Producción Sostenible De *Spirulina* spp.: Impacto Del Enriquecimiento Con Humus Sobre El Rendimiento Y Valor Nutricional A Escala Semiindustrial

Sustainable Production Of *Spirulina* spp.: Impact Of Humus Enrichment On Performance And Nutritional Value At Semi-Industrial Scale

DOI: https://dx.doi.org/10.17981/ingecuc.21.1.2025.14
Artículo de Investigación Científica. Fecha de Recepción: 05/04/2025, Fecha de Aceptación: 24/05/2025.

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To cite this paper

Y. Benavides-Escobar, J. Muelas-Calambas, V. Gómez-Prado, G. Jojoa-Cabrera & J. Hoyos-Concha "Sustainable Production Of Spirulina spp.: Impact Of Humus Enrichment On Performance And Nutritional Value At Semi-Industrial Scale," INGE CUC, vol. 21, no. 1, 2025.
DOI: *https://dx.doi.org/10.17981/ingecuc.21.1.2025.14*

INGE CUC Vol. 21. No. 1, Enero - Junio, 2025.
Barranquilla. ISSN 0122-6517 Impreso, ISSN 2382-4700 Online

Resumen

Este estudio evaluó el efecto de la adición de humus al medio de cultivo estándar en la producción de biomasa de *Spirulina spp*. El objetivo principal fue determinar si la suplementación con humus podría mejorar el rendimiento y la calidad de la biomasa en comparación con el medio estándar.

Se estableció un experimento comparativo, cultivando *Spirulina spp* en dos medios: el medio estándar convencional y el mismo medio enriquecido con una concentración específica de humus. Se monitoreó las variables, tasa de crecimiento, concentración de células (cel.mL^{-1}), concentración de biomasa (g.L^{-1}), el contenido de nutrientes, la actividad y capacidad antioxidante en el periodo de cultivo.

Los resultados revelaron que el medio de estándar suplementado con humus mostró un incremento significativo en la tasa de crecimiento y la producción de biomasa en comparación con el medio estándar. Además, se observó un aumento en el contenido de proteínas y antioxidantes, en el medio con humus.

En conclusión, la adición de humus al medio de estándar demostró ser una estrategia efectiva para mejorar la producción y la calidad de la biomasa de *Spirulina spp*. Este hallazgo sugiere que el humus puede servir como un suplemento valioso en los medios de cultivo para la producción comercial de *Spirulina spp*, ofreciendo beneficios tanto en términos de rendimiento, valor nutricional y disminución de costos en preparación del medio.

Palabras claves

Spirulina spp; medio de cultivo; humus; proteína; antioxidante

Abstract

This study evaluated the effect of adding humus to the standard culture medium biomass *Spirulina spp.* The main objective was to determine whether supplementation with humus could enhance the yield and quality of the biomass compared to the standard medium.

A comparative experiment was set up, cultivating *Spirulina spp.* in two media: the conventional standard medium and the same medium enriched with a specific concentration of humus. Variables such as growth rate, cell concentration (cells.mL^{-1}), biomass concentration (g.L^{-1}), content, and were monitored throughout the cultivation period.

The results revealed that the standard medium supplemented with humus showed a significant increase in growth rate and biomass production compared to the standard medium. Additionally, an increase in protein and antioxidant content was observed in the medium with humus.

In conclusion, the addition of humus to the standard medium proved to be an effective strategy for enhancing both the production and quality of *Spirulina spp.* biomass. This finding suggests that humus can serve as a valuable supplement in culture media for the commercial production of *Spirulina spp.*, offering benefits in terms of yield, nutritional value, and reduction of costs in medium preparation.

Key-words

Spirulina spp; Culture medium; Humus; Protein; Antioxidant

I. INTRODUCTION

Microalgae are photosynthetic microscopic organisms, and one of the oldest and most abundant forms of life on earth. They have the ability to thrive in various aquatic environments, and they are currently of high interest for study due to their potential in various food, cosmetic, agricultural, pharmaceutical, and biofuel production applications, among others [1], [2]. They have the capacity to fix carbon dioxide and treat wastewater, and are highly useful for climate change mitigation, environmental bioremediation and food security [3].

