# UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

**Escuela de Ciencias Biológicas e Ingeniería**

# TÍTULO: Bacteria associated with sessile marine invertebrates as a source of active metabolites for their application in human and animal health

Trabajo de integración curricular presentado como requisito para la obtención del título de Biólogo

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Urcuquí, Diciembre 2021

Urcuquí, 6 de diciembre de 2021

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*A mi familia.*

Maily Selena González Avilés

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# Resumen

En las últimas décadas, los invertebrados sésiles marinos han sido el foco de atención en la búsqueda de potenciales aplicaciones industriales y farmacéuticas. Esponjas y zoantidos han sido reportados como buenos candidatos para extracción de compuestos bioactivos, útiles para tratar enfermedades. Particularmente, los zoantidos bien representados en la Reserva Marina el Pelado (REMAPE), han mostrado un gran potencial de compuestos bioactivos como zoantaminas. Sin embargo, los microorganismos asociados han sido poco explorados. En este contexto, este trabajo tuvo como objetivo aislar bacterias asociadas a *Zoanthus pulchellus* mediante técnicas microbiológicas, bioquímicas, identificación molecular y análisis bioinformático para identificar cada género bacteriano. Se lograron identificar 13 aislados bacterianos, pertenecientes a las especies *Pseudovibrio denitrificans*, *Vibrio harveyi*, *Tenacibaculum mesophilum*, *Microbulbifer variabilis*, *Ferrimonas kyonanensis*, *Pseudoalteromonas luteoviolacea*, *Agarivorans litoreus* y *Bacillus sp*. Los aislados bacterianos fueron cultivados, se realizaron extractos y fracciones y se evaluaron frente a vibrios patógenos del camarón, *Vibrio harveyi* (cepa E22), *Vibrio campbellii* (cepa LM2013), *Vibrio vulnificus* (cepa S2), y *Vibrio parahaemolyticus* (cepa BA94C2, positivo para las toxinas PirA/PirB). Los extractos crudos y los sobrenadantes de cultivo no mostraron actividad microbicida hacia los vibrios evaluados. Sin embargo, cuando se evaluó la capacidad de las bacterias para excluir los vibrios patógenos en un ensayo de exclusión competitiva, usando tres concentraciones ($10^4$, $10^5$ y $10^6$ UFC $mL^{-1}$), los mejores resultados se obtuvieron con las bacterias Z2, Z13 y Z14 mostrando efectos inhibitorios hacia los vibrios patógenos. Además, evaluamos la capacidad de los extractos a dosis no microbicidas para interrumpir la formación de biopelículas en *Vibrio parahaemolyticus*, causante de la enfermedad de la necrosis aguda del hepatopáncreas (AHPND por sus siglas en inglés). Tres de estos extractos (Z1, Z3 y Z16) lograron inhibir significativamente la formación de biopelículas.

Los extractos y fracciones de las bacterias obtenidas a partir de UPLC-DAD-ELSD se evaluaron también frente a tres líneas celulares de carcinoma humano MCF–7 (cáncer de mama), Caco–2 (cáncer de colon) y Hep-G2 (cáncer de hígado). Las líneas tumorales se cultivaron en medios de cultivo estándares y la afectación por parte de las fracciones fue

determinada mediante el ensayo de viabilidad celular por reducción del compuesto Bromuro de 3-(4,5-Dimetiltiazol-2-ilo)-2,5-difeniltetrazol (MTT). La fracción (F-3) del aislado codificado Z10 (*Pseudoalteromonas luteoviolacea*) mostró un efecto citotóxico significativo ($P$ <0.05) respecto al grupo control (células sin extractos). La toxicidad se explica debido a que no se evidenció viabilidad celular, y esto fue observado para las tres líneas celulares evaluadas. Estos resultados demuestran que las bacterias asociadas a *Zoanthus pulchellus* tienen potencial para ser usadas en salud humana y animal, debido a que mostraron actividades microbicidas cuando se evaluaron frente a patógenos de camarón y de forma similar demostraron actividad citotóxica cuando se evaluaron frente a líneas tumorales.

**Palabras claves:** bacterias simbiontes, camarón, carcinoma, compuestos bioactivos, vibrios patógenos, zoantidos.

# Abstract

In recent decades, sessile marine invertebrates have been the focus of attention in the search for potential industrial and pharmaceutical applications. Sponges and zoanthids have been reported as promising candidates for the extraction of bioactive compounds, useful for treating diseases. In particular, well-represented zoanthids in El Pelado Marine Reserve (REMAPE) have shown great potential for bioactive compounds such as zoanthamines. However, the associated microorganisms have been little explored. In this context, this work aimed to isolate bacteria associated with *Zoanthus pulchellus* using microbiological and biochemical techniques, molecular identification and bioinformatic analysis techniques to identify each bacterial species. Thirteen bacterial isolates were identified, belonging to the species *Pseudovibrio denitrificans, Vibrio harveyi, Tenacibaculum mesophilum, Microbulbifer variabilis, Ferrimonas kyonanensis, Pseudoalteromonas luteoviolacea, Agarivorans litoreus* and *Bacillus spp*. Bacterial isolates were cultured, extracts and fractions were made and evaluated against shrimp pathogenic vibrios, including *Vibrio harveyi* (strain E22), *Vibrio campbellii* (strain LM2013), *Vibrio vulnificus* (strain S2), and *Vibrio parahaemolyticus* (strain BA94C2, positive to PirA/PirB toxins). The crude extracts and the culture supernatants did not show microbicidal activity towards the evaluated vibrios. However, when the ability of bacteria to exclude pathogenic vibrios was evaluated in a competitive exclusion test, using three concentrations ($10^4$, $10^5$ and $10^6$ CFU $mL^{-1}$), the best results were obtained with bacteria Z2, Z13 and Z14 showing inhibitory effects towards pathogenic vibrios. In addition, we evaluated the ability of extracts at non-microbicidal doses to interrupt biofilm formation in *Vibrio parahaemolyticus*, which causes acute

hepatopancreas necrosis disease (AHPND). Three of these extracts (Z1, Z3 and Z16) were able to significantly inhibit biofilm formation.

The extracts and fractions obtained from UPLC-DAD-ELSD were also evaluated against three human carcinoma cell lines MCF-7 (breast cancer), Caco-2 (colon cancer) and Hep-G2 (cancer of the liver). The tumor lines were cultured in standard culture media and the affectation by the fractions was determined by means of the cell viability assay by reduction of the compound 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The fraction (F-3) of the isolate encoded Z10 (*Pseudoalteromonas luteoviolacea*) showed a significant cytotoxic effect ($P < 0.05$) compared to the control group (cells without extracts). The toxicity is explained because cell viability was not evidenced, and this was observed for the three cell lines evaluated. These results demonstrate that the bacteria associated with *Zoanthus pulchellus* have the potential to be used in human and animal health, since they showed microbicidal activities when evaluated against shrimp pathogens and similarly demonstrated cytotoxic activity when evaluated against tumor lines.

**Keywords:** bioactive compounds, carcinoma, pathogenic vibrios, shrimp, symbiotic bacteria, zoanthids.

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# INTRODUCTION

The problems associated with pathogens in both human and animal health have led to the exploration of other little-explored environments in the search for new bioactive compounds. In this context, marine organisms in recent decades have been the focus of attention of many researchers, because a large percentage of the extracts evaluated have been shown to have potential industrial and pharmaceutical applications (König et al. 2005; Liu et al. 2019; Nunnery et al. 2010; Tan et al. 2013; Singh et al. 2017; Jiménez 2018). Marine biotechnology stick out the importance of product development from marine organisms as a multidisciplinary and multidimensional research (Fig. 1). Globally, more than a thousand pharmacologically active compounds of marine origin have been characterized (Carroll et al. 2019; Deshmukh et al. 2017; Jiménez 2018; Leal et al. 2016; Newman and Cragg 2016b). This illustrates the great diversity and richness of active molecules that the marine environment harbors, and much of this molecule diversity is explained by being a highly competitive environment, where, particularly sedentary species such as sponges, tunicates and zoanthids (Mayer et al. 2009; Mayer et al. 2007; Mayer and Hamann 2002; Orhan et al. 2010; Simmons et al. 2005) are the organisms commonly selected for screening for bioactive molecules. Among the most prominent biological properties recorded for marine invertebrates are cancer, viruses, bacteria, fungi, hypertension, high cholesterol, and other diseases (Wang 2006; Huang et al. 2017; Rao et al. 2006; Lavilla-Pitogo et al. ., 1998; Chen et al. 2013).

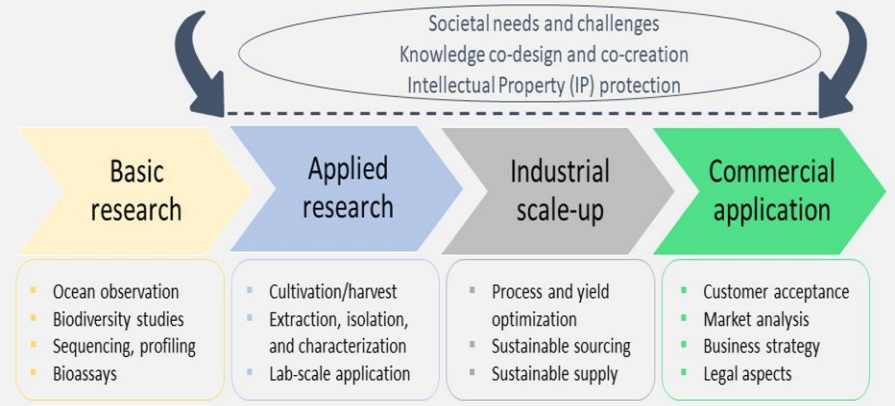



*Figure 1. A simplified depiction of the marine biotechnology pipeline, which combine research, industry and business sectors. (Rotter et al., 2021).*

Cancer is the second leading cause of death globally (Nagai and Kim, 2017; Rahman et al., 2020), so finding new molecules with antitumor activity is very promising. Another high priority area is the identification of new antimicrobial agents capable of counteracting multiresistant pathogenic bacteria (Singer et al., 2003; Magiorakos et al., 2012). This multidrug resistance requires efforts to find new effective antimicrobials from alternative sources. In the last decade, this has included the development of new antimicrobials from marine organisms. In this area, many organisms of marine origin are exposed to different challenges that stimulate the production of biologically active molecules as defense mechanisms (Gogineni et al., 2018).

Ecuador is one of the most megadiverse countries worldwide, offering a high potential for opportunities in the bioprospecting of active substances (Gaibor, 2002). Because of its geographical position, the country hosts great marine diversity, including algae, sponges, cnidarians, mollusks, crustaceans, echinoderms, fish, among others. Many of these species potentially are sources of new bioactive compounds. In particular, the zoanthids (Cnidaria, Order Zoantharia), marine invertebrates that are widely distributed throughout the Indo and Pacific oceans, and are known to biosynthesize a wide array of natural products with unique structural features and interesting bioactivity, such as zoanthamines (Behenna et al., 2008; Fukuzawa et al., 1995; Guillen et al., 2018; Rao et al., 1984). Zoanthids generate a wide range of micro-niches for microorganisms such as bacteria, viruses and fungi (Bourne and Munn, 2005), including yeasts (Bastos 2017). Several studies report a close association between microorganisms and zoanthid hosts.

Various genera of actinobacteria have been reported to be associated with zoanthids. Considering that many of them are producers of antibiotics, they produce bioactive compounds that protect the zoanthid host against pathogens (Sun et al., 2014). This symbiotic association between microorganisms and marine invertebrates can benefit hosts by protecting them from disease (for example, by producing killer yeast toxins) and providing nutrients (Bourne et al., 2016, Ritchie, 2006). These observations point to the fundamental role of associated bacteria and their bioactive potential of zoanthids symbiont bacteria as a new source of active metabolites that deserve attention for research focused on marine bioprospecting. Recently, Guillen et al. (2018) described that the zoanthid species *Zoanthus pulchellus*, present in REMAPE, registers a high concentration of zoanthamines, whose

medical applications can be broad. The objective of the present study was to isolate the bacteria associated with *Z. pulchellus* (Figure 2) and to determine the bioactive potential of the extracts by screening human carcinoma lines and pathogenic shrimp vibrios.

*Figure 2. Zoanthus pulchellus isolated from REMAPE reserve.*

# HYPOTHESIS AND OBJECTIVES

## Hypothesis

Bacteria associated with *Zoanthus pulchellus* are producers of bioactive compounds for human carcinoma cell lines and pathogenic shrimp vibrios.

## General objective

Assess the potential of symbiotic bacteria associated with the *Zoanthus pulchellus* that occurs at REMAPE, as potential sources of bioactive compounds for human and animal health.

## Specific objectives

1. Isolate and identify symbiotic bacteria associated to *Zoanthus pulchellus*.
2. Determine the bioactivity of symbiotic bacteria extracts against the pathogenic shrimp vibrios: *Vibrio harveyi*, *V. vulnificus*, *V. campbelli* and *V. parahaemolyticus*.
3. Determine the bioactivity of symbiotic bacteria fractions against human carcinoma cell lines MCF–7 (breast cancer), Caco–2 (colon cancer) and Hep-G2 (liver cancer).

# THEORETICAL BACKGROUND

## Zoanthids: characteristics and diversity

The Phylum Cnidaria is a diverse group of more than 13,000 species described so far, including jellyfish, hydras, anemones, and corals. Cnidaria comes from the Greek meaning nettle. Cnidarians have cells called cnidocytes which serve to capture and fix their prey. They are also involved in defense and movement. Cnidarians are generally colonial organisms but can also be individual (Reimer and Hickman, 2009). Cnidarians have two forms of life, in the larval stage they are polyps, and in the adult stage they are jellyfish (Low et al., 2016). They have radial symmetry and have tentacles or stinging structures called cnids. The nervous system consists of two nerve networks, and it lacks a respiratory and circulatory systems (Snyder et al., 2021).

The taxonomy of the Cnidarians is divided into two large groups, the Medusozoa Class, which in turn is divided into the Hydrozoa, Cubozoa, Scyphozoa, Staurozoa, and Myxozoa Subclasses and the Anthozoa Class that present organisms with only the polyp phase and that contains the Subclasses Octocorallia, Ceriantharia and Hexacorallia (Fig. 3) (Low et al., 2016). In particular, the Hexacorallia Subclass is an ecologically important group because in this clade is the Order Zoantharia, organisms that live in symbiosis with microalgae, dinoflagellates also known as Zooxanthellae, this symbiosis provides carbohydrates to corals, as well as some secondary metabolites of importance in human medicine (Reimer and Hickman, 2009).

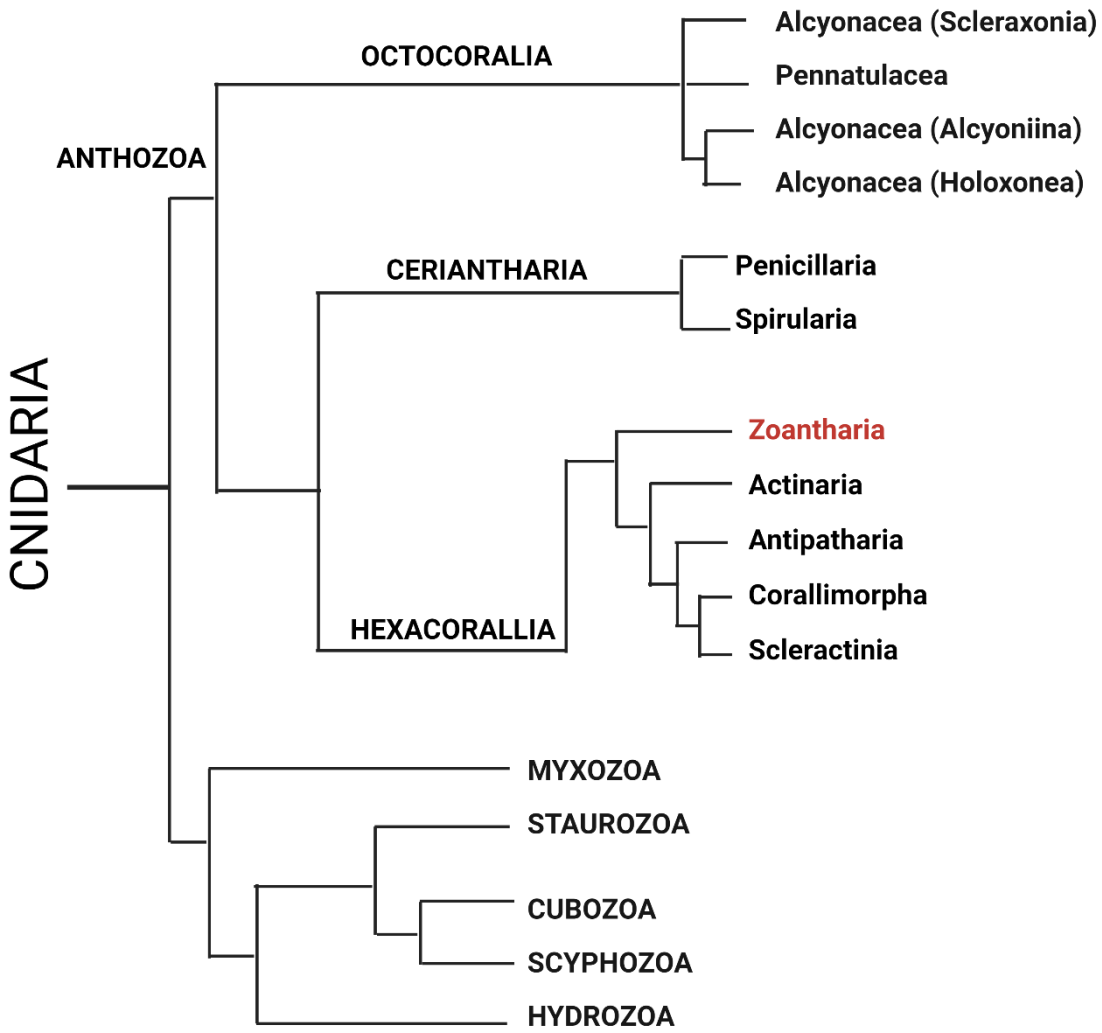



*Figure 3. Phylogenetic tree of Cnidaria phylum, taken and modified from Karla Jaramillo (2018).*

In zoanthids, polyps form colonies generated from a basal mass or stolon, from which new polyps arise. Zoantharians have a flat pharynx with a syphonoglyph, and they have numerous mesenteries with weak musculature. They have an oral disc in which there are tentacles formed in two groups. Some species incorporate sand grains or some other debris in their wall, generally have a thick cuticle, and particularly there is a great abundance of zooxanthellae algae (Santos and Reimer, 2018). Zoanthids are relatively small organisms, most of which do not exceed 1 cm in length. They have an oral disc free of tentacles except for two rings on their outer edge, which is a characteristic that distinguishes them from anemones (Low et al., 2016). Their variety of iridescent colors are a warning of their toxicity.

