal between sites. It is essential to note that bacterial diversity can vary significantly depending on the specific characteristics of the geothermal site and its proximity to the surrounding areas. Furnas may still support unique and specialised microbial communities adapted to the extreme conditions, leading to relatively higher diversity within those specific niches when compared to poorer surrounding soils.

Some previous studies have identified the microbiota of isopods, in Majed et al., 2018 the bacterial community associated with 11 specimens of terrestrial isopods belonging to six species were studied, their results demonstrated the predominance of phyla such as Proteobacteria, Actinobacteria, Firmicutes and Cyanobacteria. Similar results were shown in Delhoumi et al., 2020 and Bouchon et al., 2016. Our results are in accordance with these studies since for the hindgut in the three sites the predominant phyla were Proteobacteria, Actinobacteriota and Firmicutes. These bacterial phyla are responsible for nitrogen fixation, denitrification, carbohydrate degradation, detoxification and defensive trend against pathogens (Shao et al., 2014). Proteobacteria and Actinobacteria phyla are also associated to the degradation of lignocelluloses within the terrestrial isopod *A. vulgar* (Bredon et al., 2018). Some of the prevailing bacterial families in the hindgut were also equivalent to other studies such as *Bacillus* (*Bacillaceae*), *Pseudomonas* (*Pseudomonadaceae*) (Bouchon et al., 2016) and *Staphylococcus* (*Comamonadaceae*) (Delhoumi et al., 2020). Besides all sites had *Diplorickettsiaceae* (*Rickettsiella*) it was mostly found in Ribeirinha with 61.80%. *Rickettsiella* is an intracellular pathogen known to cause lethal disease in isopods. This pathogen is widespread, being not only detected in symptomatic hosts but also in healthy individuals and in the environment (Bouchon et al., 2016; Oliveira et al., 2021).

The hepatopancreas microbiota is predominantly similar to the hindgut, some differences are in the top families that are not the same between the two organs. In this samples the predominant families were *Entomoplasmatales Incertae Sedis, Moraxellacea, Pseudomonadaceae, Comamonadaceae, Simkaniaceae, Diplorickettsiaceae* and *Candidatus Hepatincola,* which *Entomoplasmatales Incertae Sedis* and *Candidatus Hepatincola* are found in the hindgut but not as abundant families. Studies indicate genus *Candidatus Hepatoplasma ('Hepatoplasma')* belonging to family *Candidatus Hepatincola* as hepatopancreatic symbionts, suggested to be involved in the hydrolysis of cellulose and the oxidative breakdown of lignin and tannins. It is also

reported as *Bacillus*, *Bacteroides*, *Enterococcus* and *Pseudomonas* a common bacteria found in the hepatopancreas (Bouchon et al., 2016).

It is understandable the dissimilarity of Ribeirinha to the other three sites since these isopods were widely contaminated with *Rickettsiella.* This was the only non-agriculture related site, isopods in Fajã de Baixo and Lagoa were collected in domestic compost and isopods from Furnas were collected in a garden used for agriculture activities, however with geothermal activity. According to Wang et al., 2020, Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes are mainly found in compost suggesting Firmicutes and Actinobacteria can be transfer to soil with compost application. This can explain the high abundance of this phyla in the hindgut and hepatopancreas of these isopods in all sites since their food was mainly contaminated with it. In the case of Furnas it was expected a lower diversity and bacterial core due to the geothermal field. However, it appears the temperatures and metals composition in Furnas were not enough to eliminate these bacteria. To better understand this in the future, soil microbiome, temperature and metals should be analysed in Furnas. Besides this geothermal field related bacteria were not found in isopods hindgut and hepatopancreas, this does not mean these extreme bacteria are not present in Furnas. Probably due to anthropogenic activity there are present but in lower quantities in the environment or if ingested by isopods those do not survive in the internal conditions of these invertebrates.