Currently, 37.8 million tons of algae (wet weight) are produced in the world (FAO, 2024), of which approximately 30% corresponds to *Spirulina spp,* a bluish-green cyanobacteria of high productive interest and whose annual growth is 0.3%. Its most widely used applications focus on its high nutritional value characteristics, with protein contributions of 50 to 70%, carbohydrates 12 to 20%, lipids 6 to 8%, fiber, vitamins B12, pro-vitamin A, minerals at a rate of 3.3 g kg^{-1} of magnesium; 1.2 g kg^{-1} of calcium; 1.3 g kg^{-1} of phosphorus; fatty acids such as gamma-linolenic acid and bioactive compounds with antioxidant activity such as B-carotene, phycocyanin and allophycocyanin [2], [4].

In production systems, *Spirulina spp* is characterized by having a high growth rate, accompanied by a variable metabolism capable of adapting to different environments [4], [5], [6], [7], [8]. On an industrial scale, it is generally produced in

open raceway-type systems, which facilitates operation processes by reducing costs. However, this method generates challenges related to contamination by other types of algae and microorganisms, and maintaining physicochemical parameters, so it is advisable to manage these systems in greenhouses.

Another challenge that is encountered is the adaptation to the nutritional environment. A standard formula has demonstrated high yields in obtaining biomass with values equal to 1 g/L. Alternative media have been explored, with good results, aimed at improving production efficiency and reducing costs, including the use of wastewater from industrial effluents, such as those from fish farming, which have excellent characteristics of nitrogenous components, or the use of low-cost and easily accessible commercial fertilizers and chemical products, in order to replace standard media with high quality nutrients (Zarrouk), always aimed at maintaining the nutrient balance [9], [10], [11], [12], [13], [14].

This study evaluated biomass yield and quality when using two culture media commonly used for the production of *Spirulina spp,* in a laboratory and at a semi-industrial scale, verifying their effect.

II. METHODOLOGY

The research process was carried out in two phases: phase one at the laboratory level at the facilities of Universidad del Cauca, Las Guacas campus, Faculty of Agricultural Sciences, Biotechnology Laboratory, and the second phase at a semi-industrial scale, at the company Granja Mamá Lombriz, located 3 km north of the city of Popayán. Cauca-Colombia, with an altitude of 1738 m.a.s.l., average temperature of 19°C, relative humidity of 69%.

2.1. Obtaining the Strain

The strain was collected by taking different random samples from the production reactor of the company Granja Mamá Lombriz. They were transported in previously sterilized 4L plastic bottles to start the adaptation phase in the Biotechnology Laboratory, where microscopic observation was carried out in order to inspect and assure the initial state of cell concentration.

2.2. Cultivation Conditions

2.2.1. Culture Developed in the Laboratory

An enclosed space of 6 m^2 was adapted, where a two-division shelf was used to place the experimental units, consisting of a 10 L reactor. Agitation was carried out using an LP40 RESUN aerator with air flow of 50L/min. Temperature was maintained at 26±2 °C and regulated with a K-CA18 KALLEY heater. The photo period was controlled with 2500 Lux fluorescent lamps, programmed with a timer for 12 h light and 12 h dark, with pH = 9.0 regulated using a $NaHCO_3$ solution.

2.2.2. Culture Developed in the Field

Under a greenhouse system, a circular 400 L tank was used for each experimental unit, stirred with an LP40 RESUN aerator with an air flow of 50L/min. Temperature was at 24 ± 2.06 °C, with pH = 9.0 regulated using a NaHCO3 solution. Daylight exposure was that of the environmental conditions, of approximately 10h light and 14h darkness.

