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ANTIBACTERIAL ACTIVITY OF MACROALGAE CRUDE EXTRACTS FROM PARANÁ COASTAL ISLANDS (BRAZIL) AGAINST MARINE ISOLATE OF *ENTEROBACTERIACEAE*

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Abstract: Coastal organic pollution results in eutrophication and distributional changes of microorganisms. Besides, flow of commercial antibiotics through the domestic effluents favors bacterial resistance, demanding new drugs and bioremediation processes. Macroalgae protective responses are attributed to their secondary metabolites, reason why is widely studied for bioactivities. We investigated the antibacterial activity (AA) of methanolic and aqueous extracts of four macroalgae species from Paraná coastal islands, being: *Pyropia acanthophora* (Rhodophyta), *Sargassum cymosum* (Phaeophyceae), *Ulva* sp., and *Gayralia brasiliensis* (Ulvophyceae) from Paraná coastal islands, Brazil. The crude extracts were tested against an environmental *Enterobacteriaceae* isolated and cultivated from a eutrophic estuary, and compared to the reference strain, *E. coli* ATCC 25922. The environmental strains from Paranaguá Bay sample water were isolated using Colilert®T18 and filter membrane. Gram stain and biochemical tests were performed to confirm the *Enterobacteriaceae*. The extract yields were calculated and related to antibacterial activities, evaluated by disk diffusion and broth microdilution. The investigated species showed AA only for methanolic extracts. *Ulva* and *S. cymosum* showed higher extracts yields. *Ulva* sp. inhibited *E. coli* activity in ca. of 70%, for both strains and all concentrations tested. *P. acanthophora* showed the higher inhibition value, however only at higher extract concentration and presenting the lower extract yields. *S. cymosum* extracts inhibited both strains in 70 - 80%, at higher concentration tested. Besides other studies revealed bioactivities for *G. brasiliensis*, our only species collected in a conservation unity area, their extracts showed low AA and yields in the tested conditions. Considering *Ulva* and *Sargassum* broad global distribution, and high biomasses, these are our best candidates for AA. Further molecular tests of the environmental isolate and biochemical characterization of the antibacterial compound(s) are highly recommended, aiming to explore new sustainable applications of these extracts with AA for marine biotechnological processes, products or endeavors, related to human health or marine conservation.

Keywords: marine algae, bacterial inhibition, bioactivity, southwestern atlantic ocean.

INTRODUCTION

Marine organisms such as seaweeds are a rich source of natural bioactive compounds, such as proteins, polysaccharides, and secondary metabolites. These bioactive compounds have been responsible to contribute to anti-inflammatory, antioxidant, and antimicrobial properties (Helbert, 2017; Yap *et al.*, 2019; Leandro *et al.*, 2019). Macroalgae provide a chemical arsenal of various bioactive compounds that enable their coexistence with microorganisms (Nylund *et al.*, 2005) although studies have shown that marine algae are a rich source of antibacterial agents (synopsis in Lomartire *et al.*, 2021). Marine ecosystems, as well as its organisms, is teeming with microorganisms. Kai *et al.*, (2017) identified 247 bacterial strains from ocean water samples, a significant degree of microbial diversity. The distribution of bacterial strains in the ocean (including pathogens) is a result of the availability of substrates and/or susceptible hosts, which maintains its ecological balance (Moura *et al.*, 2011; Al-Sarawi *et al.*, 2022; Grunwald *et al.*, 2022). Enterobacteria are indicators of water quality and are abundant in environments enriched with organic matter (Silva 2015; Miquelante and Kolm, 2011). These pathogenic gram-negative bacteria are resistant to higher temperatures and are one of the main cause of intestinal infections worldwide (Trabulsi and Alterthum, 2005). Although these bacteria are

more abundant in terrestrial environments and in their natural biota (guts of birds and mammals), several biogeochemical factors explain the permanence and high viability of some strains in seawater.

The seawater entry route of pathogenic microorganisms are mainly untreated domestic effluents, given by the interface between fresh water (river), brackish water (estuary) and the ocean (Pommepuy *et al.*, 2005). Through the same route of pathogens, there is also a flow of drugs that can promote a selective pressure and favors bacterial resistance in natural environments (Silva, 2015; Moura *et al.*, 2011; Hoffman *et al.*, 2015; Al-Sarawi *et al.*, 2022; Grunwald *et al.*, 2022; Su *et al.*, 2022), whose permanence turns the marine environment into a source of resistant genes (Al-Sarawi *et al.*, 2023). Antimicrobial resistance is one of the greatest threats to human health worldwide (Walker *et al.*, 2009) as it greatly limits the likelihood of treatment by compromising the effectiveness of available drugs for treatments (Ventola, 2015; Su *et al.*, 2022; Ahmed *et al.*, 2023). The natural antibacterial potential induced researchers to investigate effective compounds / drugs aiming to combat infectious diseases, which are claiming lives at alarming rates. Only in U.S. and Europe ca. of 50,000 deaths are attributed to antibiotic-resistant bacteria (ARB) diseases and if the problem with ARB remains unmonitored, it is estimated that they may lead to 10 million cases of death annually by 2050 (Rönnerstrand and Sundell, 2015). This scenario also suggests that bacteria strains are evolving and adapting for more resistant strains quickly, including due to new introduced antibiotics in marine ecosystems, while the development of novel antibiotics, mainly based in natural products, is still incipient. In contrast, marine organisms live in close association with competitive and hostile environments, as terrestrial, and humans. As defense, they produce secondary metabolites compounds in response to ecological pressure caused by competition for space, predation, and abiotic parameters abrupt variations. Some of these compounds inhibit or limit the development of microorganisms, these include macroalgae extracts (Perez *et al.*, 2016; Martic *et al.*, 2023).

In Brazil, there are listed between 800 and 900 taxa of seaweeds, classified in Chlorophyta (Ulvophyceae - green), Rhodophyta (red) and Phaeophyceae (brown algae) (Menezes *et al.*, 2015), and 139 species were listed for Paraná State (Pellizzari *et al.*, 2014). Dozens of these species have presented promising antiviral, antioxidant, and antibacterial properties (Bernardi *et al.*, 2016; Briani *et al.*, 2018; Nauer *et al.*, 2018; Cotas *et al.*, 2020; Torres *et al.*, 2021). Macroalgae are distributed from polar and tropical zones, from shallow intertidal to more than 200 m depth, from super-photic to deeper environments with low incidence of light, in areas of wide haline gradients, and environments ranging from untouched to eutrophic and contaminated. This wide distribution is due to many ways to adapt and tolerate a very broad range of abiotic conditions, or even extreme habitats (Pellizzari *et al.*, 2020). Seaweed blooms, mainly of *Ulva* and *Sargassum,* are associated with eutrophication in aquatic environments (Moura *et al.*, 2011; Fleming *et al.*, 2008) or changes in thermos-haline patterns (Calumpong *et al.*, 2021). These events, most common nowadays, also demonstrate that many of these organisms use diverse physiological strategies and biochemical arsenal to defend themselves against the presence of environmental stressors resulted from anthropic actions or abiotic changes, and the opportunistic species are very efficient in this sense. The permanence of instable or changing conditions may induce and/or stimulate the synthesis of secondary

metabolites by some seaweed species (Perez *et al.*, 2016; Yap *et al.*, 2019; Martic *et al.* 2023). Compounds such as terpenoids, steroids, phenolics, alkenes and phlorotannins are synthesized as a defense strategy in extreme and/or changeable environments (Shanmughapriya *et al.*, 2008; Makhlof *et al.*, 2023). These compounds act mainly as antibacterial (Sridhar and Vidyavathi, 1999; Nagayama, 2002; Shafay *et al.*, 2016; Perez *et al.*, 2016; Yong Li *et al.*, 2018; Shanmughapriya *et al.*, 2008; Coronel *et al.*, 2020; Hussein *et al.*, 2020; Bhruyar *et al.*, 2020; Scania and Chasani, 2021; Cmiková *et al.*, 2022).