Furnas has more bacteria genera associated then the reference sites combined. In the hindgut the predominant phylum associated to Furnas are *Proteobacteria*, *Actinobacteriota*, *Firmicutes*, *Cyanobacteria* and *Chlroflexi*. Belonging to *Proteobacteria* there are genus associated to Furnas as *Altererythrobacter*, *Ochrobactrum*, *Aliihoelaflea* and *Rickettsia*. These are all gram-negative bacteria. Besides *Rickettsia* that is an intracellular parasite the other genus has a big potential on bioremediation of polluted environments and in biotechnology to develop vitamins and amino acids. *Altererythrobacter* can also be used on production of cheese (Onraedt et al., 2005; Perlman et al., 2006). *Aliihoelaflea* does not have a lot of information about its characteristics. To *Actinobacteriota* genus such as *Brevibacterium, Brachybacterium, Rubrobacter, Sanguibacter, Dietzia, Isoptericola, Corynebacterium, Gordonia* and *Pseudokineococcus* these are gram-positive bacteria with higher potential in biotechnology for production of enzymes like proteases and lipases, bioremediation by the degradation of organic matter or elimination of soil contaminants, food preservation due to antimicrobial properties and potential as probiotics (Gharibzahedi et al., 2014; Mahmoud & Kalendar, 2016; Polivtseva et al., 2020). In *Firmicutes* genus such as *Turicibacter, Paenibacillus, Brevibacillus, Straphylococcus, Romboutsia* and *Clostridium sensu stricto 1* these are gram-positive bacteria associated to fermentation of dietary fibers and the production of beneficial metabolites, may have potential applications as probiotics, in bioremediation efforts to clean up contaminated environments by degrading pollutants, such as heavy metals, pesticides, and hydrocarbons and bioprocessing industries, including the production of enzymes, antibiotics, and biofuels (Gao et al., 2021; Gerritsen et al., 2009.; Lal & Tabacchioni, 2009; Panda et al., 2014). For *Cyanobacteria* and *Chlroflexi* the genus associated are mentioned as unknown. Regarding the hepatopancreas there was also bacteria associated to Furnas in this case belonging to phylum *Proteobacteria, Firmicutes, Verrucomicrobiota, Actinobacteriota*

and *Nitrospirota.* Starting with *Proteobacteria* the genus associated to it were *Acinetobacter* known as a gram-negative bacterium that can be used in biotechnology for the production of enzymes and bioactive compounds, they produce a variety of enzymes with industrial applications, including lipases and proteases and some strains are explored to produce antibiotics, antimicrobial agents, and other bioactive compounds (Phillips et al., 2020; Snellman & Colwell, 2004). For *Firmicutes* there was *Brochothrix, Lactobacillus* and *Steptococcus,* these are gram-positive bacteria used in biotechnological processes, including the production of enzymes and other bioactive compounds*. Brochothrix* contribute to the spoilage of food products, including meat, poultry, and dairy (Phillips et al., 2020; Russo et al., 2006; Shareck et al., 2004). For *Verrucomicrobiota* these was *Candidatus udaeobacter* is typically found in soil environments, may play a role in soil microbiota, contribute to biogeochemical cycles, such as the cycling of carbon, nitrogen, and other essential elements and as a soil bacteria interact with plants, influencing plant health and nutrient uptake (Böhmer et al., 2020; Brewer et al., 2016). As an *Actinobacteriota* there was *Blastococcus, Arthrobacter, Ornithinimicrobiur* and *Marmoricola* these are gram-positive bacteria with Biotechnological applications on production of enzymes and bioactive compounds. In the case of *Arthrobacter* it also promotes plant growth and protect plants against pathogens (Evtushenko, 2015; Manzanera et al., 2015; Stackebrandt & Schumann, 2015). For *Nitrospirota* the genus associated was *Nitrospira* a gram-negative bacteria known as nitrifying bacteria because they are responsible for the oxidation of ammonia and nitrite, it can used in agriculture as a fertilizer and facilitate nitrification and reduce ammonia levels in wastewater treatment (Latocheski et al., 2022). Most of bacteria associated to the geothermal field of Furnas still classified as unknow more studies focused on the cultivation, identification, basic physiology, taxonomy, and biotechnological potential of this thermophilic microorganisms should be done to allow comparison to microbial communities at other locations, such as Yellowstone National Park or the Rehai geothermal field of Tengchong (Hedlund et al., 2012).