Zoanthids are mainly found in marine areas with great lighting, which is important due to their association with zooxanthellae algae. They require light to carry out photosynthesis. In addition, they present other associations with other organisms such as sponges, crabs and starfish. The most important genera are *Zoanthus* and *Palythoa* due to their abundance and their ecological role (a large number of organisms associated with them) (Low, Sinniger and Reimer, 2016). Despite their great importance and ecological role of zoanthids, they have been little attended and only a few studies have described the presence of certain specimens in specific regions. In particular in Ecuador, James and Dickman (2009) published a work on the zoanthids of the Galapagos Islands associated with the dinoflagellate *Symbiodinium*. This study identified species belonging to the genus *Zoanthus* of the family Zoanthidae and species of the genus *Palythoa* of the family Sphenopidae; both genera were identified on the island of Santa Cruz and the islet Espejo, while on the island Marchena only the genus *Zoanthus* was present. An important characteristic in this work is that no non-zooxanthellate zoanthids were found in the places studied. On the other hand, the zoanthids identified here were found mainly attached to rocks. The species found were *Zoanthus sansibaricus*, *Zoanthus vietnamensis/kuroshio*, *Palythoa mutuki* and *Palythoa tuberculosa* (Fig. 4)

Another work that denotes the presence of zoanthids on continents has recently been published by Jaramillo et al. (2018), who describe the diversity of zoantarians in the El Pelado marine protected area, in Ecuador. This work is quite interesting to describe zoanthids because it combines morphological identification, molecular analysis, and identification of secondary metabolites with the purpose of identifying six species of zoanthids. *Parazoanthus darminii, Antipathozoanthus hickmani, Terrazoanthus patagonicus, Terrazoanthus sp., Zoanthus pulchellus* and *Zoanthus sociatus* were the species identified (Fig. 5).

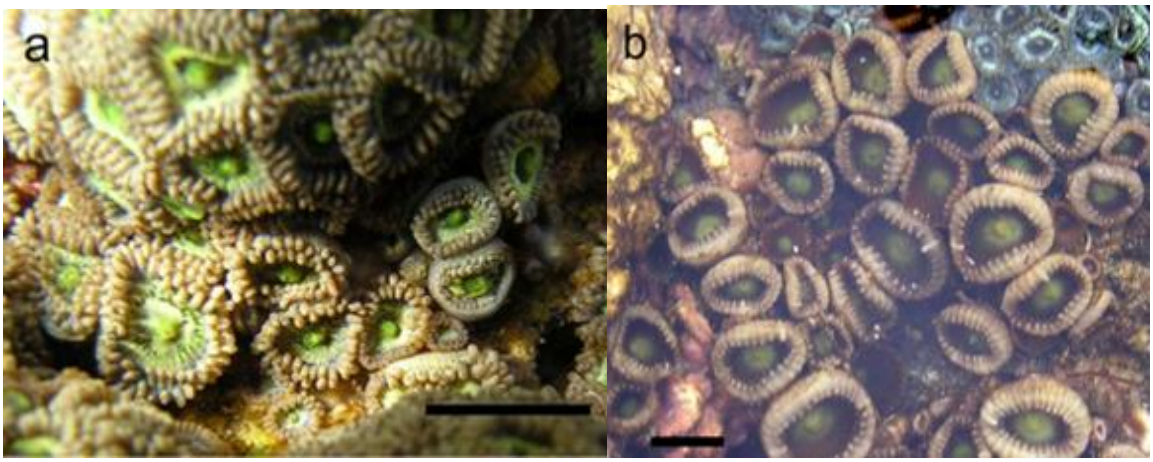



*Figure 4. Zoanthid zooxanthellated in the Galapagos, (a) Zoanthus and (b) Palythoa (James and Dickman, 2009).*

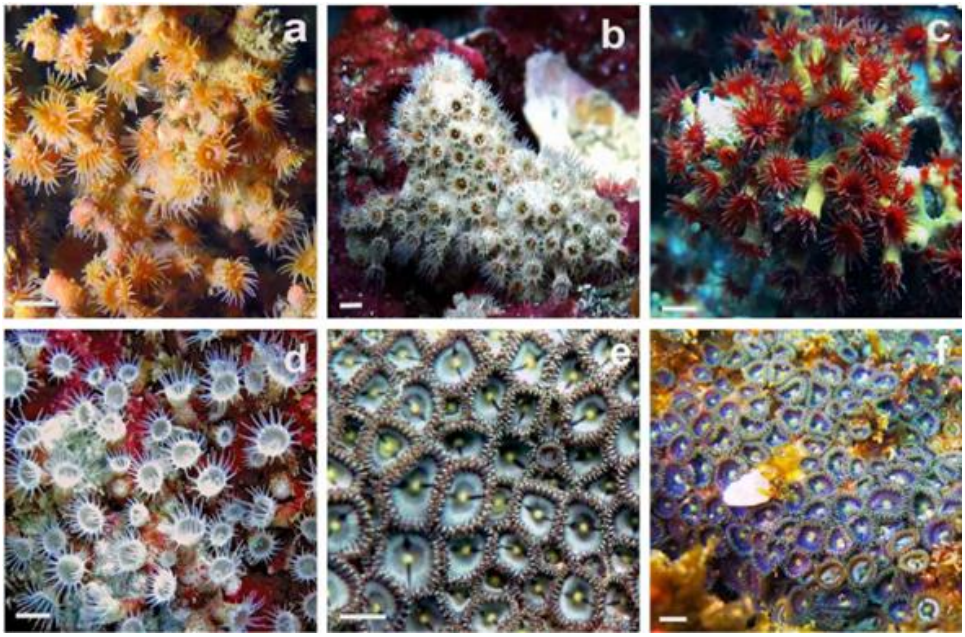



*Figure 5. Zoantharians identified in REMAPE reserve by Jaramillo et al. (2018): (a) Parazoanthus darwini, (b) Antipathozoanthus hickmani, (c) Terrazoanthus patagonichus, (d) Terrazoanthus sp., (e) Zoanthus cf. pulchellus, (f) Zoanthus cf. sociatus.*

## Chemical ecology of zoanthids

In environments where dissolved nutrients and particulate organic matter are scarce, a symbiosis between autotrophic and heterotrophic species has been seen. Sponges that feed on photosynthetically produced carbohydrates, which are high in carbon but low in nitrogen, may experience nitrogen deficiency. As a result, the idea that sea sponges provide an

ecological niche for nitrogen-fixing bacteria seems intriguing. In this way, symbiotic microorganisms may contribute to the nitrogen budget of sponges in oligotrophic environments with low nitrogen levels (e.g., coral reefs) by fixing atmospheric nitrogen $N_2$. The cyanobacteria were the first indication of N fixing in sponges with the discovery of the nitrogenase enzyme complex activity responsible for reducing N2. (Wilkinson, C. R., and P. Fay. 1979; Wilkinson, et al., 1984).

The ability of symbionts to fix nitrogen was proven in Red Sea sponges using the acetylene reduction test, and was attributed to cyanobacterial symbionts since identified nitrogenase activity in two sponge species with cyanobacteria but not in a third sponge species that did not (Wilkinson and Fay, 1979). This study adds to our understanding of the role of bacterial symbionts in sponges and shows that symbionts benefit sponges in nutrient-limited reef environments by providing fixed nitrogen. Nitrogen fixation by sponge symbionts is a possible major source of additional nitrogen to the reef ecosystem that has been overlooked thus far and deserves more study.

*Palythoa caribaeorum*, a Zoanthid, feeds primarily on diatoms and does not eat many other plankton-rich food. *Suspensivorous, P. caribaeorum* feeds primarily on tiny phytoplankton. This species is preyed upon by benthic invertebrates such as polychaetes and nektonic organisms. On the other hand, this zoanthid is an essential primary consumer in tropical reef ecosystems because it serves as an energy transfer link between the planktonic and nektonic spheres (De Santana et al. 2015).

Corals are relatively nutrient-poor. Many invertebrates, like corals, have developed a symbiotic relationship with the algae in their tissues to compensate for this lack of nutrients. Most stony corals have zooxanthellae, which are algae that live in symbiosis within the polyp cells, giving the corals shades ranging from brownish-yellow to dark brown. Zooxanthellae require sunlight to perform photosynthesis and thus obtain energy and nutrients. Part of the nutrients produced by the algae is transferred to the coral tissues and contributes to the production of calcium carbonate skeletons. The host, in return, provides the zooxanthellae with a protected environment.

When the coral-zooxanthellae connection is disrupted, bleaching occurs as a stress reaction, and zooxanthellae are ejected from the coral host or when the zooxanthellae

pigments are degraded. Because of zooxanthellae's absence, the white calcium carbonate coral skeleton can be seen through the transparent tissue, giving the coral a brilliant white or "bleached" appearance. Corals can survive for some time (days or months) without their zooxanthellae. Still, their ability to survive depends on the level and type of stress and the sensitivity of the coral. If stressors persist, corals can starve and die. Bleaching also occurs in other animals found in symbiosis with zooxanthellae, such as foraminifera, sponges, anemones, and gigantic clams.

**Zoanthids and their biotechnological potential**

Zoantarians or zoanthids, in recent years, have increased their studies due to their great ecological value, their economic importance in saltwater aquariums (due to their beautiful colors), and above all, because of the chemical diversity that has been discovered, to a great extent. Part of this chemical diversity is due to the symbiotic associations that they have with microorganisms such as bacteria, algae, and fungi, which have a very important role in the production of secondary metabolites. Compounds such as palytoxin, ecdysteroids, zoanthamines, and zoanthoxanthins have been identified (Chen et al., 2019). Although most of the studies in the search for new secondary metabolites have focused on organisms such as sponges and ascidians, zoanthids have recently gained great interest for the discovery of palytoxin, a powerful non-protein vasoconstrictor considered one of the most poisonous of the world, for vertebrates (Fig. 6) (Wu et al., 2019).

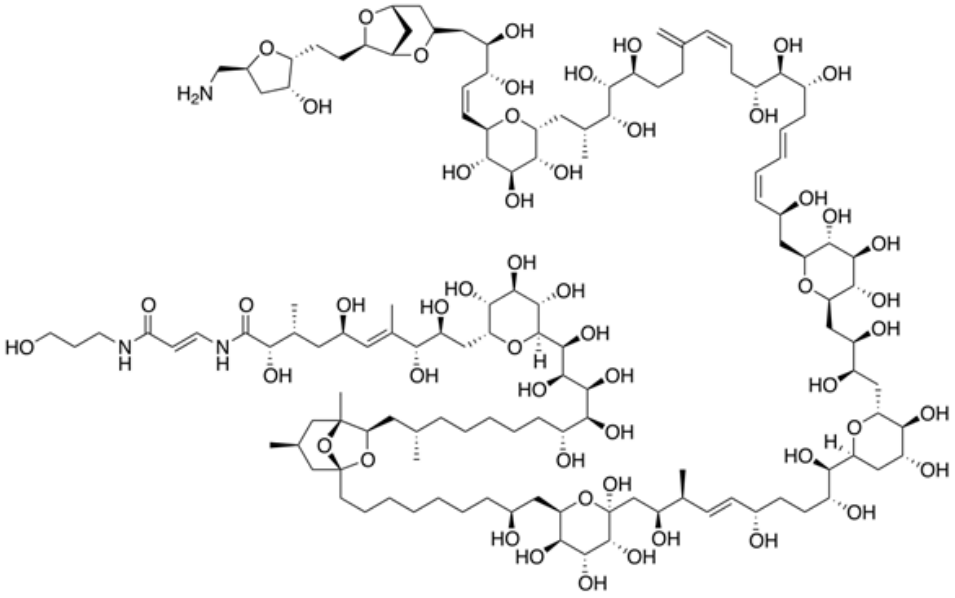


*Figure 6. Palytoxin, the most potent non-proteinaceous toxin for humans (Guillen et al., 2019).*

**Metabolites associated to zoanthids with bioactivity**

The metabolites associated with zoanthids that have been described are grouped into four families of compounds, the sterols, the palytoxins, the ecdysteroids, and the zoanthamines. Within the sterols, compounds such as palisterol, a 24-carbon ethyl sterol discovered in *Palythoa mammilosa*, have been identified. The presence of brasicasterol, dihydrobrasicasterol, gorgosterol, campesterol has also been reported in *Palythoa* sp. from Tahiti and *Palythoa tuberculosa* from the Marshall Islands. Another sterol called methylene cholesterol was found in zoanthids studied in Hawaii. On the other hand, in Japan cholesta-5,22(*E*)-dien-3β-ol and 23,24-dimethylcholesta-5,22(*E*)-dien-3β-ol obtained from *Palythoa* sp. In African zoanthids, compounds such as stigmasterol, fucosterol and crinosterol were identified, which were extracted from *Palythoa senegalensis* y *Palythoa senegambiensis.* In *P. tuberculosa* extracted from the dead sea, compounds called palysterols (AF) and 24-methylenecholest-5-en-1a,3b,11a-triol were obtained, of which palysterol A presented cytotoxic activity against breast adenocarcinoma and adenocarcinoma colon while palysterol F showed activity against breast adenocarcinoma, colon adenocarcinoma, and cervical carcinoma. In *P. caribaeroum* and *P. variabilis* collected in Brazil, various compounds were extracted such as 7a-hydroxycampesterol,5a,8a-epi-dioxycampesterol(24),24(R)-ergost-7-en-3b, 5a, 6btriol, cholest -4-en-3-one and cholesta-3,5-dien-7-one, as well as two new

compounds called (24R) -7a-hydroperoxy-ergost-5-en-3b-ol and 6b-carboxyl- ( 24R) - (8/6) -abeo-ergostan-3b, 5b-diol (Fig. 7) (Guillen et al., 2018; Elkhawas et al., 2020; Yan et al., 2021). In Zoanthus proteus and *Z. pacificus* as in Palythoa, sterols such as cholesterol, campesterol, brassicasterol, and chalinasterol were found, in addition, zoantosterol was identified from *Z. zociatus* (Chimetto et al., 2010).

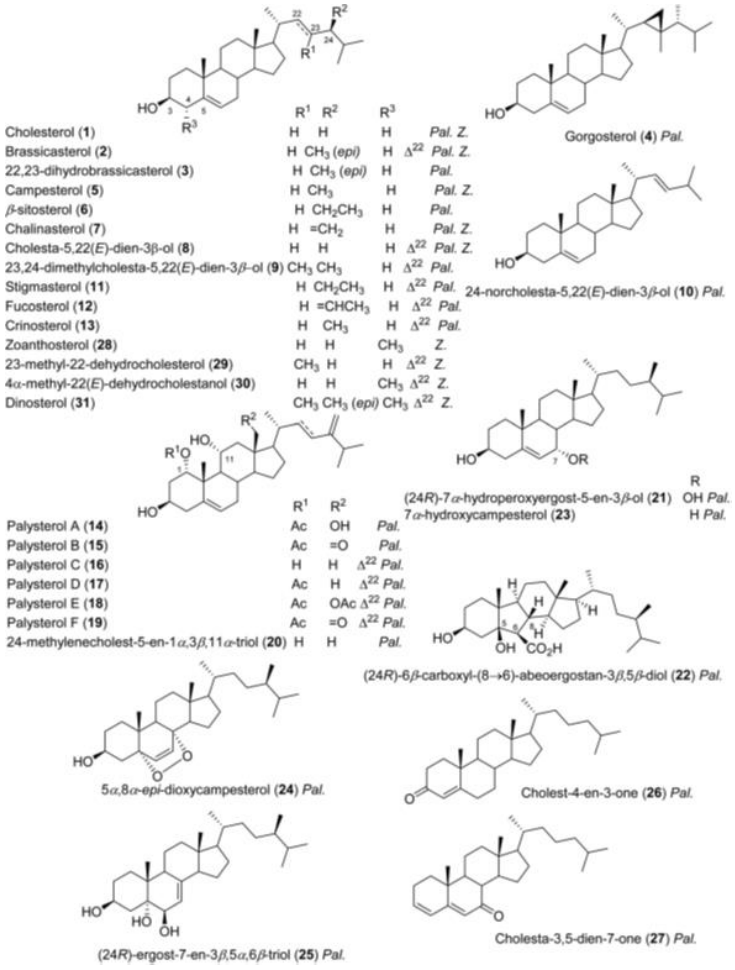
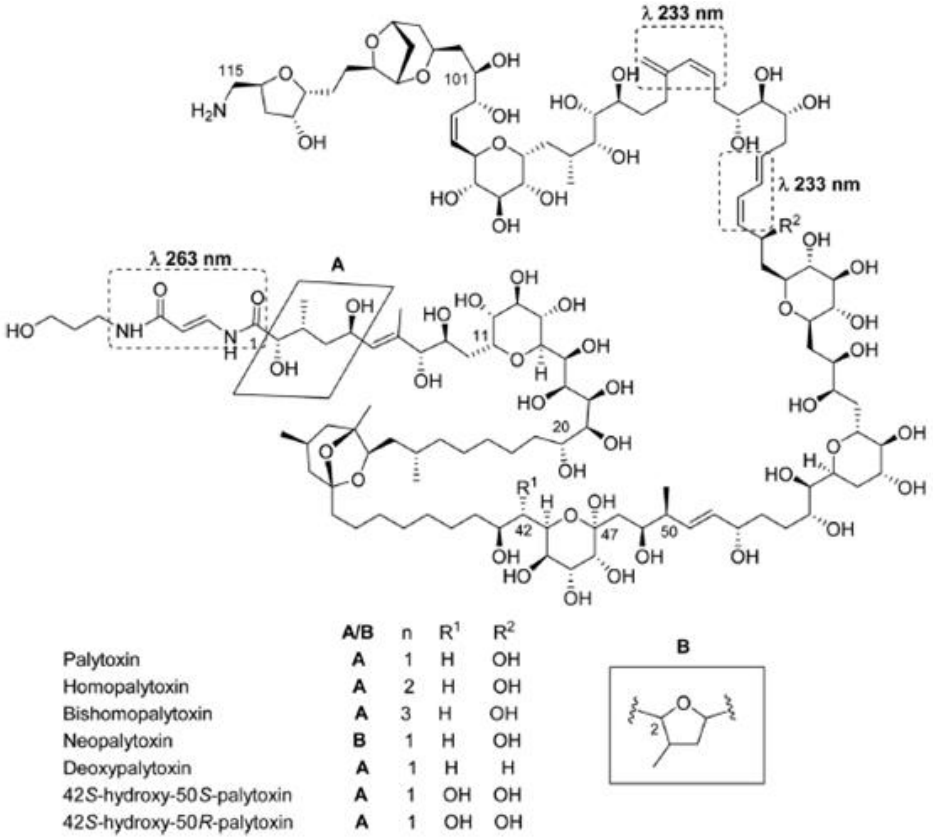



*Figure 7. Sterols found in zoanthids. (Guillen et al., 2018).*

Palytoxins are other compounds extracted from zoanthids, and have been obtained from Palythoa toxica, *P. tuberculosa, P. vestitus, P. caribaeorum, P. heliodiscus, P. margaritae, Zoantus solanderi, Z. soziatus,* and *Z. pulchellus*. The name of palytoxins is attributed because they were obtained for the first time from organisms of the genus *Palythoa*. Palitoxins are compounds considered the most toxic to humans since they exhibit an LD50 of 0.1 mg/kg if administered intravenously and 0.4 mg/kg if administered intraperitoneally.

