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Optimizing *Chlorella vulgaris* production and exploring its impact on germination through microalga-N_2-fixing bacteria consortia

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ABSTRACT

Microalgal biomass is increasingly valued in industrial and agricultural sectors due to its bioactive compounds. However, large-scale production remains costly, mainly due to nitrogen fertilizer expenses. A promising sustainable alternative is co-cultivation with N_2-fixing bacteria, capable of supplying biologically available nitrogen. In this study, *Chlorella vulgaris* was grown in synthetic medium with and without nitrogen, as well as in co-culture with three different N_2-fixing bacteria in nitrogen-free medium. Microalgal growth was assessed by dry weight, *Fv/Fm* ratio, and flow cytometry, which also allowed evaluation of population dynamics and cell viability. Biomass composition (proteins, carbohydrates, lipids, chlorophyll, and carotenoids) was analyzed under all conditions. Co-cultures in nitrogen-free medium showed comparable biomass productivity to nitrogen-supplemented controls, although *Fv/Fm* values indicated physiological stress in some cases. Moreover, the agricultural potential of the resulting biomass and supernatants was evaluated through germination bioassays using lettuce seeds. All cultures tested at 0.2 $g{\cdot}L^{-1}$ significantly improved the germination index. Also, applying the culture supernatant (biomass removed) also yielded positive effects, with GI increases exceeding 40 %. These results suggest that co-cultivation with N_2-fixing bacteria can support efficient microalgal production while generating biomass and supernatants with biostimulant potential, contributing to sustainable agriculture and circular bioeconomy strategies.

1. Introduction

Microalgae provides an eco-friendly solution for producing a wide range of products including food and feed ingredients, cosmetics, bioplastics, biopharmaceuticals, nutraceuticals, and renewable energy. They also play a role in wastewater treatment and mitigating atmospheric carbon dioxide (CO_2) levels. With their ability to produce valuable bioproducts like polysaccharides, lipids, pigments, proteins, vitamins, bioactive compounds, and antioxidants, microalgae offer immense potential for exploration in the 21st century (Brennan and Owende, 2010; Chia et al., 2022; Dang et al., 2022). With an estimated species range of 30,000–1000,000, microalgae offer numerous advantages including unique metabolisms and chemical compositions, rapid growth, high photosynthetic efficiency, no need for arable land nor potable water, and biomass harvesting could be done daily (Chisti, 2008; Rumin et al., 2020).

Despite these major advantages, its large-scale application is still limited due to high production and processing costs. For successful microalgae production, proper culture conditions, including light, pH, temperature, dissolved oxygen, and nutrient supply, are critical. Microalgae require primary inorganic nutrients such as phosphorus (P), nitrogen (N), and carbon (C) for their growth. The availability of these nutrients plays a crucial role in determining the growth rate and biomass production. When these nutrients are deficient, it negatively impacts the

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growth rate and leads to low biomass production. The nutrient supply also has a significant influence on the biochemical composition of the produced biomass (Khan et al., 2018). Nutrients are generally presented in excess, and their provision could contribute up to 5–20 % of the overall final cost of microalgae production (Acién et al., 2016). Although commercial culture media such as NutriBloom® Plus are available, bulk fertilizers are commonly used in industrial applications. For the cultivation of seawater algae, the use of macronutrients alone is feasible due to the natural abundance of essential micronutrients in seawater, which can help reduce costs (Ferreira et al., 2023; Vázquez-Romero et al., 2022). However, the use of wastewater may restrict certain applications for produced biomass, specially related to human consumption (Acién et al., 2016).

A sustainable alternative is to look for interactions between microalgae and other beneficial microorganisms that can supply the nutrients necessary for their growth. Particularly, microalgae and bacteria have a symbiotic relationship where they thrive in various conditions and interact in different ways (Fallahi et al., 2021). Microalgae play a vital role in producing oxygen (O_2) through photosynthesis and releasing dissolved organic carbon (DOC), traditionally considered a key resource for associated bacteria. In return, bacteria supply CO_2, produce essential compounds such as vitamins and hormones, and contribute to the remineralization of N and P, thereby supporting microalgae growth. Moreover, recent studies have shown that some microalgae themselves are capable of synthesizing and excreting phytohormones like indole-3-acetic acid (IAA) from tryptophan, suggesting a more complex and bidirectional chemical communication within these consortia (Sheng et al., 2010; Unnithan et al., 2014). On the other side, the antagonism/competition effect can occur when microalgae and bacteria produce a wide range of inhibitory compounds that can harm the partner's growth (Khan et al., 2018; Meyer and Nodwell, 2021). However, this competitive effect is often negligible compared to the overall synergetic relationship (Qu et al., 2021).

One powerful interaction is described between microalgae and N_2-fixing bacteria. N is abundant in the atmosphere as dinitrogen gas (N_2), but only certain prokaryotic organisms called diazotrophs can use it directly through biological N_2-fixation. Diazotrophs include N_2-fixing bacteria, which establish symbiotic interactions with various organisms for their growth. The phycosphere of green microalgae like *Chlamydomonas reinhardtii*, *Chlorella vulgaris*, and *Scenedesmus quadricauda* has been studied, revealing the presence of diazotrophic organisms such as *Rhizobium* sp. These symbiotic relationships play an important role in the growth and physiology of microalgae, potentially enhancing their industrial applications (Calatrava et al., 2023; Llamas et al., 2023). Similar systems involving cyanobacteria- N_2-fixing bacteria consortia have been studied extensively (Smith and Francis, 2016), highlighting the potential of these systems to reduce dependency on synthetic fertilizers. However, challenges remain in optimizing N transfer efficiency and ensuring sufficient nitrogen delivery (Batista and Dixon, 2019; Dixon and Kahn, 2004).

In industrial applications, a promising strategy involves combining the abilities of microalgae and N_2-fixing bacteria (Solomon et al., 2023). Microalgae release O_2 and synthesize organic compounds from CO_2 via photosynthesis, which they secrete extracellularly. These compounds serve as a valuable source of C for nitrogen-fixing bacteria, enabling them to synthesize extracellular polysaccharides and provide energy for nitrogen fixation and respiration. Simultaneously, nitrogen fixed by bacteria is transferred to microalgae, playing a crucial role in their growth. Continual circulation of these cycles allows for the concurrent utilization of CO_2 and N_2 from the air as carbon and nitrogen sources, thus facilitating more cost-effective cultivation methods (Aburai et al., 2023). By establishing a symbiotic relationship between microalgae and N_2-fixing bacteria, it is possible to replace synthetic nitrogen fertilizers achieving environmental and economic advantages. This approach reduces the cost and environmental impact of fertilizers while also tapping into the unexploited potential of this symbiotic relationship.

This paper deals with a new approach of co-culturing *C. vulgaris* microalga (well-known and FDA food and feed approved) with N_2-fixing bacteria, without an exogenous supply of N to the medium. The hypothesis of this work is that consortia between *Chlorella vulgaris* and nitrogen-fixing bacteria can generate biomass with characteristics comparable to cultures supplemented with synthetic nitrogen, and that these co-cultures, as well as their potential metabolites exuded in the supernatant, can act as biostimulants to promote lettuce seed germination. *C. vulgaris* was grown in complete synthetic and N-free media, in monoculture and in co-culture with three different N_2-fixing bacteria (*Sphingobacterium* sp. L13G8, *Microbacterium maritypicum* M13A8 and *Serratia ficaria* G8L13). For all cultures, the (i) biomass growth; (ii) population dynamics of microalga-bacteria consortia; (iii) cell viability of microalgae; (iv) biomass composition; (v) effect of microalgal cultures and supernatants *in vivo* lettuce germination were evaluated.

