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Microalgae and Cyanobacteria, a Promising Source of Antimicrobial Molecules Against Aquatic Pathogen

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Abstract

Genus *Vibrio* is involved in common pathologies of aquaculture fish species, being responsible for significant economic losses for that industrial activity. Microalgae and cyanobacteria have demonstrated to count on bioactive compounds able to diminish mortality and morbidity by their use as functional food supply or by addition of those bioactive compounds previously purified. The main goal of this work has been to evaluate the anti-microbian effect (growth of bacterial population measured as changes in optical density) of 25 microalgae extracts on the bacterial strain *Vibrio anguillarum* CECT 522T. Stock microalgae extracts were obtained from lyophilized biomass treated with methanol 99.9%. A total of thirteen extracts exhibited antibacterial activity. The highest activity corresponded to *Monochrysis lutheri*, followed by *Hemiselmis cyclopea*, *Porphyridium cruentum, Tetraselmis rubens, Cryptomonas* sp, *Navicula* sp. The anti-bacterial activity was not related to a taxonomic group, indicating species-specific or even strain-specific activity.

Introduction

Aquaculture is an emerging industrial activity of great importance for the economic development of countries (Arunkumar *et al*., 2020). Aquaculture production is currently estimated to supply almost half of the fish consumed by humans worldwide (FAO, 2020).

The high demand for aquaculture products, on many occasions, leads to an overpopulation of specimens per unit of production (Bouwmeester *et al*., 2021), allowing the appearance of infectious outbreaks that cause large economic losses, thus becoming, one of the main limiting factors for the development of the aquaculture sector (Ina-Salwany *et al*., 2019). Economic losses due to morbidity, mortality, and investment costs in treatments for the control and prevention of infectious outbreaks using drugs are so high that they may even lead to the total collapse of some facilities (Bermúdez Almada *et al*., 2017). In China economic losses are around 15-20% due to disease (Sudheesh & Cain, 2017). In Chile operating costs for pharmaceutical products are around the US $700 million per year (Flores *et al*., 2020).

Among the important pathologies of great economic and epidemiological implication in aquaculture is vibriosis. This bacterial disease is caused by some species of the genera *Vibrio* and *Photobacterium* (Istiqomah *et al*., 2020). The

significance of these genera in world aquaculture lies in the wide range of species affected, including fish, crustaceans, mollusks, corals, and rotifers of any productive level, strong commercial system or small-scale rural aquaculture farms (Mohamad *et al*., 2019).

One of the main etiological agents related to vibriosis is the species *Vibrio anguillarum*; This gram-negative pathogenic bacterium has been described as capable of causing hemorrhagic septicemia in more than 50 species of freshwater and marine fish, among which are species of great economic importance such salmon (*Salmo salar*), turbot (*Psetta maximum*), sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*), cod (*Gadus morhua*), eel (*Anguilla anguilla*) or ayu (*Plecoglossus altivelis*), as well as some bivalves and crustaceans (Behringer *et al*., 2020; Derome *et al*., 2016).

It is worth mentioning that, along with bacterial diseases and the great economic losses derived from the control or prophylactic treatment of the different pathologies associated with aquaculture, there is underlying collateral damage that derives from the excessive use of antimicrobial drugs: the appearance of multi-resistant bacterial strains with resistance genes which could be horizontally transferred to non-target organisms, including pathogens from terrestrial animals, including man (Santos & Ramos, 2018).

Currently, there is a pressing need to discover novel broad-spectrum antimicrobial active principles, whose molecules make it possible to evade current biochemical mechanisms of antimicrobial resistance, thus reducing the impact of bacterial diseases, and the increased antimicrobial resistance (Igarashi *et al*., 2018). For decades, the main sources for obtaining antimicrobial molecules have been bacteria, fungi, and actinomycetes (Hutchings *et al*., 2019); however, marine organisms have shown to be a promising natural bank of molecules with broad biological activity including antimicrobial activity (Nweze *et al*., 2020).

Microalgae (sensu lato), represents a vast group of multi-diverse microorganisms, which includes cyanobacteria and dinoflagellates. It is estimated that there are at least 200,000 species, distributed in almost all aquatic and humid ecosystems globally (Norton *et al*., 1996; Sydney *et al*., 2019).

