POLITÉCNICO DE LEIRIA | ESCOLA SUPERIOR DE TURISMO E TECNOLOGIA DO MAR

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Dissertation Report for obtaining the Master's Degree in Aquaculture

Masters project carried out under the guidance of Doctor Teresa Margarida Lopes da Silva Mouga and co-supervision of Doctor Susana Luísa da Custódia Machado Mendes

2022

Title: The effect of different culture conditions on *Gracilaria gracilis* growth, proximate composition, and bioactivities

Resumo

Gracilaria gracilis é uma alga bem conhecida, cultivada mundialmente, procurada pelo seu alto conteúdo de agar. Dependendo das condições bióticas e abióticas a que a espécie é exposta, também apresenta um perfil nutricional interessante, tornando-a numa excelente candidata para ser usada como alimento ou como suplemento alimentar. Assim, *G. gracilis* foi cultivada sob diferentes condições de pH, luz e de meios nutritivos, para avaliar o impacto destes parâmetros no crescimento e na qualidade da biomassa. O perfil nutricional da biomassa cultivada foi posteriormente avaliado. A capacidade antimicrobiana foi testada usando extração sequencial aquosa-etanólica obtida da *G. gracilis* cultivada contra estirpes microbianas de *Miccrococus luteus, Escherichia coli*, e *Bacillus subtilis*, assim como a sua atividade antioxidante. Os resultados indicam que os parâmetros ambientais têm um impacto significativo no crescimento e na qualidade da biomassa, afetando a quantidade de proteína e de hidratos de carbono na biomassa. O meio nutritivo comercial SSEP demonstrou resultados muito interessantes tanto no crescimento (8.15 ± 0.48% day^{-1}), como no perfil nutricional, tendo-se obtido o maior conteúdo proteico (19.62 ± 0.11 % dw) e o menor conteúdo lipídico (0,28 ± 0,03 % dw). Os resultados obtidos com a análise multivariada revelaram uma correlação forte entre vários dos componentes analisados, detalhando a relação entre os diferentes parâmetros medidos. *G. gracilis* também exibe atividade antimicrobiana contra *Bacillus subtilis*. Infelizmente, nenhuma atividade antioxidante significativa foi detetada nos ensaios efetuados, registando-se, porém, alguma atividade na biomassa cultivada com SSEP.

Assim, o método de cultivo afetou consideravelmente a qualidade da biomassa produzida, destacando-se a biomassa cultivada com SSEP. Como tal, para utilizar eficazmente biomassa de *G. gracilis*, é necessário otimizar os parâmetros de cultivo, para produzir biomassa nutricionalmente equilibrada, com alta capacidade antimicrobiana e antioxidante, para consumo humano.

Abstract

Gracilaria gracilis is a well-known cultivated worldwide seaweed, sought for its high agar content. Depending on the biotic and abiotic conditions to which this species is exposed, it also exhibits a very interesting nutritional profile, making it an excellent candidate to be used as a food or as a food supplement. Thus, *G. gracilis* was cultivated under different pH, light and nutrient media conditions, in order to evaluate the impact of these parameters on the growth and quality of the biomass. The nutritional profile of the cultivated biomass was evaluated afterwards. The antimicrobial capacity using the sequential aqueous-ethanolic extracts obtained from the cultivated *G. gracilis* against the microbial strains *Micrococcus luteus*, *Escherichia coli*, and *Bacillus subtilis*, as well as its antioxidant activity. The results indicate that environmental parameters have a significant impact on biomass growth and quality, affecting the amount of protein and carbohydrate in the biomass. The commercial medium SSEP showed interesting results both in growth (8.15 ± 0.48% day^{-1}) and in the nutritional value, with the highest protein content (19.62 ± 0.11 % dw) and lowest fat content (0,28 ± 0,03 % dw). Multivariate analysis results revealed a strong correlation between the analysed components, detailing the relationship between the different parameters measured. *G. gracilis* also exhibits an interesting antimicrobial activity against *Bacillus subtilis*. Unfortunately, no significant antioxidant capacity was detected in all assays, but some activity was recorded in the biomass grown with SSEP. The cultivation methods, therefore, considerably affected the quality of the biomass produced, highlighting the biomass grown with SSEP.

Thus, to efficiently use *G. gracilis* biomass, it is necessary to optimize cultivation parameters, to produce nutritionally balanced biomass, with high antimicrobial and antioxidant capacity, for human consumption.

Keywords: Seaweed; Rhodophyta; Growth Rate; Nutritional profile; Antimicrobial activity; Antioxidant activity

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1. Introduction

Due to the continued populational growth in the globe, many ecosystems have been affected by mankind's need for natural resources. The oceans, for instance, have been a great source of food for everyone since the early days of humanity, and its evolution and growth has brought about not only a need for more quantity of products such as food, but also variety as industries rush to find new sources for biochemical components and rare metabolites. However, a rising concern for sustainable practices has cast doubt on antiquated methods of recovering said products and has brought about a surge of interest in more planet-conscious alternatives. For example, many countries have depended on fishing for food, but with the rising number of mouths to feed, fisheries had to become more intensive which led to the very common problem, overfishing. This problem has raised awareness for the possibilities and reliability of aquaculture, a source of marine protein and other products that doesn't directly affect natural resources, relieving the strain that has been killing these ecosystems for years (Yarish & Redmond, 2012). In recent years, aquaculture has skyrocketed in popularity and global production. FAO has reported that in 2018 a record high 46% of global fish production belonged to aquaculture (FAO, 2021).

Macroalgae, or seaweeds, are known for their healthy nutritional profile due to a high content of metabolites such as protein, which can reach up to 47% in red seaweeds, as well a balanced content of amino acids, low lipid content, and high mineral content (Patarra et al., 2011). These photosynthetic marine organisms are also known for being a great source of carbohydrates like sugar, full of fibers, hydrocolloids, such as agars, pigments, vitamins, polyphenols, among other compounds (Vieira et al., 2018). Bioactive seaweed compounds are generally formed as the result of these marine organisms adapting to new environmental conditions or when exposed to extreme conditions, like changes in water pH, salinity, nutrient availability and light exposure (Francavilla et al., 2013). It is natural that easily farmable biomass with these attractive qualities would raise interest from different industries to develop new products and sources of valuable compounds.

Macroalgae are currently used in a multitude of different commercial applications. Seaweeds have been commonly used as food in many Asian cultures for centuries, and their recent incorporation in western cuisine has cemented food as the primary industry in seaweed production. Seaweeds' naturally healthy nutritional profile has led to many innovations in the food sector including food supplements and therapeutics as healthier replacements for existing food products (Araújo et al., 2021; Mouritsen et al., 2019; Wells et al., 2017), bioactive compounds such as pigments, vitamins and minerals (Batista et al., 2020; Ben Said et al., 2018). The pharmaceutical industry has also shown increased interest in seaweeds. Plenty of the metabolites produced by macroalgae can't be found in other organisms (Francavilla et al., 2013), turning them into a valuable source of components for many different types of medicine. Medicinal use of seaweeds dates back to 16th century China, where *Sargassum* was used to treat goiter (Fleurence & Levine, 2016), but it has since evolved into an ever innovating industry, from the use of hydrocolloids in dressings for superficial wounds to numerous supplements and multivitamins.

The numerous bioactive compounds found in seaweeds have several antibiotic, antioxidant and enzymatic properties which can all improve immune response to pathogens (Afonso et al., 2021; Fleurence & Levine, 2016). The antibacterial activity found in seaweeds is usually associated with the protein content as well as the amount of polyphenols, polysaccharides, pigments, and PUFAs, while antioxidant activity is attributed to glutathione, mycosporine-like amino acids, polyphenols, polysaccharides and pigments (Afonso et al., 2021).

Seaweed aquaculture is a widespread global industry and is considered an interesting source of biomass for several wildly different uses since they do not require arable land or freshwater, which makes it much more convenient to grow (Konda et al., 2015). World production of seaweeds has tripled since the beginning of the century, from 10.6 million tons in 2000 to 32.4 million in 2018 (FAO, 2021). Despite the impressive evolution in global seaweed production, only a small number of species are cultivated, namely *Saccharina japonica*, *Gracilaria* sp., *Eucheuma* sp., *Porphyra* sp., *Codium* sp., *Kappaphycus alvarezii* and *Sargassum fusiforme* (Araújo et al., 2021; FAO, 2021).

Seaweed aquaculture is a practice that has existed for centuries and used as a food source for even longer, mainly in Asia and Indo-Pacific countries. In 1969, global seaweed production was evenly distributed through wild harvest and cultivation, but in 2019, total cultivation output reached a staggering 34.7 million tonnes, which amounted to 97% of global seaweed production, while wild harvest remained unchanged (Cai, 2020). The distribution of total seaweed production is severely imbalanced, with 99.1% of 2019's seaweed production originating from Asia where seven of the top ten producing countries are in Eastern and South-eastern Asia. Since western civilization has only recently gained interest in seaweeds as an option in several industries, the total production of seaweeds in the Americas and in Europe contribute only 1.4% and 0.8% respectively (FAO, 2021). European seaweed production is focused mainly in France, Ireland, and Spain, where macroalgae production is still majorly dependant on wild stock harvesting while only 32% of the macroalgae production is grown in land or sea-based aquacultures (Araújo et al., 2021).

