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Molecular characterization and bioremediation effectiveness of *Limnospira platensis* cultivated in swine wastewater: biochemical analysis and assessment of the resulting biomass as a cucumber biostimulant

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The escalating global demand for water resources, combined with anthropogenic pollution, poses a significant threat to the availability of potable water. Even small-scale pig farms generate enough wastewater to pose environmental risks. Microalgal wastewater treatment offers an effective solution for pollutant recycling, providing advantages such as reduced energy consumption, lower greenhouse gas emissions, and greater cost efficiency compared to conventional methods. The biomass produced through this process has multiple valuable applications, including use as a biostimulant and biofertilizer. Consequently, this study investigates the use of the microalga *Limnospira platensis* for the biological treatment of undiluted pig slurry, intending to develop a sustainable biostimulant. Initially, a molecular characterization of the *Limnospira platensis* strain was performed to verify its identity. The research employed 150 mL bubble column photobioreactors (BCPs) with a continuous airflow of 15 mL min^{-1}, followed by the application of the resulting biomass as a biostimulant for *Cucumis sativus*. The productivity of *Limnospira platensis* was monitored daily in cultures grown in pig slurry. The results demonstrated that *Limnospira platensis* effectively treated pig slurry, producing biomass at a concentration of 1.34 g L^{-1}, and a productivity of 0.88 g L^{-1} day^{-1}. The highest removal efficiencies recorded were 99.93% for Chemical Oxygen Demand (COD), 94.83% for total phosphorus (TP), 98.93% for total nitrogen (TN), and 100% for NH_4^+ and total coliforms. The most favorable biostimulant effect of *Limnospira platensis* biomass on *C. sativus* was observed in

T_2 (0.5 g L^{-1} with centrifugation and cellular disruption), which yielded a germination index of 515.34% ± 81.81%, a shoot number of 2.20 ± 0.04, and a root number of 10.33 ± 0.17. These findings demonstrate the feasibility of using microalgae to treat wastewater with high organic loads, highlighting the significant biostimulatory potential of the biomass produced in these treatment systems.

KEYWORDS

biostimulant, *Cucumis sativus*, *Limnospira platensis*, microalgae, swine slurry

1 Introduction

Projections of climate crisis scenarios indicate an escalation in the spatial and temporal variability of the water cycle, intensifying the disparity between water supply and demand. Water availability is contingent not only on the severity of the climate crisis but also on contamination levels, particularly fecal coliforms from human and animal waste. Monitoring efforts conducted in 2016 to evaluate the presence of pathogens in surface water revealed that approximately one-third of river basins across Africa, Asia, and Latin America exhibit severe pathogen contamination, characterized by levels exceeding 1000 CFU of fecal coliforms per 100 mL (UNEP, 2016).

In a world increasingly affected by water scarcity driven by overexploitation, pollution, and climate change, neglecting the benefits of enhanced wastewater management is a significant oversight. Innovative wastewater treatment technologies are indispensable for environmental preservation and satisfying the escalating water demand. Wastewater (WW) plays a vital role in developing effective water management practices to ensure a sustainable future. Recycling and reusing wastewater are fundamental strategies to mitigate water scarcity while safeguarding environmental integrity. Conventional wastewater treatment often involves costly procedures that require substantial energy and labor, generating greenhouse gases (GHG) and sludge with limited beneficial applications (WWAP, 2017). There is an urgent need to develop advanced wastewater treatment options, especially for small and underserved communities. It is estimated that over 80 percent of wastewater—exceeding 95 percent in particular developing countries—is discharged into the environment without any treatment. When neglected, wastewater is often seen as a problem to be ignored. However, this perception is gradually shifting as water scarcity persists across many regions and the importance of wastewater collection, treatment, and reuse is increasingly recognized (WWAP, 2017).

The freshwater bodies of Ecuador are of considerable concern, as most are highly contaminated. Vinueza and colleagues (2021) identified elevated levels of pathogens in twelve rivers spanning the coastal, Andean, and Amazon regions, with the most contaminated being the Zamora River in Loja, located in the southern Andean region (*Escherichia coli*: 2.50×10^4 CFU per 100 mL; and total coliforms: 6.38×10^4 CFU per 100 mL). All rivers included in the study exceeded 1000 CFU per 100 mL (Vinueza et al., 2021). This issue is further corroborated by statistics provided by the Ministry of Public Health regarding acute diarrheal diseases (ADD), which, in 2016, reported 590,523 cases in Ecuador, predominantly affecting children and thereby diminishing the overall quality of life within the population. Children constitute one of the most vulnerable and socioeconomically disadvantaged groups due to unmet basic needs (Férez and Cañas, 2019). The primary etiological agents of ADD in Ecuador are bacteria from the family Enterobacteriaceae, including *Shigella* and *Salmonella* (27.9%), as well as Rotavirus (24.8%) and *Giardia intestinalis* (8.5%) (Nazate et al., 2022).