5.Conclusion

The active volcanic environment at Furnas act as a multifactorial stress challenge to the local soil-dwelling organisms. It has been exemplified in invertebrates such as in earthworm, *Amynthas gracilis* (Cunha et al., 2011) and moth larvae, *Pseudaletia uninpuncta* (Rodrigues et al., 2008). *P. Sexfasciatus* belonging to Furnas exhibits morphological changes in the digestive tract, more precisely in the anterior chamber of the hindgut where enzymes activity take place during digestion. In this section ventral cells had a lower height.

The microbial part of this research did not demonstrate variations between sites, in bacterial composition and diversity. However, not much is known about isopods microbiome in general. Also, the geothermal field of Furnas is inside the village, the anthropogenic presence can be a challenge to understand the micro-environment without for instance agriculture contamination with compost.

Regarding the bacteria composition of the hindgut and hepatopancreas of isopods from Furnas there was a high value of unknown bacteria families' studies focused on the cultivation, identification, basic physiology, taxonomy, and biotechnological potential of this thermophilic microorganisms should be done.

For further research, it would be essential to analyzed soil samples microbiome, do again metals, pH and temperature measurements. The soil microbiome could be compared to hindgut/hepatopancreas and feaces microbiome has in other studies to have a better idea of the isopod/environment microbiome association. Also, transcriptomics could be applied to better understand the bacteria-host association to better characterize this bacteria function on isopods fitness to this environment. Biochemical approaches would be interesting to determine different metals influence on cells. Due to the anthropogenic presence close to the geothermal field in Furnas similar studies could be done in more natural locations to mitigate also potential contamination with compost and pesticides used in agricultural practices.

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7.Supplemental Material

Table 1.2 Richness and diversity indices (mean ± SE) from hindgut tissues from 4 different locations (Furnas, Lagoa, Fajã de Baixo and Ribeirinha. Different superscript letters indicate significant differences.

Sample type: Hindgut			
Sample Location	**Chao 1 Richness Estimator**	**Shannon Diversity Index**	**Simpson Diversity Index**
Furnas	2214 ± 338.28^{a}	6.28 ± 0.59^{a}	0.99 ± 0.006^{a}
Lagoa	1659.6 ± 213.54^{b}	5.71 ± 0.31^{a}	0.98 ± 0.008^{a}
Ribeirinha	814.6 ± 124.54^{c}	3.41 ± 0.64^{b}	0.82 ± 0.155^{b}
Fajã de Baixo	1913.6 ± 401.06^{d}	5.23 ± 0.92^{a}	$0.93 \pm 0.07^{a,b}$

Table 1. 3 Richness and diversity indices (mean ± SE) from hepatopancreas tissues from 4 different locations (Furnas, Lagoa, Fajã de Baixo and Ribeirinha. Different superscript letters indicate significant differences.

Sample type: Hepatopancreas			
Sample Location	**Chao 1 Richness Estimator**	**Shannon Diversity Index**	**Simpson Diversity Index**
Furnas	$1268 \pm 288.43^{a,b}$	4.29 ± 1.13^{a}	0.86 ± 0.17^{a}
Lagoa	1618.5 ± 181.85^{a}	5.36 ± 0.07^{a}	0.98 ± 0.003^{a}
Ribeirinha	1012 ± 172.38^{b}	2.86 ± 0.25^{b}	0.71 ± 0.06^{b}
Fajã de Baixo	1678 ± 102.69^{a}	4.60 ± 0.40^{a}	0.90 ± 0.05^{a}

Table 1. 4 Bacterial community composition (%) in hindgut.