Thus, the use of seaweed extracts can be a natural, effective alternative, with less side effects and less toxicity than the synthetic antimicrobials currently available, including for resistant strains (Cmikova *et al.*, 2022; Coronel *et al.*, 2020; Hussein *et al.*, 2020). Given the need to seek efficient chemical compounds as a subsidy for antibacterial new drugs, this preliminary study aimed to evaluate the antibacterial activity (AA) of crude extracts of seaweeds, belonging to the three main taxonomical groups, against a control-reference strain of *E. coli*, in comparison to an environmental *Enterobacteriaceae* isolated from eutrophic estuary from Southern Brazilian coast.

METHODS

STUDY AREA

The Paraná coast, southeastern Brazil, southwestern Atlantic is characterized by a high diversity of ecosystems along their 90 km (Bigarella, 2001; Pellizzari *et al.*, 2014). Formed by rocky shores, sandy beaches, and by a very long estuarine Complex named Iguape-Cananéia-Paranaguá, permeated by mangroves and saltmarshes, and pristine in some areas (Lana *et al.*, 2001).

In the Southern region of Paraná coast is Matinhos City, and the Guaratuba Bay (GB) outfall, also in this area is located Farol Island (-25,8522044 S // -48,5356772 W) (Figure 1). This island receives sediment, nutrients, and freshwater input from GB, besides high degree of urbanization mainly due to the summer touristic activities in Caiobá Beach. The eutrophication influence in the nearby seawater alters the physicochemical parameters, favoring the proliferation of opportunistic green algae beds (Pellizzari *et al.*, 2014). In the Northern region, is located Pontal do Sul (-25,5645292 S // -48,3548125 W) outfall of the Paranaguá Estuarine Complex (PEC) (Figure 1) that has a floating population, between 28 and 300 thousand inhabitants during the summer season. PEC also bath Paranaguá City and its cereal harbor (3th major port in Brazil), a fact that overloads the service sector, and impact negatively several adjacent ecosystems. Records of intense ship traffic, fertilizers load and unloads, untreated domestic sewage are the main environmental issues in this area (Lana *et al.*, 1989).

In the PEC is also located Rasa Island (-25,3320709 S // -48,3986103 W) inserted in Guaraqueçaba Conservation Area (CA). The island has an area of 10.5 km² and, despite artisanal fisheries, the biodiversity is less impacted compared to Paranaguá City vicinities. However blue crabs and oysters' exploitations are the main activity for the native community (Cunico, 2016), and is probably causing local degradation in the vegetation coverage, characterized by mangroves, saltmarshes and sandbanks, despite being a CA (Fischer and Colley, 2005).

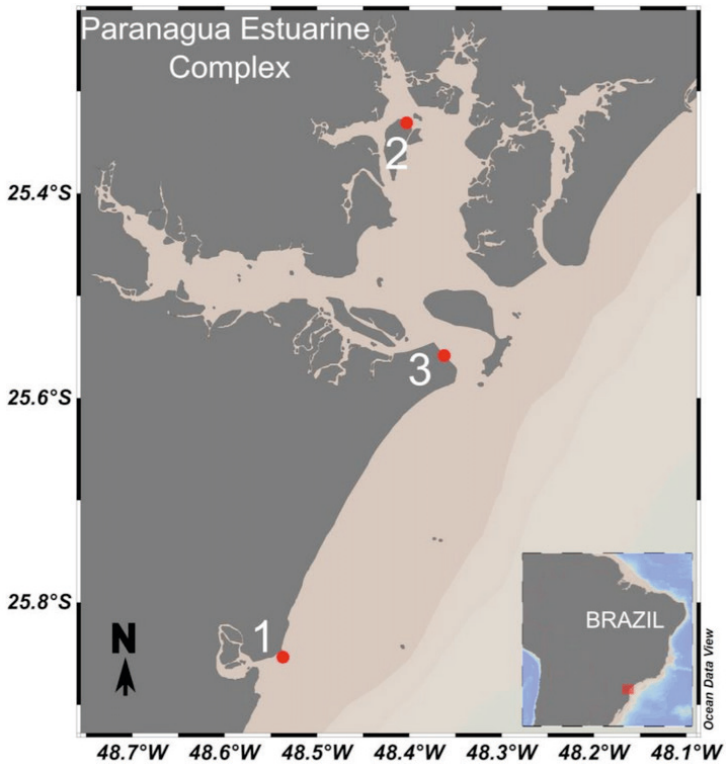

Figure 1. Seaweed sampling stations: 1. Farol Island (Caiobá Beach, Matinhos); 2. Rasa Island (Guaraqueçaba – Federal Environmental Protection Area). Seawater sampling station for bacterial strain isolation: 3. Pontal do Sul Beach. Paraná coast, Southwestern Atlantic, South Brazil.

SEAWEED BIOMASS SAMPLING

Macroalgae from the three major groups (Chlorophyta –Ulvophyceae-, Phaeophyceae and Rhodophyta) were collected at Farol island, Matinhos, being the following species, respectively: *Ulva* sp. (formerly as *U. fasciata*), *Sargassum cymosum* C. Agardh, and *Pyropia acanthophora* (E.C.Oliveira & Coll) M.C.Oliveira, D.Milstein & E.C.Oliveira 2011(Figure S1). The estuarine Chlorophyte *Gayralia brasiliensis* Pellizzari, Oliveira & Yokoya 2013 was also sampled at Rasa Island

All these samples were sorted and washed with filtered seawater in order to eliminate possible macroepiphytes and sediments. In the laboratory, the macroalgae species were rinsed three consecutive times using distilled and deionized water, and then frozen at -10°C. Subsequently, the macroalgae biomasses, for each species, were individually fractioned at samples of 500g (wet weight).

SEAWATER SAMPLING AND ANALYSIS TO ISOLATE *ENTEROBACTERIACEAE*

At Pontal do Sul beach (PEC outer sector) triplicates of 300mL of seawater were collected. Aliquots of 100mL for each sample were analyzed by kit Colilert® T-18 (IDEXX), method that uses specific chromogenic substrate for *E. coli*. The "Quanti-tray" 2000 cards were sealed with Sealer® 2000, and after incubation at 36°C, a conversion of most probable number per 100 mL (MPN/100 mL) using a specific IDEXX Quanti-Tray System table for estimating the contamination values were performed. The samples considered inappropriate for human activities (Resolution 274/2000 of the National Council for the Environment) were selected and used for culture and growth of the target environmental *Enterobacteriaceae*.