2.3. Preparation of Culture Media

Medium number one (1), developed by the company Mama Lombriz (GML), was a modified Zarrouk medium, composed of the following macronutrients: sodium bicarbonate ($NaHCO_3$), potassium nitrate (KNO_3), phosphorus oxide (P_2O_5), sodium chloride (NaCl), magnesium sulfate ($MgSO_4$), iron sulfate ($FeSO_4$). Medium number two (2), named Granja Mama Lombriz, included 15% humus (GMLH).

2.4. Initial concentration of Spirulina spp: The initial cell concentration was 9.06 x 10^5 cells/mL for each experimental unit.

2.5. Study period: The study follow-up period was 12 days, working with an inoculation percentage of 20% of *Spirulina spp* in the exponential growth phase plus 80% of the corresponding culture medium [4].

2.6. Monitoring of Spirulina spp Growth

2.6.1. Quantification of Cell Concentration

The cell concentration was measured using the Neubauer chamber counting technique, where 1 mL of culture was taken from each experimental unit, for a 20 µL sample that was taken for observation under the optical microscope (Zeiss Axio Lab A1, China). The results were expressed in cel.mL^{-1}. [15].

2.6.2. Biomass Quantification

The concentration of biomass was determined through centrifugation at 4000 rpm for 15 minutes of 15 mL of sample from each experimental unit. Subsequently, part of the supernatant was removed, and the residue was centrifuged again at 13000 rpm for 15 minutes. After removing the supernatant, the biomass was dried at 60°C to a constant weight by forced convection. The concentration of biomass was determined as grams of dry biomass per liter of culture ($(g.L^{-1})$) [16]. Cell concentration was monitored using a turbidity Secchi disc.

2.6.3. Biomass Harvesting

Performed in the Laboratory

The biomass was harvested using the natural precipitation method, leaving each reactor without agitation for a period of 12 hours. Then the water was removed, and the precipitated biomass was collected in 50 mL falcon tubes. To remove the remaining water, it was centrifuged at 4500 rpm for 20 min. The biomass obtained was frozen at -80°C and then freeze-dried for 24 hours at 10 Pa. The freeze-dried sample was crushed in mortar and stored in a desiccator for further analysis. [16], [17].

Performed in the Field

The biomass was harvested using the filtration technique, using a nylon cloth with a pore diameter of 30 µm. Subsequently, the biomass obtained was pressed, applying manual pressure. Lastly, the mixture was spread in pellets on a tray and dried at 60°C for 5 hours for subsequent weighing and chemical analysis.

In order to determine the quality of the biomass obtained from the nutritional components and bioactive compounds in the studied media (GML and GMLH), proximal analysis was performed and the antioxidant activity and capacity (DPPH, ABTS, FRAP and Folin methods) and reducing power (FRAP method) were determined.

2.7. Proximal Analysis

The proximal composition was determined according to the AOAC methodology: moisture 950.43/05, ash 991.36/05, ether extract 920.153/05, protein 968.06/05 (Kjeldahl factor: N x 6.25), crude fiber 962.09/05 and nitrogen-free extract by difference [18].

2.8. Analysis of Free Phenolic Compound Content and Antioxidant Capacity

2.8.1. Preparation of the Extract

The extraction was performed by modifying the method described by [19]. 0.1 g ± 0.0001 g of sample were weighed and two extractions were performed, the first with ethanol/ H_2O (80/20) and the second with acetone/ H_2O (70/30), then incubated at 30°C for 24 hours. The supernatant of both extractions was combined and taken to a final volume of 1 ml with deionized water, to read each sample in a dilution of 1:10 extract/ H_2O [19].

2.9. Method for Determining Free Phenolic Compound Content and Antioxidant Capacity

2.9.1. Folin (Free Polyphenols)

The determination of the free phenolic compounds was performed by the Folin-Ciocalteau spectrophotometric method reported by [19]. The results were expressed as mg gallic acid equivalents (GAE) per gram of sample (mg GAE/g sample). Each determination was performed in duplicate.