2. Materials and methods

2.1. Microorganisms and growth media

Chlorella vulgaris from the culture collection of the SABANA Demonstration Plant (Almería, Spain), was the microalga used in this work. The inoculum was prepared using 1 L spherical flasks under continuous illumination (100 ± 10 μmol $_{photons}$ $\cdot m^{-2} \cdot s^{-1}$) and with air injection (0.2 $v \cdot v^{-1} \cdot min^{-1}$). The temperature was controlled at 22 ± 2 °C. *C. vulgaris* UAL-1 was produced using modified Arnon medium (Allen and Arnon, 1955). The medium was prepared using freshwater and 0.24 $mg \cdot L^{-1}$ $NaVO_3$, 1.26 $mg \cdot L^{-1}$ $Na_2MoO_4 \cdot 2\ H_2O$, 2.86 $mg \cdot L^{-1}$ H_3BO_4, 1.81 $mg \cdot L^{-1}$ $MnCl_2 \cdot 4\ H_2O$, 0.22 $mg \cdot L^{-1}$ $ZnSO_4 \cdot 7\ H_2O$, 0.08 $mg \cdot L^{-1}$ $CuSO_4 \cdot 5\ H_2O$, 0.04 $mg \cdot L^{-1}$ $CoCl_2 \cdot 6\ H_2O$, 124 $mg \cdot L^{-1}$ $MgSO_4 \cdot 7\ H_2O$, 15 $mg \cdot L^{-1}$ $CaCl_2 \cdot 2\ H_2O$, 117 $mg \cdot L^{-1}$ NaCl, 0.03 $mg \cdot L^{-1}$ EDTA, 0.03 $mg \cdot L^{-1}$ $FeSO_4 \cdot 7\ H_2O$, 174 $mg \cdot L^{-1}$ K_2HPO_4, and 900 $mg \cdot L^{-1}$ $NaNO_3$. For the experiments, two culture media were produced. Firstly, a complete medium as previously described as a positive control. Also, the same culture medium without $NaNO_3$ consisting of the N-free medium.

The N_2-fixing bacteria tested in this work were *Sphingobacterium* sp. L13G8 (GenBank: OR304288.1), *Microbacterium maritypicum* M13A8 and *Serratia ficaria* G8L13. The bacteria tested belonged to the Laboratory of Strain-producers of BAS and Biosynthesis Culture Collection, SPC "Armbiotechnology" SNPO NAS RA, Armenia. They were isolated from the soils of Armenia in the framework of SC RA "21T-2I229" project, 2021–2024. The nitrogen-fixing ability of the bacterial strains was evaluated using a semi-solid nitrogen-free malate medium supplemented with bromothymol blue. This medium promotes microaerobic conditions, which are favorable for the activity of many diazotrophic bacteria. A positive result was indicated by a colour change from light green to blue after incubation, reflecting alkalinization of the medium due to ammonia production associated with nitrogen fixation (Melkonyan et al., 2025; Melkonyan L., 2024). For the experiments, the inoculum of the bacterial cultures was grown overnight in LB medium at 30 °C, under orbital agitation at 150 rpm.

2.2. Biomass production and experimental design

Microalga pre-culture and bacterial inoculum were transferred into a 1 L photobioreactor to get a final concentration $OD_{540\ nm} = 0.2$ of each microorganism. The reactors were then filled with the culture media until a final working volume of 600 mL and operated in batch mode for 8 days. The cultures were maintained at room temperature (22 ± 2 °C) with continuous aeration (0.2 $v \cdot v^{-1} \cdot min^{-1}$) and using continuous artificial light at 72 W (4 lamp x 18 W/865). A constant average irradiance of 134 ± 15 $\mu mol_{photons} \cdot m^{-2} \cdot s^{-1}$ was provided to the cultures. The pH was not controlled during the experiment.

For the experiments, five conditions were tested:

A - negative control – *C. vulgaris* in N-free medium.

B - positive control – *C. vulgaris* in complete medium.

C – *C. vulgaris* and *Sphingobacterium* sp. L13G8 in N-free medium.
D – *C. vulgaris* and *M. maritypicum* M13A8 in N-free medium.
E – *C. vulgaris* and *Serratia ficaria* G8L13 in N-free medium.

For each of the five treatments tested in the experiments, three replicates were performed.

2.3. Biomass growth and analytical determinations

2.3.1. Growth monitoring

The cultures´ growth was evaluated for 7 days by measuring optical density (OD) at 540, 680 and 750 nm wavelength using a Hitachi U-2000 spectrophotometer, and by dry weight, after drying at 105 ºC. pH and *Fv/Fm* ratios were also measured using a laboratory pH meter (InoLabWTW) and an AquaPen-C AP 110-C (PSI, Drásov, Czech Republic) after 15 min in the dark, respectively.

2.3.2. Flow cytometry analyses

Samples containing microalgae and/or bacteria populations were collected and immediately analyzed using the flow cytometer CytoFLEX (Beckman Coulter Life Sciences, Brea, CA, USA) equipped with a 488 nm argon laser. Forward (FSC) and Side Scatter (SSC) detectors were used to distinguish cells with different sizes and internal complexities,respectively. Fluorescence channel FITC (Fluorescein isothiocyanate, green) was used to collect data on cell viability and esterase enzymatic activity of microalgal cells using the viability dye carboxyfluorescein diacetate succinimidyl ester (CFDA). CFDA is a non-fluorescent, cell-permeant dye that is cleaved by intracellular esterases to produce a fluorescent compound retained within cells with intact membranes. Therefore, fluorescence intensity serves as an indicator of both membrane integrity and enzymatic activity. A decrease in CFDA fluorescence may indicate loss of viability or esterase function, which was used here to distinguish active from inactive microalgal populations. Data from FITC was also used in samples stained with fluorescent dye SYTO9 for identifying bacterial populations. The fluorescence channel PC5.5 (PerCP-Cyanine5.5, red) was used to identify microalgal communities since it collects fluorescence by chlorophyll emission.

All samples were diluted with the respective growth media for fitting cell concentration to a reasonable equipment speed (400–500 events/s). Dye incubation parameters were optimized with these dilutions by testing different dye concentrations and incubation times. Working stock solutions of CFDA (Invitrogen, Waltham, CA, USA) were prepared at 10 mg·mL^{-1} using pure acetone and at 0.5 mM for SYTO9 (Invitrogen, Waltham, CA, USA) using dimethylsulfoxide.

Samples were stained with 0.1 mg/mL CFDA and incubated for 30 min in the dark and at room temperature. Samples were stained with 5 μM SYTO9 and incubated for 20 min in the dark and at room temperature.

Microalgal populations were selected from high chlorophyll autofluorescence using a PC5.5/SSC density plot. Cell viability of microalgae populations was assessed by gating active and inactive cells for esterase enzymatic activity from CFDA-stained samples using a FITC/FSC density plot. Bacterial populations were gathered from low FSC and high-green SYTO9 fluorescence using FITC/FSC density plots. Cell concentration of each population was quantified (cells·mL^{-1}) and the mean/median of active microalgae population was used to describe the degree of esterase activity.