Global pork production attained 121.72 million tons in 2024 (Statista Homepage, 2025). Nevertheless, pig farming contributes to considerable environmental challenges by generating substantial waste and wastewater, as well as notable ammonia emissions into the atmosphere and nitrate runoff into aquatic ecosystems (ASPE Home Page, 2024). Smallholder pig farming does not necessitate substantial investments or extensive technical expertise. However, it is characterized by rapid growth and high productivity, making it an effective activity for improving rural incomes. Ecuador produces 206,000 tons of pork, engaging 163,000 individuals, of whom 94 percent are small-scale farmers. The pork industry accounts for approximately eight percent of the nation's Gross Domestic Product (Agrocalidad, 2023). Pig husbandry is of considerable economic significance to residents of Luz de América, a rural municipality in the Santo Domingo de los Tsáchilas Province, Ecuador, with an estimated population of around 11,504. In 2023, 6,907 pigs were vaccinated, representing 85% of the total pig population (Agrocalidad, 2023). A fattening pig produces approximately 0.008 cubic meters of manure daily, roughly 2.92 cubic meters annually (Ferreira et al., 2022). Based on these data, the estimated volume of untreated manure generated by this rural municipality is approximately 20,168.44 cubic meters per year; this volume could pose significant environmental and health risks.

Pig manure contains a variety of contaminants, including reactive nitrogen species (e.g., ammonium, nitrate), phosphorus, sediments, organic matter, and pathogens such as *E. coli* and coliform bacteria. These nitrogen compounds are primary drivers of aquatic eutrophication. It may also harbor emerging contaminants, including pharmaceutical residues, hormones, and metals such as selenium, lead, copper, mercury, arsenic, and manganese (WWAP, 2017). In rural pig farming operations, feedlots are often situated near water bodies, leading to the direct discharge of untreated slurry into rivers. Such discharges contribute to eutrophication in freshwater ecosystems, leading to algal blooms that negatively affect aquatic life (FAO, 2021).

In certain Latin American and Ecuadorian regions, the livestock sector supports rural livelihoods and promotes global economic development. Among various livestock species, pigs yield quicker financial returns for farmers due to their inherent traits, such as high fertility, efficient feed conversion, early maturity, and short generation intervals. Although pig farming requires a modest initial investment, it entails substantial water consumption and generates highly contaminated effluents containing elevated levels of organic material, metals (e.g., Cu, Fe, Zn), environmentally toxic

chemicals (N, P, K), pathogens, and pharmaceuticals (e.g., antibiotics, hormones). If not adequately treated, these effluents pose significant risks to human health and the environment (FAO, 2021).

The management of pig manure should encompass the recovery of valuable by-products, either directly—such as heat, nutrients, organic matter, and metals—or indirectly through additional conversion processes, including sludge biogas, biofuels, biofertilizers, and microalgae-derived bio-stimulants (WWAP, 2017). Furthermore, employing microalgae in photobioreactors (PBRs) for wastewater treatment offers a promising approach to purify various effluents to levels below legal standards, with energy consumption four times lower than that of conventional wastewater treatment plants (*WWTPs*) (Acién Fernández et al., 2016). This efficiency is attributed to the symbiotic relationship between photosynthetic microalgae and bacteria: the former supply oxygen to the latter and fix nutrients such as nitrogen and phosphorus, while the latter supply carbon dioxide to the former and decompose organic carbon, forming the microalgae-bacteria consortium. A significant advantage of this process is the generation of valuable biomass, which has numerous potential applications, including biofuels (Ferreira et al., 2017; 2018; 2019), animal feed (Maizatul et al., 2017), and biofertilizers (Navarro-López et al., 2020; Ferreira et al., 2023).

Nevertheless, wastewater from pig farms frequently contains excessively high levels of ammonium (~2,000 mg L^{-1}), which proves toxic to microalgal growth (toxicity threshold approximately 150 mg L^{-1}) (Acién Fernández et al., 2018; Kwon et al., 2020). Nonetheless, among the microalgae with the most significant capacity to remediate agro-industrial wastewater with high ammonia levels, the *Arthrospira, Limnospira*, and *Spirulina* groups, owing to their alkaliphilic nature, exhibit substantial tolerance to elevated ammonia levels. This was evidenced by Álvarez and Otero (2020) through the cultivation of *Arthrospira* sp. In a semi-continuous system utilizing the diluted centrate derived from the anaerobic digestion of sewage sludge, as well as cooking water from the fish canning industry, and occasionally from the dairy industry and slaughterhouse sludge (49.33–113.22 mM NH_4^+). They reduced the concentration of NH_4^+ by 80.5% ± 2.8%, from 12.9 mM to 2-3 mM, and achieved a protein concentration of 500 mg per gram of dry biomass in *Arthrospira* sp.

Consequently, this research aims to develop a circular bioeconomy model for small-scale swine farming by integrating wastewater bioremediation with the production of a high-value microalgal biostimulant. To this end, the specific research objectives are: (1) the molecular characterization and phylogenetic identification of the cyanobacterial strain *Limnospira platensis* BioMicro-001; (2) the assessment of its growth performance, productivity, and bioremediation efficiency in raw, undiluted swine slurry, including quantification of chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP), ammonium (NH_4^+), and total coliforms; (3) the biochemical characterization of the biomass produced in wastewater, with a focus on proteins, carbohydrates, lipids, and free amino acid profiles; and (4) the evaluation of the biostimulant potential of variously processed *L. platensis* extracts on Cucumis sativus (cucumber) seeds by measuring the germination index (GI), shoot number, and root number to determine the most effective treatment formulation.