The red seaweed *Gracilaria gracilis* has become the third most cultivated species of macroalgae in the world in 2018 (FAO, 2021), widely spread through the world, it lives in waters under 30ºC with a vast range of salinity. Being an agarophyte, it has a high agar content and, like other red seaweeds, has a high content of protein, polyphenols and is sought out as a valuable source of R-phycoerythrin, and arachidonic acid, which gives it a great antimicrobial and antioxidant potential (Afonso et al., 2021; Ben Said et al., 2018; Capillo et al., 2017; Francavilla et al., 2015). The booming growth of *G. gracilis* production is in part the result of a search for a replacement of *Gelidium* spp. as a viable source of agar (Dawange & Jaiswar, 2020). This genus has also displayed antimicrobial activity related to its polyphenol and fatty acid content (Afonso et al., 2021; Capillo et al., 2018; Kasanah et al., 2019). Like in many other living organisms, *G. gracilis* also commonly produces reactive oxygen species (ROS) during its metabolism but it has enzymatic and non-enzymatic cellular defence mechanisms to eliminate these compounds (Cavas & Yurdakoc, 2005). These antioxidant compounds are of great interest since the help prevent certain diseases related to oxidative stress due to an absence of sufficient antioxidant compounds or excess of ROS (Afonso et al., 2021).

The main objective of this dissertation was to optimize the in-vitro culture methodology of *G. gracilis* biomass under different pH levels, nutrient, and light sources, on the nutritional profile of the seaweed, while simultaneously assessing the effect of these different culture conditions on the antimicrobial and antioxidant properties of sequential liquid-liquid extracts of *G. gracilis*.

To this end, *G. gracilis* thalli were grown in sterile and controlled conditions and subjected to environmental changes. Proximate analysis of the biomass was then performed. To assess the antimicrobial activity, the ethanolic section of the sequential liquid-liquid extraction was challenged against common pathogens in antibiograms. The water section of the extraction was used to test the antioxidant properties of the biomass depending on the conditions of growth.

2. Materials & Methods

2.1.Sample preparation and culture

The *Gracilaria gracilis* (Stackhouse) Steentoft, L.M.Irvine & Farnham, 1995 biomass (Fig. 1) used for all assays was grown from previously collected strands, originally from Buarcos (40°9’57’’N, 8°53’5’’W) (Fig. 2, 3), harvested in 2019, according to Freitas et al. 2021.

Figure 1:*Gracilaria gracilis* biomass recovered for laboratory growth (photograph by Teresa Mouga, 11-09-2021).

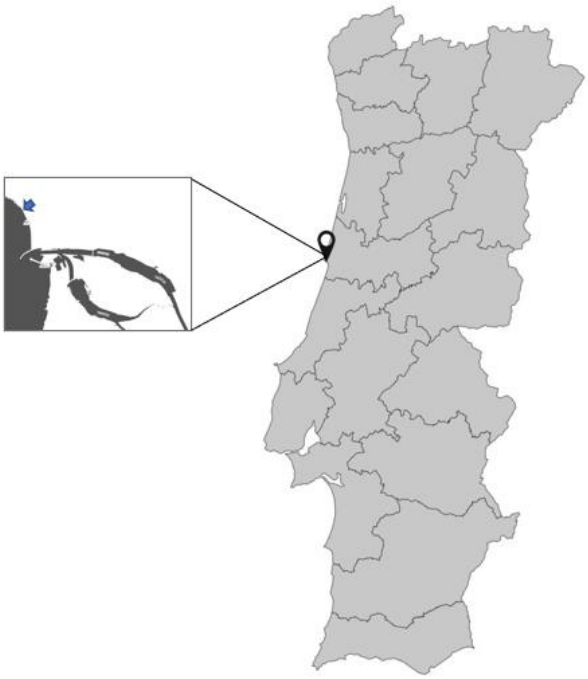

Figure 2: Collection point of wild *Gracilaria gracilis* biomass (Buarcos, Portugal).

Figure 3: *Gracilaria gracilis* harvesting location, Tamargueria beach in Buarcos, Portugal (photograph by Cláudia Moreira, 11-09-2021).

2.2. Experimental design

Ever since, the biomass has been grown vegetatively in laboratory. This biomass was aseptically cultivated from thalli in 1 L flat-bottom flasks with constant filtered airflow to maintain optimal light exposure and suspension in the seawater. This took place in a climate-controlled room (20 ± 1°C), despite some setbacks (Appendix), with daylight cool white fluorescent lamps (OSRAM Lumilux Skywhite), in a set 16:08 (light; dark) photoperiod. The seawater used for every flask was measured for pH and salinity to avoid stress (pH: 7.5 ± 0.1; salinity: 35 ± 1 psu), then sterilized by autoclave (121ºC, 20 minutes). Secondly it was supplemented, at room temperature, with full strength Von Stosch Enriched (VSE) medium specially modified for cultivating red seaweeds (Redmond et al., 2014), and 1ml/L of Germanium dioxide (GeO_2) to prevent the growth of epiphytic diatoms which would interfere with the growth of the algae. VSE medium is comprised by the macronutrients ammonium (NH_4^+) and phosphate (PO_4^{3-}), micronutrients iron, manganese, EDTA and vitamins B_{12}, thiamine and biotin. For some of the growth assays, either ammonium or both ammonium and phosphate were added to supplement the other media. The light intensity was measured using a handheld luxmeter (Konica Minolta, T-10, Tokyo, Japan) and paper towels were fashioned into barriers to accomplish optimal light intensity (16 ± 0.5 µmol photons m^{-2} s^{-1} from the white cool light lamp) and light diffusion for maximum coverage and uniformity of exposure. Finally, if by any reason, the

tips of the seaweed exhibited loss of pigment, causing discolouration which is a visual sign of stress, were promptly removed from the culture as to not be included in any assays.

For each assay, all conditions were kept as aseptic as possible, using sterile tweezers, scalpels, and scissors while the assays were set close to a Bunsen burner. For each assay, fifteen freshly cut thalli tips of similar size (ca. 10 ± 0.1 mm) were randomly selected and placed in a sterile Petri dish after being gently dried against the inside of a beaker to allow the least amount of water being weighted (Fig. 4).

Figure 4: Photograph of weighting process of *Gracilaria gracilis* thalli for assays.

After weighting, the tips were then distributed to previously sterilized 250 mL flat-bottom flasks in the climate-controlled room at 20 ± 1°C where they remained for 14 days (Fig. 5). After the growth period, as per the first time, aseptically transferred from the flask to a Petri dish for weighting.

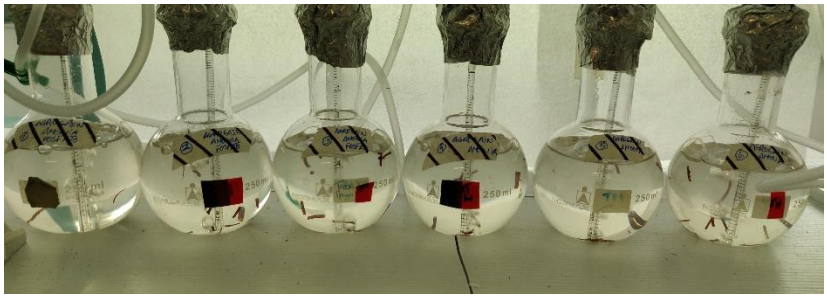

Figure 5: Photograph of thalli growth conditions during assays.

2.3. Growth Assays

Testing the growth rate of *Gracilaria gracilis* is the first step in selecting the right conditions for production. For every assay, the combined weight of the thalli was recorded before and after the trial using an analytical scale (Sartorius TE124S - 120 g x 0.0001 g). The excess water was carefully removed by gently drying on paper towels before weighting as to not affect the accuracy of the measuring. Relative Growth Rate (RGR) and Productivity assessment were calculated according to Patarra et al. (2017):

$$RGR\ (\%\ fw\ day^{-1}) = ([Ln\ (fw) - Ln\ (iw)]/t) * 100 \quad (1)$$

Where, *Iw:* initial weight, *Fw:* final weight, *T:* time in days

Several different culture conditions were tested in a total of 12 growth assays to determine the optimized conditions to sustained and thriving growth of *G. gracilis* biomass. All these assays were performed in triplicate, where the biomass was kept in the climatic controlled room (20 ± 1ºC) during 14 days with an automated photoperiod (16:08h) without being disturbed. The variables tested for the assays were the type of light used in culture, the pH of the seawater and the culture medium.