2.3.3. Microbial analyses

In addition, the Aerobic Plate Count (APC), formerly known as the standard plate count, was determined following ISO 4833 at the end of the batch mode for each culture. For this measurement, samples from the bubble columns were collected at the same time interval (09:00–10:00 am).

2.3.4. Ammonium determination

The ammonium was measured according to the Nessler method (American Public Health Association and Society, 1920) (Ammonium standard for IC: 59755).

2.4. Biomass composition

Lipid content was determined gravimetrically after Soxhlet extraction with n-hexane for 6 h, according to the Portuguese standard method NP4168 (Norma Portuguesa, 1991). The Lowry method was used for protein quantification after extraction with NaOH (0.1 M), using Bovine Serum Albumin as standard (Lowry et al., 1951). The sugars within microalgal cells were initially extracted via quantitative acid hydrolysis, following the method described by Kielkopf et al. (2020). Subsequently, the total sugar content was assessed using the colorimetric method involving the phenol-sulfuric reagent, as outlined by Dubois et al. (1956). To quantify the sugar content, a calibration curve was established using standard sugar (glucose) solutions. Moisture was determined by drying in an oven at 105 °C until constant mass. Total ash was determined by incineration at 550 °C in a muffle furnace. The pigment content (chlorophyll and total carotenoids) was calculated following the protocol described previously by Maadane et al. (2015), using a UV–Vis spectrophotometer (Genesys 10S, Thermo Fisher Scientific, Spain), after extraction 90 % (v/v) acetone. The analyses were performed in triplicate.

2.5. Germination bioassays

The germination tests were performed in Petri dishes using lettuce (*Lactuca sativa*) seeds. Each Petri dish was lined with filter paper (Whatman No. 1) and 15 seeds were placed. A volume of 5 mL of each culture and supernatant was added to the seeds. Distilled water was used as the negative control and 2.5 μM gibberellic acid (GA) was used as the positive control. The microalgal cultures were assessed at a biomass concentration of 0.2 and 0.5 g·L^{-1} in triplicate. Also, the supernatants (after removing the cells) were tested. Seeds were incubated at 20 °C in the dark for 5 days in a growing chamber (FITOCLIMA S600 PL, Aralab, Portugal). The number of germinated seeds in each Petri dish was counted, and the root and shoot lengths were measured. The germination index (GI) was determined according to (Zucconi et al., 1981) Zucconi et al., 1981.

$$GI(\%) = \frac{(G \times L)}{(Gw \times Lw)} \times 100 \quad \text{(Eq.1)}$$

where G and Gw correspond to the total number of germinated seeds and L and Lw to the root length for the tested conditions and the negative control (distilled water), respectively.

2.6. Statistical analysis

The data were analysed using PASW Statistics 28 for Windows (SPSS Inc., Chicago, IL, USA). The normality and homoscedasticity of the data within each group were checked. The data were analysed using an One-way ANOVA to assess differences between the conditions. A Tukeys' HSD test was carried out to find where the sample differences occurred and the criterion for statistical significance was $p < 0.05$. Values are the mean ± standard deviation of three replicates.

3. Results

3.1. Biomass productivity

In this study, C. vulgaris was produced in Arnon medium without nitrogen as negative control (condition A) and in Arnon medium as positive control (condition B). C. vulgaris was co-cultivated with three different N_2-fixing bacteria in medium without nitrogen source (conditions C, D and E). The growth of the microalgal monocultures and co-

cultures (with the three bacteria) were followed for 8 days by measuring dry weight (Fig. 1A). Results indicated that negative control reached 0.57 $g{\cdot}L^{-1}{\cdot}day^{-1}$ after 8 days, corresponding to the lowest concentrated culture. The final concentration of the positive control was 2.23 $g{\cdot}L^{-1}$ while the microalgal cultures with bacteria co-culturing reached from 2 up to 2.31 $g{\cdot}L^{-1}$. Fig. 1B shows the *Fv/Fm* of the cultures during the batch production. Overall, major differences were observed between the positive control and the negative control, in which the *Fv/Fm* values were significantly lower ($p < 0.05$) for the latter. Likewise, the *Fv/Fm* values of the co-culturing samples were lower than the positive control

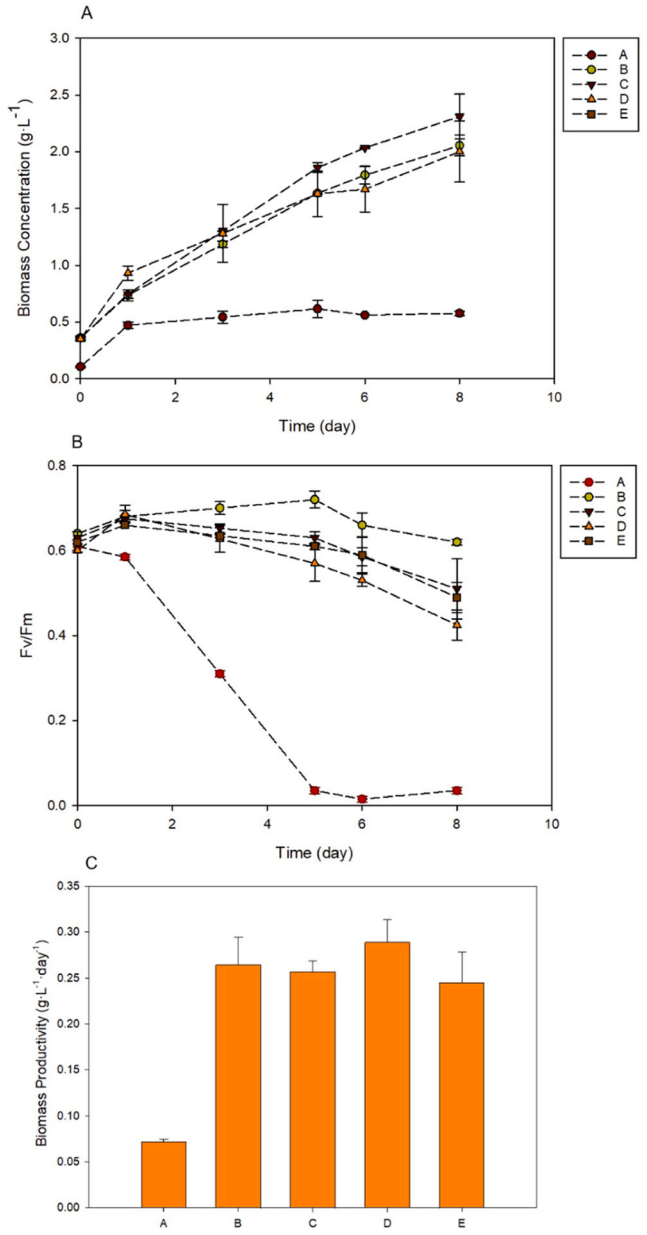

Fig. 1. Biomass concentration measured during *Chlorella vulgaris* growth using the different conditions (A), *Fv/Fm* values during the Chlorella vulgaris growth (B), final biomass productivity obtained after 8 days of batch production (C). Error bars represent the standard deviation (SD) of the independent replicates. A - negative control – *C. vulgaris*, medium without $NaNO_3$. B - positive control – *C. vulgaris*, complete medium with $NaNO_3$. C – *C. vulgaris*, medium without $NaNO_3$, using the bacterial strain *Sphingobacterium* sp. L13G8. D – *C. vulgaris*, medium without $NaNO_3$, using the bacterial strain *M. maritypicum* M13A8. E – *C. vulgaris*, medium without $NaNO_3$ but using the bacterial strain *Serratia ficaria* G8L13.