Although only a low percentage of microalgae species have been characterized (Ameen *et al*., 2021), the literature describes multiple active compounds with diverse biological activity, of which many of these molecules are related to the taxonomic groups of this study. Among the main compounds with antimicrobial activity from microalgae, it could be mentioned: organic acids, fatty acids, unsaturated hydroxylated fatty acids, glycolipids, steroids, phenols, terpenoids, toxins (such as bacterial toxin lecithinase), phycobiliproteins, exopolysaccharides (EPS) and antimicrobial peptides (AMP) (Hayes *et al*., 2017; Nájera-Arce *et al*., 2018; Pina-Pérez *et al*., 2017).

Microalgae have a wide adaptive metabolic plasticity ability to adapt their biochemical responses to their physiological state (Falaise *et al*., 2016). This quality allows through the manipulation of physicochemical factors such as nutritional stress (Lin *et al*., 2018; Lovio *et al*., 2019) temperature, irradiance (Gonçalves *et al*., 2019) pH, salinity (Jaiswal *et al*., 2020; Musa *et al*., 2019), the expression of different groups of metabolites of industrial interest can be induced (Elisabeth *et al*., 2021).

Some studies demonstrated the bactericidal and fungicidal effect of microalgae extracts on pathogens of epidemiologic interests, such as *Staphylococcus aureus*, *E. coli* and *P. aeruginosa*, as well as on different species from the genus *Vibrio* (Dewi *et al*., 2018; Jena & Subudhi, 2019; Jha *et al*., 2017; Ruiz *et al*., 2019; Schuelter *et al*., 2019). On the other hand, there is a great interest in the food industry, especially in seafood products, due to the bioactives of microalgae, where the preservative and quality improvement effect of seafood has been demonstrated (Tavakoli *et al*., 2021).

This work involves an important amount of microalgae strains (25) from different taxonomic classes, to be assayed as a possible source of new antimicrobial molecules. Antibacterial activity of different microalgal strains would be exploited to understanding the mechanism of antimicrobial action of some molecules and them optimal use in fish protection against bacterial pathogens. Additionally, this protection would not be related to synthetic antibiotics which can provoke, a) resistance of bacterial strains, and b) environmental problems due to antibiotic activity on non-target organisms. Antibacterial activity of any of those strains has not assayed before.

Materials and Methods

Microalgae and Cyanobacteria Cultures and Extracts

Microalgae strains were obtained from the Marine Microalgae Culture Collection (MMCC) at the Institute of Marine Sciences of Andalusia (CSIC), and the Microalgae Collection from the INTERREG Project ALGARED+ (Table 1). Algae were cultured in marine well water, enriched with Guillard's f/2 medium (Guillard & Ryther, 1962), and 500 $\mu g \times L^{-1}$ silicate in the case of diatoms. Cells were grown in 15 L cylindrical methacrylate columns, aerated with 0,2 µm filtered air. Cells were cultured at 20 ± 1 °C, under continuous white light (around 30 µmol quanta $\times$ $m^2 s^{-1}$) till enough biomass was reached. Then, cultures were centrifuged in a Westfalia suspension solid separator continuous flow centrifuge. Biomass obtained was washed with ammonium formiate (Sigma-Aldrich) (0.9%) to remove salt and then frozen to -80°C. After this, samples were lyophilized (Virtis Advantage Wizard 2.0, from Hucoa Erlöss).

The dry biomass of each microalgae strain was diluted in 10 $ml \cdot g^{-1}$ of 99.9% methanol, (Merck, HPLC

grade), then, was disrupted Sonic (Hielscher UP200S) at 240 W for 20 s on ice.

After this treatment, samples were stored for 24h at 4°C and then submitted to the same sonic disruption again and re-stored at 4°C for another 24 h. After this step, the samples were centrifuged and the supernatant was filtered with a syringe filter (nylon membrane pore size 0.22 μm, SinerLab). Then, the crude extracts were dried to 35°C in a rotary evaporator and then stored at -80°C till re-suspended in appropriate volumes of DMSO just before the assays assayed. Controls were treated with the same DMSO volumes, after checking the DMSO concentration used did not interfere with bacterial growth.

Bacterial Culture and Inhibitory Activity Bioassay.