Palytoxin has a molecular weight of approximately 3300 Da and its structure is presented in figure 8, including compounds derived from palytoxin, such as neopalytoxin, homopalytoxin, deoxypalitoxin, bishomopalytoxin, 42S-hydroxy-50S-palitoxin and 42S-hydroxy- 50R-palytoxin.




| | A/B | n | $R^1$ | $R^2$ |
|---|---|---|---|---|
| Palytoxin | A | 1 | H | OH |
| Homopalytoxin | A | 2 | H | OH |
| Bishomopalytoxin | A | 3 | H | OH |
| Neopalytoxin | B | 1 | H | OH |
| Deoxypalytoxin | A | 1 | H | H |
| 42*S*-hydroxy-50*S*-palytoxin | A | 1 | OH | OH |
| 42*S*-hydroxy-50*R*-palytoxin | A | 1 | OH | OH |

*Figure 8. Types of palytoxines found in zoanthids (Guillen et al., 2018).*

Other compounds obtained from zoanthids are zoanthamines, which have been identified mainly in organisms of the genus *Zoanthus*, hence the name zoanthamines. These compounds are a type of alkaloids formed by fused rings that end in an azepan ring. The first compound of this family was called zoanthamine, from there others have been discovered such as zoanthenamine, zoanthamide, norzoanthamine, oxizoanthamine, epioxyzoanthamine, norzoanthamide B, cyclozoanthamine, 3-hydroxynorzoanthamine, 3β-hydroxyzoanthemide, etc (Fig. 9).

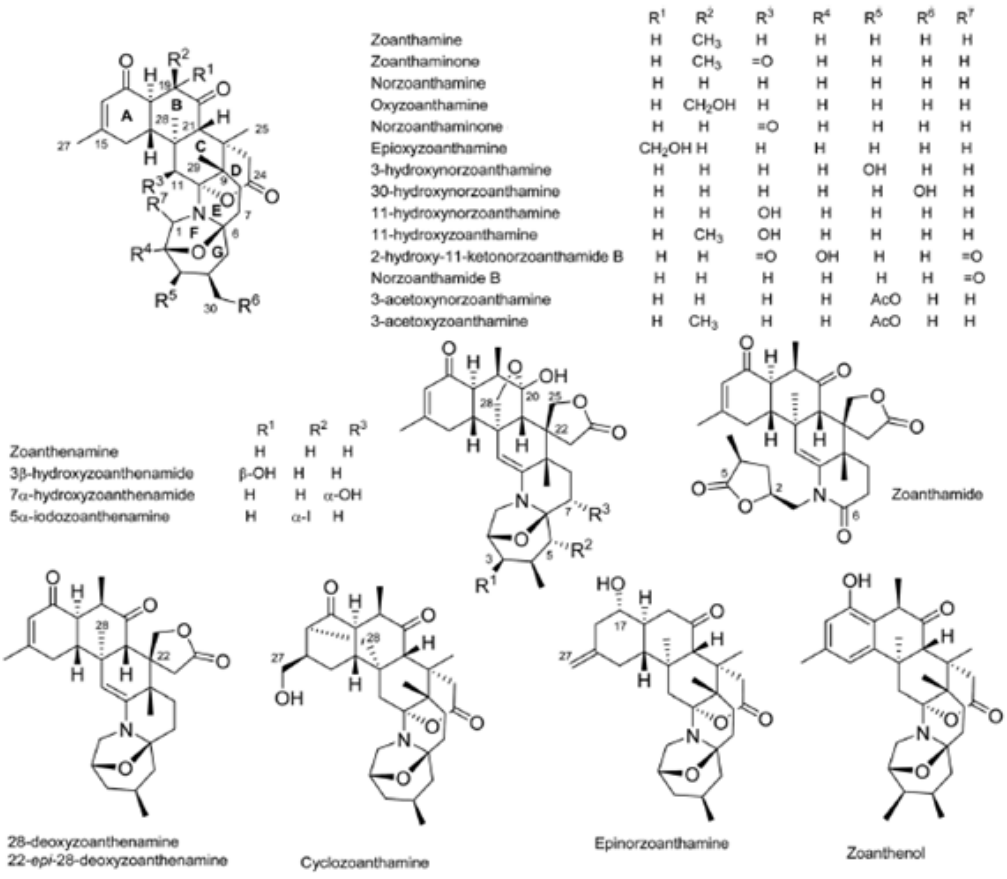
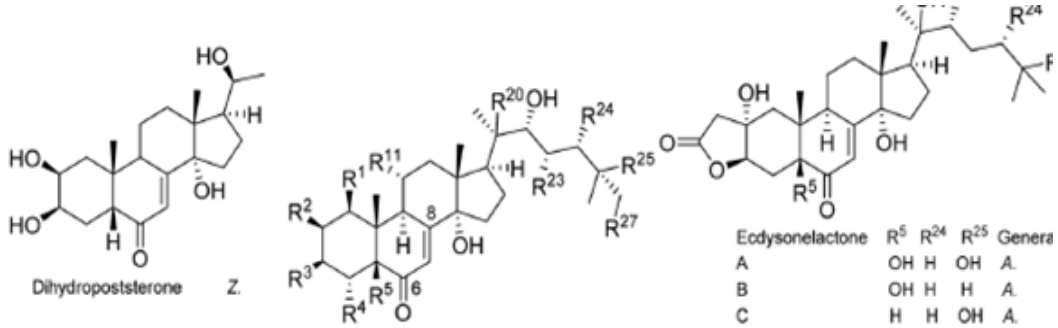



*Figure 9. Zoanthamines found in zoanthids (Guillen et al., 2018).*

Ecdysteroids are also compounds extracted from zoanthids, which, like zoanthamines, are mainly found in species of the Zoanthus and Palythoa genera. The first ecdysteroids identified were 20-hydroxyecdysone, 2-O-acetyl-20-hydroxyecdysone, and 3-O-acetyl-20-hydroxyecdysone. Subsequently, other ecdysteroids were identified such as palitoalone A and B, maquisterone, inokosterone, zoantusterone, ponasterone A, ajugosterone C, dacrihainanterone, viticosterone, 25-O-acetylintegristone A, zoanthone A, dihydropoststerone, dehydromaquisterone, epiinocosterone, turquesterone, and pterosterone (Fig. 10)




| | R1 | R2 | R3 | R4 | R5 | R11 | R20 | R23 | R24 | R25 | R27 | Genera |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 20-hydroxyecdysone | H | OH | OH | H | H | H | OH | H | H | OH | H | *Pal. Z. Par. S.* |
| 2-*O*-acetyl-20-hydroxyecdysone | H | OAc | OH | H | H | H | OH | H | H | OH | H | *Pal. Z. Par.* |
| 3-*O*-acetyl-20-hydroxyecdysone | H | OH | OAc | H | H | H | OH | H | H | OH | H | *Pal. Z. Par.* |
| Palythoalone A | H | OH | OH | H | OH | H | OH | H | $CH_3$ | H | OH | *Pal.* |
| Palythoalone B | H | OH | OH | H | OH | H | OH | H | H | H | OH | *Pal.* |
| Makisterone B | H | OH | OH | H | H | H | OH | H | $CH_3$ | H | OH | *Pal.* |
| Inokosterone | H | OH | OH | H | H | H | OH | H | H | H (*R*) | OH | *Pal. Z.* |
| Palythone A | H | H | H | OH | H | H | OH | H | H | OH | H | *Pal.* |
| 3-deoxy-20-hydroxyecdysone | H | OH | H | H | H | H | OH | H | H | OH | H | *Pal.* |
| 24-*epi*-makisterone A | H | OH | OH | H | H | H | OH | H | $CH_3$ | OH | H | *Pal.* |
| 2-deoxyecdysterone | H | H | OH | H | H | H | OH | H | H | OH | H | *Pal. Z.* |
| α-ecdysone | H | OH | OH | H | H | H | H | H | H | OH | H | *Pal. Z.* |
| Zoanthusterone | OH | OH | OH | H | H | H | OH | H | H | H | H | *Z.* |
| Ponasterone A | H | OH | OH | H | H | H | OH | H | H | H | H | *Z.* |
| Viticosterone E | H | OH | OH | H | H | H | OH | H | H | OAc | H | *Z. Par.* |
| 25-*O*-acetylintegristerone A | OH | OH | OH | H | H | H | OH | H | H | OAc | H | *Z.* |
| Ajugasterone C | H | OH | OH | H | H | OH | OH | H | H | H | H | *Z. Par. S.* |
| Dacryhainansterone | H | OH | OH | H | H | $\Delta^{10,11}$ | OH | H | H | OH | H | *Z.* |
| Zoanthone A | H | OH | OH | H | H | OH | OH | H | H | OAc | H | *Z.* |
| 24(28)-dehydromakisterone A | H | OH | OH | H | H | H | OH | H | $\Delta^{24,28}$ | OH | H | *Z.* |
| Pterosterone | H | OH | OH | H | H | H | OH | H | OH | H | H | *Z.* |
| 25-*epi*-inokosterone | H | OH | OH | H | H | H | OH | H | H | H (*S*) | OH | *Z.* |
| Turkesterone | H | OH | OH | H | H | OH | OH | H | H | OH | H | *Z.* |
| 4-dehydroecdysterone | H | OH | OH | $\Delta^{4,5}$ | | H | OH | H | H | OH | H | *Par.* |
| Gerardiasterone | H | OH | OH | H | H | H | OH | OH | H | OH | H | *S.* |

*Figure 10. Types of ecdysteroids found in zoanthids (Guillen et al., 2018).*

Finally, in the group of aminoimidazoles, pigments of the group of zoanthoxanthins have been found, such as zoanthoxanthin itself, parazoanthoxanthin A, B, D, F and G, dimethylpseudozoanthoxanthin, pseudozoanthoxanthin, palizoanthoxanthin AC, epizoantudozoanthoxanthin B, homoanthoxanthin B, homoanthozoanthoxanthin 3; these compounds have been identified in both *Zoanthus* and *Palythoa*. (Fig. 11).

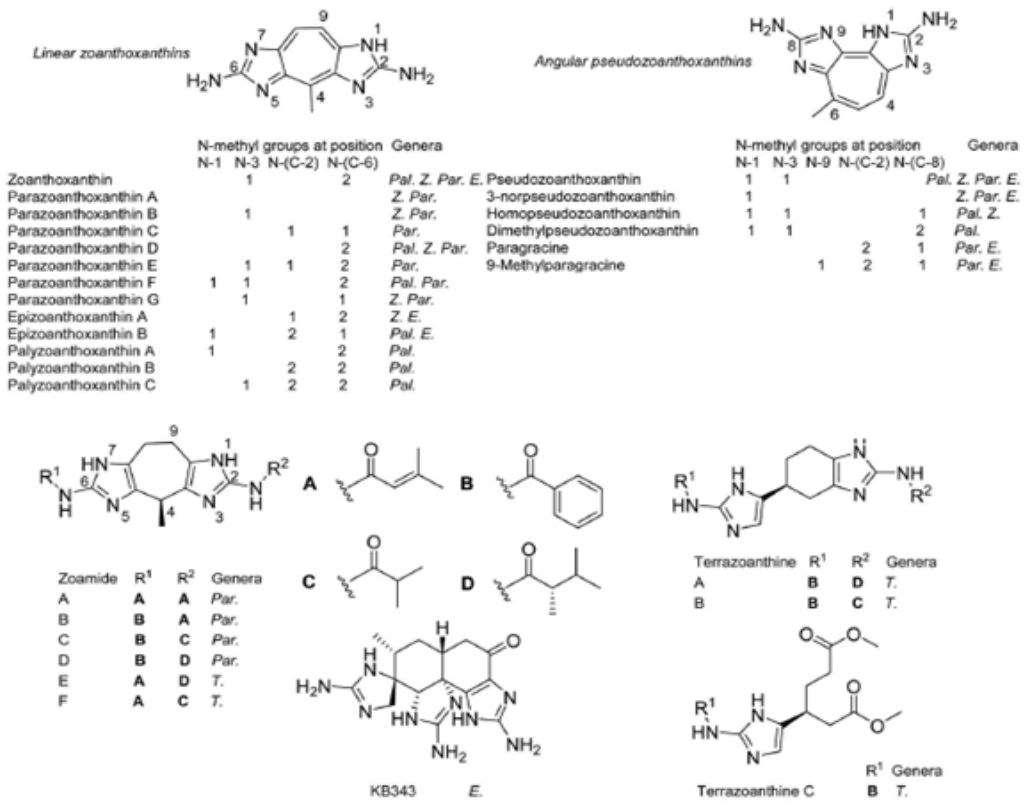



*Figure 11. Zoantoxhanthines found in zoanthids (Guillen et al., 2018).*

**Bacteria associated to zoanthids with bioactivity**

The genus *Vibrio* has many species considered human pathogens and other animals of importance to humans. It is not the exception for marine organisms. For example, there is a species called V*ibrio coralliilyticus* that is an important pathogen in corals. However, it has been observed that there are species of vibrios present in the mucus produced by some apparently healthy zoanthids such as *Palythoa caribaeorum*, *P. variabilis* and *Zoanthus solanderi*. Chimetto et al. (2008) found that the species *Vibrio harveyi, V. rotiferianus, V. campbellii, V. alginolyticus,* and *Vibrio mediterranei* were present in the three species of zoanthids already mentioned. The researchers proposed that these *Vibrio* species found in healthy zoanthids could play an important role in the production of the toxins present in the mucus that would help as a defense mechanism in these zoanthids, but they did not do any experiments to demonstrate this and the role of these vibrios. In zoanthids, it was only proposed but not proven.

Seeman et al. (2009) carried out a study to determine whether the bacterial microbiota associated with *Palythoa caribaeorum* and *Zoanthus pulchellus* was involved in the production of palytoxin (PTX), a very potent non-proteinaceous toxicant. They used the delayed hemolysis assay, which they called the palytoxin screening test. Both species (*P. caribaeorum* and *Z. pulchellus*) exhibited high hemolytic activity, which was suppressed when the red cells had been treated with ouabain supporting the assumption of the presence of PTX or PTX-like compounds.

Bacteria of the genus *Photobacterium*, such as *P. leioghnati*, *P. rosenbergi*, *P. haloterans* and *P. jeanii* were found in the mucus of the zoanthids *P. caribaeorum*. These bacteria are important since they produce antibiotics that help their coral symbiote or provide them with some competitive advantage. In addition, it can also offer to its symbiote with other compounds such as nitrogen fixation (Chimetto et al., 2008), supply of food resource (Kooperman et al., 2007) and decomposition of chitin (Ducklow & Mitchell, 1979). Given the presence of bacteria of the genus *Photobacterium*, it is thought that it can also provide protection to the studied zoanthid thanks to the antibiotics that these bacteria can produce (Chimetto et al., 2010).

**Cytotoxicity**

Pyridoacridine, a compound extracted from sponges, tunicates and anemones, was tested against different tumor cell lines, such as cells A-549 (lung cancer), H-116 (colon adenocarcinoma), PSN1 (pancreatic adenocarcinoma) and SKBR3 (carcinoma breast) and showed an excellent antiproliferative activity with an $IC_{50} \leq 5$ μg $mL^{-1}$ (Martínez-García et al., 2007). Psammaplisen A, a compound obtained from sponges, has great anticancer potential when tested against two endometrial cancer cell lines (ECC and Ishikawa) and triterpene steletin B, from marine fungi, also presented anticancer properties when evaluated against cell lines. SF-295 (glioblastoma) and K562 and KU812 (chronic myeloid leukemia); Also dibromo tyrosine, another compound obtained from sponges, showed good activity against K562 cells (Calcabrini et al., 2017). Palysterol A, obtained from species of zoanthids of the genus *Palythoa*, has been observed to show cytotoxic activity in MCF-7 (breast

adenocarcinoma) and HT-29 (colon adenocarcinoma) cell cultures with IC50 of 170-178 µM. Palisterol F (also extracted from *Palythoa* sp.) Also showed antitumor activity with MCF-7 (IC50 = 82 µM), HT-29 (IC50 = 122 µM) and HeLa (IC50 = 128 µM, cervical cancer) (Guillen et al. ., 2018) Ecteinascidin, extracted from the ascidian Ecteinascidia sp., is a compound already accepted by the Food and Drug Administration from USA, as an anticancer treatment agent and is even already on the market, bryostatin (from the bryozoan (Bugula neritin) is in phase III while dehydrodidemnin B (Aplidium albicans), dolastatin 10 (Dolabella auricularia) and kahalalida F (Elysia rufescens) are in clinical phase II. Other compounds such as bengamide (Jaspidae sp.) and hemiasterlin (sponges) are in phase I and diazonamide (Diazona angulata), thiocoraline (Micronomonospora marina) and vitilevuamide (Didemnum cuculiferum and Polysyncranton lithostrotum) are only in the preclinical phase (Agrawal et al., 2017; Dou and Dong, 2019; Watters, 2018 ).