2.9.2. ABTS

The first method evaluated the stabilization of the 2,2-azinobis radical (3-ethylbenzothiazoline-6-sulfonic acid), for which 135 µL of the extract was mixed with 4 mL of the ABTS solution, then stirred and left in reaction for 30 min in darkness. The absorbance was measured at a wavelength of 729.7 nm using a Shimadzu UV 1800 spectrophotometer, Japan [20]. Each determination was performed in duplicate. Results were expressed as milligrams of ascorbic acid equivalent per gram of sample (mg EAA/mg sample).

2.9.3. DPPH

A second method was used to measure the stabilization of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) [21]. 3900 µL of the radical solution was mixed with 100 µL of extract. The reaction was left for 30 min in the dark and then the absorbance was measured at 517 nm (spectrophotometer, Shimadzu UV 1800, Japan). Each determination was performed in duplicate and results were expressed as mg EAA/mg sample.

2.9.4. FRAP

For the analysis of the reducing power, the FRAP reagent was prepared with 2.5 mL of TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) 0.01 M, 2.5 mL of $FeCl_3$

0.02 M and 25 mL of 0.3 M sodium acetate buffer, pH 3.6. The reaction was carried out by mixing 1800 µL of deionized water and 60 µL of extract. The reaction was left at 37 °C for 30 min in the dark, after which the absorbance was measured at 595 nm using a Shimadzu UV 1800 spectrophotometer, Japan [22]. Each determination was performed in duplicate. Results were expressed as mg EAA/mg sample.

2.10. Experiment Design

The experiment was conducted under a completely randomized experimental design (DCA), in which two treatments (T_1 GML and T_2 GMLH) were evaluated. Each treatment had two replicates, using as response variables cell concentration ($cel.mL^{-1}$) and biomass ($g.L^{-1}$).

2.10.1. Statistical Analysis

The information obtained was analyzed using the Student T test, with $\alpha = 0.05$, inputting the data in the SPSS version 23 statistical software.

III. RESULTS

3.1. Monitoring and Evolution of Growth Stages

In order to assure the seeding process, an initial inoculum concentration of 9.06×10^5 $cel.mL^{-1}$ was determined. In addition, observation was made by optical microscopy identifying helical morphological characteristics and the bluish-green coloration of *Spirulina spp* [24], [25] (See fig. 1).

In Figure 2, the growth behavior of *Spirulina spp* in the GML and GMLH culture media is observed. In the first 24 hours the cells are in the adaptation phase, then the exponential phase begins, reaching its maximum concentration on day 10. During this phase the process of nutrient absorption possibly occurs, increasing cell division (increase in biomass), followed by the dormancy phase where cellular decrease was observed, possibly due to nutrient deficiency [26], [27].

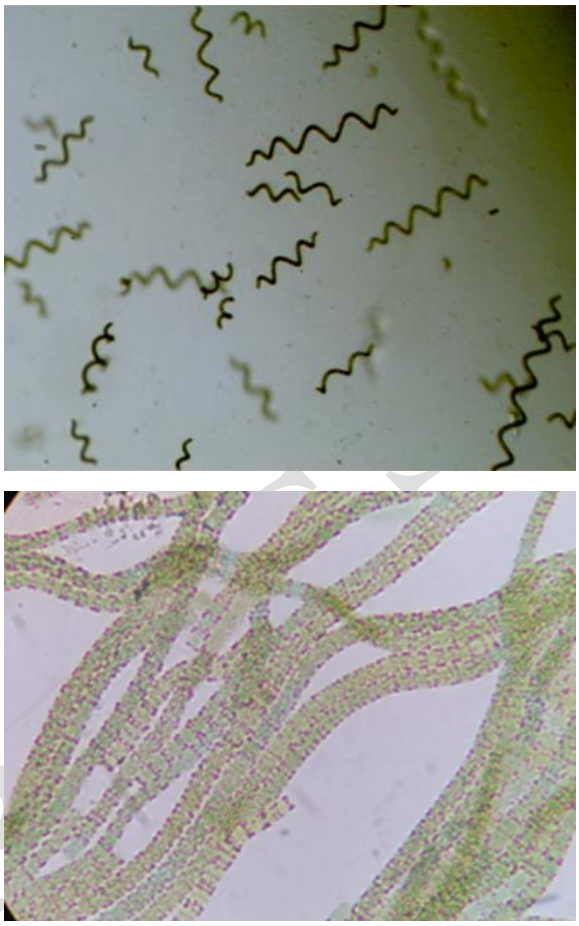

Figure 1. Morphological structure of *Spirulina spp* (Photo: Biotechnology laboratory)