Table 1**.** Summary of *Gracilaria gracilis* growth assays performed.

Assay	Light	pH	Culture media
A (Control)	White Cool Florescent	7.5 ± 0.1	VSE
B	White Cool Florescent	7.5 ± 0.1	SSEP 0.01 g/L + Ammonia + Phosphate
C	White Cool Florescent	9	VSE
D	White LED	7.5 ± 0.1	VSE
E	White Cool Florescent	7.5 ± 0.1	SSEP 0.1 g/L
F	White Cool Florescent	7.5 ± 0.1	SSEP 0.01 g/L
G	White Cool Florescent	7.5 ± 0.1	SSEP 0.01 g/L + Ammonia
H	White Cool Florescent	7	VSE
I	White Cool Florescent	7.5 ± 0.1	AgroGain 1mL/L
J	White Cool Florescent	7.5 ± 0.1	AgroGain 2mL/L
K	White Cool Florescent	7.5 ± 0.1	AgroGain 1mL/L + Ammonia
L	White Cool Florescent	7.5 ± 0.1	AgroGain 1mL/L + Ammonia + Phosphate

Assay A was used as control as it has already been significantly studied and the Von Stosch Modified Enriched Medium (VSE) was designed for red seaweeds and pH was kept at seawater standard (7.5 ± 0.1). Assay H was preformed to simulate the acidification of the ocean, and assay C de basification of the medium. Both were performed to assess if said shift in pH would affect the biomass growth and its biochemical composition in any form. The pH of the seawater was adjusted using small quantities of HCl and NaOH. In assay D the light sourced was changed from White Cool Florescent light to a White LED, which has a low light intensity (1000 lux), to examine the difference in photosynthetic activity related to growth. Acadian™ Soluble Seaweed Extract Powder (SSEP) is derived from fresh *Ascophyllum nodosum* biomass, which is rich in natural growth promoters and nutrients that benefit the growth of biomass. This culture media was used in assays E and F in different concentrations to determine how it affected both the growth and biochemical composition of the algae, despite the growth seen in these assays, the growth of the biomass slowed abruptly after the 14 days. Assays G and B were preformed to test the effects of supplementing the SSEP with ammonia in the form of NH_4Cl and ammonia and phosphate in the form of ($Na_2HPO_4 \cdot 12H_2O$) usually used in VSE medium, surpassing the abrupt reduction in growth after the end of the 14 days. AgroGain™, derived from *Kappaphycus alvarezii*, is an agricultural bio stimulant used for its increase in photosynthetic activity and crop metabolism. This medium was tested as with SSEP in assays I through L.

After the assays, the biomass obtained was weighed to assess the Relative Growth Rate, to determine which assays were viable for future production and further analysis. It is important to note that assays such as the one with Agrogain at 1mL/L of seawater and VSE at pH 7, despite showing significant growth in the first 14 days, the biomass either did not survive or displayed subpar growth after the end of the assays. This meant that those specific conditions did not yield enough biomass for further parameter analysis, thus were discarded. As such, the biomass selected for further investigation are shown in Table 2 and will be referenced as such.

Table 2. Selected assays for biochemical analysis.

Assay	Light	pH	Culture media
A (Control)	White Cool Florescent	7.5 ± 0.1	VSE
B	White Cool Florescent	7.5 ± 0.1	SSEP
C	White Cool Florescent	9	VSE
D	White LED	7.5 ± 0.1	VSE

These assays were then upscaled to 1L flat-bottom flasks, and then onto 2L flat-bottom flasks, for about a month, until enough biomass was obtained to allow the proximate biochemical composition. This biomass was then softly dried with paper towels, weighted and frozen at -20ºC for future use.

2.4. Proximate Biochemical Composition

To determine the nutritional viability of the cultured *Gracilaria gracilis* biomass, the proximate biochemical composition was calculated, including the primary metabolites: total protein, total lipid, and carbohydrate content. Moisture and ash were also calculated to estimate the amount of minerals in the biomass. Antimicrobial and antioxidant were also tested, for which sequential extracts were produced from frozen *G. gracilis* biomass from the selected assays and tested against several prominent pathogens. The previously frozen *G. gracilis* from the selected assays were dried in a Laboratory oven (FD115 Binder, Tuttlingen, Germany) at 25ºC for 48h. It was then ground to powder and stored in a zip lock bag to avoid contact humidity. All proximate composition assays were performed in triplicate.

2.4.1. Moisture and ash content

For the determination of moisture and ash content, the official AOAC standard methods were used (AOAC & Latimer, 2016). Moisture content was preformed using fresh *G. gracilis* biomass. The crucibles used were weighted when empty, the biomass was placed and weighted in crucibles after being thoroughly dried using paper towels. By subtracting the weight of the empty crucible first to the weight of the crucible with the sample we get the fresh weight of biomass. The sample was then placed in a laboratory oven (FD115 Binder, Tuttlingen, Germany) at 105 ºC for 48h. After the drying process, the crucibles were placed inside a desiccator until the they reached room temperature, at which point they were again weighted, the moisture content calculated using the following formulas, and was expressed as a percentage of fresh weight (fw):

% Dry weight = (dry weight /fresh weight) x 100 (1)

% Moisture = 100 - % Dry weight (2)

To determine the ash content of the samples, the crucibles used in the moisture procedure are placed in a muffle furnace at 525ºC for 5h (Nabertherm, B170, Germany). By this time the sample has lost all humidity and is extremely brittle, it is then moved again into a desiccator and weighted until the mass stabilizes. The ash content is then able to be calculated using the final weight of the sample by following formulas:

% Ash in dry weight = (Incinerated weight / Dry weight) x 100 (3)

% Ash in fresh weight = (Incinerated weight /Fresh weight) x 100 (4)

For these assays, moisture is expressed in percentage of fresh weight and ash content is expressed in percentage of dry weight.

2.4.2. Total Protein content

The total protein content was determined using the Kjeldahl method (AOAC & Latimer, 2016) which consists of three steps: digestion, distillation, and titration. In theory, the determination of total protein content is achieved by multiplying the amount of nitrogen by a conversion factor of 5, specific for seaweeds (Angell et al., 2016). This calculation is extremely accurate given that the conversion factor is based on the quantification of total amino acids.

In the first step, 0.5 g of dried and ground *G. gracilis* biomass were weighted using a small analytical scale and then placed in Kjeldahl digestion tubes where a catalyst was added. The digestion was promoted by adding 25 mL of a 97% solution of Sulfuric acid was then added to the tube where the biomass was mineralized by transforming nitrogen into ammonium salt. The digestion tubes were then moved to the Kjeldahl digestor (Digestor2006, Foss, Hillerød, Denmark) where the temperature rose to 220 ºC. After reaching this temperature, a water tap connected to the digestor was then turned on and the temperature was maintained for 30 minutes. The temperature was risen to 400 ºC after this time and maintained for 90 more minutes. When time was up, the digestor was turned off until the tubes were safe enough to handle. The resulting product of the digestion process was a transparent liquid. After the tubes and liquid cooled enough, 80 mL of distilled water were delicately and gradually added to the tubes.

The second step to the procedure is the distillation of the samples. For this step, several Erlenmeyer flasks were prepared with 30 mL of 4% boric acid solution containing bromocresol green and methyl red. While the product of the digestion is still in liquid form, the tubes were individually transferred to the Kjeldahl distiller (Kjeltec2100, Foss, Hillerød, Denmark) with its corresponding Erlenmeyer flask. The equipment was previously cleaned before the samples were placed inside and the NAOH used for the distillation level was checked. Distillation of each tube takes 6 minutes in which the colour of the boric acid turns from its distinctive reddish purple to grey, meaning the reaction occurred successfully.

The third and final step of the procedure is the titration. After the distillation, the Erlenmeyer flask is then placed under a burette containing HCl (0.1 M) in a support. The titration is then executed slowly into the Erlenmeyer flask until the solution starts turning pink, at which point the value of HCl used is noted. After this step, the quantification of protein (% of dry weight) is made using the formula below:

$$\text{Total Protein Content (\%)} = \frac{[(V_a - V_b) \times \text{n} \times 5 \times 0.014]}{m} \times 100 \quad (5)$$

Where: V_a = Volume of HCl used in titration (mL); V_b = Volume of HCl in white titration (mL); n = Concentration of HCl used in titration (0.1 $mol.L^{-1}$); m = Initial mass of sample (g)

Calculations of total protein content were made for each individual sample, using the average of volumes spent in both white titrations.