# METHODOLOGY

## Sample collection

Zoanthids in this study were sampled under Exemption Permit Number Nº 005-17 IC-FAU-DPSE/MA, by Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM). There were no endangered or protected species involved. Nine zoanthids samples were collected at a depth of between 50 cm in rocky intertidal zones between San Pedro de Manglaralto and Ayangue, Province of Santa Elena (Fig. 12). This samples collection were collected in sterile Ziploc plastic bags filled with fresh seawater and transferred to the laboratory for processing.

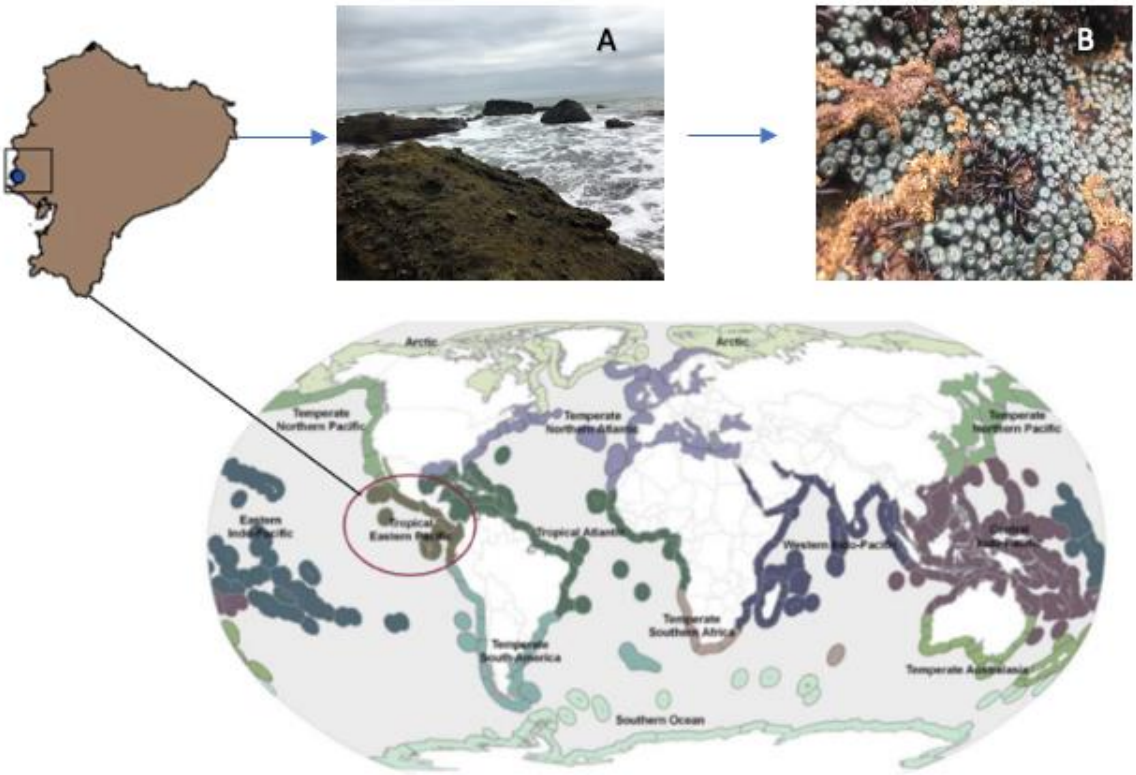



*Figure 12. Study area of specimens collected (Karla Jaramillo, 2018). A) Sample collection site in REMAPE, San Pedro de Manglaralto; B) Zoanthids collected in REMAPE.*

**Isolation of bacteria and growth conditions**

The zoanthids samples were washed with sterile seawater in order to remove endobionts, macroscopic epibionts, sand and detritus. Five grams of tissue taken from different parts of the organism and these were macerated and diluted in 45 mL sterile natural seawater (NSW). Later one hundred (100) µL of dilutions of serial dilutions ($10^1$ a $10^5$) were made in triplicate using the spread plate technique (Phadale & Kumar, 2018) and placed in Petri dishes containing Marine Agar (MA; Difco 2216). Plates were incubating for 72 hours at 27°C in a period of two days. After incubation, several strains were selected for morphological differences. Subsequently, each strain was purified, by strain seeding, on individual plates. Once the strains were obtained, they were incubated at 27°C for two days. The isolated strains were re-cultivated in 1 mL of Luria-Bertani (LB) broth at 2% NaCl to later be incubated at 30°C for 24 hours. The culture was diluted in a 70% glycerol solution to be stored in a -80°C freezer for later use.

**Biochemical characterization of isolated strains**

All zoanthids isolates were characterized by performing various biochemical tests according to Kalimutho et al., 2007. Bacterial cultures were examined for their Gram reaction by conventional staining methods. The biochemical assays evaluated included catalysis reaction, oxidase presence, amino acid (arginine, ornithine, and lysine) utilization, carbohydrate (celabiose, D-galactose, D-arabinose, D-sucrose and D-mannitol) utilization, use of citrate as a carbon source, the formation of diacetyl (Voges-Proskauer), and indole production. Oxidative/fermentation test (O/F) in order to determine the metabolism of bacterial isolates when using glucose as a source of substrate (Fig. 13).

*Figure 13. Illustration of minuterized biochemical tests.*

## Molecular identification of isolated strains

### DNA extraction and amplification

The sixteen bacterial isolates were identified by molecular analysis by amplifying the 16s rRNA genes using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5'-TACGGYTACCTTGTTACGACTT-3′). Bacterial DNA was extracted following the procedure described by Anand et al. (2006) with slight modifications. Fresh individual colonies were suspended in 500 µl of buffer solution (100 Mm NaCl, 10 Mm Tris-HCl pH 8.0, 1mM EDTA) and 80 µL of 10% SDS, placed in a water bath at 55 °C for one hour, 600 µL of phenol and centrifuged at 13,000 rpm for 10 min. The supernatant was recovered, 1 volume of chloroform-isoamyl (24: 1) was added, centrifuged at 13,000 rpm for 10 minutes. 2.5 volumes of cold 100% ethanol and 0.5 volumes of 5M ammonium acetate were added to the supernatant. The solution was stored at -20 ° C overnight. The solution was centrifuged at 13,000 rpm for 15 min and the supernatant was removed. Two washes were carried out with 300 µL of cold 70% ethanol. The pellet was allowed to dry for 2 hours at 45 °C and the DNA was resuspended in 50 µL of Milli-Q water. The amplified products were sent to Macrogen (Seoul, Korea) for sequencing. Subsequently, the sequences were aligned with the National Center for Biotechnology Information (NCBI) BLASTn public database based on > 98% identity.

### Phylogenetic analysis

Database search and comparisons from zoanthids strains were done with the BLASTn search using the NCBI database. The gaps of the sequences obtained using BioEdit software version 4.7.3 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) were edited. The sequences were then assembled using the GENEIOUS software, multiple sequence alignments obtained from the NCBI were performed in MEGA v.10 (Kumar et al., 2016) using ClustalW. By using the Maximum Likelihood (ML) and Bayesian Inference (BI) in a MEGA v.10 the phylogenetic tree was generated based on 16S rRNA gene sequences. The GTR+I+G model was determined in jModelTest 0.1.1 as the most appropriate, based on both the Bayesian and Akaike information criterion, and was used for ML and BI analyses. Bootstrap resampling was performed on 1000 replicates.

## Production of bacterial biomass

The 16 bacterial isolates were grown by individual Petri dishes in MA, and incubated at 27 °C for 48 h. Subsequently, individual colonies of zoanthids were transferred to test tubes with 10mL of LB medium in NSW, and incubated for 48 h at 27 °C. Then the bacterial culture was centrifuged (4000 g, 10 min, 4 °C). Then the pellets were resuspended in NSW to later be stored at 25 °C (room temperature). The final concentration (CFU $mL^{-1}$) and bacterial viability were determined by plating the bacterial suspension in MA, as described previously.

## Preparation of extracts and fractions

Bacterial extracts were prepared as described by Dash et al., 2009 with slight modifications. Aliquots of 1 mL of the previously prepared bacterial stocks were inoculated in 10 mL of LB medium and incubated at 28 °C for 24 hours. This inoculum was transferred to flasks containing 500 mL of LB and incubated at 28 °C for 24 hours. Subsequently the inoculum were transferred to 1L flasks. Once the stationary phase was reached after 3 days

of bacterial growth, the bacterial culture was centrifuged at 4000g for 15 minutes, and the microbial pellet was collected. The obtained cell pellets were extracted with a mixture of $CH_3OH/CH_3CH_3$ (1:1) and placed in a sonicator bath 2 times for 5 minutes each time at a frequency of 40 kHz. It was left to rest for 30-60 minutes at temperatures below 30 °C and protected from light. The samples were filtered and the extract was placed in a round bottom balloon.

The organic phase was recovered and the solvent was evaporated on a rotary evaporator (Buchi, RII, Switzerland). Solid Phase Extraction (SPE) was carried out with SPE C18 cartridges (Thermo Scientific, HYPERSEC, United States), using five different eluents, 10 mL each: $H_2O$ (F1), $H_2O$:$CH_3OH$ 1:1 (F2), $CH_3OH$ (F3) (JT Baker, Trinidad and Tobago), $CH_3OH$:$CH_2Cl_2$ 1:1 (F4), and $CH_2Cl_2$ (F5) (SupraSolv®, Merck, Germany). Fractions F2 and F3 were evaporated to dryness. The SPE residues were recovered with the same solvent used in the elution for each fraction, filtered through 0.22 µm filters (Merck Millipore Co.), and injected into the Ultra-High Efficiency Liquid Chromatography with Diode Array Detector and Evaporative Light Scattering Detection (UPLC-DAD-ELSD) (Waters, H-CLASS 1, Singapore). Chromatographic runs were performed with a reversed phase column (C18) of dimensions 50 x 2.1 mm and 1.7 µm in particle size (ACQUITY, Waters, Ireland). The mobile phase consisted of acetonitrile (Sigma-Aldrich, South Korea) and grade I water, acidified with 0.1% trifluoroacetic acid (Sigma-Aldrich, France). A gradient of water (A) and acetonitrile (B) was performed for 0-2 minutes 10% B, from 2-10 minutes B increased to 100%, from 10-12 minutes B was maintained and finally from 12-15 minutes B dropped to 10%. The absorbance was recorded at 254 nm.

**Animal health evaluation**

The pathogenic shrimp vibrios evaluated were*Vibrio harveyi* (strain E22), *Vibrio campbellii* (strain LM2013), *Vibrio vulnificus* (strain S2), and *Vibrio parahaemolyticus* (strain BA94C2, positive to PirA/PirB toxins). The antimicrobial activity of the extracts was evaluated against two pathogenic shrimp vibrios, including *Vibrio harveyi* (strain E22), and *Vibrio vulnificus* (strain S2) and the other two pathogens were not evaluated due to the lack of biological material. This test consisted of determining the antibacterial effect of the crude

extracts using disc diffusion method (Kirby-Bauer). Subsequently, we also evaluated the capacity of the supernatant of the bacteria when they were cultured in liquid medium by well agar diffusion method modified by Al Atya et al. (2015), against the four pathogenic vibrios. In addition, the exclusion capacity of isolates was also evaluated to exclude the four pathogenic vibrios Al Atya et al. (2015) with modifications. We also evaluated the ability of the extracts at non-microbicide doses to interrupt the formation of biofilms of pathogenic *Vibrio parahaemolyticus* (strain BA94C2, positive to PirA/PirB toxins), using the method described by Domínguez et al. (2018). The bacterial biofilm biomass was stained with violet crystal (CV) and quantified spectrophotometrically following the methodology described by Djordjevic et al. (2002), with slight modifications.

**Antimicrobial activity**

The pathogenic vibrios were spread in Tryptone Soy Broth (TSB; Oxoid CM129) at 2% NaCl and adjusted to a concentration of $1x10^8$ by absorbance using calibration curves ($OD_{0.3}$) which simulates a concentration of $c$ One hundred (100) µl of the bacterial inoculum were spread by extension in Petri dishes of Marine Agar. Six mm diameter wells were punched on the prepared agar plates with a sterile hollow glass rod. Then, fifty (50) µL of the crude extracts were added to the wells. Oxytetracycline was used as a positive control at a concentration of 10.0 µg $mL^{-1}$, and 2% Saline Solution was used as a negative control. Each assay was performed in duplicate. The plates were incubated for 48 h and 72 h at 28 °C. Following incubation, measuring inhibition zones around the well containing the extracts. According to Zidour et al. (2017), only inhibition zones greater than 5 mm are considered positive reactions.

**Microbicidal effect of secreted products from isolates**

The bacteria isolated from the zoanthids were cultured for 24 hours in LB medium. They were centrifuged (18000 g, 10 min, 4 °C), then the supernatant was transferred to another container, and these supernatants (secreted products) were filtered with sterile filtered (0.22 µm filters; Merck Millipore Co.), to remove any residue. Sterile Mueller-Hinton agar plates were placed. Six mm diameter wells were punched on the prepared agar plates as

mentioned above. Then, fifty (50) µL and one hundred (100) µL of the filtrate was added to the wells. Oxytetracycline was used as a positive control at a concentration of 10.0 mg $mL^{-1}$, and 2% NaCl was used as a negative control. Each assay was performed in duplicate. The plates were incubated for 48 h and 72 h at 28 °C. Following incubation, the results were interpreted by measuring the inhibition zone diameter around the well containing the supernatant.

**Exclusion capacity of isolates**

The exclusion capacity of bacteria isolated from zoanthids towards pathogenic vibrios was evaluated by a well agar diffusion method previously described by Al Atya et al. (2015). The vibrio suspensions were prepared, adjusted, and spread on the MA as described above. Two hundred (200) µL of each bacterial suspension were evaluated at three different concentrations ($10^4$, $10^5$ and $10^6$ CFU $mL^{-1}$) while the pathogen evaluated was at a concentration of $10^5$. Then, were spread on wells of MA plates, previously carried out under the same conditions described above. Subsequently, 2% NaCl was used as a control. The plates were incubated for 48 h and 72 h at 28 °C. Following incubation, antibacterial activity was assessed by measuring exclusion zones around the well containing the microbial isolation.

**Biofilm formation and inhibition**

Vibrios suspensions ($1\times10^6$ CFU $mL^{-1}$) were prepared as previously described. A volume of twenty (20) µL of bacterial suspension was transferred to each well in a 96-well microplate containing 180 µL of LBb 2% NaCl sterile at different sublethal concentrations (10.0 µg $mL^{-1}$) of each isolate evaluated. A control was performed containing bacterial suspension + LB + 2% NaCl without extracts form bacterial isolated, with three replicates for each concentration. The plates were incubated at 27 °C for 36 h. The cells were removed, and the generated biofilms were carefully washed twice using 200 µL of PBS (pH 7.2). The biofilms were dried at 50 °C for 30 min and stained with 220 µL of 0.1% violet crystal (w/v)

(Merck_ C0775) per well for 15 min. After rinsing the plates to remove excess dye, they were dried at room temperature (26 °C). Then, 220 µL of ethanol was used to dissolve the impregnated violet crystal (CV). Then, 150 µL of the solubilized product was transferred to a new 96-well plate, and the optical density was read at 590 nm ($OD_{590}$). The data were transformed to (%) biofilm formation considering 100% biomass of untreated bacterial biofilms (negative control). The following formula was used to calculate the level of biofilm inhibition:

$$Percent\ inhibition = \left[\frac{OD\ Control\ x\ OD\ Test}{OD\ Control}\right] x100$$

**Human health evaluation**

**Human cell lines and cell culture conditions**

The three tumor lines used in the present study were, MCF–7 (breast adenocarcinoma ATCC®-CRL3435), Caco–2 (colon adenocarcinoma ATCC®-HTB37), and Hep-G2 (hepatocyte carcinoma ATCC®-HB8065) from cell culture laboratory of the National Center for Aquaculture and Marine Research (CENAIM) and kept at 37 °C with 5% $CO_2$. MCF–7 cell line was cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 100 µM MEM nonessential amino acids, 1 mM sodium pyruvate, 100 U $mL^{-1}$penicillin, 100 µg $mL^{-1}$ streptomycin, and 0.01 mg $mL^{-1}$. Meanwhile, Caco–2 cells were cultured in EMEM medium with 10% FBS, 2 mM glutamine, 50 U $mL^{-1}$ penicillin, and 50 µg $mL^{-1}$ streptomycin. Finally, Hep-G2 cells were cultured in EMEM medium supplemented with 10% FBS, 2 mM glutamine, 100 U $mL^{-1}$ penicillin, 100 µg $mL^{-1}$ streptomycin, and 1 mM sodium pyruvate.

**MTT reduction cell viability bioassay**

Fractions were evaluated against three tumor lines mentioned above by the MTT colorimetric assay ((3- (4,5-dimethylthiazol-2-yl) −2,5-diphenyltetrazolium bromide)), previously described by Cautain et al. (2015) (Fig. 14). The cells were seeded ($1.0x10^4$ cells/well) in 96-well plates and incubated at 37 °C and 5% CO2 for 24 h. Subsequently, the medium was removed, and the adherent cells were incubated with 90 µL of complete medium and 10 µL of fractions (final concentration 10 µg $mL^{-1}$). Cells were also cultured without F2 or F3 as a negative control. As a positive control, doxorubicin (50 µM) was used instead of the peptide. The plates were incubated at 37 °C and 5% $CO_2$ for 48 h. After the treatment with fractions, the cells were incubated with MTT (5 mg $mL^{-1}$) for 2 h, and then the formazan crystals were dissolved with 200 µL of dimethyl sulfoxide (DMSO). The plate was analyzed in a Varioskan LUX detector at 620 nm. Cell viability was calculated considering 100% viability at the average of the 3 replicates of the negative control. The results were expressed in function of the mean ± standard deviation (SD).