($p < 0.05$). The biomass productivity of *C. vulgaris* under different conditions is shown in Fig. 1C. The biomass productivity achieved by cultures was influenced by the tested conditions ($p < 0.05$). The biomass reached by the condition D was 0.29 $g{\cdot}L^{-1}{\cdot}day^{-1}$, while it was 0.28 $g{\cdot}L^{-1}{\cdot}day^{-1}$ for the condition B (positive control). Conditions C and E reached 0.26 and 0.25 $g{\cdot}L^{-1}{\cdot}day^{-1}$, respectively, while the biomass productivity of the condition A (negative control) was significantly lower (0.07 $g{\cdot}L^{-1}{\cdot}day^{-1}$). Statistically significant differences were observed between condition A (negative control) and the rest of the conditions, but not between condition A (positive control) and the co-cultures.

3.2. Flow cytometry

The growth of microalgae and bacteria was monitored by flow cytometry. Fig. 2A shows the growth of the microalgae in the positive control (condition B) and negative control (condition A), as well as the co-cultures with bacteria (conditions C, D and E). The results showed that from day 3, the number of cells in the negative control was significantly lower than the other conditions ($p < 0.05$). In addition, the number of microalgae cells at the end of the experiments was lower in the co-cultures with bacteria than in the positive control ($p < 0.05$). In condition B, the microalgal cells reached 3.5×10^8 $cell{\cdot}mL^{-1}$, while it decreased to 2.3×10^8 $cell{\cdot}mL^{-1}$ in condition C, 2.5×10^8 $cell{\cdot}mL^{-1}$ in condition D, and 2×10^8 $cell{\cdot}mL^{-1}$ in condition E.

The growth of the bacteria is shown in Fig. 2B. The results showed that the bacteria in conditions C and D on day 0 were higher than the other conditions ($p < 0.05$). Initially, the lowest number of bacteria was measured in the positive and negative control since *C.vulgaris* was not axenic. At the end of the experiment (day 8), no significant differences were observed between the positive control and the co-cultures with bacteria, while bacteria were lower in the negative control ($p < 0.05$). At the end of the experiment, cell viability of microalgae populations were assessed by distinguishing between active and inactive for esterase enzymatic activity with CFDA-stained samples (Fig. 3C). For this purpose, the mean emission intensity of the CFDA marker attached to the cells was determined. The results showed that the CFDA intensity was significantly lower in condition A, while no significant differences were found between condition B, C, D and E ($p < 0.05$).

3.3. Aerobic plate count

In addition to flow cytometry studies, the aerobic plate count (APC) was employed to assess the bacterial populations in the samples. On the first day, APC was higher in the microalgal co-cultures with bacteria compared to the positive and negative controls ($p < 0.05$). However, at the end of the trial, no significant differences were observed between positive control and negative control. The highest APC values were observed in conditions C, D and E, corresponding to the co-cultures. The results showed that on day 1 of the experiment, Log (CFU·mL) varied between 7.3 and 8.1 in the negative and positive control, respectively. In the case of co-cultures, this value ranged from 8.8 to 9.1. After 8 days of the experiment, Log (CFU·mL) values decreased in all conditions, with higher values observed in conditions C, D, and E (microalga-bacteria co-cultures). Apart from the microbial analyses, at the end of the batch mode production, the levels of $N\text{-}NH_4^+$ in the culture medium were measured (after biomass removal). The results revealed significant differences between the conditions ($p < 0.05$) (Fig. 4). It is observed that under conditions A and B, these values were below 1 $mg{\cdot}L^{-1}$, whereas under conditions C, D, and E, they ranged from 6.9 to 8.5 mg $N\text{-}NH_4^+{\cdot}L^{-1}$.

3.4. Biomass composition

The produced biomass was characterized to determine the biochemical composition (proteins, carbohydrates, lipids, and ash)

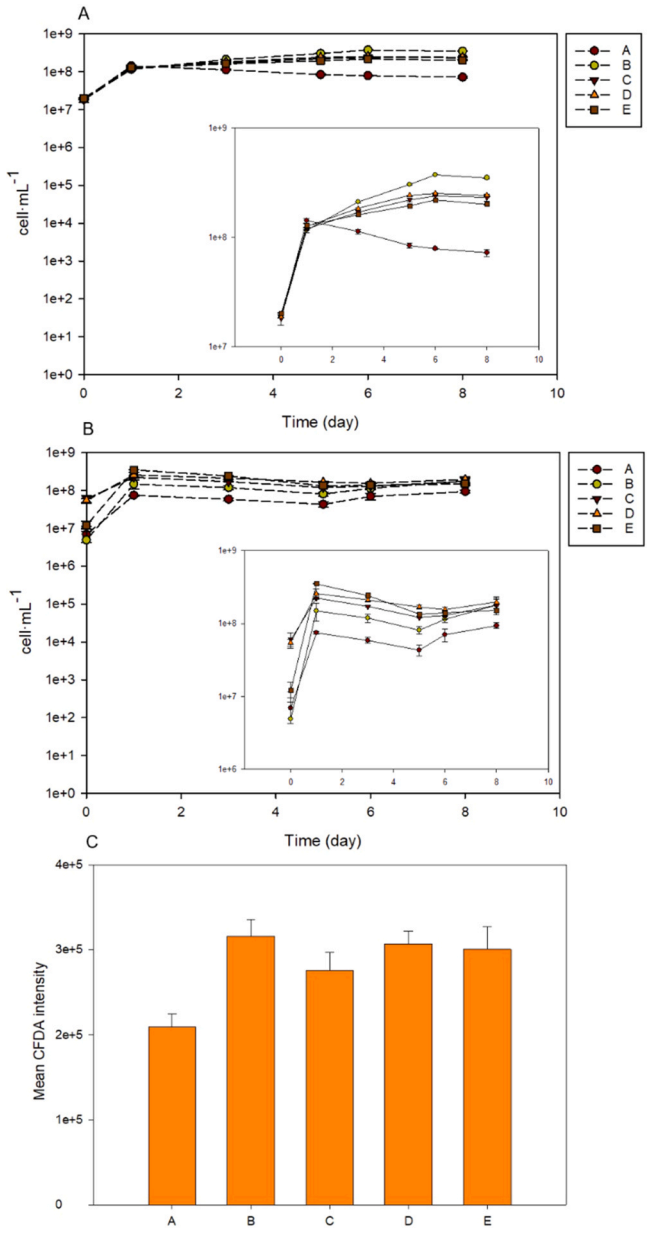

Fig. 2. Cell density of *C. vulgaris* during batch production using Flow cytometry (A), Growth dynamics of bacterial cells during batch production using SYTO9 green dye (B), mean CFDA fluorescence intensity as a function of esterase enzymatic activity (C). Error bars represent the standard deviation (SD) of the independent replicates. A - negative control – *C. vulgaris*, medium without $NaNO_3$. B - positive control – *C. vulgaris*, complete medium with $NaNO_3$. C – *C. vulgaris*, medium without $NaNO_3$, using the bacterial strain *Sphingobacterium* sp. L13G8. D – *C. vulgaris*, medium without $NaNO_3$, using the bacterial strain *M. maritypicum* M13A8. E – *C. vulgaris*, medium without $NaNO_3$ but using the bacterial strain *Serratia ficaria* G8L13.