2.4.3. Total Lipid content

Lipid content was determined using the Folch method (Folch et al., 1957) with some modifications. Approximately 1g of dried and grounded biomass was weighted inside a falcon tube which in turn is filled with 0.8 mL of water. Then 5 mL of Folch reagent, consisting of chloroform ($CHCl_3$) and methanol (CH_3OH) solution (2:1 v/v) was added and the solution was homogenized in a vortex for 5 minutes, for each sample. After this time, 1.2 mL of 0.8% sodium chloride (NaCl) is mixed in the solution by using the vortex for one more minute. The solution was then centrifuged at 4637g for 10 minutes at 4ºC. During this step, columns were made to filter the lower phase into an evaporating flask. For these, P5000 pipette tips were stuffed with hydrophobic cotton making an approximately 1 cm tall filter, after this 5 mL of chloroform were added. After centrifuging the solution again, the lower phase was then extracted from the falcon tube and passed through the cotton and into the previously weighted evaporating flasks leaving only the lipidic components of the sample in the flask itself. The flaks were then placed in a rotatory evaporator (Heidolph, Laborota 4000, Germany) at 45 ºC until most of the moisture was removed, at which point they were involved in aluminium foil and placed in a laboratory oven (FD115 Binder, Tuttlingen, Germany) for 1 hour at 40 ºC, and finally in a desiccator overnight. At this stage, the flasks are completely dry and are weighted in an analytical scale. Total lipid content was calculated using the formula below:

Total lipid content (% dry weight) = $\frac{Fw-Iw}{Sw} \times 100$ (6)

Where: S_w = Biomass initial dried weight (g); F_w = Mass of flask with lipid residue (g); I_w = Mass of flask (g)

2.4.4. Total Carbohydrates

For the calculation of total carbohydrates, an adaptation of Dubois et al. (1956) method was used. The theorical fundament behind this method consists in the quantification of carbohydrate monomers through colourimetry using spectrophotometry. These monomers are produced by the dehydration of simple sugars, polysaccharides, and methyl-esters with free reductor groups by using sulfuric acid. Sulfuric acid hydrolyses these compounds into quantifiable monomeric subunits. Five milligrams of ground and dried *G. gracilis* biomass were added to 3 mL of 1M sulfuric acid and the samples were placed in a water bath at 90 ºC for 1 h. The samples were then cooled at room temperature and 0.5 mL of phenol 5 % and 2.5 mL of 96% sulfuric acid were added before placing the samples in a centrifuge at 1700 g for 10 minutes at 4ºC. The absorbance was read in a UV-Visible spectrophotometer (Evolution201, Waltham, Massachusetts, USA), at 485 nm. A galactose solution was used as standard for the calculations of total carbohydrate content. The total carbohydrate content was expressed as percentage of dry weight, according to Equation 4.

Total carbohydrates (% dry weight) = $\frac{Carbohydrate.mL-1}{dw} \times 100$ (7)

Where: dw = Dry *G. gracilis* mass

2.5. Sequential Extraction of *Gracilaria gracilis* biomass

For the antimicrobial and antioxidant assessment of *Gracilaria gracilis* biomass, Afonso et al. (2021) guidelines for sequential aqueous-ethanolic extraction procedures were used. Sequential extraction means the sample is subjected to multiple consecutive extractions and filtrations. For these assays, 6 g of dry ground *G. gracilis* were stirred with 60 mL of MiliQ water in an aluminium foiled covered beaker, at

40ºC for 30 minutes. The extract was then filtered through filter paper (Whatman No.1) and followed up with a second aqueous extraction and filtering. The sample was then extracted using ethanol 100% following the same procedure. Both the aqueous and ethanolic extracts were safely stored at 4ºC. Prior to its use, the sequential ethanolic extract used in the antimicrobial activity assays was dissolved in absolute ethanol to a final concentration of 100 $mg.mL^{-1}$. For the antioxidant activity assessment, aqueous extracts were used and were not rehydrated.

2.6. Antimicrobial Assays

In the antimicrobial assay, the microbial strains of *Micrococcus luteus* (DSM 1605), *Escherichia coli* (DSM 301), and *Bacillus subtilis* (DSM 10) obtained through DSMZ biobank, were selected to assess the antimicrobial activity of the ethanolic extract of *Gracilaria gracilis* biomass cultured in different conditions. For the strains to be used, they were first incubated in sterile test tubes with the appropriate grown media and temperature (Table 3) and placed in a temperature controlled shaking incubator (Stuart SI500) at the optimal temperatures for 24 – 48 hours at 140 rpm. A positive and negative control were also created as to compare the efficacy of the extracts in preventing the growth of the different microbial strains. The antibiotic Chloramphenicol (Oxoid) was used for the positive control. The negative control was simply the dissolving agent for the extract, which in this case was absolute ethanol.

Table 3: Reference table for temperature, medium and antibiotics for each strain used in the antimicrobial assay. (NB: Nutrient Broth; Chlo: Chloramphenicol)

Strain	**Temperature (ºC)**	**Medium**	**Antibiotic**
Micrococcus luteus	30	NB	Chlo
Escherichia coli	37	NB	Chlo
Bacillus subtilis	28 – 30	NB	Chlo

2.6.1. Disk diffusion method

The disk diffusion method used was performed according to Bauer et al., (2018) to determine the antimicrobial abilities of the ethanolic sequential extract from *G. gracilis* biomass. After incubating, the strains were suspended in a saline solution comprised of 0.85 % NaCl to reach a final density of 1 x 10^8 cfu.mL^{-1} which was measured using a bench top turbidimeter (0.5 standard) (VWR Chemicals). Sterile Petri dishes with previously sterilized Muller Hinton Agar (MHA) (VWR Chemicals) were uniformly spread with the now suspended cultures using a sterile cotton swab. Sterile paper diffusion disks (6 mm) were then carefully placed in the agar and embedded with 20 µl of each extract (100 mg.mL^{-1}) and the corresponding positive and negative control. These MHA plates were then incubated according to the optimal temperature of each microbial strain until the cultures developed. The inhibition halos were then measured (mm) from the centre of the diffusion disk to the edge of the halo.

2.7. Antioxidant activity

2.7.1. Reduction of DPPH radical

The 2,2-diphenyl-1-picryl-hydrazyl-hydrate compound (DPPH) consists of free stable radicals in the form of a dark coloured crystalline powder which acts as an indicator of chemical reactions involving oxidizing radicals. DPPH is simultaneously a radical and radical capturing compound which makes it useful in determining the radical nature of a given reaction. The method belonging to (Brand-Williams et al., 1995) allows the assessment of the free stable radical's reduction in the absence of light and at room temperature of red seaweed extracts through the quantification of lipid peroxidation products. When a DPPH solution is mixed with an antioxidant it forms a non-radical form of DPPH which loses its characteristic violet colour or even turning a pale yellow detectable by spectrophotometry. DPPH is soluble in organic solvents and detects the presence of apolar antioxidant substances. To start, 3.6 mg of *Gracilaria gracilis* dry aqueous extract were added to a test tube and then mixed with 6 mL of phosphate

buffer with pH 5.5, where the final concentration was 0.6 $mg.mL^{-1}$. This solution was homogenized using an ultrasonic cleaning bath (VWR, USC600TH, Radnor, Pennsylvania, USA) for 20 minutes while keeping the bath temperature as low as possible.

When the solution was properly homogenized, 1mL of the solution was transferred to a new test tube which was then mixed with 0.5 mL of phosphate buffer and 0.5 mL of acetonitrile. A DPPH solution was prepared by adding 2.5 mg of DPPH to 25 mL of acetonitrile, 1 mL of this solution was added to the sample solution and mixed using a vortex. The solutions were kept in a dark cabinet after vortexing for 30 minutes until ready to be read in a UV-Visible spectrophotometer at 517 nm, after executing a baseline with phosphate buffer and acetonitrile in a 1:1 ratio. The DPPH radical reduction capability of each extract was calculated in percentage of DPPH radical reduction through the formula below:

$$DPPH\ radical\ reduction\ (\%) = \frac{Abs\ white - Abs\ sample}{Abs\ white} \times 100 \qquad (8)$$

Abs: Absorbance read at 517 nm

2.8. Statistical Analyses

All assays were performed in triplicate (n = 3) except for the control VSE modified medium that was n = 6. To optimize *Gracilaria gracilis* culture, all assays were tested through a one-way Analysis of Variance (ANOVA), preceded by normality and homogeneity of variance validation to meet the assumptions of parametric statistical analysis (Zar, 2010). Whenever homogeneity of variances was not achieved, Kruskal-Wallis test was used (Zar, 2010). Whenever applicable, the multiple comparisons tests were carried out, according to the fulfilment or not of the variance analysis assumptions, that is, Tukey's and Games-Howell's test, respectively (Zar, 2010). All differences were considered significant at p-value < 0.05. Data were expressed as mean ± standard deviation (SD). Calculations were performed with SPSS Statistics 27 (IBM Corporation, New York, EUA).