ENVIRONMENTAL *ENTEROBACTERIACEAE* CULTURE

After 10-12 hours of incubation for confirming the presence of total coliforms and environmental *Enterobacteriaceae* by the Colilert® T-18 method (IDEXX), the remaining aliquot of the sampled seawater, kept under refrigeration, was filtered in a vacuum pump, using a cellulose nitrate membrane filter, pore size 0.45um. The filter was removed and placed in a sterilized Petri dish, moistened with 1mL of distilled water and homogenized using a Drigalski inoculation loop.

The resulting aliquot was transferred to be inoculated in Petri dishes containing MacConkey growth medium (Kasvi) and then kept in a bacteriological kiln – BOD (SL 101-SOLAB) at 35±2°C for 24-36 hours. After bacterial growth the rosaceous and isolated growth colonies were added to test tubes containing 1mL of saline solution (0.85% NaCl) and then subjected to Gram stain aiming to confirm the presence of gram-negative environmental bacteria.

Subsequently, a new inoculation was carried out in new plates containing MacConkey growth medium, incubated at 35°C ± 2°C for 24 hours and subjected to serial subcultures. The samples were submitted to an instantprov biochemical test (NewProv®) for confirmation at the family level.

MACROALGAL CRUDE EXTRACTS

The frozen seaweed biomass was lyophilized (Alpha 2-4 LDplus - Christ) and ground in a mortar and pestle. The dry powder material was divided into five subsamples (n = 5) for sequential extractions in solvents of increasing polarity by simple maceration: methanol and hot water.

METHANOLIC EXTRACT

The lyophilized algal biomass was eluted in the methanol solvent PA (SYNTH) in the proportion of 80 mL of solvent for each 5g of biomass, in 5 repetitions (n=5). The solution was stored in a beaker protected from light at room temperature for 24 hours. Afterwards filtering was performed on a cellulosic fiber filter. The extract resulting from the filtering was deposited into another beaker and kept in a gas exhaust hood at 25°C until the solvent had completely evaporated. The procedure was repeated 3 times on consecutive days.

AQUEOUS EXTRACT

To the algal biomass resulting from the methanolic extraction process, 160mL of Milli-Q® water (Direct Water Purification System) were added. Afterwards filtering was performed using cellulosic fiber filter. The solution was stored in a beaker protected from light at 35-40° in a heated chamber until the total evaporation of the water.

The extract yield percentages were calculated based on the dry mass of the seaweed and the final weight of the extract used in the extraction, according to the formula: Yield (%) = Weight of the Extract (g) / Weight of the dry biomass (algae) x 100. The dried crude extracts were stored in amber glass at -20◦C.

ANTIBIOGRAMS

Antibacterial activity (AA) of the seaweed crude extracts was determined by performing antibacterial assay and calculating the percentage of bacterial inhibition of each extract for both selected strains.

PREPARING THE BACTERIAL INOCULUM

Two strains of bacteria were used: the environmental isolated and cultivated *Enterobacteriaceae,* and the reference strain of *E. coli* (ATCC – American Type Culture Collection 25922). An aliquot of bacterial growth from each sample was cultured in BHI (Brain Heart Infusion) growth medium. Three colonies of each culture were diluted in saline solution (0.85%) until obtaining a turbidity standard of 0.5 on the McFarland scale (ANVISA, 2020).

DISK DIFFUSION ANTIBIOGRAMS

Two types of tests were performed. In the first, extracts impregnated on discs with three different antibiotics were used as a control and tested. Solvent control was prepared by incubating the bacterial cultures in DMSO 0,5%. In the second, a range of seven antibiotics were tested against the inoculum isolated from the environment, and against the *E. coli* strain ATCC 25922. A stock solution was used where a 5 mg aliquot of each crude extract was diluted in 10% DMSO to a final concentration of 5 mg/mL. A swab was immersed separated in both bacterial strains dilutions and seeded across the entire surface of the culture medium of the Petri dish (150 mm) containing the Mueller Hinton Agar (MHA) medium.

The disks, containing macroalgae extracts and reference antimicrobials (azithromycin, neomycin and amoxicillin) were inserted into the already inoculated culture medium, with the aid of tweezers, and arranged at three cm from each other. All the samples (treatments and controls) were tested in triplicates, in independent experiments. The plates were incubated at 35±2ºC for a period of 18 to 24 hours in a BOD® chamber (Marconi). The diameter of the halos was measured using a digital carbon fiber caliper (Nobrand).

A complementary test of disk-diffusion susceptibility was performed to compare the patterns previously observed in the two tested bacterial strains using the antibiotics Ampicillin, ciprofloxacin, nitrofurantoin, cephalothin, gentamicin, ofloxacin, penicillin discs (CEFAR brand). The halos measurements were interpreted according to CLSI (Clinical & Laboratory Standards Institute).

ANTIBIOGRAM

Following the previous inoculum preparation, a new dilution was obtained until the concentration of 5x104 CFU / mL. A 5µL aliquot of the bacterial suspension (5x104 CFU / mL) was added to the culture medium (Mueller Hinton broth) containing serial dilutions of the methanolic and aqueous extracts (at concentrations of 0.4mg/mL, 0.8mg/mL and 1.6mg/mL), separately, in a 96-wells plate.

The entire plate was incubated from 18 to 24 h at 35±2°C. The absorbance readings were performed using a microplate reader (SpectraMax® mxe) at 600 nm. For the control, only the culture medium with the dilution solvent was used, without the inoculum, and for the positive control, the culture medium was used with the dilution solvent and the bacterial inoculum. The mean and standard deviations of the analyzed triplicates were calculated, and graphs were generated from these results using the GraphPad Prism software, version 8.0.1.

The formula used to calculate the CFU/mL of the bacteria is stated as Equation (a) while the percentage of antibacterial effect based on the CFU/mL was calculated using the Equation (b) stated below:

(a). CFU/mL = No. of colonies x dilution factor 0.01 mL

(b). Percentage of antibacterial effect (%) = CFU/mL nontreated sample – CFU/mL treated sample x 100%
CFU/mL nontreated sample

STATISTICAL ANALYSIS

Extract yields (%) calculation for both extractors were expressed in Mean ± standard deviations SD (n = 5). Data were compiled in spreadsheets (Microsoft Office Excel® 2023) and statistical analysis and graphs were generated by Software R (R Core Team, 2023). Data were tested for normality (Kolmogorov-Smirnov test) and homoscedasticity (Bartlett test) prior to one-way analysis of variance (ANOVA) to compare samples ($p < 0.05$). When differences were identified, Tukey's post-hoc multiple comparison test was applied. Statistical analysis was applied for both extracts and among the seaweed species tested, letters suggest significant differences ($p<0,05$).

After exploratory analyzes to verify data distribution and identify outliers, data of antibacterial assays were compiled and analyzed using R studio software (2023). Statistical analysis was performed using SPSS version 20.0 and GaphPad Prism version 5.0. The significance of difference was considered to include values of establishing alpha of $p=0.05$, using the JAMOVI (2022) and Vegan (2022) software. The graph was generated in the Sciplot (2020) package in R.