(Table 1). The carbohydrate values varied from 41.7 % to 49.7 % in the different conditions. The highest content was detected in condition E, with significant differences compared to the others. On the other hand, protein content ranged from 32.1 % to 38 % in conditions B, C, D, and E, while it was 13.7 % in condition A (negative control). The percentage of lipids in the produced biomass was also measured, with the highest value detected in condition B at 31.6 %. In the other conditions, it ranged from 13.2 % to 17.2 %. Moreover, the pigment contents were assessed in the biomass under the different conditions. The chlorophyll content of the biomass was affected by the treatment conditions

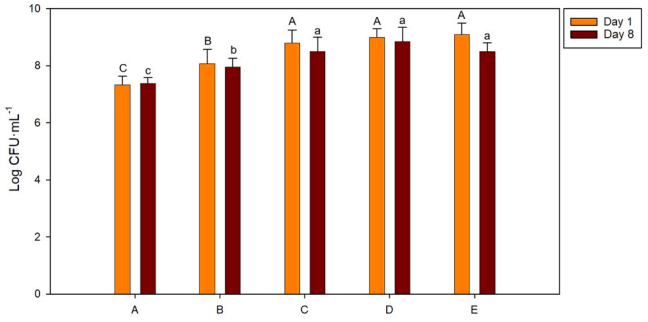

Fig. 3. Aerobic Plate Count measured at the first and final day of *Chlorella vulgaris* growth under different conditions. Error bars represent the standard deviation (SD) of the independent replicates. A - negative control – *C. vulgaris*, medium without $NaNO_3$. B - positive control – *C. vulgaris*, complete medium with $NaNO_3$. C – *C. vulgaris*, medium without $NaNO_3$, using the bacterial strain *Sphingobacterium* sp. L13G8. D – *C. vulgaris*, medium without $NaNO_3$, using the bacterial strain *M. maritypicum* M13A8. E – *C. vulgaris*, medium without $NaNO_3$ but using the bacterial strain *Serratia ficaria* G8L13.

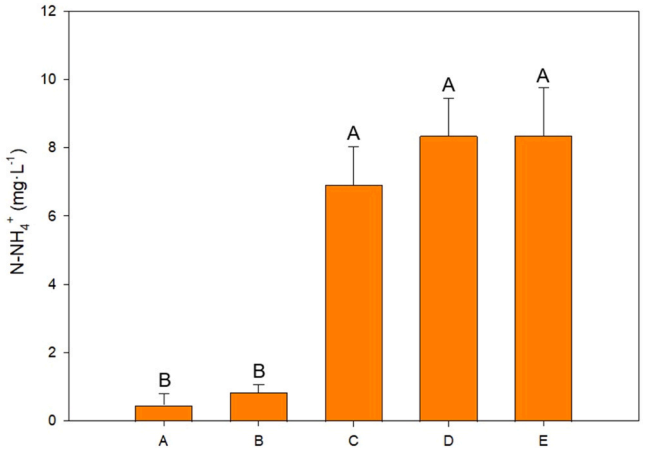

Fig. 4. Ammonium concentration ($N\text{-}NH_4^+$) measured at the end of the experiments for the different conditions. Error bars represent the standard deviation (SD) of the independent replicates. A - negative control – *C. vulgaris*, medium without $NaNO_3$. B - positive control – *C. vulgaris*, complete medium with $NaNO_3$. C – *C. vulgaris*, medium without $NaNO_3$, using the bacterial strain *Sphingobacterium* sp. L13G8. D – *C. vulgaris*, medium without $NaNO_3$, using the bacterial strain *M. maritypicum* M13A8. E – *C. vulgaris*, medium without $NaNO_3$ but using the bacterial strain *Serratia ficaria* G8L13.

($p < 0.05$). Pigment analyses reveleased chlorophyll content decreased from 705.8 mg·100 g^{-1} in the positive control culture (B) to 144.4 mg·100 g^{-1} in the negative control (A). The chlorophyll content of the biomass in the conditions C, D and E was 474.4, 307.8 and 465.1 mg·100 g^{-1} respectively. Related to the carotenoids content, it was also affected by the treatment conditions ($p < 0.05$). The carotenoids content was 112.4 mg·100 g^{-1} in the positive control culture (B), while it decreased up to 9.4 mg·100 g^{-1} in the negative control (A). The carotenoids content of the biomass in the conditions C, D and E was 95.4, 55.6 and 85.7 mg·100 g^{-1} respectively.

3.5. Germination index

Fig. 5A illustrates the impact of microalgal cultures on the GI of lettuce seeds, while Fig. 5B demonstrates the influence of supernatant (after removing the biomass) on the GI. Microalgal extracts were evaluated at concentrations of 0.2 and 0.5 g·L^{-1}. Overall, all the microalgal cultures tested, conditions A, B, C, D, and E, at 0.2 g·L^{-1} showed a statistically significant increase in the GI of the seeds compared to distilled

Table 1
Macromolecular composition of produced biomass under different conditions.

	A	B	C	D	E
Carbohydrates (%)	41.7 ± 2.6^{B}	43.5 ± 1.1^{B}	42.0 ± 1.8^{B}	44.0 ± 2.2^{B}	49.7 ± 0.9^{A}
Proteins (%)	13.7 ± 0.3^{D}	38.1 ± 1.5^{A}	36.2 ± 0.7^{AB}	33.0 ± 1.5^{BC}	32.1 ± 1.8^{C}
Lipids (%)	31.6 ± 2.5^{A}	13.4 ± 1.7^{B}	15.6 ± 1.2^{B}	17.3 ± 2.1^{B}	13.2 ± 1.9^{B}
Ashes (%)	10.1 ± 1.5^{A}	5.0 ± 0.8^{B}	6.1 ± 0.7^{B}	5.8 ± 0.7^{B}	5.0 ± 0.5^{B}
Chlorophylls (mg·100 g^{-1})	144.4 ± 3.8^{D}	705.8 ± 41.3^{A}	474.4 ± 21.9^{B}	307.8 ± 15.4^{C}	465.1 ± 21.7^{B}
Total carotenoids (mg·100 g^{-1})	9.4 ± 0.4^{D}	112.4 ± 7.3^{A}	95.4 ± 5.5^{B}	55.6 ± 3.4^{C}	85.7 ± 4.7^{B}

Results are the average of three determinations ± SD. Different letters in the same row indicate significant differences ($p < 0.05$).
A - negative control – *C. vulgaris*, medium without $NaNO_3$
B - positive control – *C. vulgaris*, complete medium with $NaNO_3$
C – *C. vulgaris*, medium without $NaNO_3$, using the bacterial strain *Sphingobacterium* sp. L13G8.
D – *C. vulgaris*, medium without $NaNO_3$, using the bacterial strain *M. maritypicum* M13A8
E – *C. vulgaris*, medium without $NaNO_3$ but using the bacterial strain *Serratia ficaria* G8L13.