In addition, to identify patterns in the relation between the parameters under study (namely, ash content, total protein content, total carbohydrates, DPPH radical reduction, total lipid content, growth rate, moisture content) and assays (namely, control VSE medium, Soluble Seaweed Extract Powder medium, VSE with pH=9 and VSE with LED lights), principal component analysis (PCA) was performed. This multivariate technique was used to identify the components (that is, the core parameters) that explain the correlations within the measured data. Therefore, by means of PCA, it was possible to achieve associations between parameters, reducing the dimension of the original data. The information provided by the principal components highlight the most meaningful parameters, describing the whole data matrix and an ording data reduction with minimum loss of original information. Through a linear combination analysis, the positions of original variables in the diagram represent their relevantly interrelations. Thus, principal components effectively represent the original measured data. As a result, if the parameters are closely positioned, their correlation is strong and positive. In contrast, if the parameters are in an opposite position, then those parameters are negatively correlated. Hence, graphical representation of PCA, which plots simultaneously the objects (points) and the variables (vectors), is very useful to detect possible associations between variables and objects. Moreover, the association between objects and variables can be determined depending on their relationship and proximity within each group. Although the results for the first two components (PC1 and PC2) were presented, the others were also analysed. Calculations were performed with CANOCO version 4.5 package (Copyright Petr Smilauer©2012–2019, Ithaca, New York, USA).

3. Results

3.1.Relative Growth Rate

As previously mentioned, not all assays were fully investigated due to lack of survival of the biomass or viability (Fig. 6). Even so, the relative growth rate showed statistically significant differences (ANOVA, p-value<0.05, Fig. 7) in the light source used between the selected assays. Of the selected assays, White Cool Light used in VSE medium, as a control solution, SSEP and pH assays, exhibited significantly higher growth percentages than the assay using white LED as a light source (ANOVA, Tukey, p-value < 0.001, Fig. 7). In the last assay, using LED as the light source, showed the lowest yield of biomass with a RGR of only 2.35 ± 0.67 %, while Assay B (SSEP) showed the highest growth with 8.15 ± 0.48% when compared to the other assays, despite not being statistically significant (p-value > 0.05, Fig. 7). Assays A and C both showed average and similar RGR values with 7.15 ± 3.63 and 6.68 ± 0.69% respectively.

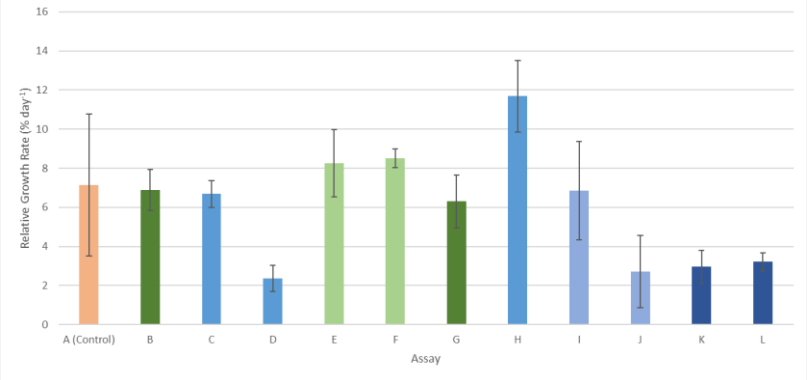

Figure 6: Average Relative Growth Rate (% dw^{-1}) of all tested conditions. Assay 1: Control Von Stosch Enriched (VSE) medium; Assay 2: VSE with pH = 7; Assay 3: VSE with pH = 9; Assay 4: VSE with LED white light; Assay 5: Soluble Seaweed Extract Powder (SSEP) at 0.1g/L; Assay 6: SSEP 0.01 g/L; Assay 7: SSEP 0.01 g/L + Ammonia; Assay 8: SSEP 0.01 g/L + Ammonia + Phosphate; Assay 9: AgroGain 1mL/L; Assay 10: AgroGain 2mL/L; Assay 11: AgroGain 1mL/L + Ammonia; Assay 12: AgroGain 1mL/L + Ammonia + Phosphate. Values are presented as mean ± SD (n=3).

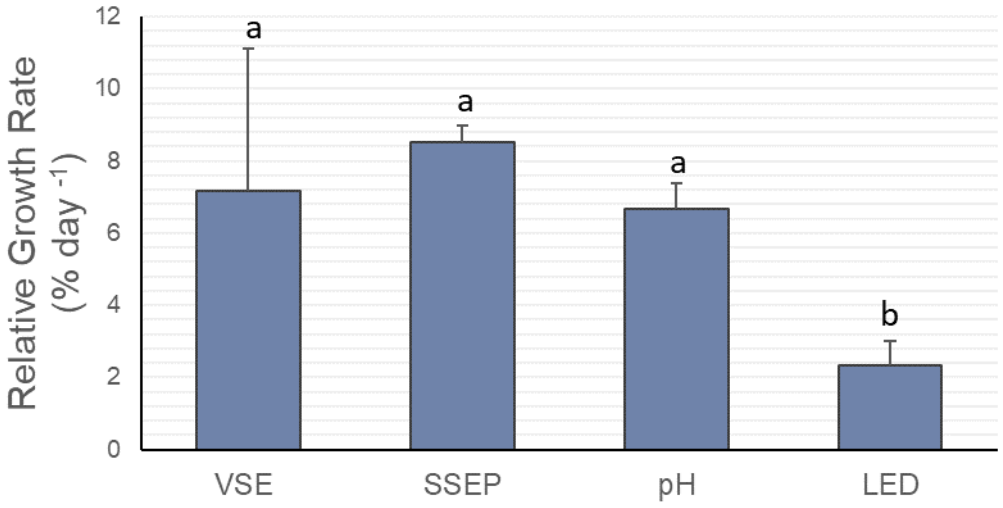

Figure 7: Relative Growth Rate (RGR) of *Gracilaria gracilis* cultured in different conditions: A: control Von Stosch Enriched modified medium (VSE) with White Cool Light (WCL); B: Acadian Soluble Seaweed Extract Powder (SSEP)(WCL); C: VSE medium with pH = 9 (pH)(WCL); D: VSE medium with white LED light (LED) Values are presented as mean ± SD (n=3). Different lowercase letters represent statistically significant differences (p-value < 0.05).

3.2. Proximate Composition

3.2.1. Moisture content

Gracilaria gracilis biomass grown under the four tested culture conditions showed average to high moisture percentages of the fresh biomass. However, it was determined that the biomass originating from the LED assay exhibited significantly lower humidity (75.41 ± 1.51%) when compared to the other assays which showed moisture contents at least 10% higher (ANOVA, Tukey, p-value < 0.001, Fig. 8). The remaining assays did not show significant differences (p-value > 0.05, Fig. 8) between them, with the SSEP trial being the one to show higher moisture at 86.1 ± 0.4 % compared to the control and pH assays, 85.5 ± 1.0 and 85.8 ± 0.67 respectively.

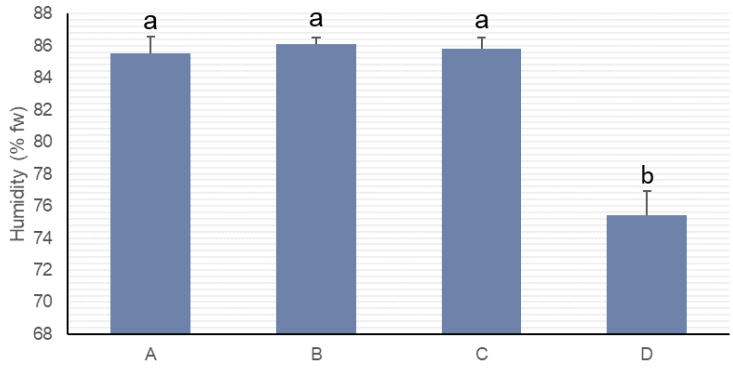

Figure 8: Moisture content (% fw) of *Gracilaria gracilis* cultured in different conditions: A: control Von Stosch Enriched modified medium (VSE) with White Cool Light (WCL); B: Acadian Soluble Seaweed Extract Powder (SSEP)(WCL); C: VSE medium with pH = 9 (pH)(WCL); D: VSE medium with white LED light (LED). Values are presented as mean ± SD (n=3). Different lowercase letters represent statistically significant differences (p-value < 0.05).

3.2.2. Ash content

Ash content in dry weight biomass was high in all assays, but with no statistically significant differences between them (ANOVA, p-value > 0.05, Fig. 9). Similarly, to moisture, the LED assay also displays the lowest percentage of ash out of all assays (28.8 ± 9.01 %). The SSEP trial, likewise, had the highest ash content of all assays (46.96 ± 2.05 %), followed by the pH assay (36.35 ± 1.72 %) and the control (VSE) assay (34.20 ± 2.43 %).