Phylum	***Acidobacteriota***	1.68	0.29	0.83	0.07
	Actinobacteriota	24.01	34.59	16.95	10.84
	Chloroflexi	1.65	0.85	1.08	0.17
	Cyanobacteria	0.91	5.23	5.56	2.96
	Firmicutes	13.65	23.13	18.66	29.45
	Myxococcota	1.72	0.55	1.06	0.12
	Planctomycetota	2.42	1.06	1.71	0.65
	Proteobacteria	45.98	30.06	37.98	53.30
	Verrucomicrobiota	2.12	2.54	12.42	0.71
	Unknown	5.86	1.70	3.74	1.74
Family	***Streptomycetaceae***	4.56	20.64	3.95	1.71
	Bacillaceae	9.45	15.82	5.75	1.83
	Paenibacillaceae	5.11	6.41	13.13	1.68
	Rhodobacteraceae	7.98	5.69	4.74	5.70
	Comamonadaceae	9.91	4.92	4.64	1.70
	Diplorickettsiaceae	7.31	6.85	13.15	61.80
	Moraxellaceae	9.11	3.92	6.67	3.44
	Pseudomonadaceae	9.13	3.54	4.43	1.72
	Simkaniaceae	2.53	3.25	16.76	0.97
	Unknown	34.91	28.98	26.78	19.44

Table 1.5 Bacterial community composition (%) in hepatopancreas.

Hepatopancreas: Percentage (%)		**Furnas**	**Lagoa**	**Faja de Baixo**	**Ribeirinha**
Phylum	***Acidobacteriota***	0.50	0.41	0.64	0.26
	Actinobacteriota	8.43	20.04	12.71	7.51
	Bacteroidota	2.10	1.50	1.09	0.08
	Chloroflexi	0.35	0.63	0.73	0.31
	Cyanobacteria	1.00	1.36	1.77	0.36
	Firmicutes	30.14	19.77	12.10	26.24
	Myxococcota	0.27	0.42	0.70	0.32
	Proteobacteria	50.40	51.90	57.55	61.63
	Verrucomicrobiota	1.13	1.66	10.28	0.28
	Unknown	5.70	2.30	2.44	3.01
Family	***Bacillaceae***	3.41	5.87	2.53	1.13
	Entomoplasmatales Incertae Sedis	29.26	1.97	0.72	22.67
	Rhodobacteraceae	9.08	4.21	4.00	1.44
	Candidatus Hepatincola	2.77	0.30	0.15	16.82
	Comamonadaceae	10.69	5.76	14.37	1.19
	Diplorickettsiaceae	3.99	7.93	9.65	44.04
	Moraxellaceae	12.25	15.59	26.52	1.32
	Pseudomonadaceae	5.29	11.71	4.96	1.26
	Simkaniaceae	0.52	2.01	14.31	0.09
	Unknown	22.73	44.65	22.80	10.04

Table 1.6 DESeq2 plots additional information, comparing microbial composition in hindgut; graph (A).