RESULTS

SEAWATER ANALYSIS

Water quality analyzes using Colilert method showed that all water samples collected at Pontal do Sul (Canto das Pedras Beach) showed microbiological contamination equal, or higher than 1,000 MPN of *E. coli* / 100 mL. The sample used for cultivation of the environmental bacteria strain had an IDEXX Quanti-Tray System Standard Table conversion ca. of 1,100 MPN of *E. coli*.

YIELD OF SEAWEED EXTRACTS

Table 1 shows the yields (%) of methanolic, and aqueous extracts obtained from *Ulva* sp. *Pyropia acantophora* e *Sargassum cymosum* sampled at Ilha do Farol, a site with more intense urban activity, and from *Gayralia brasiliensis* sampled at Ilha Rasa, a Conservation Area. The yield percentage of the aqueous crude extracts were higher, in general, when compared to methanolic extracts. The red seaweed *Pyropia* showed the higher value of yield in aqueous extract (75.05 ± 5.19%). On the other hand, the yields of methanolic extracts samples was low, and did not show statistical difference ($p < 0.05$), ranging from 6.78 ± 0.58 to 11.34 ± 2.35%.

ENVIRONMENTAL *ENTEROBACTERIACEAE* CULTURE

After three subcultures in McConkey growth medium, and Gram staining for each subculture, the Gram-negative strain was confirmed in all cultures, where *E. coli* is inserted. In the biochemical test was confirmed that the cultivated environmental strain belongs to the *Enterobacteriaceae* family, whose was compared with the reference strain (ATCC 25922).

Table 1. Extract yields (%) of lyophilized crude extracts of the macroalgae species from Paraná coast (Mean ± SD; n = 5). Statistical analysis was applied for extracts, letters indicate significant differences (p<0,05) among species according to one-way ANOVA and Tukey post-hoc test.

Macroalgae taxa	Extract	Mass of Extract Obtained (g)	Yield (%)
Ulva	Methanol	0.41 ± 0.16	9.12 ± 1.51[c]
	Aqueous	1.51 ± 0.42	33.69 ± 3.39[b]
Sargassum	Methanol	0.57 ± 0.12	11.34 ± 2.35[b]
	Aqueous	2.05 ± 0.12	39.1 ± 11.14 [c]
Pyropia	Methanol	0.33 ± 0.03	6.78 ± 0.58[c]
	Aqueous	3.90 ± 0.37	75.05 ± 5.19[a]
Gayralia*	Methanol	0.8	8
	Aqueous	**	**

ANOVA (One-way)

Effect	SS	Degree of freedom	MS	F	p
Intercept	6.593001	1	6.59	3559.4	0.00
	1.106610	5	0.22	119.47	0.00
Error	0.044459	24	0.00	**	**

** There were not replicates data available for this species, nor aqueous extract.

Table 2. Inhibition halos expressed in millimeters (mm) and measured in the antibiograms performed using antibiotics, negative control, seaweed crude aqueous and methanolic extracts, collected at Farol Island – Paraná, Southeastern Brazilian coast, against the environmental isolate (Enterobacteriaceae).

Antimicrobial	Inhibition Halo Diameter
Antibiotics	
Azithromycin	9.8
Neomycin	8
Amoxicillin	7.5
Methanolic Extract	
Ulva sp.	5.1
Pyropia acanthophora	6.9
Sargassum cymosum	4.7
Aqueous Extract	
Ulva sp.	0
Pyropia acanthophora	0
Sargassum cymosum	5
Control	
DMSO 10%	0

DISK DIFFUSION ANTIBIOGRAM *VS* ENVIRONMENTAL ISOLATE STRAIN

From the measurement of the resulting inhibition halos (Table 2), there was antibacterial activity for the extracts of *P. acanthophora* and *Ulva* sp. However, the halos were observed for both extracts (aqueous and methanolic) only for *S. cymosum*. The mean values of inhibition halos for methanolic extracts of *P. acantophora* and *Ulva* sp. were 6.9 mm and 5.1mm, respectively. *S. cymosum* showed inhibition halos for aqueous and methanolic extracts of 5.0 and 4.7mm, respectively.

The inhibition halos were formed for the commercial antimicrobial azithromycin measuring 9.8 mm, neomycin 8.0 mm, and 7.5 mm for amoxicillin. Considering the mean value (8.5 mm), none of the tested seaweed extract reached the potential expected for commercial antibacterial used here. However, these are crude extracts suggesting that improvement in extraction process may be potential alternatives and or competitive even if compared to commercial compounds.

SUSCEPTIBILITY TEST OF CONTROL, AND ENVIRONMENTAL *ENTEROBACTERIACEAE* STRAINS

The inhibition halos observed in the susceptibility test are presented in Table 3. The environmental marine isolated strain obtained in this study was resistant only to nitrofurantoin (300μg) and penicillin G (10μg); and both strains (control ATCC 25922 and environmental) were sensitives to the other commercial antibiotics tested. However, the environmental strain showed halo formation of 13 mm for nitrofurantoin, if compared to the absent halo of the control strain (ATCC 25922), suggesting that the environmental strain can be less resistant compared to the control. Both control and environmental strains were resistant to penicillin G (10μg), showing no halo formation.

MICRODILUTION ANTIBIOGRAM

The aqueous extracts of *Ulva* sp., *P. acanthophora,* and *S. cymosum* had low or did not show antibacterial activity at the concentrations tested against reference *E. coli* and Gram-negative environmental isolates. Aqueous extracts of the macroalgae *G. brasiliensis* were not obtained.

The methanolic extract of *Ulva* sp. reduced significatively the bacterial activity of both strains, and in all tested concentrations (Figure 2). At the higher extract concentrations tested (1.6 mg/mL), in average it reduced the cell viability, or inhibited the strain, in 73% for the environmental *Enterobacteriaceae* isolate, and in 70% against the reference strain (Table 4). And even the lower concentrations tested (0.4 mg/mL), in average reduced the cell viability in 66.5% for the environmental *Enterobacteriaceae* isolate.

Considering *P. acanthophora* extracts, was observed a reduction in cell viability (Table 4), only at the higher concentration (1.6 mg/mL) of methanolic extract; in this species was obtained the higher mean inhibition rate among species tested, inhibiting 91% for the environmental strains, and 75% for the reference / control strain (ATCC 25922) strain. The mean inhibition rate at the concentration of 1.6mg/mL of the methanolic extract of *S. cymosum* was 81% for the reference / control strain (ATCC 25922), and 69% for the environmental strain. The methanolic extract of the green macroalgae *G. brasiliensis,* the only seaweed sampled at a Conservation Area, showed low antimicrobial activity (AA) compared to the other species extracts, inhibiting 40% for the environmental strains only in the lower extract concentration tested (0.4 mg/mL), and 31% for the reference strain, only at higher concentration tested (1.6 mg/mL).

Univariate statistical analysis is presented in the Figure 3. The standard deviations

and means demonstrate different inhibition behavior (or AA) among samples. *Ulva* sp. differs from the other species since it presents lower absorbance values (higher AA), as well as *G. brasiliensis*, showed higher absorbance values and therefore lower AA. The extracts of *P. acanthophora* and *S. cymosum* showed similarity in the results of average absorbance, in these extracts the AA were higher only at the higher extract concentration used (1.6mg/mL).