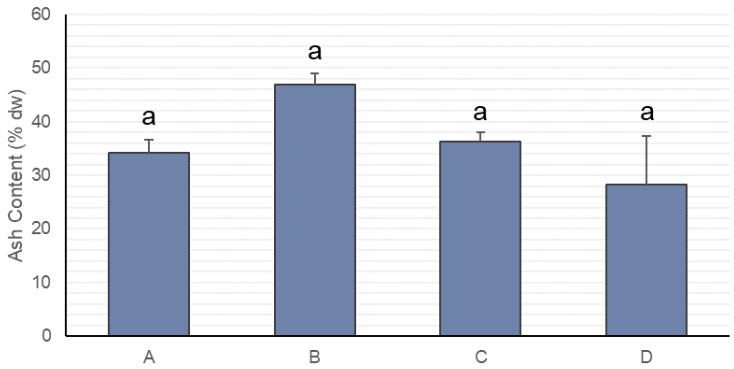

Figure 9: Ash content (% dw) of *Gracilaria gracilis* cultured in different conditions: A: control Von Stosch Enriched modified medium (VSE) with White Cool Light (WCL); B: Acadian Soluble Seaweed Extract Powder (SSEP)(WCL); C: VSE medium with pH = 9 (pH)(WCL); D: VSE medium with white LED light (LED). Values are presented as mean ± standard deviation (n = 3).

3.2.3. Total Protein Content

The protein content (% dw) of *G. gracilis* grown in different conditions was high in all assays, as is among the expected values for red seaweeds. *G. gracilis* protein content ranged from 16.13 ± 0.31 % in the control assay using Von Stosch Enriched modified medium (VSE), to 19.62 ± 0.11 % in the Acadian Soluble Seaweed Extract (SSEP). The control media (VSE) showed statistically significant differences when compared to both the SSEP medium (Kruskal-Wallis, Games-Howell, p-value = 0.002, Fig. 10) and LED assay (Kruskal-Wallis, Games-Howell, p-value = 0.042, Fig. 9), while the pH assay differed significantly from SSEP (Kruskal-Wallis, Games-Howell, p-value = 0.042, Fig. 10).

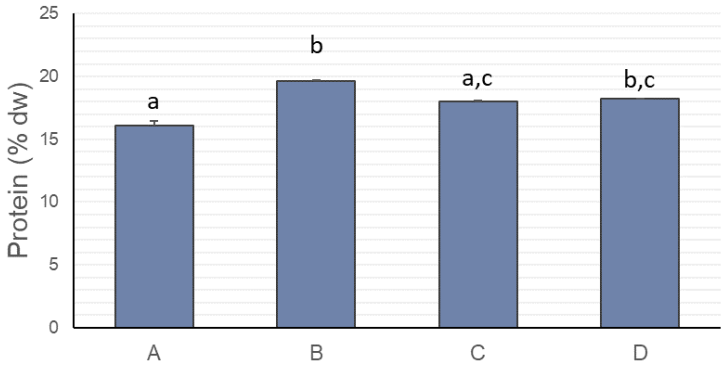

Figure 10: Total Protein Content (% dw) of *Gracilaria gracilis* grown under different conditions: A: control Von Stosch Enriched modified medium (VSE) with White Cool Light (WCL); B: Acadian Soluble Seaweed Extract Powder (SSEP)(WCL); C: VSE medium with pH = 9 (pH)(WCL); D: VSE medium with white LED light (LED) Values are presented as mean ± SD (n=3). Different lowercase letters represent statistically significant differences (p-value < 0.05).

3.2.4. Total Lipid Content

As it was expected for seaweeds, total lipid content is always extremely low. Of the four tested conditions, *G. gracilis* biomass grown using Acadian Soluble Seaweed Extract contained significantly less fat than all other assays (ANOVA, Tukey, p-value = 0.003, Fig. 11) with 0.28 ± 0.03 %. On the other hand, biomass grown with Von Stosch Enriched modified medium using white LED as a light source exhibited the highest lipid content, despite not having significant differences from the control and pH assay, with 0.40 ± 0.03 % (ANOVA, p-value > 0.05, Fig. 11). The change in pH seemed to have no effect of total lipid content as the control assay and pH assay had identical results with 0.38 ± 0.02 % for both assays.

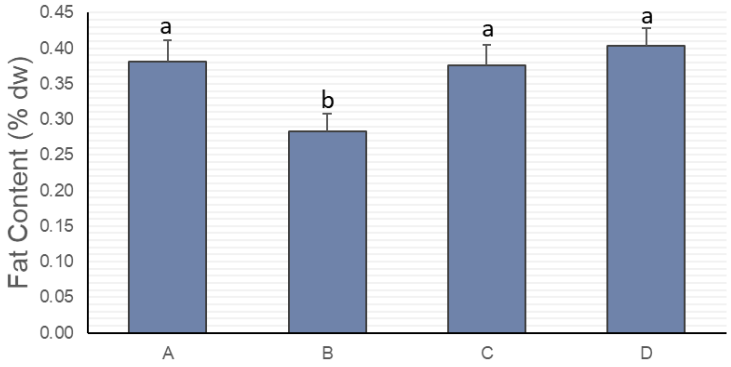

Figure 11: Total Lipid Content (% dw) of *Gracilaria gracilis* cultured in different conditions: A: control Von Stosch Enriched modified medium (VSE) with White Cool Light (WCL); B: Acadian Soluble Seaweed Extract Powder (SSEP)(WCL); C: VSE medium with pH = 9 (pH)(WCL) D: VSE medium with white LED light (LED). Values are presented as mean ± SD (n=3). Different lowercase letters represent statistically significant differences (p-value < 0.05).

3.2.5. Total Carbohydrates

Carbohydrate contents fell within expected results, although with significant discrepancies between assays (ANOVA, Tukey, p-value = 0.010, Fig. 12). The assay with the lowest amount of carbohydrates was the control assay with 12.66 ± 0.20 %, which is significantly lower than the values observed in the assay using SSEP (21.44 ± 2.33 %) and VSE using white LED (19.73 ± 1.63 %) (ANOVA, Tukey, p-value = 0.009, p-value = 0.028, respectively, Fig. 12). Total carbohydrate content in the pH trial was intermediate with no statistically significant differences when compared to the rest of the tested conditions (ANOVA, p-value > 0.05, Fig. 12).

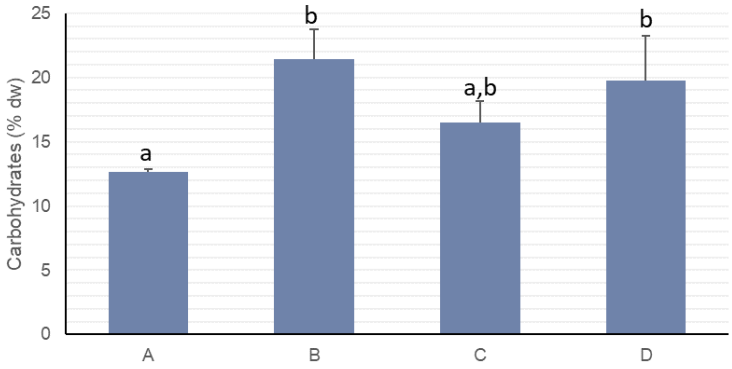

Figure 12: Total Carbohydrates (% dw) of *Gracilaria. gracilis* cultured in different conditions: A: control Von Stosch Enriched modified medium (VSE) with White Cool Light (WCL); B: Acadian Soluble Seaweed Extract Powder (SSEP)(WCL); C: VSE medium with pH = 9 (pH)(WCL); D: VSE medium with white LED light (LED). Values are presented as mean ± SD (n=3). Different lowercase letters represent statistically significant differences (p-value < 0.05).

3.3. Antimicrobial Assays

Of the three strains challenged against the ethanolic sequential extract of *G. gracilis* biomass, *Bacillus subtilis* was the only one showing interesting results. *Escherichia coli*, and *Micrococcus luteus* showed no inhibition halos, thus exhibiting the inability to impair the growth of these strains (Table 4).

Table 4: Antibacterial activity observed from *Gracilaria gracilis* ethanolic sequential extracts when challenged against *Escherichia coli*, *Bacillus subtilis*, and *Micrococcus luteus*. The inhibition halo is expressed in mm and results shown as mean ± standard-deviation using three replicates for *B. subtilis* and six replicates for the other microorganisms. Assay A: Control VSE modified medium (VSE), Assay B: Soluble Seaweed Extract Powder (SSEP), Assay C: VSE medium with pH = 9, and Assay D: VSE with LED white light (LED).

Bacteria	Assay	Inhibition Halo (mm)
Escherichia coli	A (Control)	0
	B	0
	C	0
	D	0
	Chloramphenicol	14 ± 1.15
Bacillus subtilis	A (Control)	7.67 ± 0.47
	B	8.33 ± 0.47
	C	0
	D	8 ± 0.41
	Chloramphenicol	27.67 ± 1.24
Micrococcus luteus	A (Control)	0
	B	0
	C	0
	D	0
	Chloramphenicol	13.42 ± 0.84

Thusly, the assays pertaining to these strains were repeated as to eliminate possible doubts of user error, but still no growth inhibition was observed. For *B. subtilis* however, inhibition halos were observed measuring from 7.67 ± 0.47 to 8.33 ± 0.47 mm in extracts from the control VSE and SSEP assays respectively. The LED assay also showed inhibition halo measuring an average of 8 ± 0.41 mm. No statistically significant differences were observed between these assays (ANOVA, p-value > 0.05). Finally, the pH trial surprisingly did not produce inhibition halos being the only extract to not impair the growth of *B. subtilis* in the agar plates.