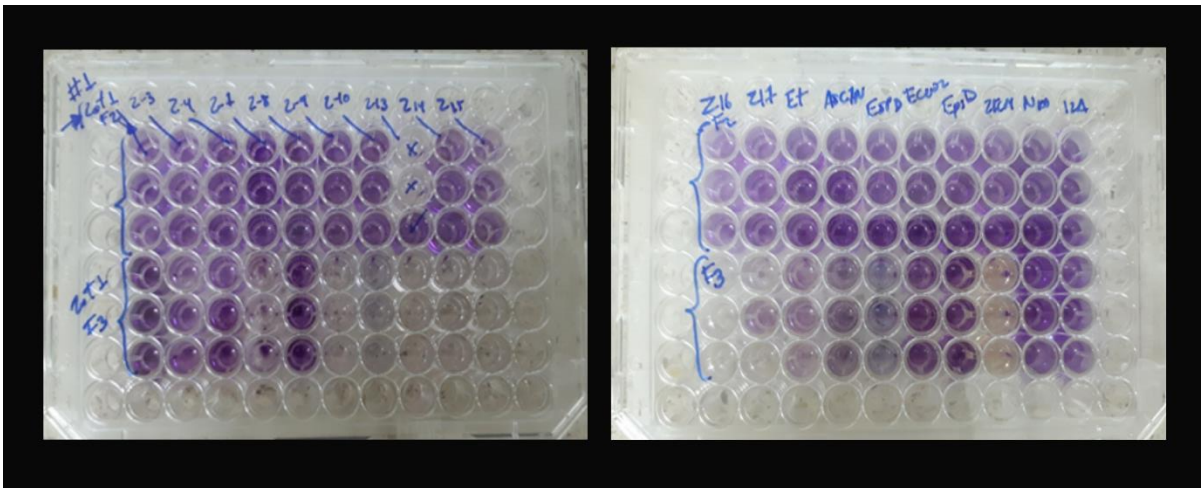



*Figure 14. Colorimetric analysis of the extracts diluted in F2 and F3.*

# RESULTS AND DISCUSSION

## Isolation and characterization of bacterial isolates

In all 16 samples processed, a large number of culturable bacteria was evidenced in the MA culture medium (Fig. 15). Different morphotypes were selected. The isolates were reseeded by depletion in individual MA plates. Of the 16 morphotypes initially selected, 13 were isolated, due to the similarity of the morphotypes of the colonies (Fig. 16). With the Gram stain, it was possible to determine that the majority (11/16) isolates belong to the Gram-negative ones. As for only a single strain of Gram-positive bacteria was evidenced. Previous studies showed that in zoanthids, there is a greater number of Gram-negative bacteria interacting with other organisms in the sea (Webster and Taylor (2011); Sun et al. (2014); Ramesh et al. (2021)).

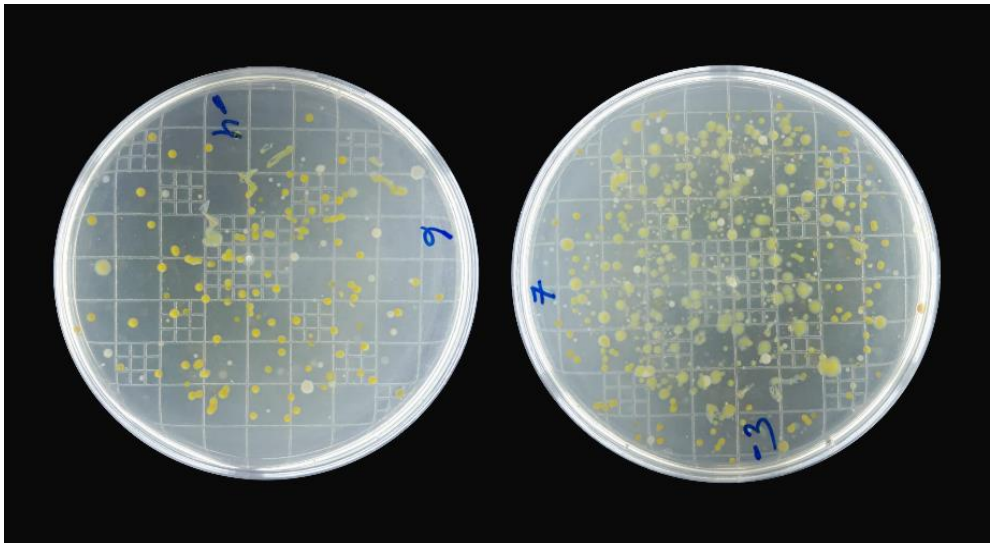


*Figure 15. Different morphotypes of colonies grown on marine agar.*

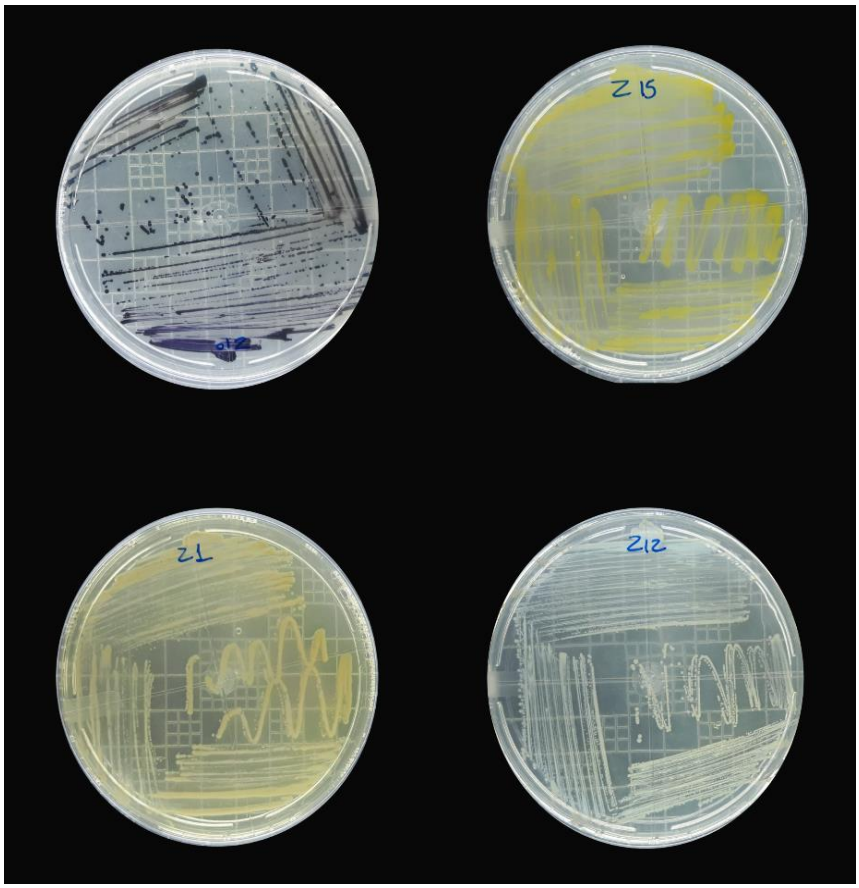



*Figure 16. Isolates reseeded by depletion in individual marine agar plates*

In biochemical tests, a marked difference in carbohydrates and amino acids was observed among the isolates. All isolates also used citrate as a carbon source. However, strain Z-10 was the only isolate that did not use citrate (Table 1). This similar result was also observed by Vynne et al. (2011) and Vynne, Manson and Gram (2012).

*Table 1. Biochemical characterization of bacteria isolated from Z. pulchellus.*

| **Biochemical characteristics** | **Z1** | **Z2** | **Z3** | **Z4** | **Z7** | **Z9** | **Z10** | **Z12** | **Z13** | **ZOT 14** | **Z14** | **Z15** | **Z16** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gram staining | - | - | - | - | - | - | - | - | - | + | - | - | - |
| Manose | + | + | + | - | + | + | - | + | + | ND | + | - | + |
| Arabinose | + | - | - | - | - | + | + | + | - | + | + | - | + |
| Cellobiose | - | + | + | - | - | + | - | + | + | - | - | - | - |
| Galactose | - | + | + | - | - | + | + | ND | + | - | - | - | - |
| Sucrose | + | + | + | - | - | + | - | ND | + | + | - | - | + |
| O/F an. | - | + | + | - | - | + | - | ND | + | - | - | - | - |
| O/F ar. | - | + | + | + | - | + | + | ND | + | + | - | + | - |
| NB | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Indole | + | + | + | + | - | - | - | + | + | - | - | - | + |
| Mannitol | + | + | + | + | - | + | - | ND | + | + | - | + | + |
| Arginine | - | - | - | + | - | + | - | ND | - | - | - | - | - |
| Citrate | + | + | + | + | + | + | - | + | + | + | + | + | + |

ND: not determined

## Genotypic identification of bacteria

All bacteria showed> 98% similarity with the analyzed sequences available in the NCBI GenBank database (Table 2). Molecular identification through 16s rRNA gene sequencing identified bacterial species: Z1, *Pseudovibrio denitrificans*; Z2, *Vibrio harveyi*; Z3, *Vibrio harveyi*; Z4, *Tenacibaculum mesophilum*; Z7, *Microbulbifer variabilis*; Z9, *Ferrimonas kyonanensis*; Z10, *Pseudoalteromonas luteoviolacea*; Z12, *Agarivorans litoreus*; Z13, *Vibrio harveyi*; ZOT14, *Bacillus sp*; Z14, *Microbulbifer variabilis*; Z15, *Tenacibaculum mesophilum*; Z16, *Pseudovibrio denitrificans*.

*Table 2. Identity percentages of bacterial isolates of the 16s rRNA ribosomal gene.*

| Zoanthid | Strain | NCBI percent identity (%) | | |
|---|---|---|---|---|
| *Zoanthus pulchellus* | Z1 | 99.45%<br>*Pseudovibrio denitrificans*<br>NR_113946.1 | 99.45%<br>*Pseudovibrio denitrificans*<br>NR_029112.1 | 99.06%<br>*Pseudovibrio japonicus*<br>NR_041391.1 |
| | Z2 | 99.86%<br>*Vibrio harveyi*<br>NR_113784.1 | 99.86%<br>*Vibrio harveyi*<br>NR_043165.1 | 99.42%<br>*Vibrio galatheae*<br>NR_147758.1 |
| | Z3 | 99.92%<br>*Vibrio harveyi*<br>NR_113784.1 | 99.92%<br>*Vibrio harveyi*<br>NR_043165.1 | 99.60%<br>*Vibrio galatheae*<br>NR_147758.1 |
| | Z4 | 98.16%<br>*Tenacibaculum mesophilum*<br>NR_113841.1 | 98.80%<br>*Tenacibaculum lutimaris*<br>NR_043080.1 | 98.16%<br>*Tenacibaculum mesophilum*<br>NR_024736.1 |
| | Z7 | 99.71%<br>*Microbulbifer variabilis*<br>NR_041021.1 | 99.13%<br>*Microbulbifer epialgicus*<br>NR_041493.1 | 98.56%<br>*Microbulbifer echini*<br>NR_156859.1 |
| | Z9 | 98.38%<br>*Ferrimonas kyonanensis*<br>NR_041387.1 | 98.30%<br>*Ferrimonas kyonanensis*<br>NR_113998.1 | 98.06%<br>*Ferrimonas futtsuensis*<br>NR_114007.1 |
| | Z10 | 100%<br>*Pseudoalteromonas luteoviolacea*<br>NR_114237.1 | 99.63<br>*Pseudoalteromonas luteoviolacea*<br>NR_026221.1 | 98.28%<br>*Pseudoalteromonas rubra*<br>NR_026223.1 |
| | Z11 | 98%<br>*Ferrimonas kyonanensis*<br>NR_041387.1 | 98%<br>*Ferrimonas kyonanensis*<br>NR_113998.1 | 98%<br>*Ferrimonas futtsuensis*<br>NR_114007.1 |
| | Z12 | 98.52%<br>*Agarivorans litoreus*<br>NR_134691.1 | 98.45%<br>*Agarivorans gilvus*<br>NR_117238.1 | 98.40%<br>*Aliagarivorans marinus*<br>NR_044585.1 |

| | | | | |
|---|---|---|---|---|
| | Z13 | 99.78%<br>*Vibrio harveyi*<br>NR_113784.1 | 99.78<br>*Vibrio harveyi*<br>NR_043165.1 | 99.35%<br>*Vibrio galatheae*<br>NR_147758.1 |
| | ZOT14 | 100%<br>*Bacillus stratosphericus*<br>NR_118441.1 | 99.93%<br>*Bacillus aerius*<br>NR_118439.1 | 99.93%<br>*Bacillus altitudinis*<br>NR_042337.1 |
| | Z14 | 99.78%<br>*Microbulbifer variabilis*<br>NR_041021.1 | 99.21%<br>*Microbulbifer epialgicus*<br>NR_041493.1 | 98.63%<br>*Microbulbifer echini*<br>NR_156859.1 |
| | Z15 | 98.16%<br>*Tenacibaculum mesophilum*<br>NR_113841.1 | 98.80%<br>*Tenacibaculum lutimaris*<br>NR_043080.1 | 98.16%<br>*Tenacibaculum mesophilum*<br>NR_024736.1 |
| | Z16 | 99.47%<br>*Pseudovibrio denitrificans*<br>NR_113946.1 | 99.47%<br>*Pseudovibrio denitrificans*<br>NR_029112.1 | 98.72%<br>*Pseudovibrio japonicus*<br>NR_041391.1 |

The phylogenetic tree of the isolated strains of zoanthids is displayed in Figure 17. Sequence lengths of 900–1200 Bp were used to generate the tree. These strains showed coincidences of at least three species identified with the GENBANK sequences. We used BLAST analysis to determine the species name of the closest strain. In this way, was possible to identify them at the species level, except for ZOT14 (*Bacillus sp.*) who can only be identified at the genus level. This result agrees with Sacchi et al. (2002) who mentions that in *Bacillus sp*., some bacteria could not be identified at the species level because there is an obstacle to the use of 16S rRNA gene sequencing to identify and differentiate some *Bacillus* species. In *Bacillus* is necessary to evaluate the reference genes for each isolated genus to identify at the species level. (Miranda et al., 2007).

In this characterization, three bacterial isolates of the genus *Vibrio* were identified. *Vibrios* are generally pathogenic in humans and animals. However, it confers protection to their host in many sessile organisms. For example, *V. maritimus* and *V. variabilis* have been identified in the zoanthid *Palythoa caribaeorum* (Chimetto et al., 2011) and *V.*

*communis* in *Palythoa communis* and *Palythoa caribaeorum*. Several bacterial isolates obtained in this study are *Pseudovibrio denitrificans, Bacillus sp.* and *Pseudoalteromonas luteoviolacea* (Figure 17 and Table 2). These genus of our bacterial isolates have been reported as useful in aquaculture (Domínguez et al., 2019; Kuebutornye et al., 2019; Radjasa et al., 2005) and also in human health (Rodrigues et al., 2017; Lefevre et al., 2016; Sannino et al., 2018), indicating that they could be used as potential strains for human and animal health.

For instance, the genus *Bacillus* has been used in aquaculture as a probiotic being a good option to improve the use of food, the immunological response, stress response, and disease resistance (Kim et al., 2021; Kewcharoen et al., 2019; Chien et al., 2019). Also, the genus *Pseudovibrio* promises to be a natural marine product with biotechnological value due to its secondary metabolites with antagonistic activities (Penesyan et al., 2011; Bondarev et al., 2013; Taylor et al., 2007; Romano 2018). Furthermore, several studies report that the genus *Pseudoalteromona* inhibits the growth of pathogenic shrimp bacteria, which is why these bacteria have been used as possible biological control agents (Wang et al., 2018; Louis et al., 2018; Ringo, 2020).

On the other hand, *Bacillus* has numerous applications in biomedicine, since this genus exhibits potential anticancer activities that can be used to treat human diseases (Almaki et al., 2020; Mostafa et al., 2019). In addition, in the last decade there has been a growing interest in the genus *Pseudovibrio* since it has been reported as a source of active metabolites that has relevant pharmaceutical characteristics (Hassan et al., 2020; Romano, 2018; Crowley et al., 2014). In the same way, the genus *Pseudoalteromona* has been associated with a range of bioactive compounds that inhibit human carcinoma cell lines (Ivanova, 2004; Bowman, 2007; Yan et al., 2006).

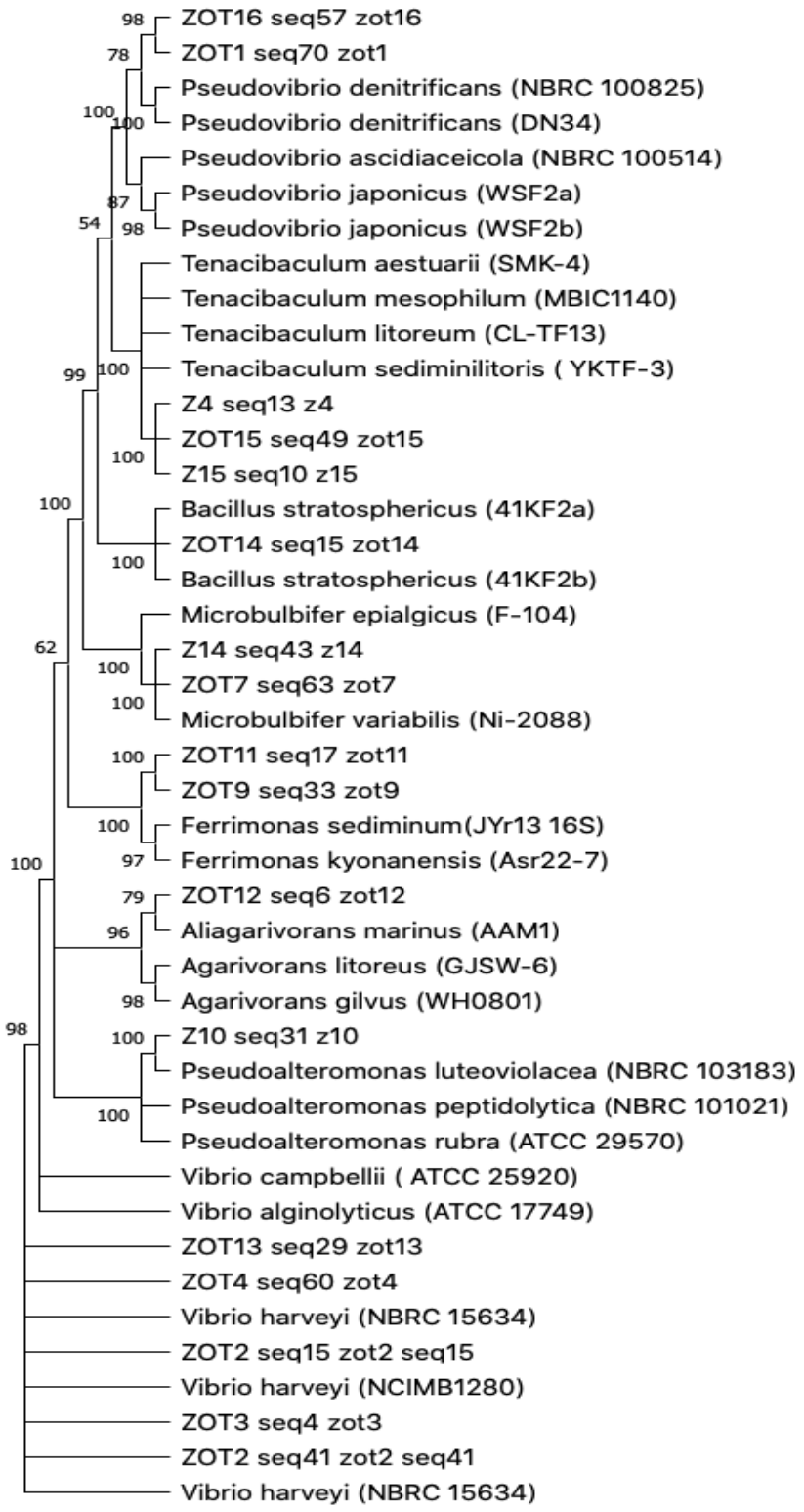



*Figure 17. Phylogenetic analysis of the sequences obtained in this study with GenBank sequences. The tree was elaborated with the Maximum Likelihood method and the GTR + G + I model with a total of 1000 repetitions (Bootstrap).*

**Extracts and fractions**

The crude extracts were obtained with 100% $CH_3OH$, from 10mL an extract biomass greater than 100 mg $mL^{-1}$ was obtained for each of the isolates. The same ones that were fractionated by chromatography. In figure 17, the chromatograms of fraction F3 of Z1 can be observed. Pattern recognition analysis was employed. Once the baseline was corrected, we proceeded with the Principal Component Analysis (PCA) with data centered on the mean, and scaled. Centering in the middle corrects for signals produced by chemical artifacts, such as impurities or sudden changes in mobile phase. While scaling promotes that all variables have the same factor of importance. There was a difference in the profiles, which shows that there are different molecules or compounds typical of each bacterium, as illustrated in the schedules (Figs. 18-21). In the present study, the evaluation of the extracts for animal health was considered. Regarding human health, the evaluation of the fraction F-2 and F-3 was considered.