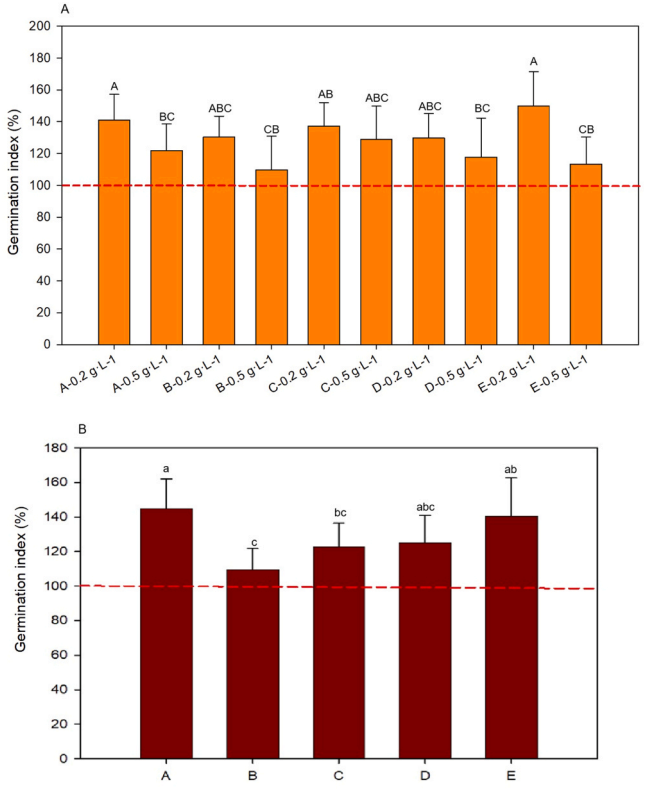

Fig. 5. Germination index of microalgal extracts at a biomass concentration of 0.2 and 0.5 g·L^{-1} (A), and the germination index of the resulted supernatants after removing the cells. Error bars represent the standard deviation (SD) of the independent replicates. A - negative control – *C. vulgaris*, medium without $NaNO_3$. B - positive control – *C. vulgaris*, complete medium with $NaNO_3$. C – *C. vulgaris*, medium without $NaNO_3$, using the bacterial strain *Sphingobacterium* sp. L13G8. D – *C. vulgaris*, medium without $NaNO_3$, using the bacterial strain *M. maritypicum* M13A8. E – *C. vulgaris*, medium without $NaNO_3$ but using the bacterial strain *Serratia ficaria* G8L13.

water. The highest growth percentage was observed in conditions A (negative control) and E at 0.2 g·L^{-1}, increasing the germination index by 40 % and 50 % compared to distilled water, respectively. Regarding the supernatant, statistically significant differences were observed in conditions A, C, D, and E, whereas the germination results obtained in condition B (positive control) were statistically like distilled water. Application of the supernatants of conditions A and E led to an increase in the GI by more than 40 %.

4. Discussion

4.1. Biomass productivity

In microalgal cultures, atmospheric CO_2 can serve as a carbon source, but nitrogen needs to be supplied separately. Conversely, when N_2-fixing bacteria convert atmospheric nitrogen into inorganic forms, a carbon source becomes necessary. A mutually beneficial system emerges when microalgae secrete organic matter while N_2-fixing bacteria release inorganic nitrogen. This cooperative coexistence signifies a sustainable cultivation approach, where both resources are utilized harmoniously (Aburai et al., 2023).

Although this idea might seem new in the context of modern biofertilization strategies, the co-cultivation of microalgae with N_2-fixing bacteria has actually been studied for a long time. In the 1980s, *Chlamydomonas* (a green alga) could live in symbiosis with *Azotobacter*, a N_2-fixing bacterium. In this system, the algae provided organic compounds from photosynthesis, while the bacteria supplied N, even when no N was added to the medium (Gyurjan et al., 1986). These early studies were key to understanding how algae and bacteria can interact in environments without nitrogen.

More recently, efforts such as those by Ambrosio and Curatti (2021), introduced timed N-NH_4^+ release using engineered *Azotobacter vinelandii*, while Barney et al., (2015),enhanced nitrogen excretion through genetic modifications, demonstrating that co-cultures with *Chlorella sorokiniana* could thrive when supplemented with carbon sources.

These ideas were addressed in this study to investigate the potential co-cultivation of C. vulgaris with N_2-fixing bacteria without the need to add inorganic nitrogen to the medium. The results showed that the use of C. vulgaris and N_2-fixing bacteria co-cultures (C, D, and E) allowed a biomass concentration of more than 2 g·L^{-1} in N-free cultures. These results were significantly higher than those obtained in the negative control (A), in which neither inorganic nitrogen nor N_2-fixing bacteria were added. Furthermore, the biomass concentration achieved in the co-cultures was like the one achieved for positive control (B), which was supplied with synthetic nitrogen in the form of sodium nitrate.

The monitoring of the maximum photosynthetic efficiency of the PSII, known as Fv/Fm, showed significant differences between the co-cultures (C, D, and E) and the positive control (B). A decrease in these values was observed during production with bacteria, indicating some form of stress in the cultures. Additionally, a substantial decline in Fv/Fm values was observed in the negative control (A). The optimal fluorescence value for chlorophylls, typically falls between 0.6 and 0.8 for the majority of common microalgal strains (Masojídek et al., 2013). Values below this range could indicate that the microalgae are experiencing specific stress factors. Among these stressors, one of the most significant is the combined impact of intense light exposure and other excessive environmental conditions, such as elevated dissolved oxygen levels, extreme temperatures, or nutrient deficiencies (Malapascua et al., 2014). In this study, nitrogen limitation was likely the cause of the decline in Fv/Fm values in condition A. In the case of the co-cultures, three hypotheses are proposed for the potential decline in Fv/Fm: (i) the bacteria may not fix enough nitrogen to meet the needs of C. vulgaris, (ii) the bacterial co-culture could act as a stressor due to the production of certain compounds that affect the microalga, or (iii) a combination of both factors.

Regarding biomass productivity, results indicated that it is possible

to combine microalgae and N_2-fixing bacteria to produce microalgal biomass without nitrogen supplementation. Particularly, the co-culture of *M. maritypicum* M13A8 and C. vulgaris (condition D) reached the same productivity as the positive control (condition B). A critical aspect of this approach revolves around the utilization of energy required for N_2 fixation, which appears to be sourced from the exudates produced by microalga (Ortiz-Marquez et al., 2012). When cultivating microalgal cells in photobioreactors, it is observed that exudates can account for as much as 17 % of the total CO_2 assimilated by the microalgae, depending on the specific conditions of the culture and the selected strain. These exudates from microalgae can result in energy wastage within the system and also create a fertile environment for the growth of unwanted organisms (Hulatt and Thomas, 2010). Co-cultivating microalgae with N_2-fixing bacteria offers a solution by effectively redirecting these exudates towards bacterial growth and N supply for the microalgae. Recent studies have shown that *Chlorella sorokiniana* can release carbon compounds such as glycerol, lactate, and myo-inositol, which are readily utilized by growth-promoting bacteria like *Azospirillum brasilense* in mutualistic systems (Palacios et al., 2023). Moreover, these interactions may involve a reciprocal nutrient exchange, whereby the microalga also benefits from bacterial metabolites. Although in this study the release of glycerol or other photosynthates did not quantify, these findings support the plausibility that *Chlorella* may be releasing photosynthetically derived compounds as part of a symbiotic interaction. Additionally, the growth of the selected bacteria may have the potential to inhibit the growth of other potentially harmful bacterial groups, thereby promoting a more controlled microbial environment.