3.4. Antioxidant activity

After the analysis of the antioxidant activity provided by aqueous/ethanolic sequential extracts of *G. gracilis*, only a very tiny percentage of DPPH radical reduction was verified. DPPH tests showed antioxidant activity on all assays, even if minimal. The control VSE assay showed the least activity with 1.15 ± 0.73 %, followed by the LED and pH assays with 1.41 ± 0.23 % and 1.47 ± 0.27% respectively. The extract obtained from *G. gracilis* biomass cultured using SSEP showed the largest percentage of antioxidant activity with 5.09 ± 3.14 % (Fig. 13).

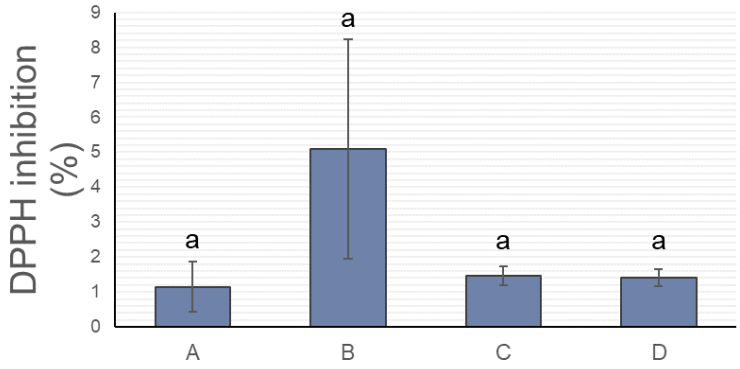

Figure 13: DPPH radical reduction (%) of *Gracilaria gracilis* extract that was cultured in different conditions: A: control Von Stosch Enriched modified medium (VSE) with White Cool Light (WCL); B: Acadian Soluble Seaweed Extract Powder (SSEP)(WCL); C: VSE medium with pH = 9 (pH)(WCL); D: VSE medium with white LED light (LED). Values are presented as mean ± SD (n=3).

3.5. Principal Component Analysis

The results achieved by the PCA, revealed that the first factorial plan explains 92.7% of the total variance of the data, with the first component (PC1) holding 70.6% and component two (PC2) holding 22.1% of this variability (Fig. 14). Additionally, the pattern found allowed us to observe a strong correlation between ash content, total protein content, total carbohydrates, and DPPH radical reduction. These parameters showed a negative correlation with the total lipid content, showing that their increase determines the decrease in total lipid content. Growth rate and moisture content presented a null correlation with the group formed by ash content, total protein content, total carbohydrates, and DPPH. Although with a low intensity, growth rate and moisture showed a negative correlation with total lipid content. The SSEP assay determined the occurrence of high values for ash content, total protein content, total carbohydrates, and DPPH radical reduction and with lower expression for the growth rate and moisture content. The other assays showed average to low results in most parameters and average (VSE810 and VSE912) to high (VSE812) for total lipid content.

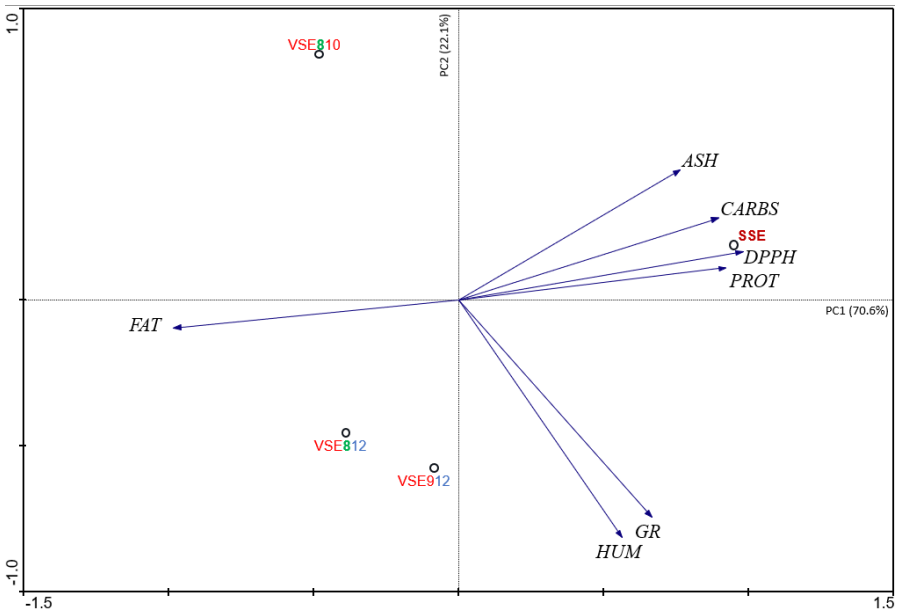

Figure 14: Principal Component Analysis (PCA) diagram of the parameters tested on *G. gracilis* biomass and subsequent aqueous extracts. Vectors represent the different parameters analysed (GR – Growth rate; PROT - Total protein content; FAT – Total lipid content; CARBS – Total carbohydrates; HUM – Moisture content; ASH – ash content; DPPH – DPPH radical reduction) with biomass grown under different conditions (VSE812 – Control assay; SSE – SSEP assay; VSE912 – pH assay; VSE810 – LED assay.

4. Discussion

4.1. Relative Growth Rate

Several significant differences were observed between the several media conditions used in the culture of *Gracilaria gracilis* regarding Relative Growth Rate (RGR), proximate composition, and antimicrobial activity of the extracts produced using *G. gracilis* biomass. RGR is a crucial indicator as growth rates dictate the possibility of efficiently producing high quality biomass yields to maximize profits in the commercialization of products derived from the biomass. The LED trial was the only one to show statistically significant differences between the other growth conditions tested during this study (p-value < 0.05). This trial displayed the lowest rate of growth when compared to the others, inferring that the light source used during culture is the main abiotic factor affecting *G. gracilis* growth. This reduction in growth could be linked to observations made by Fethi and Ghedifa (2019) in which it was deduced that an over-increase in light intensity could reduce the ability of the algae to preform photosynthesis efficiently. Photosynthesis is among the most sensitive physiological processes preformed in photosynthetic organisms since excessive light can destroy the photosystem II through photo-oxidation. Thusly, photo-oxidation reactions due to excessive light exposure can occur inside the cell, resulting in a decrease of growth and, in extreme cases, even the demise of the affected biomass. Beyond their intensity, LED lights also display an intense emission peak around 450 – 480 nm and a smaller but wider peak between 530 and 600 nm, while white fluorescent light's peaks are around 550 nm and 620 nm (Fethi & Ghedifa, 2019). The two main pigments contained in *G. gracilis* are chlorophyl *a* which has an active wavelength at 430 and 680 nm (Hsieh-Lo et al., 2019) and R-phycoerythrin which has two peaks at 495 nm and 566 nm (Rossano et al., 2003). Correlating the different peaks exhibited by the two light sources used with the absorption peaks of the pigments present in *G. gracilis*, it is possible to deduce that the cool white light is absorbed more efficiently by the biomass, consequently promoting greater growth.

Despite a lack of statistical significance, it is important to point out that the trials using the SSEP extract exhibited the highest growth rates among the other trials using white cool light (RGR = 8.514 % day^{-1}). It is very interesting to compare the growth rates obtained in this trial with the regular Von Stosch Enriched (VSE) modified medium (RGR = 7.152 % day^{-1}) and VSE at pH = 9 (RGR = 6.680% day^{-1}) since these rates are similar and in some cases higher than those reported in other studies (Bezerra & Marinho-Soriano, 2010; H. Wu et al., 2015) which used different species of *Gracilaria.* The VSE formula used for this study was specifically modified and optimized for red seaweeds (Freitas et al., 2021) and contains essential compounds for the development of red seaweeds, including the macronutrients ammonia and phosphate, the micronutrients iron and manganese, and the vitamins biotin, thiamine, and $B_{12.}$ The downside is that VSE is highly expensive, and time-consuming to prepare which makes it a very poor choice for large scale production of *G. gracilis*, inciting interest in the discovery of new and cost-effective alternatives. With the RGR results observed in the present study, it seems the commercial medium SSEP might be a very appealing and viable option since it also contains macronutrients such as ammonia, phosphate, and nitrate but also hormones and growth promoters which derive from the seaweed used in its production, all of which benefit the culture of *G. gracilis*. Ali et al., (2020) obtained similar results when using an *Ascophylum* marine plant extract enriched with potassium to test the growth of *Kappaphycus alvarezii*. Given these results, commercial seaweed extracts should be considered as an alternative to laboratory made nutrient media.