The *Vibrio anguillarum* strain (CECT 522T) was supplied by the Biochemical and Molecular Biology Laboratory of the Chemical Department at the University of Huelva (Spain). The inoculum was stored at -80°C in marine agar and glycerol. Before experiments, the *V. anguillarum* strain was quickly thawed, re-seeded in agar Mueller Hinton (MH) (OXOID) prepared with filtered seawater and incubated at 26°C for 24 h, and then inoculated in MH broth and also incubated at 26°C for 24 h. Then, culture was adjusted to a MacFarland 0.5 optical patron, measuring optic density to adjust 0.08-0.1 DO at 595 nm.

Bioassays were carried out in 96 multi-well plates (NunClon) filled with 180 μL of optically adjusted *V. anguillarum* culture plus 20 μL of microalgae extracts diluted in 2% DMSO. To increase the possibility of observing any antimicrobial effect present in the extracts, the experiment was carried out by testing the maximum concentration obtained from each microalgae respectively during the extraction process with 99.9% methanol.

Experiments were carried out with five replicate per microalgae extract, and the control was inoculated with 20 μL of 2% DMSO. Readings for OD were performed at time 0 and 24 h in a Multi-plate Spectrophotometer reader TECAN Genios 2000 (measuring wavelength 595 nm, band width 10 nm, reference wavelength 570 nm, number of flashes 25, no settling time. The incubation experimental time was 24h.

Data Analysis

Differences in *V. anguillarum* growth in response to the microalgae alcoholic extracts inoculation were analyzed with the Kruskal-Wallis test (P=<0.05) using the IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY. USA).

Results

Microalgae strains and their taxonomic group, as well as the final concentration of the crude extract used in the bioassay can be seen in Table 1.

The results of this study showed that thirteen methanolic extracts showed statistical differences related to the growth of *V. anguillarum* after 24 h, measuring such divergences in DO at 595 nm. Likewise, within these significances, two groups can be differentiated.

The first group showed high significant (P<0.001) statistical differences with controls: *Hemiselmis Cyclopea, Porphyridium cruentum, Tetraselmis rubens, Cryptomonas* sp*., Navicula* sp. and *Monochrysis lutheri*. This last strain showed the highest significant

Table 1. Microalgae strains and their taxonomic group. Final concentration of the crude extract used in the bioassay

Species (or strain)	Taxonomic group	Final extract concentration(mg mL^{-1})
Ostreopsis cf. ovata	Dinophyta	0.237
Chaetoceros gracilis	Bacillariophyta	0.149
Gyrodinium dorsum	Dinophyta	0.212
Tetraselmis striata	Prasinophyta	0.199
Nannochloropsis sp	Eustigmatophyta	0.240
Chlorococcum sp	Chlorophyta	0.153
Amphora sp.	Bacillariophyta	0.602
Synechococcus sp	Cyanophyta	0.213
Chroomonas salina	Cryptophyta	0.137
Chlamydomonas reinhardtii	Chlorophyta	0.177
Navicula sp	Bacillariophyta	0.155
Diacronema vlkianum	Haptophyta	0.203
Ochromonas sp	Chrysophyta	0.240
Monochrysis lutheri	Chrysophyta	0.140
Dunaliella salina	Chlorophyta	0.161
Anabaena sp	Cyanophyta	0.172
Cryptomonas sp	Cryptophyta	0.146
Porphyridium cruentum	Rhodophyta	0.257
Tetraselmis rubens	Prasinophyta	0.244
Hemiselmis Cyclopea	Cryptophyta	0.316
Picochlorum maculatum	Trebouxiophyceae	0.149
Isochrysis galbana	Haptophyta	0.159
Nannochloropsis gaditana	Eustigmatophyta	0.192
Picochlorum oklahomensis	Trebouxiophyceae	0.306
Phaeodactylum sp	Bacillariophyta	2.940

differences with controls (approximately 17% more bacterial growth inhibition than others). A second group, showed significant (P<0.05) statistical differences with controls: *Phaeodactylum* sp*., Picochlorum oklahomensis, Nannochloropsis gaditana, Ochromonas* sp., *Chlamydomonas reinhardtii, Gyrodinium dorsum, and Chaetoceros gracilis.* The other 12 microalgae extracts showed no significant inhibitory effect on bacterial growth or stimulate bacterial proliferation, as was observed with the strain *Dunaliella salina* (Figure 1).

Discussion

In this study, antibacterial activity on *V. anguillarum* for methanol extracts of 13 from the 25 microalgae assayed strains has been stated. Although the most effective extracts were obtained from organisms including the classes Chrysophyta, Rhodophyta, and Prasinophyta, other organisms from those classes did not show the same activity, pointing out a species-specific (perhaps a strain-specific) antibacterial capacity. The possibility of activation of this capacity when microalgae and cyanobacteria are previously exposed to the pathogen should be checked in further studies.