2 Materials and methods

2.1 *Limnospira platensis*: a key factor in this study

The microalgae utilized in this research are obtained from the Cell Biology Laboratory strain bank at the University of the Armed Forces-ESPE (UFA-ESPE) in Santo Domingo de los Tsáchilas, Ecuador. Filamentous cyanobacteria were isolated from a brackish water body located in Guayas Province (Estero Salado) (2°17′88.8″ S; 79°88′36.5″ W). This strain is preserved in Zarrouk standard medium (pH 9.9) at 22 °C ± 2 °C and subjected to an illumination intensity of 30 μmol photons m^{-2} s^{-1} under a 12-h light: 12-h dark cycle.

2.2 DNA barcoding and phylogenetic analysis

Genomic DNA was extracted using the methodology described by Morin et al. (2010), with some modifications. A 2 mL aliquot of the axenic liquid culture sample was transferred into 2 mL Eppendorf tubes. Cells were isolated via centrifugation for 10 min at 3500 rpm. The cell lysis process consisted of sonicating the sample on ice for 15 min, followed by incubation in a thermostated bath at 70 °C for 1 h. Afterward, the supernatant was discarded. The resultant pellet was resuspended in 500 μL of TES buffer (100 mM Tris, pH 8.0; 10 mM EDTA; 2% SDS). Subsequently, 5 μL of Proteinase K solution (50 mg mL^{-1}) was added, and the mixture was incubated for 30 min at 37 °C. Next, 140 μL of 5 M NaCl and one-tenth of the volume of 10% CTAB were incorporated, followed by incubation for 10 min at 65 °C. For phase separation, an equal volume of chloroform: isoamyl alcohol (24:1) was added. The tubes were placed on ice for 5 min and then centrifuged at 15,000 rpm for 10 min at 4 °C. 225 μL of 5 M ammonium acetate was added to the supernatant, and the mixture was centrifuged again at 15,000 rpm for 10 min at 4 °C. Half the volume of isopropanol was added to the supernatant, which was incubated at −20 °C for 24 h. Subsequently, the tubes were centrifuged for 5 min at 4 °C at 15,000 rpm, and the supernatant was discarded. The pellet was resuspended in 400 μL of cold ethanol and centrifuged again. The supernatant was discarded, and 200 μL of ultrapure water was added to the DNA pellet. Three absorbance measurements were taken at 280 nm, 260 nm, and 320 nm to quantify the DNA concentration using a spectrophotometer, with ultrapure water serving as the blank.

The reactions were conducted in a final volume of 10 μL, comprising 2X GoTaq® Green master mix (5 μL, at a 1x final concentration), along with primers 8F (AGA GTT TGA TCC TGG CTC AG) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) (refer to Table 1). Each primer was utilized at a final concentration of 0.5 μM. Furthermore, 2 μL of DNA was added, with ultrapure water used to bring the total reaction volume to 10 μL. Amplifications were carried out using a thermal cycler under

TABLE 1 Primers used in PCR amplification.

Primer	Sequence (5′-3′)	T_m (°C)	Size of product
8F	AGA GTT TGA TCC TGG CTC AG	54.3 °C	1505 bp
1492R	GGT TAC CTT GTT ACG ACT T	49.4 °C	

Melting temperature (T_m) was calculated using the salt-adjusted melting temperature formula available at http://oligocalc.eu/

the following conditions: initial denaturation at 95 °C for 2 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min; with a final extension at 72 °C for 5 min.

Following PCR amplification, the products were purified and sequenced at a commercial facility (Macrogen, Rockville, MD, USA). All sequences were analyzed using MEGA X. After alignment, the sequences were trimmed at both ends to generate a consensus sequence, which was subsequently uploaded to GenBank. The sequence was then subjected to BLAST analysis to determine similarity. For phylogenetic analysis, various accessions were aligned with MUSCLE in MEGA X. Phylogenetic reconstruction was performed using the maximum likelihood method with 1000 bootstrap replicates. The optimal model for the analysis was identified by MEGA X (Kumar and Chaurasia, 2018).

2.3 Culture medium and experimental setup

The wastewater utilized as an alternative culture medium is obtained from the distribution channel of the experimental pig farm at the Universidad de las Fuerzas Armadas-ESPE. The pig slurry exhibits the following initial chemical characteristics: pH 8.83, total nitrogen (TN) 71.47 ± 0.02 mM, total phosphorus (TP) 0.63 mM, ammonium 7.32 mM, chemical oxygen demand (COD) 16,266.67 ± 450.90 mg L^{-1}, an electrical conductivity (EC) of: 11.33 ± 3.45 mS cm^{-1}, and total coliforms 1.17 × 10^7 CFU mL^{-1}.