For the statistical analysis, data were taken from the day of peak production, or the phase of greatest exponential growth chosen for the harvest, which was the moment with greatest availability of nutrients [27], [28].

3.2. Phase One (1) Analysis

The Student's T analysis indicates that statistically significant differences were found with $\alpha = 0.05$ between the means of cell concentrations and biomass for the GML and GMLH treatments in the 10 L reactors at the laboratory level. The values for day 10 show cell concentration of $1.95 \times 10^6 \pm 2.2$ x104 and $3.23 \times 10^6 \pm 1.7 \times 10^4$ $cell.mL^{-1}$ and biomass 0.38 ± 0.002 $g.L^{-1}$ and 0.51 ± 0.01 $g.L^{-1}$ (See Fig. 2).

These results are in line with what was reported by [28], where the values are $5x10^6$.

This result may possibly be linked to the greater contribution of nitrogen, micronutrients and the

decrease of phosphorus in the humus medium taking into account what was reported by [13], who states that at concentrations of 120 mg/L of phosphates (PO_4^{3-}) the growth of *Spirulina spp* is optimal, which probably caused the GMLH treatment to obtain better performance in cell number and biomass when the concentration of (PO_4^{3-}) for GML was 159 mg/L and for GMLH 104 mg/L per [29].

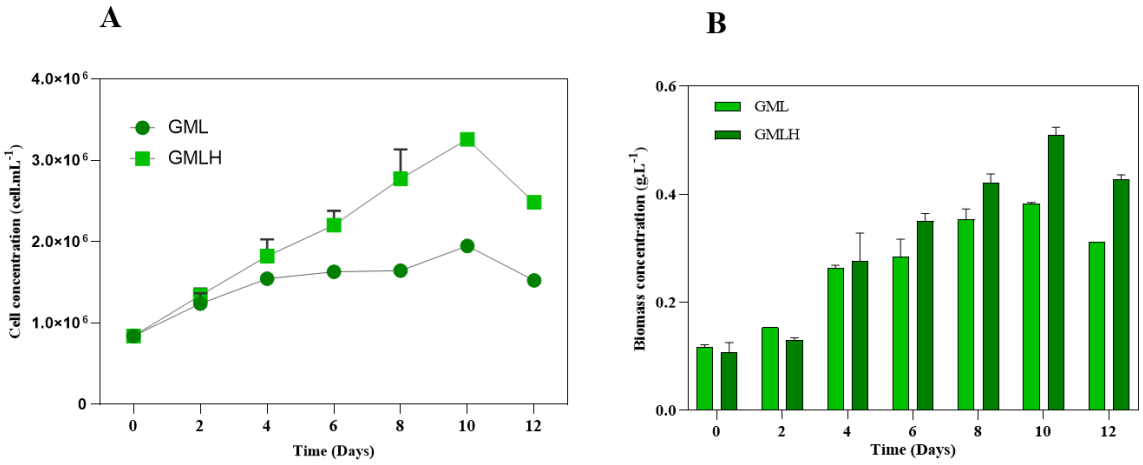

Figure 2. Cell concentration and biomass of GML and GMLH culture media of *Spirulina spp* in 10 L reactors, A. Cell concentration cell. mL^{-1}, B. Biomass concentration $g.L^{-1}$