Literature about antibacterial activity of microalgae extracts is scarce. Duff *et al*., (1966) demonstrated the inhibitory effect of extracts from *Monochrysis lutheri, Hemiselmis* sp*., Cryptomonas* sp. and *Tetraselmis* sp., on different bacterial strains, mainly from marine environments. Some active antibacterial metabolites from *M. lutheri* correspond to hydrocarbons, terpenoids, phenols (Metting & Pyne, 1986), phospholipases (Antia & Bilinski, 1967), and some lipidic compounds such as digalactosildiacil glicerol (GL) and phosfatidilcoline (PL) (Khomova *et al*., 1994). Bashir *et al*. (2018) already demonstrated the bacteriostatic capacity of methanolic extracts of *C. reinhardtii, P. purpureum P. lutheri* and *Isochrysis sp*. on bacteria of epidemiologic interest such *as M. luteus, P. aeruginosa* and *S. aureus.*

Used as food supplements, microalgae have demonstrated a protective role for cultured fishes. Thus, when *M. lutheri* was added to composing feds, juvenile turbot *(Scophtalmus maximus)* survival increased (Spektorova *et al*., 1986). Cultures salmons (*Salmo salar*) also demonstrated higher resistance to infections due to *Vibrio sp*. when microalgae *T. suecica* was included in the diet. (Falaise *et al*., 2016).

Nowadays, alternatives to traditional antibiotic treatments are considered due to environmental and epidemiological consequences derived from the use of those substances. Some proposals include the use of bacteriophages, probiotics, enzymes or antimicrobial peptides (AMPs) (Ghosh *et al*., 2019; Ture *et al*., 2022).

Even some studies of antimicrobial activity through synergistic treatments with plant extracts have proven to be a good starting point to motivate future studies with microalgae extracts; which could contribute to a lower environmental effect than other substances (Bartual *et al*., 2014; Cheesman *et al*., 2017).