The growth of microalgae was assessed using graded concentrations of pig slurry (10%–100%) to reduce adaptation time at the beginning of the cultures. For the control experiment, the Zarrouk standard medium was employed, comprising the following constituents: 214.3 mM $NaHCO_3$; 29.4 mM $NaNO_3$; 2.9 mM K_2HPO_4; 5.7 mM K_2SO_4; 17.1 mM NaCl; 0.8 mM $MgSO_4{\cdot}7H_2O$; $CaCl_2{\cdot}2H_2O$ 0.5 mM; $FeSO_4{\cdot}7H_2O$ 0.04 mM; EDTA 0.3 mM; H_3BO_3 0.046 mM; $MnCl_2{\cdot}4H_2O$ 0.009 mM; $ZnSO_4{\cdot}7H_2O$ 0.0008 mM; Na_2MoO_4 0.00009 mM; and $CuSO_4{\cdot}5H_2O$ 0.0003 mM (Sigma-Aldrich, St. Louis, MO, USA) (Zarrouk, 1966). The experimental treatments included 100% swine slurry. Each cultivation was conducted in quadruplicate.

2.4 Photobioreactor setup and culture conditions

Microalgae were batch-cultured in four bubble column photobioreactors, each with a culture volume of 0.15 L. The initial cell density was set at 0.44 ± 0.01 g L^{-1}. The reactors were subjected to artificial illumination with a 12-h light/12-h dark photoperiod, providing 100 μmol photons m^{-2} s^{-1} irradiance using white LED lamps. The culture temperature was maintained at 24 °C ± 2 °C. Mixing was facilitated by a continuous airflow of 0.1 volumes of air per volume of reactor per minute (15 mL), which also helped maintain dissolved oxygen concentrations within the 100% ± 10% saturation range. To accurately assess the effectiveness of *Limnospira platensis* in treating pig slurry, twelve photobioreactor units were used: four units contained cultures in Zarrouk medium, four others contained cultures in pig slurry, and the remaining four housed pig slurry under culture conditions (including continuous airflow, light cycles, etc.) without *L. platensis*.

Daily samples were collected from the photoreactors to measure biomass and calculate productivity. COD, TN, TP, ammonium, and total coliform analyses were performed on days 0, 5, and 10. Various analytical techniques were used to determine growth parameters and chemical and microbiological composition. This included daily measurements of dry biomass. The percentages of total nitrogen and phosphorus removal were also calculated to evaluate nutrient removal efficiency from the medium.

The COD was determined using method 410.4, officially approved by the United States Environmental Protection Agency (USEPA). Total nitrogen (TN) was quantified using an adaptation of the chromotropic acid method (Clarke and Jennings, 1965). Phosphorus concentration was determined using modifications of the vanadate-molybdate method initially developed by Tandon, Cescas, and Tyner (Tandon et al., 1968). Ammonium concentration was measured using the Nessler method (Krug et al., 1979). These analytical determinations will be carried out using the validated methodologies mentioned above, along with the HI-83224 photometer, which incorporates a tungsten lamp and a silicon photocell equipped with a narrowband filter (HANNA® Instruments). EC and pH were measured using an EC214 conductivity meter (HANNA® Instruments) and a pH meter (Apera Instruments AI209).

2.5 Analytical methods for assessing growth, biomass composition, and nutrient removal efficiency

2.5.1 Determination of microalgal growth and productivity

The dry weight of the biomass (g L^{-1}) was measured according to the method described by Zhu and Lee. Fiberglass filters (Whatman® GF/F, 47 mm diameter, 0.7 μm pore size) were dried at 90 °C in an oven for 24 h to prevent moisture absorption. The dried filters were stored in a desiccator with silicon dioxide (SiO_2), and their initial weight was recorded using an analytical balance. Five milliliters of homogeneous culture were extracted and filtered from each sample,

and 5 mL of a 0.5 M ammonium formate (HCO_2NH_4) solution was added to remove salts from the medium. Each filter was returned to the oven at 72 °C for 24 h. After drying, the filters were reweighed using an analytical balance. The total dry weight was determined by subtracting the initial weight of the dry filter from the weight of the filter with the dry biomass, which provided the dry weight of the biomass in grams per liter (g L^{-1}) of culture (Zhu and Lee, 1997).

Based on biomass production data, biomass productivity (Pb) was calculated using Equation 1 proposed by Mohd Apandi et al. (2021):

$$Pb = \frac{x_2 - x_1}{t_2 - t_1} \quad (1)$$

where:

Pb represents biomass productivity, in g L^{-1} d^{-1}.

x_1 and x_2 denote the biomass concentrations at times t_1 and t_2, respectively.

t_1 and t_2 indicate the corresponding times.

To determine the nutrient removal efficiency (Re), calculate the percentage difference between the initial (Ci) and final (Cf) concentrations of each nutrient in the culture, utilizing Equation 2.

$$Re = 100 - \frac{Cf\ x\ 100}{Ci} \quad (2)$$

where:

Re is the nutrient removal efficiency (in percentage).

Cf is the final concentration of the nutrient.

Ci is the initial concentration of the nutrients.

This equation assesses the percentage decrease in nutrient concentration in the microalgae culture over the course of the experiment, illustrating nutrient removal efficiency from the wastewater.