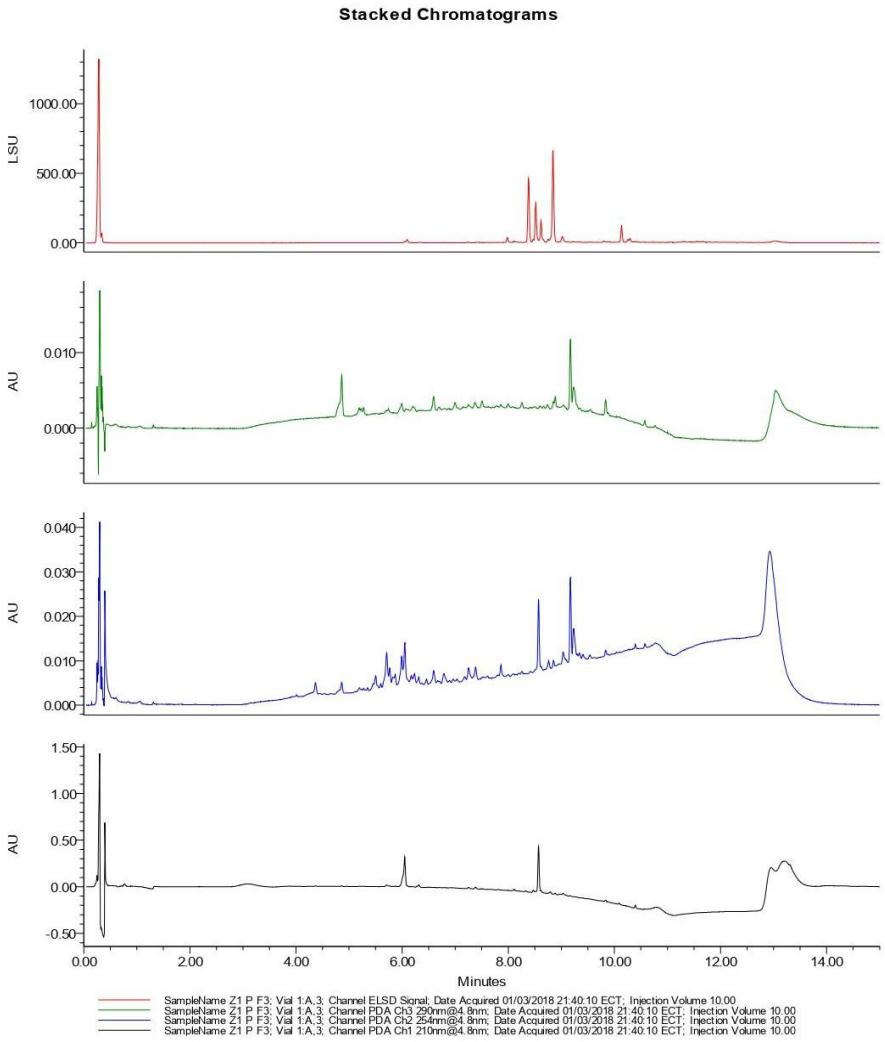



*Figure 18. Chromatogram of F-3 of Z1.*

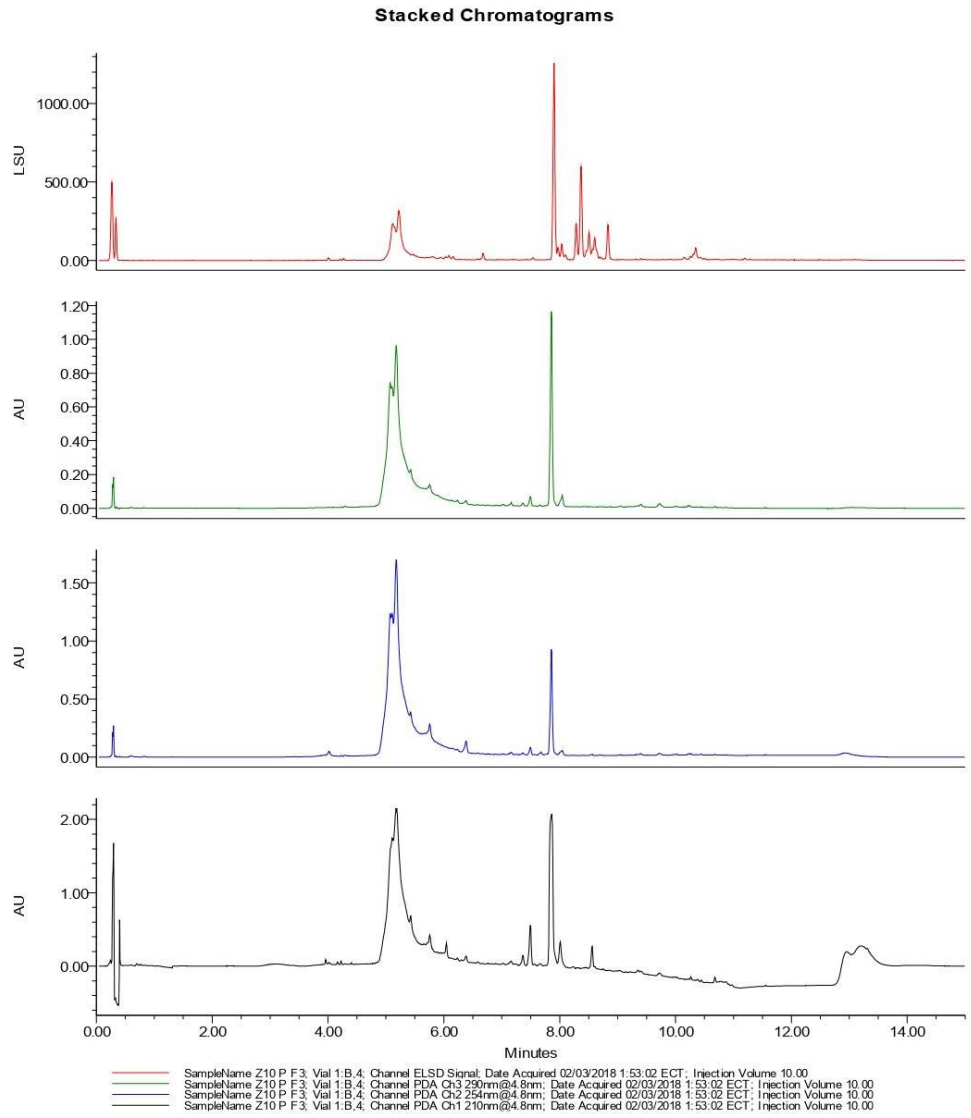



*Figure 19. Chromatogram of F-3 of Z10.*

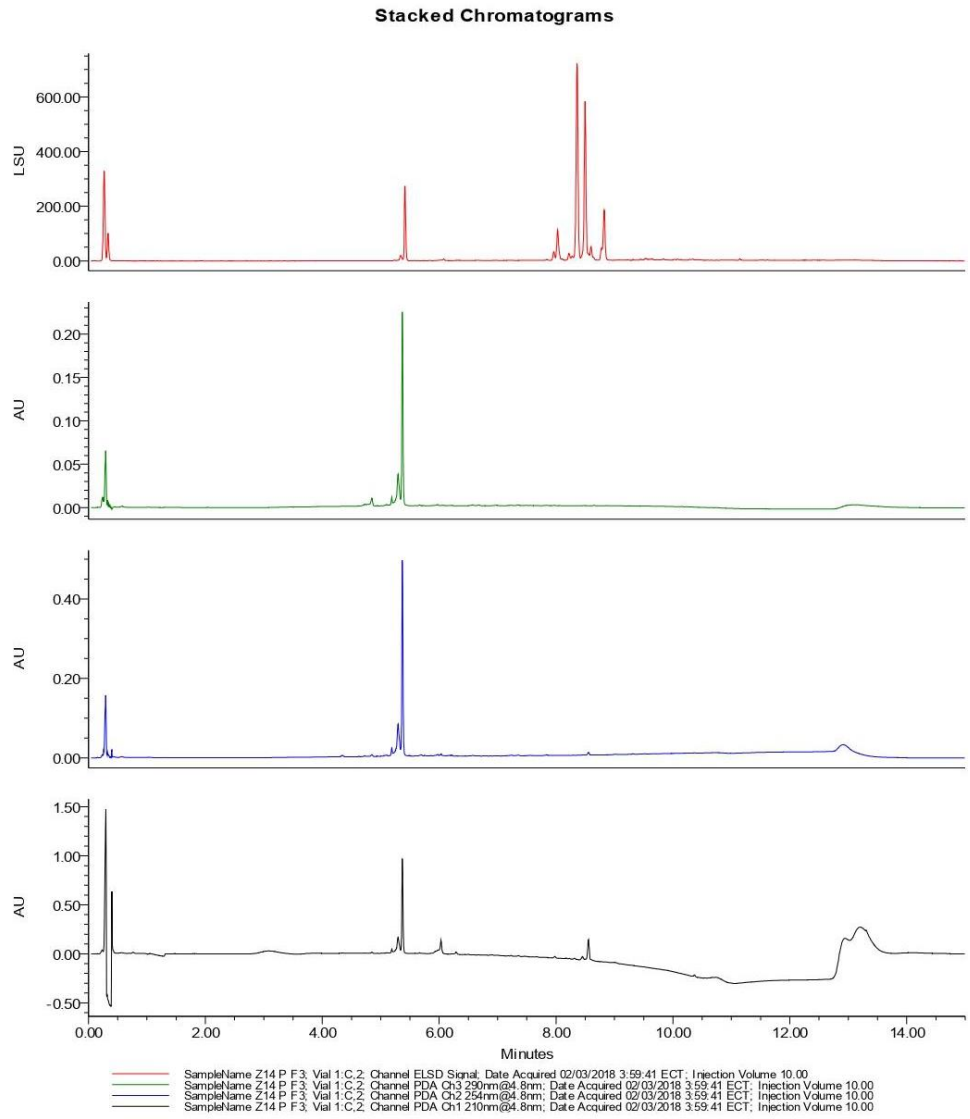



*Figure 20. Chromatogram of F-3 of Z14.*

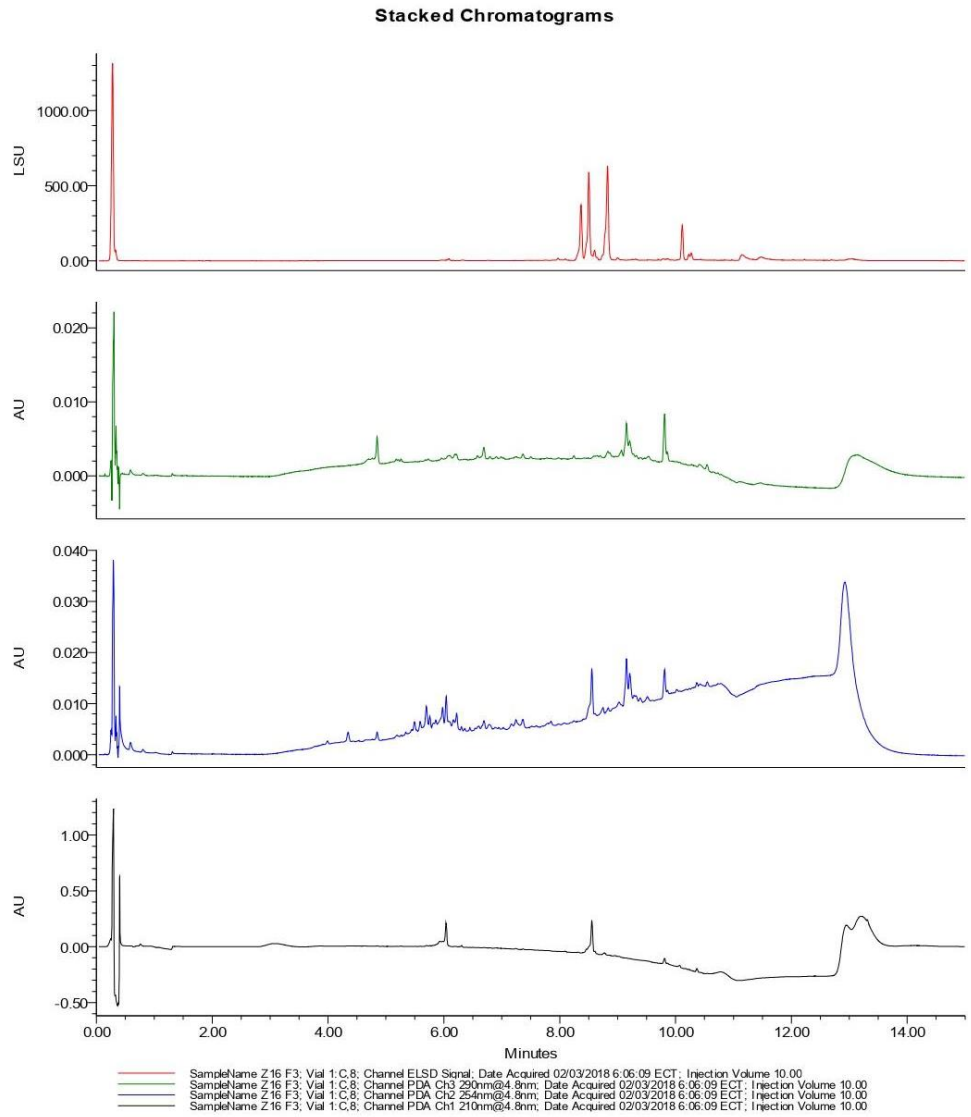



*Figure 21. Chromatogram of F-3 of Z16.*

**Animal health: assessment against pathogenic shrimp vibrios**

The most frequent bacterial pathogens in shrimp cultures in aquaculture are *Vibrio spp.*, such as *Vibrio harveyi* (Mirbakhsh, Akhavan Sepahy, Afsharnasab, Khanafari, & Razavi, 2014), V*ibrio alginolyticus* (Siddique, Hasan, & Hossain, 2017) and *Vibrio campbellii* (Wang et al., 2015; Alexpandi et al., 2021). Vibrios are adapted to the cultivation conditions of tropical peneids (Zheng et al., 2017) where they are favored by high

temperatures, abundance of organic matter and the presence of exuvia, for which they have a high affinity. To help mitigate vibriosis in culture systems, you will need bacteria of marine origin, which have beneficial characteristics, with metabolic diversity. In this study *Zoanthus pulchellus* was a screening source to select bacteria with antimicrobial properties against pathogenic Vibrios in aquaculture (Fig. 13). The selection of *Z. pulchellus* is due to its production of secondary metabolites such as zoanthamines and ecdysteroids (Guillen et al., 2018; Jaramillo et al., 2018; Monte-Martínez et al., 2019), indicating that the associated microbiome it could help the production of this metabolite.

**Antimicrobial activity from extracts and secreted products of isolated bacteria**

The presence of inhibition zones was not observed, we can conclude that the extracts do not have a microbicidal effect for the evaluated vibrios. Similarly, in the secreted products (supernatant), no inhibition zones were observed, and exclusion zones were observed, it being possible to explain that the supernatants of the bacteria were not filtered correctly, or there was contamination. So, that safety could not be totally guaranteed (Fig. 22).