3.3. Phase Two (2) Analysis

The Student's T-analysis indicates that statistically significant differences were found with $\alpha = 0.05$ between the means of cell concentrations and biomass for GML and GMLH treatments, taken on day 10, in the 400L reactors at a semi-industrial level, with values of $5.2x10^6 \pm 1.3x10^4$ and $5.8x10^6 \pm 1.5x10^4$ cel.mL^{-1}; 0.50 ± 0.013 and 0.55 ± 0.017 $g.L^{-1}$ (See Fig. 3). The treatment with humus incorporation demonstrated a greater biomass production of 10% compared to the other medium, indicating that natural environmental factors such as luminosity can have a positive impact because sunlight provides a complete light spectrum with all the wavelengths necessary for photosynthesis, allowing the production of different bioactives such as phycocyanin, chlorophyll and carotenoids essential for *Spirulina spp.* It should be noted that *Spirulina spp* has evolved over centuries to make use of sunlight and adapt to its natural variations such as exposure to ultraviolet rays, thermoregulation, among others, which allow it to create better quality biomass in its pigments and nutritional composition [3].

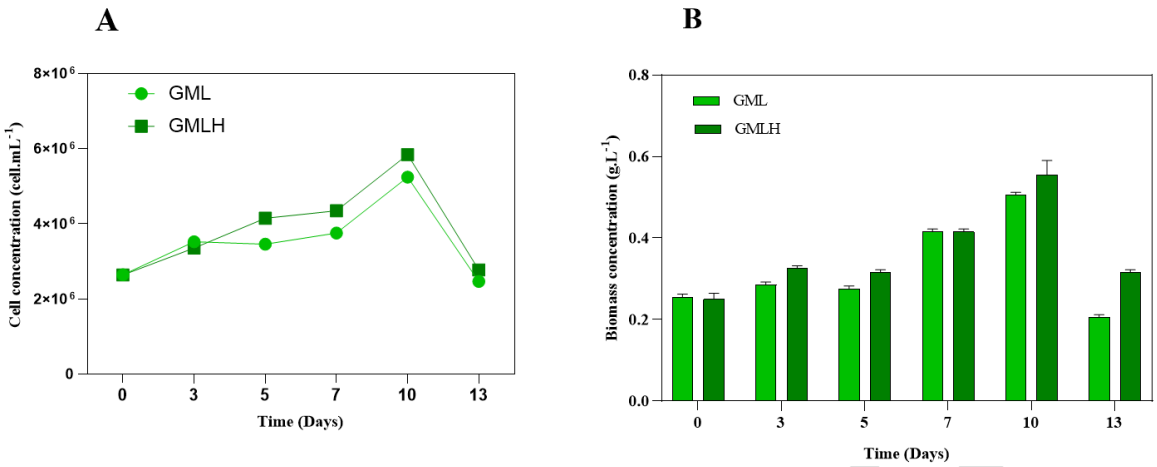

Figure 3. Cell concentration and biomass of GML and GMLH culture media of *Spirulina spp* in 400 L reactors, A. Cell concentration cell. mL^{-1}, B. Biomass concentration $g.L^{-1}$.

3.4. Proximal Analysis, Free Phenolic Compound Content and Antioxidant Capacity of Biomass

The chemical composition of the biomass harvested from MGL and MGLH media is summarized in Table 1. These results are similar in most of the components and display a higher value for protein compared to those reported in the study by [31], where it was found that for every 100 g of dry biomass of *Spirulina spp* there were: moisture 11.51 ± 0.04, protein 51.02 ± 0.79, ash 11.72 ± 0.12 and lipids 7.7 0 ± 0.10. The results obtained can be associated, as in laboratory production, to the adequate phosphorus content delivered by the MGLH treatment in the culture medium, taking into account that the production of microalgae is aimed at human consumption, where the aim is for protein content to prevail in greater concentration than the other nutrients. This was also reported by [32], who indicates that in order to obtain products such as ethanol, phosphorus levels must be lowered so that the microalgae can adapt by accumulating carbohydrates and lipids, which is a requirement for the production of biofuels.

Table 1.