DISCUSSION

Macroalgae are a unique source of natural marine products due to their ability to thrive in the dynamic marine environment. In this article, we explored preliminary data of antibacterial activity (AA) in *Ulva* sp. (formely as *U. fasciata*), *P. acantophora, S. cymosum* and *G. brasiliensis*, which are common and potential edible, and or pharmaco-cosmetical used seaweeds for several commercial purposes (Pellizzari and Reis, 2011). The World Health Organization's 'Global Action Plan on Antimicrobial Resistance (www.who.int/antimicrobial-resistance/global-action-plan/en/) encourages researchers to investigate and propose new treatment options, and our results suggest that these seaweeds may be potential novel sources for antibacterial compounds.

In the present study, the higher extract yields obtained for aqueous extracts of the tropical species tested suggest that probably the matrix of secondary metabolites is mainly composed of carbohydrates, majority compound in many seaweed species (Thakur *et al.* 2022, Sasaki and Yoshikuni, 2022). There were no statistical differences among methanolic extract yields, suggesting similar polarity matrix of secondary metabolites. In turn, the methanolic extract of *Pyropia* sp. showed the lower yields suggesting probably a higher concentration of volatile compounds, as already discussed by Jiménez-González *et al.* (2023). Similarly, Afrin *et al.* (2023) tested different methods of extraction for distinct seaweed species and showed that distinct laboratorial procedures may have a significant influence on the final yield rate of the crude extracts. Although our results did not demonstrate significant effectiveness of aqueous extracts for antibacterial activity, there is still room for further investigations for exploring other bioactive properties of aqueous extracts, such as antioxidant and antitumor activity as reported by Qiu *et al.* (2022) and Agena *et al.* (2023).

Among all the macroalgae crude extracts tested through microdilution, only the methanolic extracts exhibited inhibitory potential against the reference *E. coli* strain (ATCC 25922) and the cultivated *Enterobacteriaceae* isolated from the environment, suggesting the presence of antimicrobial compounds in the four tested seaweed species. However, the methanolic extract of *Ulva* sp. (conspicuous marine Chlorophyte) reduced significatively the bacterial activity of both strains, and in all tested concentrations (Figure 2). Also, this species showed the higher yields (Table 1), followed by *Sargassum*. Therefore *Ulva* sp. extract could be the best candidate for future studies against *E. coli*, as already reported by Alves *et al.* (2016). Besides, Yap *et al.* (2019) observed a positive correlation between the phenolics, antioxidant, and antibacterial activities from *Caulerpa* spp., also a green seaweed.

Ulva sp. showed a higher constancy among the inhibitory values and tested concentrations, maintaining similar means between 70 and 75% of inhibitory rates for both bacterial strains tested. Also, the statistical analysis of variance (Figure 3) reveals that the deviations and homogeneity of the results also suggest this species as the most promising crude extract,

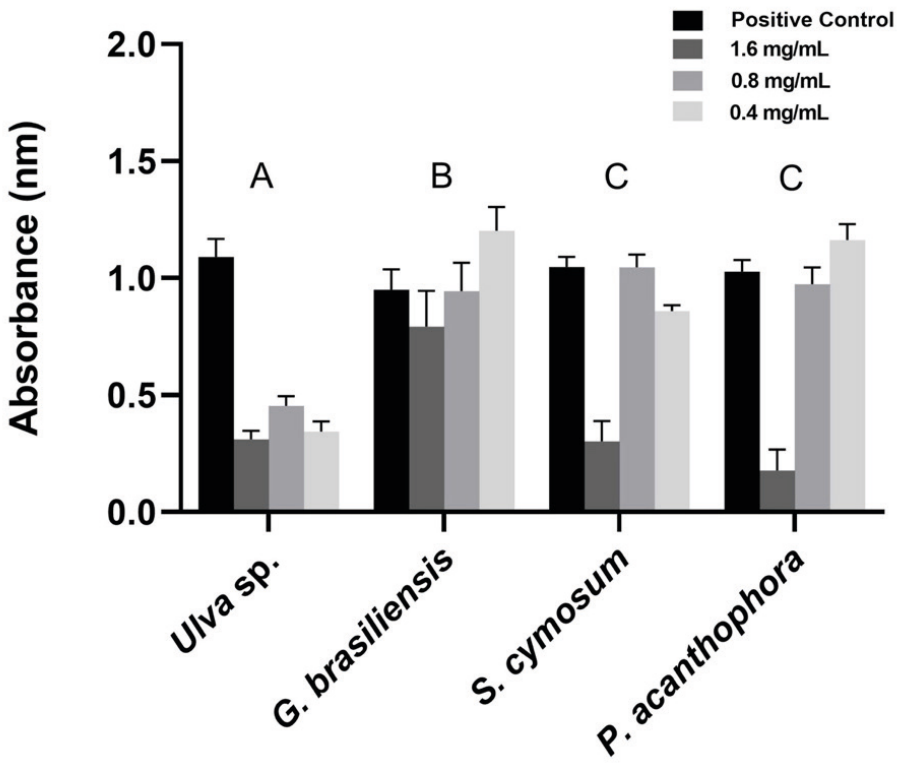

Figure 3. Univariate ANOVA and ranking by significance among methanolic extracts, at different concentrations. *Ulva* sp. showed a different behavior (higher AA) compared to the other seaweed extracts. *P. acanthophora* and *S. cymosum* showed higher AA only at the higher concentration tested. The margin of error is expressed by upper bars.

until now, in terms of antibacterial activity (AA). Furthermore *Ulva* sp. is a globally conspicuous and an opportunistic species, occurring in high biomass in many locations that show severe eutrophic conditions; besides studies suggest tendency to increase still more biomasses facing climate change scenarios (Pellizzari *et al.* 2014, 2020; Gao *et al.*, 2017). This species is commonly associated with high nitrogenous compounds supply, which suggests a relationship with microorganisms, including Enterobacteria (Alves *et al.*, 2016; Elizondo-González *et al.* (2018).

Hamouda *et al.* (2023) studied AA of *Ulva*/Nanocellulose and *Ulva*-Ag/Cellulose nanocomposites and both blended with fluoride against bacteria causing dental decay. Abeer *et al.* (2023) investigated synergistic antibacterial effects of *Ulva lactuca* methanolic extract alone, and in combination with different antibiotics, on multidrug- resistant *Klebsiella pneumoniae* isolate concluding that using 2.5 µg/mL of *Ulva* methanolic extract + gentamicin (4 µg/mL), *U. lactuca* extract has the power to aid antibiotics in reducing the growth of pathogenic dental *K. pneumoniae*.

P. acanthophora extract showed at higher extract concentration (1.6 mg/mL) the lower absorbance recorded, and consequently the higher inhibitory activity, mainly for the environmental isolate(s). However, *Pyropia* showed, considering methanolic extract, the lower yield rate among the species tested. In contrast and comparing our data with another Rhodophyte study, Amorin *et al.* (2012) screened the antimicrobial effects of crude sulfated polysaccharide extracted from the rhodophyte *Gracilaria ornata* in

Ceará, NE Brazil, against the growth of several bacterial strains (*Bacillus subtilis, Staphylococcus aureus, Enterobacter aerogens, E. coli, Pseudomonas aeruginosa, Salmonella choleraesuis* and *Salmonella typhi*), and only the growth of *E. coli* was inhibited.