2.5.2 Biochemical composition and safety assessment of *Limnospira platensis*: a focus on amino acids, NP content, and pathogen load

The protein fraction of the biomass was quantified employing the methodology established by Lowry et al. (1951), with modifications introduced by Herbert et al. (1971). The procedure comprised several steps: 10 mL of culture was centrifuged, and the resulting pellet was treated with 2 mL of 1 M NaOH. The sample was sonicated at 20 kHz and 4 °C for 15 min, then incubated in a water bath at 95 °C for 1 h to denature the proteins. After cooling to ambient temperature, the samples were centrifuged at 3,800 g for 15 min to eliminate insoluble residues. Subsequently, 100 mL of the supernatant from each sample was mixed in triplicate with 400 mL of distilled water, 300 mL of 1 N NaOH, and 2 mL of saturated copper tartrate solution. These mixtures were incubated for 10 min before adding 400 µL of diluted Folin-Ciocalteu reagent (1:1 v/v). The mixtures were then vortex-mixed and allowed to react for an additional 30 min. Ultimately, the protein concentrations in each sample were determined spectrophotometrically at 750 nm. The data were quantified by interpolation against the bovine serum albumin (BSA) standard curve (Lowry et al., 1951; Herbert et al., 1971).

The carbohydrate concentration in the biomass was quantified using the phenol-sulfuric acid method, as described by Dubois et al. (1956). A volume of 1 mL from each culture sample was collected and centrifuged. The resultant pellet was resuspended in 1 mL of 1 N NaOH, homogenized with a vortex mixer, and sonicated at 20 kHz for 20 min at 4 °C. Following sonication, the samples were centrifuged at 8,000 g for 15 min at 4 °C. Subsequently, a 1: 10 dilution was prepared in triplicate within test tubes, comprising 100 µL of supernatant from each sample and 900 µL of distilled water. 25 µL of 80% phenol and 2.5 µL of concentrated sulfuric acid were added to each tube. The mixtures were thoroughly homogenized using a vortex mixer and cooled for 30 min. Subsequently, the carbohydrate concentration was determined by UV-Vis spectrophotometry at 485 nm, using glucose as the reference standard.

The lipid concentration in the biomass was quantified using the method initially proposed by Bligh and Dyer (1959) and subsequently refined by Marsh and Weinstein (1966). Initially, 15 mL of biomass from each culture was dried to attain a dry weight of 5 mg. Subsequently, 3 mL of methanol and 1.5 mL of chloroform were incorporated into the dried biomass. The mixture was homogenized and sonicated at 20 kHz and 4 °C for 20 min, then incubated in a water bath at 50 °C for 30 min. After cooling, the samples were centrifuged at 3,900 rpm for 10 min to facilitate phase separation. To the supernatant, 1.5 mL of chloroform and 1.5 mL of distilled water were added; the mixture was then centrifuged. The supernatant was then discarded. 0.5 mL of acetone was added to the pellet to eliminate residual compounds. The solution was evaporated under a continuous nitrogen flow until the volume was reduced to 1 mL of chloroform. Further analysis was performed according to the Marsh and Weinstein protocol, in which 2.5 mL of concentrated H_2SO_4 was added to 100 µL of the chloroform-resuspended sample in triplicate. Carbonization was conducted at 200 °C for 15 min. After cooling, 3 mL of distilled water was added to each tube, and the lipid concentration was quantified spectrophotometrically at 375 nm, using palmitic acid as the standard.

The total coliform count (TC) in biomass and pig slurry was assessed using Petrifilm® *E. coli*/Coliform Count Plates (NEOGEN®, Lansing, MI, USA). Initially, 10 mg of dry biomass was mixed with 10 mL of sterile 0.9% saline solution, and serial dilutions were performed for pig slurry. The Petrifilm® *E. coli*/Coliform Count Plate was placed on a flat surface; the top film was lifted, and, holding the pipette vertically over the inoculation area, 1 mL of the sample suspension was dispensed into the center of the bottom film. The plates were incubated at 42 °C ± 1 °C for 24 ± 2 h. All red colonies were counted, regardless of gas production. The colony count in one or more representative squares was used to approximate the total coliform count, and the mean value per square was calculated (AOAC International and Latimer, 2023).

To determine the total nitrogen and phosphorus content of the biomass, 5 mg of biomass was weighed and manually ground for 10 min using a sterile mortar and pestle. The biomass was then homogenized with 25 mL of sterile distilled water. Ultrasonic pulses (Portable Ultrasonic Cell Disruptor, UCD-P01, Biobase©) at 20 kHz were applied to the microalgal suspension for 20 min at 4 °C to facilitate cell disruption and the release of intracellular components. The mixture was centrifuged for 10 min at 5,000 rpm. The resulting supernatant was vacuum-filtered through a glass fiber filter (Whatman® GF/F, 47 mm diameter, 0.7 µm pore size) to remove any residual particles that could interfere with the analysis (APHA, 2017).