Proximal analysis of algae production

Treatments		
% of Components	GML	GMLH
Humidity	11.99 ± 0.07	11.29 ± 0.41
Lipids	7.71 ± 0.02	7.94 ± 0.07
Protein	69.62 ± 0.53	74.94 ± 0.07
Fiber	9.18 ± 0.21	8.01 ± 0.22
Ash	7.20 ± 0.04	7.42 ± 0.03

In terms of antioxidant activity and free polyphenols, it was found that the biomass of the GML and GMLH media showed antiradical activity (DPPH and ABTS methods) and reducing power (FRAP method). Additionally, it was observed that the content of phenolic compounds was greater in the GML medium compared to the GMLH medium, being 42.42 ± 0.60 and 30.10 ± 0.16 mg GAE/g respectively. These concentrations are higher than those obtained by [33] of 26.64 ±0.16 mg GAE/g. The higher values reported in this study may be associated with the interrelationship that was generated between the culture medium and the conditions of the natural environment, which enable the improvement of the quality and content of

pigments. *Spirulina spp* offers high potential as a source of phenols for human consumption [34].

The *Spirulina spp* grown in GMLH displayed increased antioxidant activity in the DPPH and FRAP assays (See Table 2), suggesting a greater ability to neutralize free radicals and reduce iron in this medium. However, the GML medium exhibited significantly higher activity in ABTS and a higher polyphenol content, indicating that this medium favors the accumulation of specific antioxidant compounds [33]. These differences reflect the influence of the culture medium on the expression and concentration of metabolites with antioxidant activity. The polyphenol content is higher in the GML medium, which may be related to cellular stress, the breakdown of cell walls and the greater availability of nitrogen sources. Therefore, the depletion of nutrients makes its consumption simple, increasing the production and synthesis of antioxidant phenolic compounds [35], [36], [37].

Table 2.

Free phenolic compounds and antioxidant capacity

Contents (g/100g)	GML	GMLH
DPPH (mg AAE/g)	45.25 ± 0.37^{B}	51.16 ± 0.24^{A}
ABTS (mg AAE/g)	26.82 ± 0.25^{A}	7.76 ± 0.04^{B}
FRAP (mg AAE/g)	34.06 ± 0.09^{B}	37.35 ± 0.50^{A}
Polyphenols (mg GAE/g)	42.42 ± 0.60^{A}	30.10 ± 0.16^{B}

IV. CONCLUSIONS

The addition of humus to the nutrient medium for the production of *Spirulina spp* at a semi-industrial level increased biomass production by 10% per production cycle, reducing the cost of preparing the medium by 2.1%, a finding that shows the potential of humus as a nutritional supplement and enhancer of microalgae growth.

It is necessary to continue studying alternative media, extending the possibility for experimentation with wastewater effluents with a high content of nitrogenous components. A promising path is to improve sustainability in the aquaculture and biotechnology industry to explore other sources of organic nutrients, such as fish farm wastewater, that can be used for the same purpose, considering its high content of organic and inorganic components derived from the feeding systems. The elements they generate can cause eutrophication problems, but a circular economy could be created if they are used for the production of microalgae.

V. CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Y. Benavides-Escobar: methodology, validation, formal analysis, research, writing; **J. Muelas-Calambas:** methodology, validation, formal analysis, research; **Víctor Gómez-Prado:** conceptualization, methodology, research; **G. Jojoa-Cabrera:** methodology, validation, formal analysis, research, writing, review and editing, data curation, **José Hoyos-Concha:** project management, background acquisition, supervision, visualization, resources, formal analysis, conceptualization.

ACKNOWLEDGEMENTS

This research was funded by the General System of Royalties (SGR) and executed by Reddi Colombia within the framework of the Fusioni3 Cauca project – "Strengthening innovation in SMEs based on the research results of Higher Education Institutions (HEIs) in the department of Cauca" with BPIN No. 2021000100371. The authors thank Universidad del Cauca and the company Granja Mamá Lombriz for their valuable support as allies in the development of this research, as well as Reddi Colombia for its management and accompaniment as a facilitator of the research process.

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