At higher concentration, *S. cymosum* crude extract also showed lower absorbances, and consequently higher inhibitory activity, mainly for the control/reference strain tested (ATCC 25922). Also, *Sargassum* showed the higher extract yields among all the species tested and was the only species that showed considerable extract yields for both tested solvents, suggesting a diversity of compounds with wide polarity, and consequently a high diversity of bioactive compounds. However, the standard deviations for these extracts are high, suggesting that new essays at the same concentrations could provide a more concise result, and that *Sargassum* also may be a potential candidate for marine enterobacteria inhibitory promising activity.

Similarly, and in accordance with our results, *Sargassum* species are also widely studied for their secondary metabolites against bacteria due to abundant distribution in a broad range of environmental conditions. Scania and Chasani (2021) reported that *Sargassum polycystum* extracts were the most efficient extract in inhibiting the growth of *E. coli*. Elizondo-González *et al.* (2018) demonstrated positive results using *Sargassum* sp. as bioremediation alternative in an integrated multitrophic aquaculture shrimp farming, whose main issue is to control organic matter, nitrogen and phosphorus compounds, and bacteria from fecal pellets.

In India, Sutharshan *et al.* (2021) tested different concentrations of ethanolic and methanolic extracts from *Sargassum* sp. and obtained promissing results for antibacterial activity against drug-resistant *E. coli*, human uropathogenic *E. coli*, and verotoxin-producing *E. coli*. Shanmughapriya *et al.* (2008) reported that the Sargassaceae family had prevalent microbial activity compared to other families of macroalgae. The dichloromethane extracts of *Sargassum dentifolium* tested by Shanab (2007) using disk-diffusion method showed halos of 11mm (maximum) for *E. coli*.

Regarding the extract concentrations tested, in the present study we obtained high enterobacteria inhibitory results at concentrations much lower than those observed by Coronel *et al.* (2020). These authors suggested AA for *Sargassum filipendula* extract from Brazil, using concentration of 156.25 mg/mL, obtaining higher AA mainly against *E. coli* reference strain.

Similarly to *Sargassum*, *G. brasiliensis* extracts showed AA against the reference strains (ATCC 25922), however, in lower inhibition rates if compared with the other species tested. Regarding *G. brasiliensis*, this result is surprising because it is widely reported in literature that the species holds several promising bioactivity compounds. Preliminary biochemical analyzes support the nutraceutical and cosmetic uses of *G. brasiliensis* (Pellizzari and Reis, 2011), as well as bioactivity against the herpes simplex virus (Cassolato *et al.*, 2008), rheological characteristics (Nasatto, 2016) and antioxidant properties (Bernardi *et al.*, 2016). Furthermore, polysaccharides obtained from seaweeds of *Monostroma* Complex, including *Gayralia* spp., are used for several industrial applications, including recent Brazilian patent obtained by Duarte-Noseda *et al.* (2021) for chemical compounds (methylcellulose and sulfated heterorhamnana) of *G. brasiliensis* that modulate neovascularization.

The bioactive compounds present in algal species distributed among the three major groups, enables different biological activities, dependent on different extractions protocols according to their polarity (Mattos,

Table 3. Inhibition halos measurements, expressed in mm, resulted from the susceptibility tests with antimicrobials used for classification of the degree of resistance of the cultivated environmental bacteria, and the reference strain (ATCC25922). Where: S= Sensitive (up to 16mm), R= Resistant (0-15mm).

Antibiotic	Reference *E.coli* ATCC25922	Resistance	Environmental Isolate *Enterobacteriacea*	Classification
ampicillin (10µg)	18	S	18	S
ciprofloxacin (5µg)	21	S	48	S
nitrofurantoin (300µg)	absence of halo	**R**	13	**R**
Cephalothin(30µg)	17	S	33	S
gentamicin	18	S	33	S
ofloxacin (5µg)	23	S	21	S
penicillin G (10µg)	absence of halo	**R**	absence of halo	**R**

Table 4. Percentages of antibacterial effect of methanolic crude extracts of algal species against *E.coli* reference strain and an environmental isolated Enterobacteriaceae, from Paraná coast, Southeastern Brazilian coast.

Methanolic Extracts Seaweed Species	Inhibition (%)					
	E. coli (ATCC 25922)			Environmental Strain Enterobacteriaceae		
	Extracts concentration (mg/mL)					
	0.4	**0.8**	**1.6**	**0.4**	**0.8**	**1.6**
Ulva sp.	**70.50 ±2**	56.04 ±2	**69.64 ±2**	**66.50 ±4**	60.76 ±4	**73.08 ±4**
Pyropia acantophora	10.05 ±8	2.99 ±7	**75.25 ±5**	20.67 ±4	11.24 ±3	**90.89 ±1**
Sargassum cymosum	11.06 ±12	0.13 ±8	**80.47 ±3**	18.26 ±2	3.5 ±3	**66.99 ±5**
Gayralia brasiliensis	13.51 ±3	10.51 ±14	30.90 ±13	39.95 ±2	9.67 ±1	1.6 ±3