4.2. Flow cytometry and aerobic plate count

Apart from the biomass determination, the C. vulgaris and bacteria cells were monitored by flow cytometry. Results demonstrated that the microalga cells concentration was higher in the positive control (condition B) than in the co-cultures with bacteria. This result could indicate that N_2-fixing bacteria do not produce enough N to support the growth of Chlorella or that there is some limitation due to another factor. However, the co-cultures showed better results than the negative control (condition A), with no external N source added. Therefore, these findings appear promising for making this strategy viable without relying on the addition of chemical fertilizers in conventional production, particularly considering that the bacterial strains studied have been shown to release sufficient amounts of ammonium, potentially supporting a stable mutualistic interaction with Chlorella through reciprocal nutrient exchange. Flow cytometry analysis of bacterial count revealed no significant differences between the positive control (condition B) and the co-cultures (conditions C, D, and E) at the end of the experiment. This outcome was unexpected, given the initial inoculation of N_2-fixing bacteria in the co-cultures, suggesting two potential explanations. Firstly, it's plausible that the microalgae's exudates in the control culture facilitated the growth of bacteria already present, aligning the bacterial counts with those in the co-cultures. Alternatively, or when considering both possibilities together, it's possible that the inoculated N_2-fixing bacteria did not persist in the culture. The latter hypothesis gains strength considering that no carbon source was added to the culture, which could have limited the growth and maintenance of the bacteria. Therefore, further research is necessary, focusing on monitoring the N_2-fixing bacteria throughout the microalgae growth process. The result is a final biomass that doesn't show a difference in bacterial content whether it was produced solely with chemical fertilizers or with N_2-fixing bacteria. This statement refers to the number of bacteria, not to specific types, as genetic analyses have not been conducted to distinguish the bacterial groups present.

Esterase activity is commonly used as a metric for assessing cellular enzyme activity (Hyka et al., 2013). CFDA dye can be used as microalgae viability parameter that enters the cell membrane through passive diffusion. Once inside, active esterases break down CFDA, creating a fluorescent compound that remains within the cell when its membrane is intact. However, if the cytoplasmic membrane is compromised, both the unaltered substrate and the breakdown products are released, and the cell does not retain the stain. Thus, CFDA intensity serves as a suitable indicator of cell viability (Dias et al., 2032). Results showed that no significant differences were found between condition B, C, D and E in CFDA intensity. This result indicates that the *Chlorella* cells presented a similar viability in the positive control and in the co-cultures. Therefore, it might be concluded that *Chlorella* presented a similar proportion of live and functional cells existing in the population, while low values were obtained in the condition A (negative control) as expected.

Flow cytometry analyses are complemented with the aerobic colony count on the first and eight days of condition. The results showed that on the first day, the mesophilic aerobic bacteria were higher in the microalgal co-cultures with bacteria compared to the positive and negative controls. Also, the APC values observed in conditions C, D, and E, which corresponded to the co-cultures were higher than the controls. In all conditions, the final APC values were lower than on day 1, demonstrating a drop in bacterial growth. This can be explained by the fact that the bacteria grew during the initial hours; however, due to the absence of any carbon source, their concentration decreased by the end of the assay.

Thus, the growth results of the five conditions, indicates that the use of Chlorella and N_2-fixing bacteria co-cultures allows for considerable productivity, with values comparable to the positive control. However, flow cytometry data reflect that in all co-cultures, the microalgal cell concentration is lower than the positive control, and furthermore, the Fv/Fm values are also lower than those of the positive control. As mentioned, there might occur two limitations: one stemming from the lack of N and another related to the possibility that bacteria may excrete some harmful compound for the microalga. Therefore, the ammonium concentration present in the cultures at the end of the assays after removing the biomass was determined. Results showed that the concentrations in the outflow of the co-cultures ranged from 6.9 to 8.5 N-NH_4^+mg·L^{-1}. Since no ammonium was added to the cultures, the detected levels could only be due to the action of N_2-fixing bacteria or cellular decomposition. Lower values, below 1 mg N-NH_4^+·L^{-1}, were measured in the positive (B) and negative controls (A). These residual values may be attributed to the mineralization of intracellular nitrogen compounds released through cell lysis or microbial degradation processes occurring during the cultivation period. Overall, the elevated ammonium levels observed in the co-cultures suggest that N_2-fixing bacteria contributed to nitrogen availability, which in turn may have supported algal growth.

4.3. Biomass composition

After analyzing the growth difference between cultures solely with Chlorella and co-cultures, the composition of the produced biomass was evaluated. Chlorella is composed by significant quantities of carbohydrates, proteins (primary metabolites), lipids, pigments, and various other constituents (such as dietary fibre, ash, moisture, and insoluble substances). Additionally, they have notable levels of secondary metabolites and minerals. The biochemical composition is highly influenced by supplemented nutrient levels, especially P and N. The N level greatly affects the metabolic pathway involving nutrient assimilation, and carbohydrate fixation, and even determines the conversion of the dominant fraction of proteins into other biochemical compositions, including lipids (Huang et al., 2021). The data showed that the most significant differences were observed in the condition A (negative control, absence of nitrogen). In this case, the carbohydrate composition remained like the positive control, but the percentage of proteins drastically decreased, while the lipid content more than doubled compared to the positive control.

These results were expected, as the lipid content of microalgae can be increased by environmental stressors such as nitrogen and phosphate

limitation, and certain metal components. Depletion of nitrogen alters cellular carbon flux, favoring lipid synthesis over protein synthesis (Ratomski and Hawrot-paw, 2021). For instance, the lipid content of C. vulgaris can increase from 20 % or less to up to 40 % under nitrogen deprivation (Mujtaba et al., 2012). In condition D and E (co-cultures), a decrease in protein content compared to the positive control was observed, while condition C showed the same content. Regarding lipid content, it increased in conditions A compared to the positive control, an expected result as the N limitation provokes lipid induction, while conditions C, D and E (co-cultures) exhibited comparable lipid content. However, in the literature, the increase in lipid content when conducting co-cultures with N_2-fixing bacteria in the absence of nitrogen has been previously reported. For instance, the co-cultivation of Chlorella vulgaris with the N_2-fixing bacteria Mesorhizobium sangaii under nitrogen limitation enhanced lipid production (Wei et al., 2019). These results indicate certain differences in the biomass produced solely with the cultivation of C. vulgaris compared to that produced with co-cultivation in conditions D and E, while the composition of condition C could be comparable with the positive control. These differences may stem from potential stressors faced by the algae, but they could also result from a variety of interactions and complex responses to environmental conditions and microbial community dynamics. For instance, bacteria may secrete compounds that affect the growth or physiology of the algae, thereby influencing the accumulation of lipids or carbohydrates (Fuentes et al., 2016).