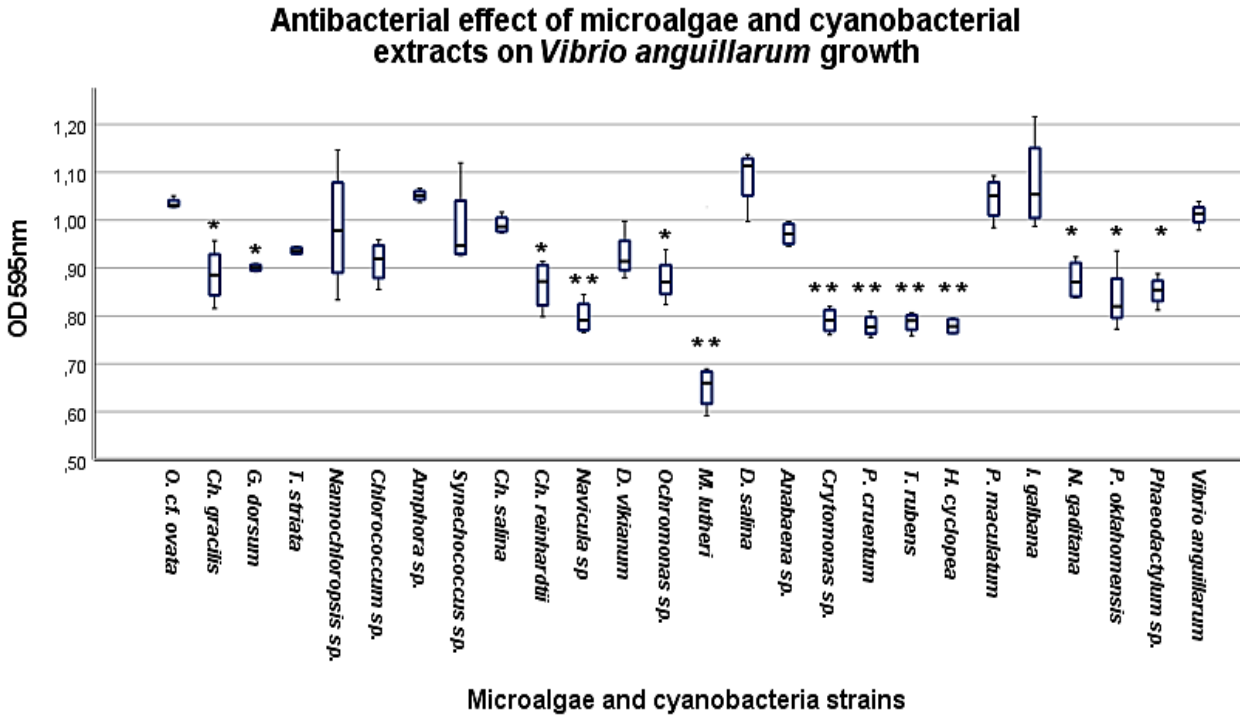

Figure 1. Effect of microalgae and cyanobacteria alcoholic extract on *Vibrio anguillarum* growth after 24 h culture in multi-well plates. Control corresponds to *Vibrio anguillarum* inoculate with 20 µL of 2% DMSO. Statistical significance according to the Kruskal-Wallis test **(P=<0.001); *(P=<0.05) in the inhibition of bacterial growth in comparison with the control is signed with an asterisk.

Most of the used strains in this study were isolated from coastal environments. Those biocenosis are quite active in physic-chemical and biological terms (Rojas Jirón *et al*., 2019), which could imply a better capacity of organisms inhabiting those biotopes to combat microbe pressure (Bartual *et al*., 2020; Fuentes *et al*., 2016; Salcedo & Coello, 2018). This is also an interesting point to be checked in order to test if strains of the same species belonging to ecosystems with different grades of physical-chemical (or even biological) pressure show different antibacterial activity.

Extraction with methanol permits the integration of a wide range of polar and non-polar compounds present in the microalgae and cyanobacterial biomass, which is an excellent first approach for a vast number of taxa. However, to achieve a greater range of extraction of compounds, it would be convenient to carry out successive fractions with different solvents of extreme and intermediate polarities, in this way a greater range of biological activity could be obtained (Cepas *et al*., 2019).

In addition, it is considered that the application of techniques such as super-critical fluids (Sánchez-Camargo *et al*., 2017) or microwave-assisted extraction (Kapoore *et al*., 2018) could be a needed improvement for this type of study.

Our results stated that within the group of extracts that does not show an inhibitory effect against *V anguillarum*, there is a treatment (*D. salina*) that imply an OD of 14.4% above the growth threshold of the control. Faced with this behavior, some hypotheses may arise, within which we could support two of them. The first could be related to the molecular profile of the methanolic extracts of the microalgae *D. salina*. Some studies, such as that of Ambrico *et al*., (2020) have characterized the composition of some extracts of this microalgae, using solvents such as Methanol: chloroform, Hexane and ethanol. Within the main components of the extracts can be found, proteins (probably including free amino acids), carbohydrates, fatty acids, among others. This nutritional cocktail could favor the growth of *V. anguillarum*, in the event that there is no inhibitor agent, or if the latter is found in very low concentrations within the extracts.

On the other hand, it has been shown that some substances, with a certain degree of toxicity for some organisms, can exert a stimulating effect on growth when they are present at very low concentrations. This phenomenon it is known as Chemical Hormesis (in the case of chemical agents) and was reviewed by Calabrese and Baldwin, (2000); It is currently the subject of rigorous study to understand in more detail this effect on different organisms and toxic agents (Agathokleous *et al*., 2021).

Due to the above, it could be presented that, some organic molecules that register antibacterial activity present in the extracts of *D. saline*, for example those described by Cakmak *et al*., (2014) and Little *et al*., (2021), could be found in very low concentrations, and this can exert a stimulating bacterial proliferation, effect on the development of *V. anguillarum* as observed in our experiment.

Ethical Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Author Contribution

All authors have read and agree to the published version of the manuscript. Conceptualization, C.T.-B and I.M.-G.; methodology, C.T.-B, R.F and A.P.; software, C.T.-B and J.A.L.-R.; validation, C.T.-B, I.M.-G and J.A.L.-R.; formal analysis, C.T.-B, I.M.-G and J.A.L.-R.; investigation, C.T.-B, I.M.-G and.; data curation, C.T.-B, and J.A.L.; writing—original draft preparation, C.T.B, I.M-G and J.A.L.-R.; writing—review and editing, C.T.B, I.M-G, M.P-G and J.A.L.-R; supervision, I.M-G.; funding acquisition, I.M-G.

Conflict of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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