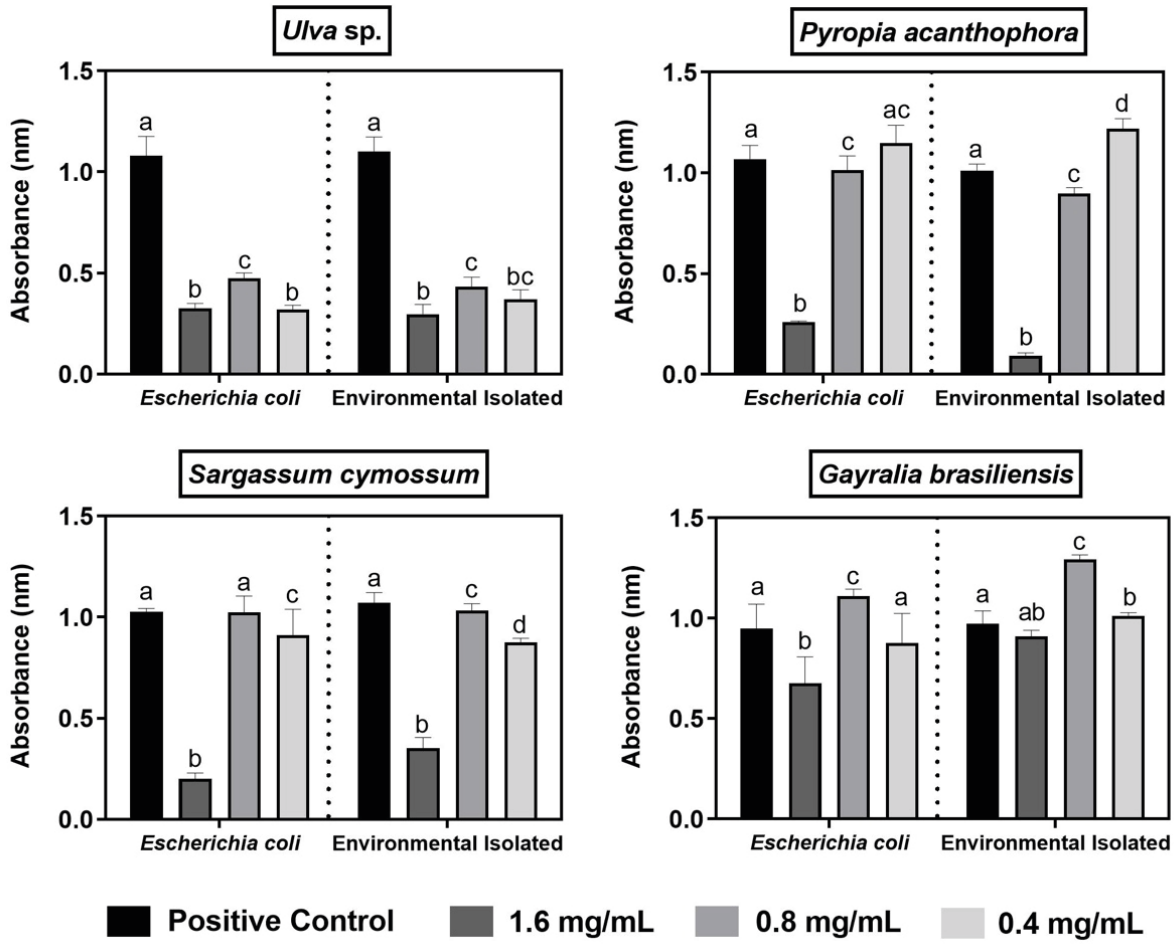

Figure 2. Absorbance readings for different concentrations of methanolic extracts of *Ulva* sp. (formerly as *Ulva fasciata* Delile) (Chlorophyta), *P. acanthophora* (Rhodophyta) and *S. cymosum* (Phaeophyceae), sampled at Farol Island, and *G. brasiliensis* collected at Rasa Island, a Conservation Area. Different letters mean statistical difference ($p \leq 0,05$). Lower absorbances mean higher bacterial inhibition.

2013). Perez *et al.* (2016) in a revision study suggested that substances isolated from seaweeds that present high AA belong mainly to polysaccharides, fatty acids, phlorotannins, pigments, lectins, alkaloids, terpenoids and halogenated compounds.

Lima-Filho *et al.* (2002) suggested that the AA depends on the algal species and the success of the extraction method, i.e., the bioactive compounds available in the specific taxa and the chemicalextractor, as well as its concentration, are essential for searching satisfactory results. Corroborating this hypothesis, Shanmughapriya *et al.* (2008) tested methanolic extracts and obtained positive results for AA, different from what was observed in the ethanolic extracts in the same study, suggesting that the extraction method has a definitive effect on the isolation of bioactive compounds. In our study, despite the better extract yields, the aqueous extracts did not show any inhibitory activity.

Lima-filho *et al.* (2002) analyzed extracts of hexane, chloroform, and ethanol from six marine macroalgae from (Rhodophyta and Phaeophyceae) on the north coast of Ceará (Brazil) against Gram-negative pathogenic strains, including *E. coli.* None of the extracts tested showed antibacterial activity against *E. coli,* the authors highlight the importance

of the extraction method and substance, especially when liposoluble extracts have shown pharmacological properties. Likewise, Shanmughapriya *et al.* (2008) state that organic solvents are more efficient than water-based methods in antimicrobial activity of macroalgae, which may explain why the aqueous extracts did not show antibacterial activity in the present contribution.

Coronel *et al.* (2020) suggests that the antimicrobial activities of algae are attributed to phenolic compounds and some proteins. Since the methanol extraction method can precipitate and degrade proteins, it may suggest that the bioactive compounds responsible for the antibacterial activity are the phenolics. Aqueous solvents usually extract proteins and carbohydrates. Thus, the observed AA of the tested species here are probably attributed to the presence of phenolic compounds in the methanolic extracts. Among the different solvents used in studies of this nature, 80% methanol is the most suitable solution for the extraction of phenolic compounds, reported in the literature as one of the main bioactive responsible for inhibiting bacterial growth (Vieira *et al.*, 2010). Secondary metabolites of total phenolics include terpenoids, phlorotanins and phenol, as the mainly compounds responsible for the AA of seaweed extracts against pathogenic microorganisms. Our study indicates that the extract yields is not directly related to the AA, and further investigation concerning phenolic compounds concentrations and characterization in our extracts can be promising.

In addition, and using other method to evaluate inhibitory activity in our study, the antibiograms by disk diffusion, was not possible to observe inhibition for all species tested. The methanolic extracts of *P. acanthophora*, *Ulva* sp. and *S. cymosum* showed an inhibition halo (6.9mm, 5.1mm and 4.7mm respectively) against the environmental isolate of *Enterobacteriaceae*. Differing from the aqueous extracts, and the microdilution test that did not show inhibition activity, the antibiogram for the *S. cymosum* extract showed inhibitory activity (5mm). This result may be attributed to the extract concentrations used, suggesting further essays since the chemical profile of the extracts produced here are not known.

The environmental marine isolate obtained in this study was resistant to nitrofurantoin (300µg) and penicillin G (10µg). However, this strain showed halo formation of 13 mm for nitrofurantoin, if compared to the absent halo of the control strain (ATCC25922), suggesting that the environmental strain can be less resistant compared to the control. Both control and environmental strains were highly resistant to penicillin G (10µg), showing no halo formation. Ciprofloxacin (5µg) showed the best results as antibiotic against the *Enterobacteriaceae* environmental isolate.

Bhuyar *et al.* (2020) and Coronel *et al.* (2020) suggested antimicrobial effect using antibiotic concentrations higher than we used in the present study. Bhuyar *et al.* (2020) suggested minimum concentration for Gram-negative bacteria of 25µg/mL, and that to inhibit *E. coli* in disk diffusion, the inhibition halo varied according to antibiotic concentration, from 3mm at 50mg/mL, to 10mm at the 100mg/mL concentration. In our study we obtained satisfactory results using lower concentrations.

Considering extract concentrations, Fayzi *et al.* (2020), investigated AA of methanolic extracts from conspicuous macroalgae from Morocco (*Bifurcaria sp., Corallina officinalis* and *Ulva fasciata,* the former was taxonomically repositioned currently as *U. lactuca)* against human pathogenic bacteria (*Staphylococcus aureus, Escherichia coli, Bacillus subtilis* and *Pseudomonas aeruginosa). Bifurcaria* (Phaeophyceae) and

U. fasciata (Chlorophyceae) showed the better results for disk diffusion. When tested *in vitro*, the same extracts showed activities at higher concentrations tested (7.5 mg/mL). In our study we observed satisfactory results using maximum concentration of 1.6 mg/mL, suggesting that in higher concentrations our extract could be highly inhibitory even for resistant strain tested.