These interactions between algae and bacteria could play a significant role in determining biomass composition and productivity in co-culture systems, emphasizing the importance of understanding microbial dynamics to optimize biotechnological production processes. Therefore, characterizing and understanding the composition of the biomass produced is crucial for selecting the appropriate application of it or vice-versa (imposing the culture conditions to achieve the interesting composition). Significant changes were also observed in chlorophyll and carotenoid content among the positive and negative control, and co-cultures. The lowest pigment values were observed in the negative control, as expected. In the co-cultures, pigment concentrations were lower than in the positive control, suggesting a possible nitrogen limitation or some effect resulting from co-cultivation with bacteria. Previous studies have shown that low nitrogen levels not only compromise protein production but also lead to dramatic changes in C. vulgaris cell physiology. Nitrogen limitation results in higher lipid and pigment contents and lower protein (Griffiths et al., 2014).

4.4. Microalgal application to agriculture

Related to the applications of produced biomass, microalgae and cyanobacteria generate an array of metabolites that, owing to their biological properties, find applications in agriculture as biofertilizers, biostimulants, or biopesticides. Biostimulants are materials, that, when applied in small quantities, promote the growth and quality of food crops/vegetables/fruits, improve nutrient content, stimulate defense responses against abiotic stress, and enhance the overall resistance of plants (Ammar et al., 2022; Parmar et al., 2023). Furthermore, they do not leave behind harmful chemicals and are safe for both people and the environment, making them a green choice instead of synthetic plant protection products. Among biostimulants, algae biomass or extracts as contain many groups of active compounds including peptides and amino acids as well as plant growth-promoting substances, e.g., plant hormones (cytokinins, auxins, gibberellins, abscisic acid and ethylene). Notably, phenolic compounds, terpenoids, FFAs, polysaccharides, carotenoids, and phytohormones stand out as they have been recognized as promoters of plant growth (Ferreira et al., 2023; Gonçalves, 2021).

Microalgae can be used as a biostimulant by applying the cells in suspension or the extract of their biomolecules to the soil, seeds or foliar. This method, known as wet microalgae, is a simple and cost-effective way to utilize microalgae in agriculture. Wet microalgae can be applied from the germination stage of seeds and throughout the cultivation process. The effectiveness of using microalgae extracts or living cells is extensively studied and has shown promising results in enhancing soil fertility and promoting plant growth (Osorio-Reyes et al., 2023). Currently, there is a diversity of studies regarding whether seed germination promotion can be attributed to both the biomass of algae, i.e., the intracellular material, and the extracellular material of algae cultivation which may contain metabolic exudates such as amino acids, vitamins, and organic compounds (Rupawalla et al., 2022). Also, in full microalgal culture application, avoiding cell rupture or hydrolysis methods yielded higher germination rates due to optimal amino acid and polyamine concentrations in undisrupted biomass, while pre-treatment could inhibit seed growth by increasing compound concentrations (Ferreira et al., 2022; Navarro-López et al., 2020).

In this context, experiments were conducted to evaluate the germination index of lettuce seeds using the different cultures produced (positive control, negative control, and co-cultures with bacteria) without cell rupture. Additionally, the GI was assessed using culture supernatant after removing the microalga biomass. Comparing these data poses a challenge because the supernatant was applied as is, with only the biomass removed, while the cultures were diluted to 0.2 and 0.5 $g{\cdot}L^{-1}$. Related the cultures, the better results of the germination index were obtained from cultures A, and E, at a concentration of 0.2 $g{\cdot}L^{-1}$, compared to distilled water, while a higher concentration (0.5 $g{\cdot}L^{-1}$) yields poorer results. In general, it is observed that applying cultures at 0.2 $g{\cdot}L^{-1}$ yields better results than applying them at 0.5 $g{\cdot}L^{-1}$. Regarding the application of supernatants after biomass removal, the supernatant resulting from the growth of *C. vulgaris*, except for those from treatment B, increases the germination index. These findings indicate that using the supernatant from condition B yields similar results to distilled water application, whereas utilizing supernatants from other conditions (A, C, D, and E) significantly enhances the germination index, obtaining the best results with the supernatant from treatment A. This effect is possibly due to the presence of extracellular exudates from algae in the culture medium, which likely contribute to enhancing plant nutrition (Kholssi et al., 2019).

For conditions C, D, E (co-cultures, nitrogen absence), a similar hypothesis is plausible along with the microbial interaction effects, since N_2-fixing bacteria are used in the cultivation process.

Previous work indicated that utilizing specific strains of *Microbacterium* spp. through seed priming could alleviate the impact of *Botrytis cinerea* on lettuce seedlings. Furthermore, it can induce a significant growth-promoting effect during the early stages of plant development (Suárez-Estrella et al., 2023). In this context, it may be comparable to the results obtained from culture D, which involves a co-culture of *C. vulgaris* with *M. maritypicum* M13A8. The bacterium tested in D, *Serratia ficaria* G8L13, is particularly interesting for its potential applications in agriculture. Zafar-ul-Hye et al., (2017) and Kulkova et al., (2024)) reported that strains of this genus and species enhance plant growth through various mechanisms, including the synthesis of phytohormones, the secretion of exoenzymes, the production of siderophores, and phosphate solubilization. Thus, further studies are necessary in this regard to draw definitive conclusions. Considering the positive results of the supernatants, they could be valuable for potential agricultural applications, towards zero waste and thereby adding value to the biomass produced for alternative purposes like inclusion in animal feed. However, due to the expense of separation operations, direct application of the culture in agriculture may be viable and yield positive outcomes.

5. Conclusions

The results obtained in this study demonstrate the feasibility of producing *C. vulgaris* using N_2-fixing bacteria without the addition of inorganic N to the culture medium. The data indicate that comparable biomass production can be achieved to standard production when

supplementing the medium with N. However, the concentration of microalgal cells and the photosynthetic apparatus are compromised. This could be due to insufficient N_2-fixation by the bacteria to support microalgal growth, as no C source was provided to sustain bacterial growth, or due to some form of adverse microbiological interaction. The composition of the biomass produced in the co-cultures is similar in some parameters to the control, such as the percentage of carbohydrates and lipids. However, the protein content decreases in one of the co-cultures, and the pigment content is significantly lower than the control in all co-cultures. Additionally, it was observed that the produced co-cultures could be applied in agriculture, improving the germination index of lettuce seeds, and yielding better results than the control *C. vulgaris* culture. These results are promising, allowing an important saving of synthetic N, which is produced by highly-pollutant and intensive-energy methods, such as Haber-Bosch, therefore with a very positive economic and environmental impacts. Nonetheless, further studies are still necessary to fully achieve the total potential of co-culturing.

CRediT authorship contribution statement

Francisco Gabriel Acién: Writing – review & editing, Supervision, Project administration, Funding acquisition. **Luisa Gouveia:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Tomas Lafarga:** Writing – review & editing, Supervision, Project administration. **Ana Sanchez-Zurano:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Daniel Figueiredo:** Writing – original draft, Visualization, Software, Methodology, Investigation, Data curation. **Silvia Vilaró-Cos:** Writing – original draft, Visualization, Software, Methodology, Investigation, Data curation, Conceptualization. **Alice Ferreira:** Writing – review & editing, Methodology, Investigation, Data curation. **Lusine Melkonyan:** Methodology, Investigation.

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Declaration of Competing Interest

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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Data availability

Data will be made available on request.

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