GUT	OTUs	baseMean	log2FoldChang	lfcSE	stat	pvalue	padj	domain	phylum	class	order	family	genus
	TACGTAG(	104.2857788	-23.55346754	3.454542877	-6.81811411	9.22434E-12	7.95343E-10	Bacteria	Actinobacteriota	Actinobacteria	Propionibacteriales	Nocardioidaceae	#N/A
	TACAGAG(	96.25464178	-23.43638694	3.254792498	-7.200577903	5.99579E-13	1.16318E-10	Bacteria	Cyanobacteria	Cyanobacteriia	Chloroplast	#N/A	#N/A
	TACGTAG(	64.99900203	-22.86242672	3.015885816	-7.580667214	3.43782E-14	8.9056E-12	Bacteria	Actinobacteriota	Actinobacteria	Kineosporiales	Kineosporiaceae	Pseudokineococcus
	TACGTAG(	67.33161353	-22.74169905	3.000035624	-7.580476334	3.44289E-14	8.9056E-12	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	#N/A	#N/A
	TACGGAG(	74.50355696	-22.64970267	2.919720558	-7.757489874	8.66268E-15	6.72224E-12	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	#N/A
	TACGTAG(	56.00547545	-22.60685373	3.232106337	-6.994464716	2.66274E-12	3.17757E-10	Bacteria	Actinobacteriota	Rubrobacteria	Rubrobacterales	Rubrobacteriaceae	Rubrobacter
	TACGTAG(	50.95289315	-22.49610593	3.191678532	-7.048362076	1.81036E-12	2.80968E-10	Bacteria	Actinobacteriota	Actinobacteria	Corynebacteriales	Nocardiaceae	Gordonia
	TACGTAG(	43.08106725	-22.31646728	3.454776795	-6.459597423	1.04982E-10	7.40599E-09	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium sensu stricto 1
	TACGTAG(	39.6036018	-22.20597865	3.179493162	-6.984125306	2.86636E-12	3.17757E-10	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	#N/A	#N/A
	TACAGAG(	67.7917805	-21.74955394	3.454631488	-6.295766715	3.05884E-10	1.97805E-08	Bacteria	Proteobacteria	Gammaproteobacteria	Diplorickettsiales	Diplorickettsiaceae	Rickettsiella
	TACGTAG(	27.16780414	-21.67702124	3.455010153	-6.274083225	3.517E-10	2.09938E-08	Bacteria	Actinobacteriota	Actinobacteria	Corynebacteriales	Corynebacteriaceae	Corynebacterium
	TACGTAG(	18.01007788	-20.88650828	3.455330175	-6.044721407	1.49668E-09	8.29591E-08	Bacteria	Actinobacteriota	Actinobacteria	Streptomycetales	Streptomycetaceae	#N/A
	TACGTAG(	44.33776272	-20.68509231	2.989808109	-6.918535087	4.56338E-12	4.42648E-10	Bacteria	Firmicutes	Clostridia	Peptostreptococcales-Tissierellale	Peptostreptococcaceae	Romboutsia
	TACGTAG(	26.64509933	-19.64884336	3.455022969	-5.68703697	1.29262E-08	6.68718E-07	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Microbacteriaceae	#N/A
	TACGTAG(	301.6070377	-11.11115055	2.191630384	-5.069810417	3.98212E-07	1.93133E-05	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
	TACGTAG(	216.3029147	-10.63130068	1.621644741	-6.555875284	5.53164E-11	4.29256E-09	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Micrococcaceae	#N/A
	TACGTAG(	111.5671794	-9.675982237	2.271689003	-4.259378034	2.04997E-05	0.000883763	Bacteria	Firmicutes	Bacilli	Staphylococcales	Staphylococcaceae	Staphylococcus
	TACGTAG(	52.81646077	-8.598243596	2.15247538	-3.994583946	6.4808E-05	0.002394808	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	#N/A
	GACAGAG	47.63755301	-8.449195327	2.520916303	-3.351636592	0.000803354	0.02078009	Bacteria	Cyanobacteria	Cyanobacteriia	Chloroplast	#N/A	#N/A
	TACGAAG(	47.26442898	-8.437991623	2.50558022	-3.367679692	0.000758036	0.020284	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Ochrobactrum
	CACGGAG	45.8753179	-8.395135777	2.681011414	-3.131331606	0.001740155	0.035262611	Bacteria	Cyanobacteria	Cyanobacteriia	Chloroplast	#N/A	#N/A
	TACGTAG(	45.19261059	-8.371157553	2.254578879	-3.712958385	0.000204851	0.006766935	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Promicromonosporaceae	Isoptericola
	TACGTAG(	43.24871573	-8.308247539	1.846434461	-4.499616809	6.80761E-06	0.000310747	Bacteria	Actinobacteriota	Actinobacteria	Streptomycetales	Streptomycetaceae	#N/A
Furnas	TACGTAG(	42.03908162	-8.268931262	2.704682256	-3.05726532	0.002233665	0.040309856	Bacteria	Firmicutes	Bacilli	Brevibacillales	Brevibacillaceae	Brevibacillus