4.2. Proximate analysis

The biochemical analysis of *G. gracilis* cultivated under different conditions showed significant differences among trials. Regarding humidity, it is considered an important analytical parameter since it allows to determine the yield (fw) of the samples (Patarra, et al. 2011). Seaweeds usually present high humidity content (Marsham et al., 2007; Paiva et al., 2014). The Assay D (LED trial) exhibited the lowest values when compared to all other trials, with a humidity level of 75.41 ± 1.51 %, comparable to those we found for wild populations (Freitas et al., 2021), whereas the other culture conditions showed reasonably higher levels with no significant differences among them, also comparable to our previous results. Therefore, the seaweed presenting the highest yield of fresh weight

was *G. gracilis* cultivated in assay D, with 24.59%. These results similarly correlate with research made by Inácio et al. (2021).

Ash content is directly associated with the mineral content in seaweeds, giving a rough estimate of its content in the differently cultivated algae. The LED-grown algae (Assay D) displayed the lowest value (28.28 ± 9.005 %) once again, values quite similar to the values obtained by our previous work (Freitas et al., 2021) and by other authors (Nathan et al., 1978; Neto et al., 2018), while SSEP-grown algae (Assay C) possessed the highest ash content with 46.96 ± 2.05%. These high ash values are usually recorded for brown seaweeds, which are known to hold the highest mineral content among seaweeds (Pereira, 2016). Hence, it is hypothesized that these results may derive from the additional mineral content in this SSEP medium, and its ability to hold mixtures of enriched trace metal in a soluble form (S. Wu et al., 2010), which can easily be incorporated into the growing *G. gracilis* biomass.

As to the protein content, the SSEP and VSE control trials (Assay B and A, respectively) presented the highest and lowest protein content, respectively (19.62 ± 0.11% and 16.13 ± 0.31%). These values are comparable to those found by other authors (Francavilla et al., 2013; Rodrigues et al., 2015) for *G. gracilis* and for red seaweeds in general. As found by Freitas et al. (2021) the protein content of cultivated biomass is rather high when compared to the wild biomass, meaning that the nutrient culture media provide plentiful nitrogen required for the protein synthesis. Again, the composition of the SSEP medium seems to show more adequate properties, not only in growth but also in altering the biochemical composition of the cultured seaweed by providing higher protein content, which is most desirable in a nutritional perspective and economic value.

Carbohydrate content was lower than that usually obtained for red seaweeds (Pereira, 2016, Freitas et al., 2021). Our data revealed significant differences between assays with results for VSE control trial showing the least amount of carbohydrates, while the highest values were observed in the SSEP assay (21.44 ± 2.33 %).

Completing the analysis with lipidic content, all the trials delivered very low percentage, as expected, since seaweeds usually show very low lipid content (Pereira, 2011, 2016). The values presently obtained are even lower than those found by Neto et al. (Neto et al., 2018), reaching a maximum of 0.40 ± 0.03 % for the LED assay (Assay D). Therefore, these results demonstrated that the SSEP medium could produce a high protein and extremely low-fat algae which may contribute for the development of a low energy, healthy product worthy incorporating in the human diet.

4.3. Antimicrobial and antioxidant capacities

As to the antimicrobial activity observed in *G. gracilis* ethanolic extracts, it was coherent with previous studies for *G. gracilis* (Fethi & Ghedifa, 2019), *G. changii* (Hsieh-Lo et al., 2019), *G. corticata*, and *G. edulis* (Ghedifa et al., 2021; Rossano et al., 2003). No activity was found in 2 of the 3 strains of pathogens, with results showing only a positive antimicrobial effect against the strain *Bacillus subtilis*, a gram-positive human pathogen. (Bezerra & Marinho-Soriano, 2010; Fethi & Ghedifa, 2019). Out of all extracts, only the one obtained from Assay C (VSE at pH = 9) exhibited no antimicrobial activity which suggests that the compound responsible for this inhibition of growth was either suppressed or completely removed by the increase in pH, whereas the choice of light source or culture media did not influence the antibacterial activity observed against this strain.

The gram-negative bacteria *Micrococcus luteus* and *Escherichia coli* displayed no kind of inhibition in bacterial spread, which was also reported in previous studies (Lima-Filho et al., 2002; Sasidharan et al., 2009). As reported by Torres et al., 2019, the methanolic extracts of *Gracilaria* are active against *Staphylococcus aureus*, an organism which we have not tested. Although better results have been obtained with other solvents, such as methanol, acetone, chloroform, or diethyl ether, among others, we decided not to use them for these are not green solvents, and therefore unsuitable to be used in potential food products. We are aware however, regarding the antimicrobial activity, that extract optimization is required to improve our results.

DPPH free-radical activity has been widely used to assess the antioxidant activity of seaweed extracts (Souza et al., 2012). Unfortunately, the results obtained through sequential extraction portion with MiliQ water as a solvent were disappointing when compared to other studies using the same seaweed and solvent extraction procedure (Afonso et al., 2021). Other authors have found both greater antioxidant activity with aqueous extracts using a variety of different seaweeds such as *Agarophyton vermiculophyllum, Chondrus crispus, Porphyra purpurea,* and *Asparagopsis taxiformis* (Nunes et al., 2018; Sabeena Farvin & Jacobsen, 2013), while others have found greater activity with ethanolic solvents and have correlated this activity with the total phenolic content of each specimen (Afonso et al., 2021; Chakraborty et al., 2015; Chan et al., 2015; Marinho et al., 2019).

Thus, although we could find that SSEP seems a better culture medium for the growth and development of *G. gracilis* in laboratory conditions, as well as providing a better biochemical composition of the biomass, further antioxidant assays are required to better display this seaweed's natural antioxidant properties.

4.4. PCA analysis

The PCA was a key in order to understand the relationship between the different components analysed in this study. By determining the existence of a strong correlation between total protein content, ash content, total carbohydrates, and DPPH radical reduction, as well as a negative correlation between these and total lipid content, it was possible to observe how *G. gracilis* exhibits a very desirable high protein, low fat nutritional profile, making it a very healthy option as a food product. These components also positively correlate with SSEP, which displayed extremely interesting results with high value of protein, ash content, total carbohydrates and DPPH, although with a low expression for growth rate. However, through the evaluation of the relative growth rate, we can safely state that it is the most successful culture medium among the ones tested, since it produced the most amount of biomass while increasing beneficial nutritional aspects and lowering fat content.

5. Conclusion

Seaweed cultivation is a rewarding activity in expansion, that aims to provide the market with sustainable and efficient biomass production. Yet, the optimization of the cultivation process is still required to guarantee not only the highest yields, but also the best biomass quality. Progress towards a successful *Gracilaria gracilis* production is presented, substituting the common laboratory Von Stosch Enriched modified medium by a commercial AcadianTM Seaweed Soluble Extract Powder Medium, which proved to increase not only the relative growth rate of cultured seaweeds, but also to significantly improve their protein and ash content. Thus, the usage of this commercial medium seems to be rewarding in a nutritional and economical perspective, not only because it is readily prepared, but because its composition significantly improves the algal biomass.

Replacing the traditional cold fluorescent white light sources with white LED sources did not prove to be a promising alternative, probably due to different absorption peaks of photon emissions. Further studies with different LED lights, namely blue, green, and red, will probably produce different results according to the wavelengths of the emitted light. The selective use of wavelengths is a topic currently explored in seaweed metabolite production. This is something worth considering in future studies as LED sources are currently being regarded as a worthy alternative to traditional lighting in indoor seaweed cultivation, for being an economical and efficient technology.

The cultivation conditions significantly affected the antimicrobial capacity of the biomass extracts of *G. gracilis*. Although our results were modest, it was interesting to observe that the recorded activity against *B. subtilis* was completely suppressed in *G. gracilis* culture at pH = 9, although this pH value did not negatively influence its nutritional profile. Thus, the pH interfered with the ability to produce antimicrobial agents, which is information that may certainly be pertinent when optimizing the production of *G. gracilis*. Monitoring pH values in culture must thus not be overlooked when the objective is obtaining an end-product to be incorporated into a healthy human diet, that will ideally be not only nutritionally rich but also provide a boost to the human immune system.

Finally, the study resulted in no noticeable antioxidant activity in any condition, however, it is known that *G. gracilis,* like so many other seaweeds, has the potential for such activity. Thus, its absence in this study should encourage further experimentation, primarily by replacing the extraction solvent from MiliQ water to ethanol, as this has resulted in greater activity in other studies.

In future studies, there should be a focus on expanding the variety of abiotic factors tested in order to fine tune the culture procedure, like the color of the light source since different wave lengths are absorbed differently during photosynthesis. There should also be an in-depth analysis of the effect the different culture conditions have on specific bioactive compounds such as R-phycoerythrin and perhaps even agar yield and quality as this species exhibits great potential for the harvesting of these products. Ultimately, further research on the internal alterations in the seaweed are required for a more accurate optimization of the growth and quality improvement of *G. gracilis.*

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