Total nitrogen was measured using an adaptation of the chromotropic acid method (Clarke and Jennings, 1965), while phosphorus concentration was determined using a modification of the vanadate-molybdate method of Tandon, Cescas, and Tyner (Tandon et al., 1968). Concentrations were evaluated using established methodologies with the HI-83224 photometer, which employs a tungsten lamp and a silicon photocell with a narrowband filter (HANNA® Instruments).

The free amino acid content was quantified using a Beckman Coulter P/ACE MDQ capillary electrophoresis system with a UV detector. A micellar analysis buffer of pH 9.28 and a 75 mm diameter polyamide-coated fused silica capillary were employed. The sample was introduced into the system at 0.2 psi for 5 s. The composition of the analysis buffer comprised 40 mM SDS, 30 mM sodium borate, and 30 mM lithium chloride. Derivatization was conducted for 30 min using a 20 mg mL^{-1} concentration of 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl). Detection was performed by calculating the area under each peak. The ratio of the amino acid mixture (Table 2) to NBD-CI remained consistent across all analytical processes (Omar et al., 2017).

Calibration curves were established for each amino acid. The respective concentration ranges for these curves, expressed in mg mL^{-1}, are as follows: Hyp (0.00765-0.12240); Pro (0.0045-0.0720); Met (0.04025-0.64400); Ala (0.01354-0.21670); Lys (0.0134-0.2144); Ser (0.02299-0.36790); Gly (0.00541-0.08660); Trp (0.03428-0.54850); Arg (0.04866-0.77860). Each calibration solution was analyzed via capillary electrophoresis under the specified conditions. The recorded response variable was the peak area corresponding to each analyte.

An ethanolic extract of amino acids and subsequent derivatization were executed for sample preparation according to the methodology outlined by Omar et al. (2017). This protocol was modified to accommodate the dry microalgae biomass matrix employed in this study. Approximately 60 mg of the sample on a dry basis was combined with 0.5 mL of 80% ethanol in a 2 mL Eppendorf tube. The mixture was subjected to an ultrasonic bath for 20 min, followed by centrifugation at 6,000 rpm for 5 min. The supernatant was collected and transferred to a separate Eppendorf tube, and the solvent was evaporated using a LABTRON LVC-A10 vacuum concentrator centrifuge at 60 °C. The residual phase, containing free amino acids and other compounds, was reconstituted in 200 µL of 0.1 M Borax. Derivatization was performed by mixing 40 µL of the reconstituted sample with 10 µL of NBD-Cl (20 mg mL^{-1}), then placing the mixture in a thermostatic bath at 58 °C for 30 min. Subsequently, the sample was injected into the capillary electrophoresis system under the specified conditions (Omar et al., 2017). Biochemical characterization analyses were conducted in triplicate for each photobioreactor.

TABLE 2 Composition and concentrations of the standard amino acid solution.

L-amino acid	Concentration (mg mL^{-1})
Lysine (Lys)	0.488
Hydroxyproline (hyp)	0.444
Proline (pro)	0.500
Methionine (met)	0.211
Alanine (ala)	1.000
Serine (ser)	0.500
Glycine (gly)	0.111
Arginine (arg)	0.911
Tryptophan (trp)	0.311

2.6 Obtaining extracts of *Limnospira platensis* and their evaluation in *Cucumis sativa*

Cucumber (*Cucumis sativus*) was selected as the model species for the *in vitro* biostimulant bioassay because of its well-established sensitivity and reliability under controlled laboratory conditions. Its seeds germinate rapidly and uniformly, providing a consistent, reproducible system for quantifying treatment effects on early developmental parameters. Furthermore, the clear morphological responses of its seedlings—including radicle and hypocotyl elongation—are easily measurable and serve as validated indicators of biostimulant activity. This model system is therefore ideal for efficient, standardized screening of algal extracts (Navarro-López et al., 2020; Xie et al., 2022).

Three primary effects of extracts derived from the microalga *Limnospira platensis* were identified: (i) seed germination index (gibberellin-like effects), (ii) number of roots (auxin-like effects), and (iii) number of shoots (cytokinin-like effects) using *C. sativus* seeds. These three bioassays were conducted utilizing 8 *L. platensis* extracts (T_1-T_8), obtained from fresh biomass of the microalga cultivated with pig slurry, employing various post-processing strategies, and two extract concentrations (Ext.C) (0.1, 0.5 g L^{-1}) (refer to Table 3). All treatments were performed in quadruplicate, along with their respective controls (distilled water).

In each assay, 100 *C. sativus* seeds were tested: 25 seeds were placed in four sterilized Petri dishes (Pyrex® 100 × 20 mm) on Whatman® filter paper No. 5. Subsequently, 2 mL of sterile distilled water (control) and the same volume were administered to the treatments at the beginning of the trial and on the seventh day with eight different *L. platensis extracts* (T_1-T_8) (Table 3). On day fifteen, the treatments with extracts at 0.1 g L^{-1} reached a final concentration of 0.4 mg per Petri dish, while the treatments with extracts at 0.5 mg L^{-1} reached a final concentration of 2.0 mg per evaluation unit. After applying the different extracts, the plates were sealed with Parafilm and stored in a growth chamber at 25 °C in the dark for 72 h. Subsequently, they were exposed to a 12:12 photoperiod (12 h of light and 12 h of darkness) with a light intensity of 50 µmol m^{-2} s^{-1}. The second treatment was administered on the seventh day, and the plates were resealed. The growth chamber was the LDC700BXPRO DAY-ILD200LED model (FDM Environment Makers, Rome, Italy) (Navarro-López et al., 2020; Xie et al., 2022). The germination index was calculated employing Equation 3 (Zucconi et al., 1981). The lengths of germinated seeds were measured with an electronic digital caliper (ADORIC® 150 mm, Adoric Technologies LTD., Tel Aviv, IL).