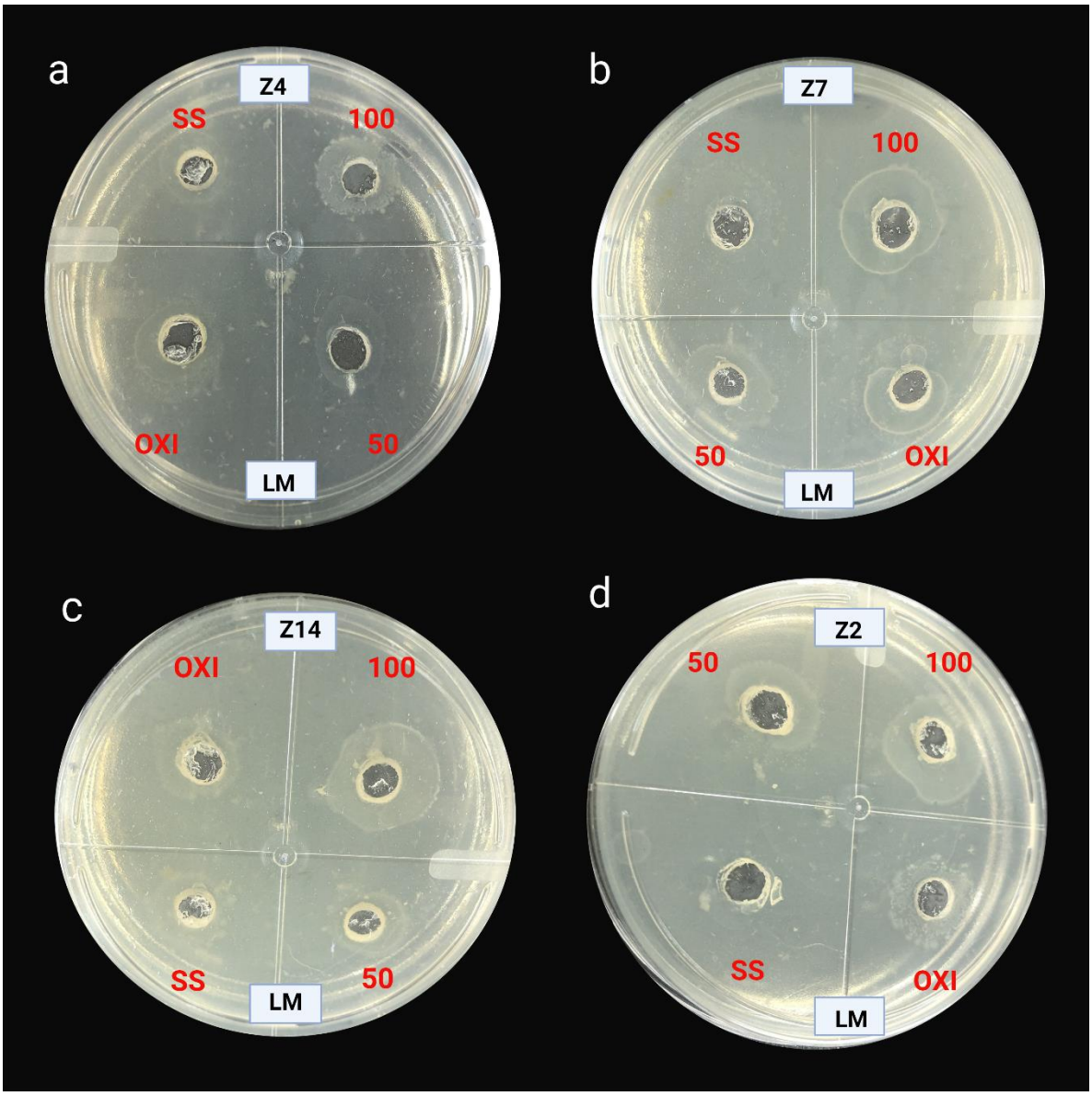



*Figure 22. Exclusion zone of bacterial isolates at volumes of 50 µL, 100 µl, 2% NaCl saline (SS) and oxytetracycline at 10.0 mg $mL^{-1}$ against Vibrio campbellii (strain LM2013), causing bioluminescence in shrimp cultures. a) Bacterial isolate Z4 (*Tenacibaculum mesophilum), b) Bacterial isolate Z7 (Microbulbifer variabilis), c) Bacterial isolate Z14 (Microbulbifer variabilis) d) Bacterial isolate Z2 (*Vibrio harveyi).*

**Exclusion capacity of isolates**

In the competitiveness study, only 3 bacterial isolates, Z2 (*Vibrio harveyi*), Z13 (*Vibrio harveyi*) and Z14 (*Microbulbifer variabilis*), showed positive inhibition effects against pathogenic shrimp vibrios (*Vibrio harveyi, V. vulnificus, V. campbelli* and *V*

*parahaemolyticus*). Strains Z2, Z13 and Z14 can inhibit *Vibrio harveyi* at various concentrations ($10^4$, $10^5$ and $10^6$ CFU $mL^{-1}$). However, to inhibit *V. vulnificus*, the best bacterial isolate is the Z14 strain at the highest concentration (1x$10^6$ CFU $mL^{-1}$). Likewise, in the evaluation against *Vibrio parahaemolyticus* and *Vibrio campbelli*, strains Z13 and Z14 and Z2 showed inhibition of the pathogen, although it is evident that Z2 obtained better inhibition results (23 mm) at a concentration of (1x$10^6$ UFC $mL^{-1}$) (Figs. 23 and 24).

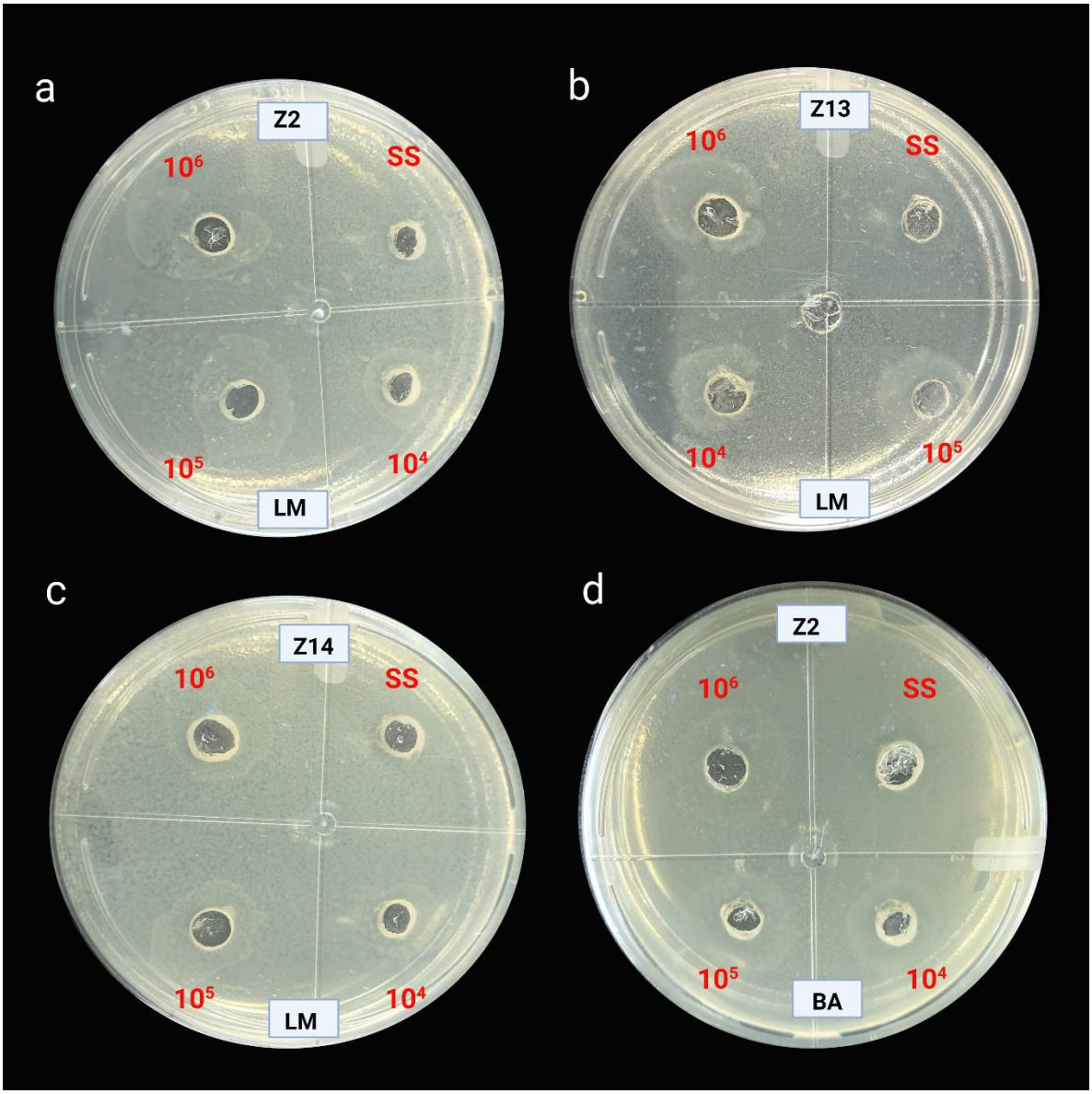



*Figure 23. Exclusion zones of bacterial isolates at concentrations of $10^4$, $10^5$ and $10^6$ CFU mL, a) Z2 (Vibrio harveyi) vs Vibrio campbellii (strain LM2013) (LM), b) Z13 (Vibrio harveyi) vs Vibrio campbellii (strain LM2013) (LM), c) Z14 (Microbulbifer variabilis) vs Vibrio campbellii (strain LM2013) (LM), d) Z2 (Vibrio harveyi) vs Vibrio parahaemolyticus (strains BA94C2) (BA).*

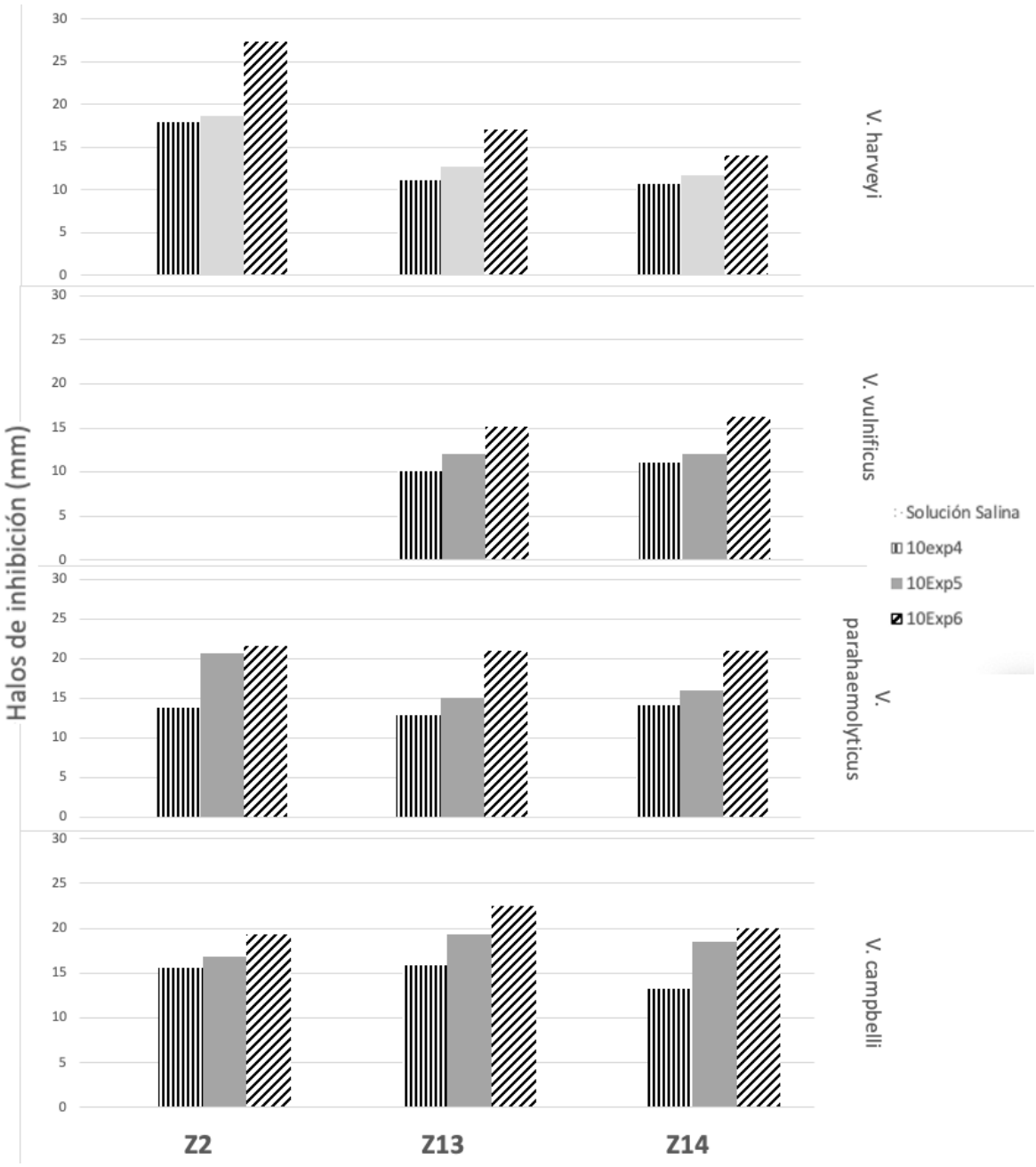



*Figure 24. Inhibition effect (mm) of bacterial isolates of Zoanthids against pathogenic shrimp Vibrios.*

These results show that *Vibrio harveyi* can be used as a probiotic against shrimp pathogens. Several studies show that *Vibrio sp*. serves to counteract vibriosis in cultivation systems. Thus, we have that *Vibrio alginolyticus* showed good probiotic properties against the shrimp pathogen *Vibrio harveyi* (Gullian et. Al, 2004). Likewise, Austin et al. (1995) and

Vandenberghe et al. (1999) highlight the probiotic capacity in larviculture of *Vibrio alginolyticus* to reduce the disease caused by *Aeromonas salmonicida*.

**Biofilm formation and inhibition**

Biofilm formed by the pathogen is difficult to eliminate (Hanning et al., 2008). *Vibrio* species form biofilms to adapt and survive in different environmental conditions (Yildiz & Visick, 2009). In this study, the percentage of inhibition of biofilm formation of *Vibrio parahaemolyticus* was analyzed. The three extracts Z1, Z4 and Z16 were able to significantly inhibit biofilm formation when compared to the control (Fig. 25). The extracts of strains Z4 and Z16 decreased biofilm formation by 32 and 38% respectively. Interestingly, the Z1 strain extract reduced biofilm by almost 50% of its production.

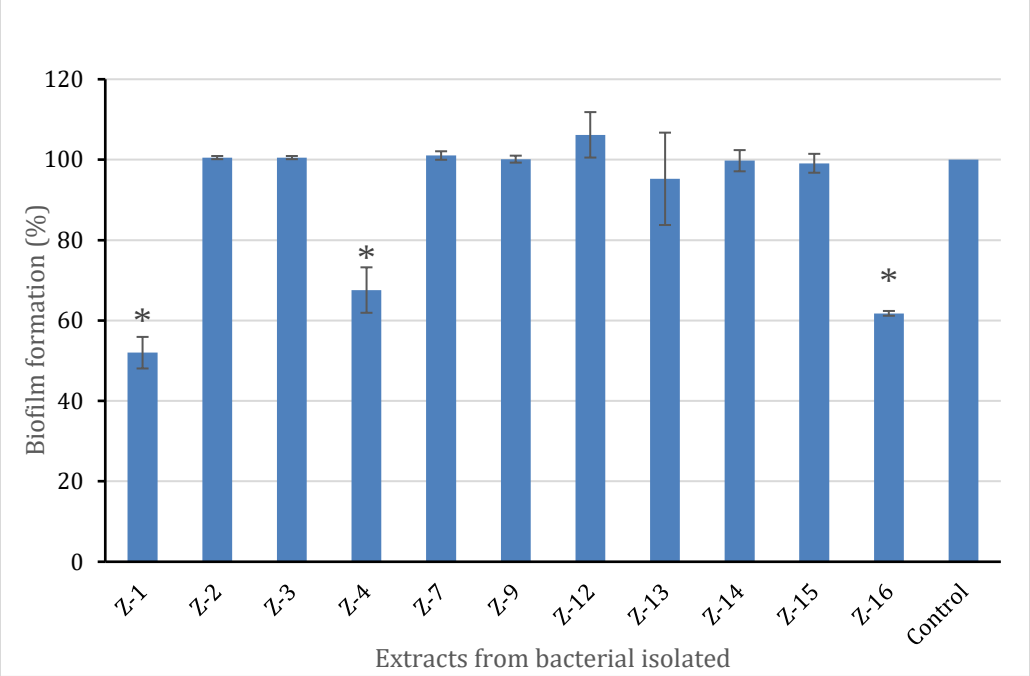



*Figure 25. Percentage of inhibition of extracts from isolated bacteria.*

Furthermore, figure 26 shows a boxplot of the data, obtaining a $P_{value} 4.01e^{-013}$ with 99% confidence in the treatments described. Which states that there were significant differences in the interruption of biofilm formation in extracts Z1, Z3 and Z16. With the

results obtained, it can be hypothesized that our extracts can be used for aquaculture farming systems for biofilm inhibition.

These results are consistent with studies conducted by Suhartono et al. (2019a) who used ethanol extracts of *M. oleifera* to inhibit biofilm formation of *V. alginolyticus*. Suhartono et al. (2019b) reported the ability of an ethanolic extract of Waru (*Hibiscus tiliaceus*) leaves to inhibit *V. alginolyticus* biofilm formation. Additionally, Karnjana, Soowannayan, & Wongprasert, (2019) investigated the ethanolic extract from *Gracilaria fisheri* and furanone to inhibit the biofilms formation of *V. harveyi* and *Vibrio parahaemolyticus*. Furthermore, extracts from marine *Bacillus spp*. could impair the biofilm formation of *Vibrio spp.* (Nithya & Pandian, 2010)

To sum up, Z1, Z4 and Z16 extracts considerably hampered the biofilm formation of *Vibrio parahaemolyticus*. These extract products can act as a natural alternative antibacterial agent to control shrimp pathogenic bacteria.