The data present here regarding the environmental isolate of estuarine *Enterobacteriaceae* is unprecedent and will aid to establish a baseline for further studies. Human contact with free and resistant bacteria in the marine environment increases the risk of infections. Rima *et al.* (2022) reports that environmental bacterial biofilms participate in 65% of microbial human diseases and 80% of chronical infections. Fleming *et al.* (2008) studied microbial contamination that affects the security of seafood, as well as commercial and recreational uses for coastal waters. Pommepuy *et al.* (2005) demonstrates that marine contamination is caused by rivers and or sewage outfalls and that the supply of nutrients, light and temperature allow the high viability and permanence of enterobacteria such as *E. coli* and *Enterococcus* including in estuarine waters. In accordance to this, Maciel *et al.* (2019) investigated the occurrence of *C. albicans* and other opportunistic yeasts in sand and seawater samples from beaches in Brazil to assess their correlation with *Escherichia coli,* and to characterize the pathogenic potential of the yeast isolates. The authors found that up to 70% of seawater samples could be classified as inappropriate for primary contact recreation in relation to *E. coli* densities. Among the 144 opportunistic yeasts evaluated, 61% displayed resistance or dose- dependent sensitivity to at least one tested drug, and 40% produced proteinase. Strains of *C. albicans* and *Kodamaea ohmeri* exhibited the highest rates of adhesion to buccal epithelial cells. The identification of opportunistic and pathogenic yeast species in seawater and sand samples from Brazilian beaches suggest a potential risk to the human health in these environments for recreational purposes.

Pommepuy *et al.* (2005) suggested that contamination by *E. coli* in an estuarine environment usually decreases proportionally with river and tidal flows, or when non treated effluents / fecal loads decrease. Dionisio *et al.* (2000) scan the classical indicators of faecal pollution in a Portuguese estuary, using total coliforms (TC), faecal thermotolerant coliforms (FC), fecal streptococci (FS) and somatic coliphages, as well as physico-chemical parameters aiming to stablish different degrees of organic pollution. Relationships between fecal indicators and several pathogenic microorganisms (*Salmonella, Pseudomonas aeruginosa* and *Candida albicans*) were also established in these recreational marine zones. The results obtained for these authors indicate that none of the indicators tested may be considered as a unique and universal index for the presence of pathogens in water. However, fecal streptococci, enterococci and *E.coli* indexes showed a higher and significant relationship with sewage discharges. Therefore, the persistence of positive results for *E. coli* observed in Pontal do Sul beach samples, where we isolated the *Enterobacteriaceae*, may suggest that the load of effluents being dumped into the sea, may be a chronical environmental issue in Paranaguá Estuarine Complex (PEC), even in the mouth of the estuary that receives between tides high exchange rates of seawater. Associated biological and abiotic parameters are recommended to be monitored in the area aiming to control organic pollution in this fishery and recreational zone.

Considering the coexistence of microorganisms and runoff of antibiotic by effluents in the environment, the permanence

of considerable levels of bacteria on the analyzed beach may facilitate the emergence of resistant bacteria, as observed also by Pommepuy *et al.* (2005) and Guardabassi and Kruse (2010). Marine macroalgae are potential source of diverse and unique compounds, including antimicrobials, being promising in the search for new bioactive compounds, in addition to being excellent bioindicators of environmental quality. The present results also raise data for further applied experiments involving human health, seafood quality and processes of bioremediation against *Enterobacteriaceae* from non-pristine or organic polluted environments, focused mainly in *Ulva* and *Sargassum* species. Further essays related to chemical composition of the extracts and to isolate the active components are recommended. Then these macroalgae bioactive compounds alone, or in combination with other antimicrobials, could improve biomedical applications, or to provide a baseline for new approaches. Considering the results obtained specifically for the environmental isolate of *Enterobacteriaceae*, this could be a starting point for new potential line of research, as use of antibacterial algal extracts for bioremediation in aquaculture farms, in hydroponic agriculture plants, or even in small coastal water bodies.

The main focus and unprecedent feature of the work was its environmental relevant potential of testing the algal extracts against a bacterial strain isolated from a polluted area. Although the preliminary character of the study, the research field here explored is relevant considering the increasing urge for the development of new antibiotics with unique mechanism of action, and that natural products are a continuing source of drug candidates. Further investigation of chemical characterization of the crude active extracts, tests in lower concentrations, and experiments to to identify and characterize the environmental isolated Enterobacteriaceae strain, including the sequencing of the 16S rRNA gene, are highly recommended. Thus, among the species tested, *Ulva* sp. (formerly as *U. fasciata*) and *S. cymosum* are the most promising candidates to inhibit Gram-negative marine environmental *Enterobacteriaceae*. Both seaweed species are very abundant, mainly for causing huge blooms, or *green / brown tides* worldwide, and these biomasses could be a novel source for antibacterial compounds. Finally, this contribution opens a new window for studies to build the understanding on action mechanisms and for identifying the specific compound(s) responsible for the desired antibacterial effects, with potential for multiple uses in marine conservation and applied biotechnology.

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SUPPLEMENTARY MATERIAL

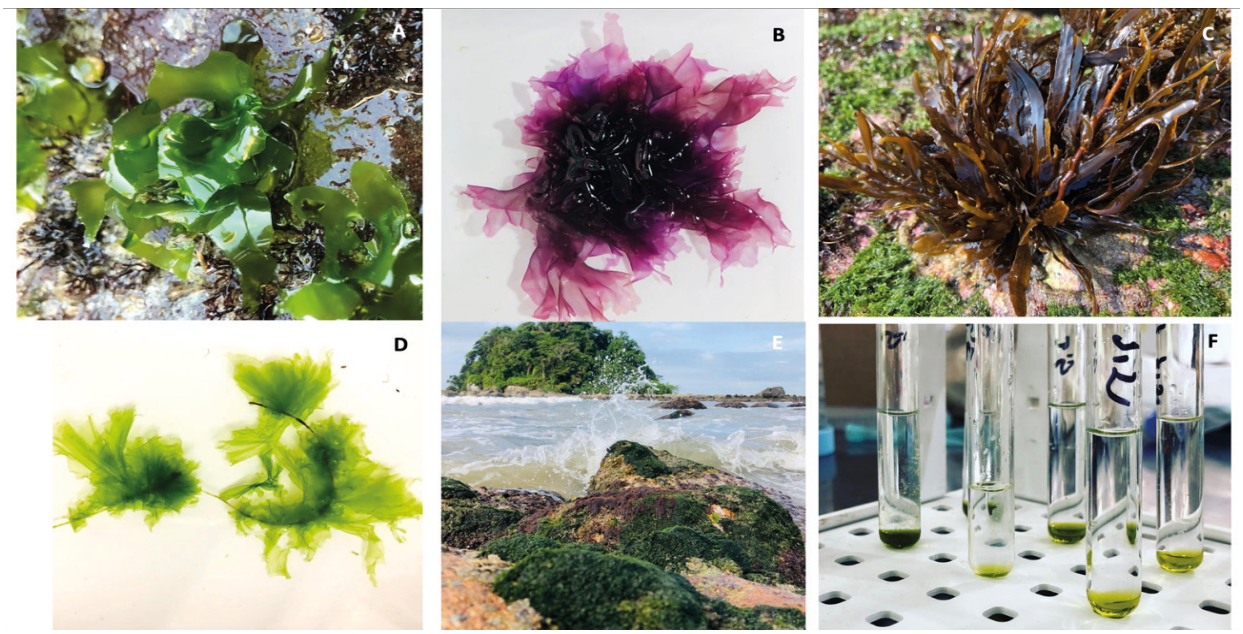

Figure S1. – Seaweed species analyzed. A. *Ulva* sp. (formerly as *U. fasciata*); B. *Pyropia acantophora*; C. *Sargassum cymosum*; D. *Gayralia brasiliensis*; E. Farol Island, Paraná Coast, Southern Brazil, one of the sampling sites; F. Laboratorial essay of the species crude extracts.