TABLE 3 Post-processing of fresh microalgal biomass with or without centrifugation and ultrasonic cell disruption. Two concentrations for each treatment (0.1, 0.5 g L^{-1}, depending on the bioassay) were analyzed.

Fresh wet biomass of *Limnospira platensis*							
With centrifugation				Without centrifugation			
With cell disruption (WC-WCD)		Without cell disruption (WC-NCD)		With cell disruption (NC-WCD)		Without cell disruption (NC-NCD)	
T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8
0.1 g L^{-1}	0.5 g L^{-1}	0.1 g L^{-1}	0.5 g L^{-1}	0.1 g L^{-1}	0.5 g L^{-1}	0.1 g L^{-1}	0.5 g L^{-1}

$$GI\ (\%) = \frac{G\,x\,L}{G_W\,x\,L_W}\,x\,100 \quad (3)$$

where:

GI is the germination index (in percentage).

G is the number of germinated seeds for the treatments.

L is the length of the germinated seeds for the treatments.

G_W is the number of germinated seeds in the negative control.

L_W is the length of the germinated seeds in the negative control.

2.7 Reproducibility of results and statistical analysis

The experimental design guarantees consistent results. Four photobioreactors were used for each treatment to cultivate *Limnospira platensis* and assess its growth in pig slurry, yielding a total of 8 experimental units. Analyses were conducted in triplicate for each photobioreactor.

In applying *L. platensis* biomass extracts, eight treatments (T_1–T_8) were evaluated, each with four replicates, for thirty-two experimental units. Prism version 10.6.1 (GraphPad Software, Inc.®, CA, USA) was utilized for statistical analyses. A standard distribution analysis was conducted across treatments to identify the appropriate parametric test, employing the Lilliefors modification of the nonparametric Kolmogorov-Smirnov test. A one-way repeated measures ANOVA and a Tukey *post hoc* test were also performed.

3 Results

3.1 Identification of selected strain

The microalgae selected for this study were based on prior research in agro-industrial wastewater treatment (Álvarez and Otero, 2020; Álvarez et al., 2020). Favorable results were obtained using the modified DNA extraction protocol, particularly regarding the quality and concentration of the isolated DNA, which are suitable for PCR analysis and subsequent verification via electrophoresis. DNA quality assessment by 1% agarose gel electrophoresis revealed distinct bands with the 8F and 1492R primer pairs.

Once the sequences were obtained, they were aligned, and a consensus sequence was manually derived using MEGAX. The resulting consensus sequence was subsequently analyzed for DNA sequence similarity against the GenBank database using BLAST, revealing a match with a cyanobacterium and demonstrating homology ranging from 99.33% to 100% with cyanobacteria of the genus *Limnospira platensis* (see Table 4). The DNA sequence of the *L. platensis* isolate BioMicro-001 was uploaded to GenBank under accession PP700501 (https://www.ncbi.nlm.nih.gov/nuccore/PP700501.1).

Phylogenetic analysis confirmed the identification of *L. platensis* by obtaining a bootstrap value of 94 in a clade with most accessions identified as *L. platensis* or *Arthrospira platensis* (Figure 1).

3.2 Growth of *Limnospira platensis* cultivated on swine slurry

The growth of *Limnospira platensis* is illustrated in Figure 2. The findings indicate that cultivation of *L. platensis* in pig slurry was approximately 10% lower (1.34 ± 0.08 g L^{-1}) than in cultures using the standard Zarrouk medium (1.48 ± 0.08 g L^{-1}), both measured after 9 days of cultivation (Figure 2a). *Limnospira platensis* grown in Zarrouk exhibits an exponential phase between days one and six, while in pig slurries, a more linear growth is observed, without a marked exponential phase. An extended adaptation period was observed in cultures cultivated in pig slurry from days 2–7.

A notable distinction was observed in productivity, with results demonstrating a 17.76% increase in cultures growing with Zarrouk medium (1.07 ± 0.08) compared to those grown with pig slurry (0.88 ± 0.05) (Figure 2b).

3.3 Nutrient removal

As anticipated, the cultural conditions, including continuous airflow and a 12/12 light cycle, contributed to nutrient depletion in raw pig slurry. Nevertheless, *L. platensis* demonstrated superior nutrient removal capabilities and offered the additional benefit of generating biomass, which has potential application as a plant biostimulant.