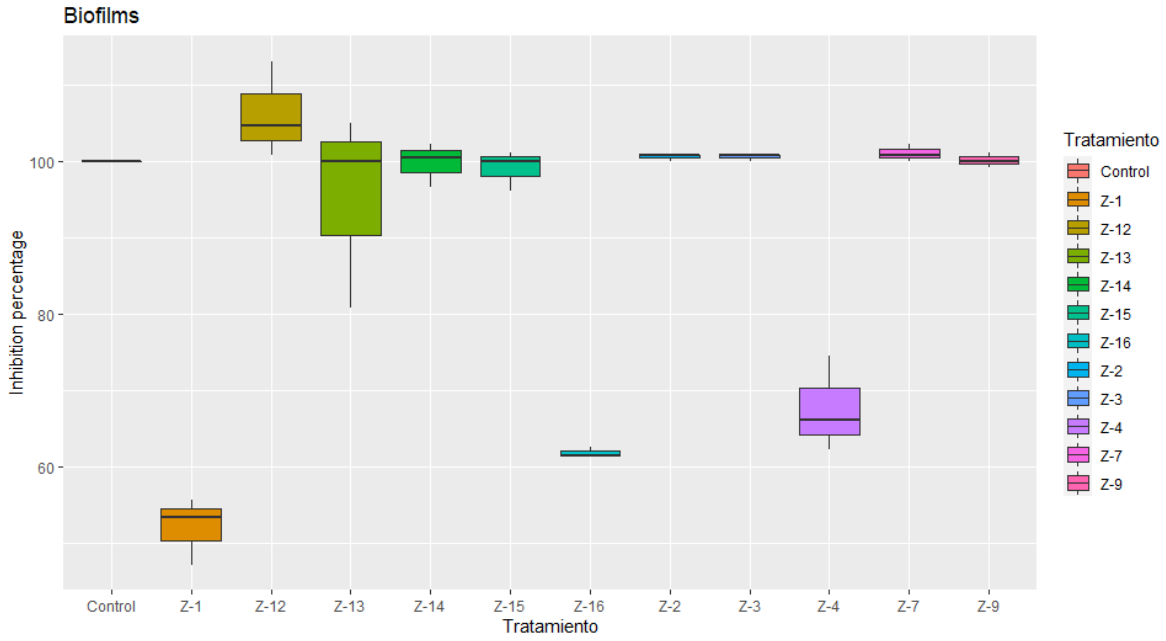



*Figure 26. Boxplot of the inhibition percentages of the extracts from isolated bacteria. The graph shows a significant difference with* $P_{value}$ *4.01* $e^{-13}$

**Human health: carcinoma cell lines assessment**

As initial screening, 10 µg $mL^{-1}$ of the fractions (F2 and F3) obtained from each bacterium were evaluated at a single final concentration against three tumor cell lines MCF-7, Caco-2 and Hep-G2 (Fig. 27). Through the analysis of cell viability, it was evidenced that nine F-3 significantly affected the viability of the three tumor lines, showing a marked cytotoxic activity (Fig. 18). However, no cytotoxic activity was evidenced for the F- 2. The F3 that showed cytotoxic activity against the three tumor lines were retested at six different concentrations (0.315, 0.625, 1.25, 2.5, 5.0, 10.0 µg $mL^{-1}$). Where only the F-3 from the bacteria encoded as Z-10 (*Pseudoalteromona luteoviolacea*) showed cytotoxic activity for the three tumor cell lines, dependent on the concentration (Figs.28 and 29).

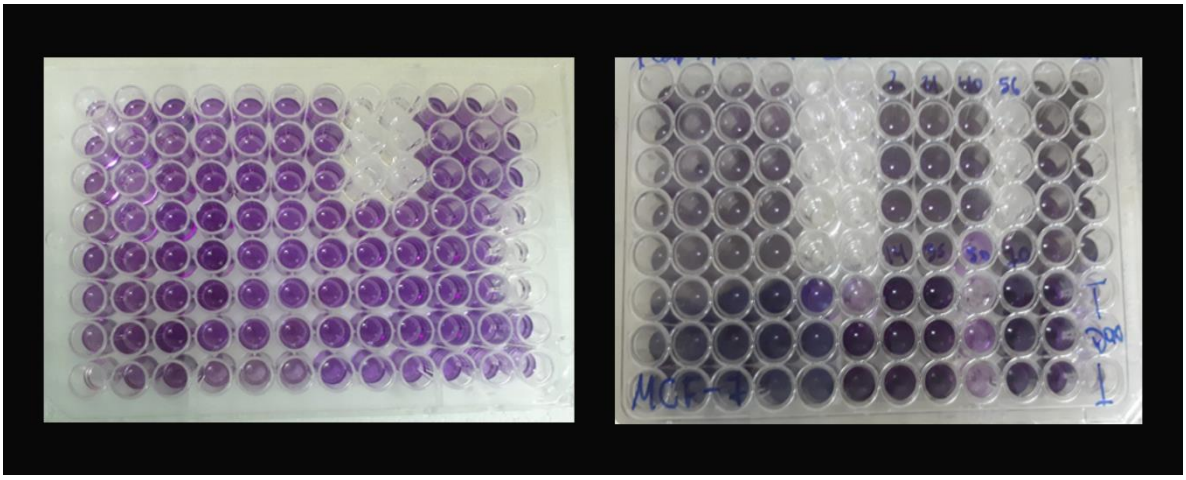


*Figure 27. Inhibition assays against Hep-G2 (top) and MCF-7 (bottom) cell lines.*

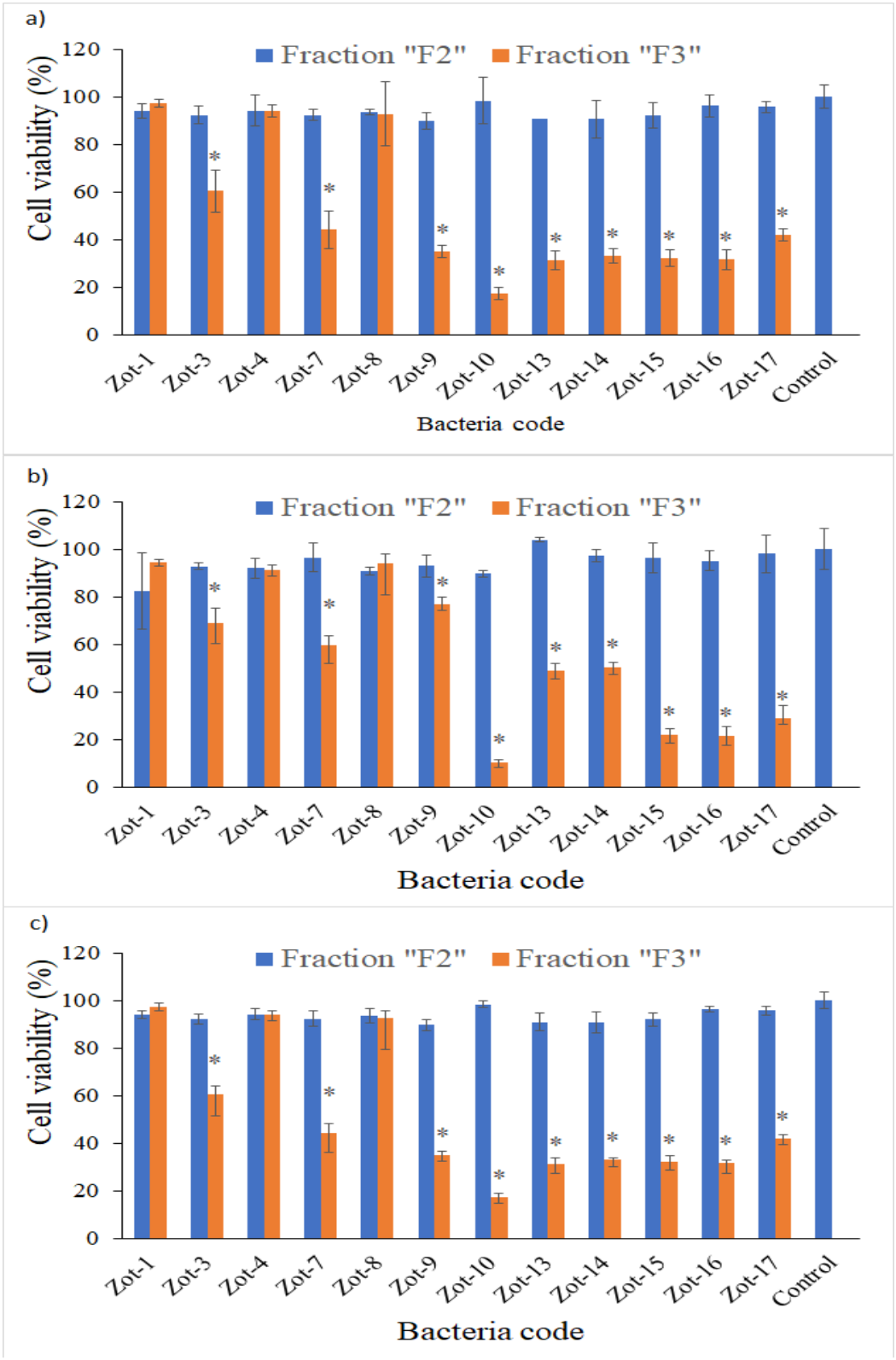



*Figure 28. Cytotoxic activity of the fractions. A) against the MCF-7 cell line, in B) against the Caco-2 cell line and C) against the Hep-G2 cell line. The data are represented as mean ± SD of three repetitions and correspond to the percentage of normalized cell viability against the mean value recorded in negative control samples. Asterisks (*) denote statistical differences (P <0.05) compared to the negative control.*

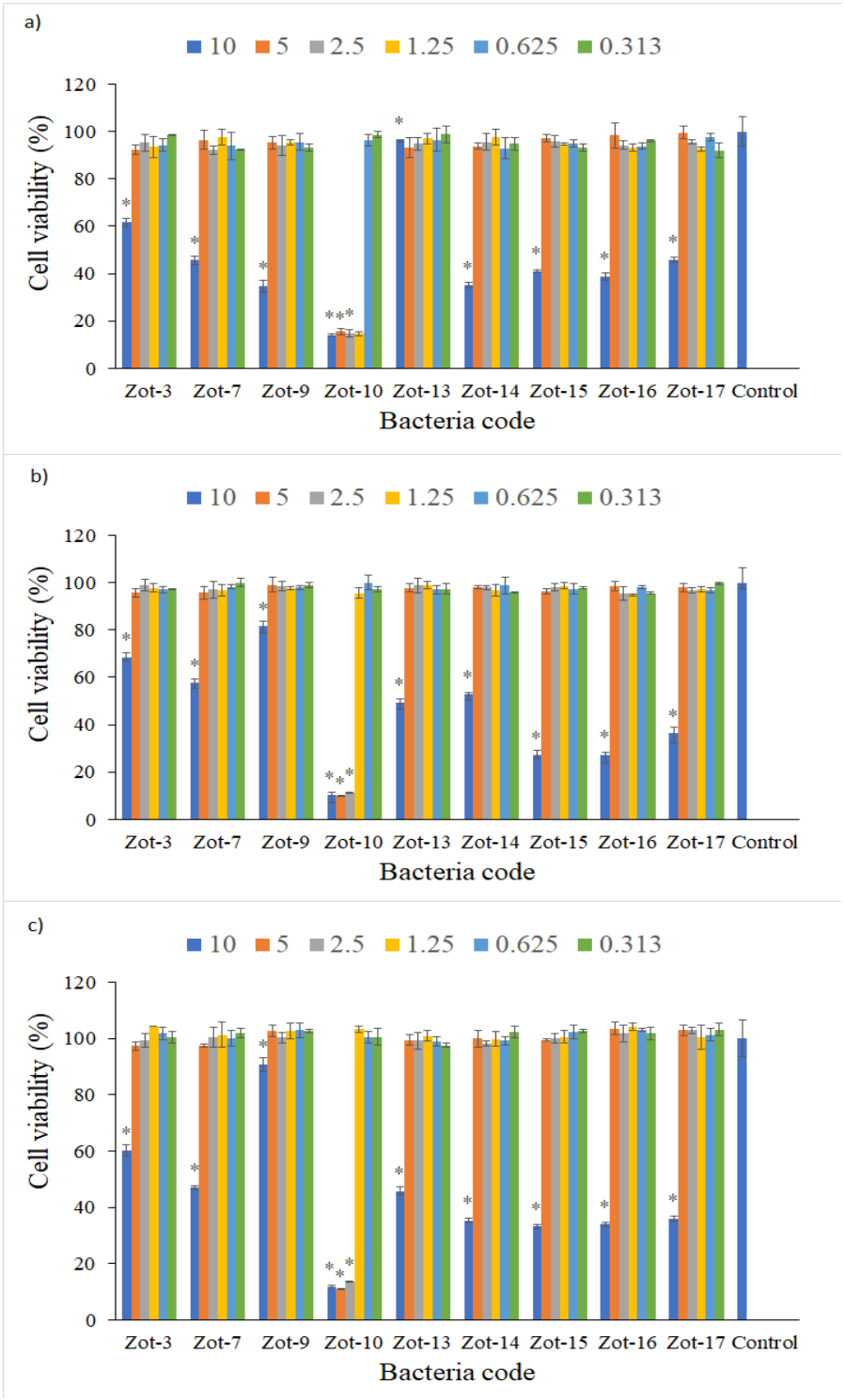



*Figure 29. Cytotoxic activity of the fractions at six different concentrations. A) Against the MCF-7 cell line, in B) against the Caco-2 cell line and C) against the Hep-G2 cell line. The data are represented as mean ± SD of three repetitions and correspond to to the percentage of normalized cell viability against the mean value recorded in negative control samples. Asterisks (*) denote statistical differences (P <0.05) compared to the negative control*

Hu et al. (2017) identified two new compounds which they called Neoantimycins A and B and which were observed to possess cytotoxic activity against the cell lines SF-268 (glioblastoma), NCI-H460 (lung cancer) and MCF-7 (adenocarcinoma of the breast) although in this case their activity was mild. In this work, extracts were found that showed strong cytotoxic activity against the MCF-7 cell line, therefore said extract has great potential as a possible anticancer agent. Furthermore, the study by Hu et al. (2017) identified these new compounds from the bacterium *Streptomyces* antibiotics in marine sediments.

Lai et al. (2021) extracted secondary metabolites from the sponge *Theonella sp*., within which teonelasterol L,5'-O-acetyl-20-deoxyuridine and 5'-O-acetylthymidine were obtained. The metabolites were tested against various cancer cell lines such as MCF-7, MDA-MB-231, T-47D, HCT-116, DLD-1, K562 and Molt 4 and it was observed that they presented antiproliferative activity against these cell lines. Again, as already mentioned in previous studies, the MCF-7 cell line was also inhibited by the extracts obtained in the present work.

Fahmy and Abdel-Tawab (2021) extracted secondary metabolites from *Streptomyces* sp. associated with sponges. In this work, no compounds were identified as such, since extracts (as in this work) were evaluated. They were evaluated for antiviral activity against HSV-1, Cox-B4 and hepatitis A viruses and a good antiviral activity was determined. The extract was also evaluated for anticancer activity against MCF-7, Hep-G2 and HCT-116 cell lines and showed good cytotoxic activity. In this project, extracts tracts were evaluated against MCF-7, Caco-2 and Hep-G2 cell lines, as in the work of Fahmy and Abdel-Tawab (2021) and, like them, promising results were also obtained against these cell lines.

Notably, the studies mentioned against cancer cell lines are mainly of secondary metabolites obtained from sponges, while in the present project, they are of bacteria associated with zoanthids.

# CONCLUSIONS

Sixteen zoanthids were analyzed, from which thirteen bacterial strains were isolated and identified, these include Z1, *Pseudovibrio denitrificans*; Z2, *Vibrio harveyi*; Z3, *Vibrio harveyi*; Z4, *Tenacibaculum mesophilum*; Z7, *Microbulbifer variabilis*; Z9, *Ferrimonas kyonanensis*; Z10, *Pseudoalteromonas luteoviolacea*; Z12, *Agarivorans litoreus*; Z13, *Vibrio harveyi*; ZOT14, *Bacillus sp*; Z14, *Microbulbifer variabilis*; Z15, *Tenacibaculum mesophilum*; Z16, *Pseudovibrio denitrificans*. Although most of them were identified at the species level, *Bacillus* spp. it could not be identified at the species level since it is a more complex organism and needs other studies.

In the evaluation of isolated strains in zoanthids against shrimp pathogens, three isolates were obtained as possible probiotic strains in aquaculture. Two of these isolates (Z2 and Z13) belong to *Vibrio harveyi*, the other belongs to *Microbulbifer variabilis*. Both genus previously reported as useful in aquaculture. The isolate Z16 belonging to *Pseudovibrio denitrificans* presented bioactivity only as an extract. Furthermore, such extracts of bacterial isolates helped to interrupt the formation of the biofilm of the pathogen (*V. parahemolyticus*). Observations of these bioactivity responses in controlling vibriosis in culture systems support the importance of continuing research in shrimp with these *Zoanthus pulchellus* isolates.

In this work it was shown that the fractions have cytotoxic activity against the MCF-7, Caco-2 and Hep-G2 cell lines. Nine fractions (F-3) significantly affected the viability of the three tumor lines, where only the F-3 of the bacteria encoded as Z10 (*Pseudoalteromonas luteoviolacea*) showed cytotoxic activity against the three tumor lines.

Finally, it is important to mention that although many studies of bioactive compounds with cytotoxic activity have been carried out, the vast majority of them are based on compounds obtained from sponges and spices associated with them, in addition to studies where they are identified bacteria associated with zoanthids but not the metabolites obtained from them. Therefore, the present study increases the knowledge of bioactive compounds with the cytotoxic activity of bacteria associated with zoanthids.

# RECOMMENDATIONS

Carry out the identification of the bioactive compound isolated from *Pseudoalteromona luteoviolacea* through mass spectrometry, to identify the bioactive compound present in this strain.

Use the bacterial strains and the supernatants of bacterial isolates obtained in this investigation in shrimp larvae to know if there would be a toxic effect on these organisms.

Identify which molecules cause this antimicrobial activity evidenced in the extracts.

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# SUPPLEMENTARY MATERIAL

Table 1. Difco™ Marine Agar 2216 Medium Components

| **Components** | **Approximate Formula Per Liter** |
|---|---|
| Peptone | 5.0 g |
| Yeast Extract | 1.0 g |
| Ferric Citrate | 0.1 g |
| Sodium Chloride | 19.45 g |
| Magnesium Chloride | 8.8 g |
| Sodium Sulfate | 3.24 g |
| Calcium Chloride | 1.8 g |
| Potassium Chloride | 0.55 g |
| Sodium Bicarbonate | 0.16 g |
| Potassium Bromide | 0.08 g |
| Strontium Chloride | 34.0 mg |
| Boric Acid | 22.0 mg |
| Sodium Silicate | 4.0 mg |
| Sodium Fluoride | 2.4 mg |
| Ammonium Nitrate | 1.6 mg |
| Disodium Phosphate | 8.0 mg |
| Agar | 15.0 g |