	TACGTAG(	41.25717892	-8.240770161	2.312021329	-3.564314074	0.000364809	0.010484879	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Brevibacteriaceae	Brevibacterium
	TACGAAG(	40.40279973	-8.212305278	1.980507946	-4.146565175	3.375E-05	0.001378422	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	#N/A
	TACGAAG(	40.33587575	-8.209315954	2.214224912	-3.707534817	0.000209287	0.006766935	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Aliihoeflea
	TACGTAG(	39.99073157	-8.197392837	2.101847643	-3.900088983	9.61573E-05	0.003391732	Bacteria	Firmicutes	Bacilli	Paenibacillales	Paenibacillaceae	Paenibacillus
	TACGTAG(	39.12988229	-8.165839131	2.652005094	-3.079118947	0.002076138	0.038359116	Bacteria	Firmicutes	Bacilli	Bacillales	#N/A	#N/A
	TACGTAG(	30.34324284	-7.79786468	2.271720921	-3.432580389	0.000597867	0.016569446	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Brevibacteriaceae	Brevibacterium
	TACGTAG(	28.84733356	-7.726794574	1.917154628	-4.030345003	5.5695E-05	0.002160968	Bacteria	Firmicutes	Bacilli	Paenibacillales	Paenibacillaceae	#N/A
	TACGTAG(	28.18195881	-7.692045934	2.323652432	-3.310325515	0.000931875	0.021913189	Bacteria	Actinobacteriota	Actinobacteria	Corynebacteriales	Dietziaceae	Dietzia
	TACGTAG(	27.30086249	-7.647411913	2.079527009	-3.677476599	0.000235553	0.007311555	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Sanguibacteraceae	Sanguibacter
	GACAGAG	26.48386695	-7.600500797	2.512155296	-3.025490028	0.002482306	0.042278382	Bacteria	#N/A	#N/A	#N/A	#N/A	#N/A
	TACGAAG(	26.20267055	-7.587378393	2.289300258	-3.314278398	0.0009188	0.021913189	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Ochrobactrum
	TACGTAG(	25.97583881	-7.575705288	2.095858588	-3.614607078	0.000300803	0.008977827	Bacteria	Firmicutes	Bacilli	Bacillales	#N/A	#N/A
	TACGTAG(	24.54642086	-7.493217239	2.482235845	-3.018737021	0.002538308	0.042278382	Bacteria	Firmicutes	Bacilli	#N/A	#N/A	#N/A
	TACGTAG(	49.85245096	-7.355390956	2.387755188	-3.080462768	0.002066792	0.038359116	Bacteria	Actinobacteriota	Rubrobacteria	Rubrobacterales	Rubrobacteriaceae	Rubrobacter
	TACGTAG(	20.75086449	-7.250641676	2.402637831	-3.017783863	0.002546304	0.042278382	Bacteria	Firmicutes	Bacilli	Erysipelotrichales	Erysipelotrichaceae	Turicibacter
	TACGTAG(	20.36975295	-7.224935206	2.23367167	-3.234555599	0.001218322	0.027011939	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Microbacteriaceae	#N/A
	TACGTAG(	17.26260072	-6.986949404	2.209709272	-3.161931522	0.001567264	0.033783249	Bacteria	Chloroflexi	Chloroflexia	Thermomicrobiales	JG30-KF-CM45	#N/A
	TACGGAG(	16.3319595	-6.906767243	2.194580697	-3.147192196	0.001648466	0.034573226	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Altererythrobacter
	TACGTAG(	102.8716987	-6.279739705	2.12495563	-2.955233331	0.003124327	0.049479132	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Dermabacteraceae	Brachybacterium
	TACGAAG(	122.5011412	-6.09940487	1.962774985	-3.107541576	0.001886504	0.036598187	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	#N/A
	TACAGAG(	336.5901054	-5.616986751	1.72784734	-3.250858234	0.001150572	0.026260117	Bacteria	Cyanobacteria	Cyanobacteriia	Chloroplast	#N/A	#N/A
	TACGTAG(	212.9494442	-5.287938721	1.691617339	-3.125966256	0.001772219	0.035262611	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	#N/A
	TACGTAG(	151.5516615	-2.925229662	0.969878706	-3.01607783	0.002560675	0.042278382	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Brevibacteriaceae	Brevibacterium
No Geothermal activity	TACGAAG(	17.44713703	8.508963373	2.555506469	3.329658319	0.000869526	0.021766203	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	#N/A
	TACGGAG(	18.28474339	8.576648364	2.888062653	2.969689164	0.002981012	0.04819303	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium

Table 1.7 DESeq2 plots additional information, comparing microbial composition in hepatopancreas; graph (B).

HEPATO	OTUS	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	domain	phylum	class	order	family	genus
	TACGTAGGTG	172.3259814	-24.28297674	3.07516167	-7.896487842	2.86872E-15	2.57898E-12	Bacteria	Firmicutes	Bacilli	Lactobacillales	Listeriaceae	Brochothrix
	TACGAAGGGC	71.56248472	-23.00078284	3.146633889	-7.309646961	2.67845E-13	8.02643E-11	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	#N/A
	TACGGAGGGT	50.16131167	-22.56478494	3.086836071	-7.310004295	2.67134E-13	8.02643E-11	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	#N/A	#N/A
	TACAGAGGGT	13.61325429	-20.74782487	3.402209188	-6.09833897	1.07176E-09	2.40879E-07	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter
	TACGTAGGTG	194.9387095	-10.49162474	2.572891576	-4.077756262	4.54724E-05	0.005839955	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	#N/A
	TACGTAGGGT	72.2433796	-9.059121597	2.51164708	-3.606844954	0.000309943	0.020748291	Bacteria	Actinobacteriota	Actinobacteria	Propionibacteriales	Nocardioidaceae	Marmoricola
	TACGTAGGGT	63.90968835	-8.881999132	2.052863317	-4.326639314	1.51402E-05	0.002360905	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Intrasporangiaceae	Ornithinimicrobium
	TACGTAGGGC	45.69711413	-8.398973108	2.416681127	-3.475416353	0.000510061	0.030569658	Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	Micrococcaceae	Arthrobacter
Furnas	TACGTAGGTG	35.49994015	-8.033680253	2.458730073	-3.267410416	0.001085362	0.048787027	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus
	TACGGAGGGC	30.48049542	-7.814839161	2.370640401	-3.296509735	0.000978942	0.04838931	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	#N/A
	TACGTAGGGC	27.78164103	-7.677146999	1.917636894	-4.003441436	6.24277E-05	0.00623583	Bacteria	Actinobacteriota	Acidimicrobiia	IMCC26256	#N/A	#N/A
	TACGTAGGTC	733.4437981	-7.65346902	2.092691453	-3.657237195	0.000254948	0.019099887	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
	TACGAAGGTG	19.99936694	-7.207582434	1.884075459	-3.825527475	0.000130492	0.010664786	Bacteria	Nitrospirota	Nitrospiria	Nitrospirales	Nitrospiraceae	Nitrospira
	TACGTAGGGT	16.24218352	-6.904534112	1.708275747	-4.041814749	5.30391E-05	0.005960272	Bacteria	Actinobacteriota	Actinobacteria	Frankiales	Geodermatophilaceae	Blastococcus
	TACGTAGGGT	11.14291037	-6.365420395	1.938189463	-3.284209577	0.001022688	0.04838931	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	#N/A
	TACAGAGGTC	9.888035193	-6.180350667	1.71865924	-3.59603028	0.00032311	0.020748291	Bacteria	Verrucomicrobiota	Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	Candidatus Udaeobacte
	TACGTAGGTG	511.253182	-5.505979113	1.392726018	-3.953382819	7.7054E-05	0.006927155	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	#N/A
No	TACGAAGGGT	113.1844064	3.817109195	1.138776889	3.351937708	0.000802481	0.042437069	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
Geothermal	TACGGAGGGT	104.7641368	4.982356729	1.153902201	4.317832765	1.57569E-05	0.002360905	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanella
activity	TACGGAGGGT	20.37309559	8.728850067	2.536598268	3.441163773	0.000579218	0.032544802	Bacteria	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	Legionella