The removal efficiencies (REs) under the specified culture conditions were as follows: COD 96.60%, NT 89.63%, PT 40.00%, ammonium 6.24%, and total coliforms 86.58%. For *L. platensis* subjected to identical parameters and sequence, the RE values were: 99.93%, 98.93%, 94.83%, with both ammonium and

TABLE 4 Blast analysis of the five most similar accessions from the GenBank database, based on the percentage identity of the 16S ribosomal RNA gene sequence derived from the isolate BioMicro-001 (GenBank accession number PP700501, queried on 2nd October 2025).

Organism	Isolate	Accession number	Query cover	E value	Identity
Arthrospira platensis	CBN3	KC195870	100%	0.0	100.00%
Limnospira platensis	RCC10818	PQ037850	100%	0.0	99.93%
Limnospira platensis	CENA597	CP185278	100%	0.0	99.93%
Arthrospira platensis	NIES-46	LC455668	100%	0.0	99.93%
Arthrospira platensis	NIES-39	AP026945	100%	0.0	99.93%

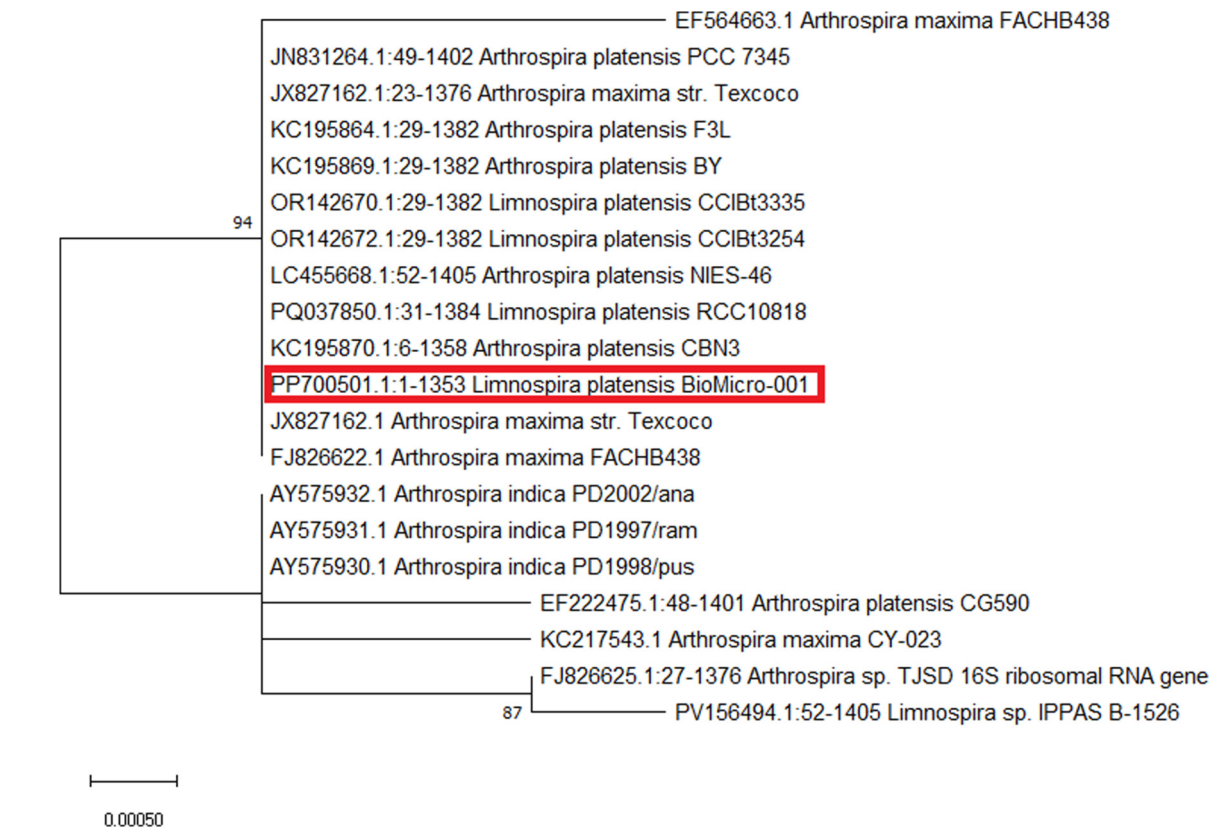


FIGURE 1
Phylogenetic tree of the *Limnospira platensis* strain. The result is highlighted in a red box.

total coliforms exhibiting 100% RE. The initial nutrient concentrations in pig slurries, along with the final concentrations following treatment and their corresponding standard deviations, are outlined in Table 5. When comparing removal efficiencies across culture conditions, *Limnospira platensis* demonstrated increases of 3.33% in COD, 9.30% in TN, 54.83% in TP, 93.76% in NH4+, and 13.42% in total coliforms. It is also noteworthy to highlight the potential application of biomass as a plant biostimulant within the matrix, as mentioned earlier.

A complete removal of ammonia was accomplished by *L. platensis*, despite the elevated concentration (292.80 ± 25.05 mM) of this compound in undiluted pig slurry.

3.4 Valorizing pig slurry–Biochemical composition, amino acids, nitrogen and phosphorus content, and hygienic safety of *Limnospira platensis* biomass

Microalgae are characterized by their capacity to recycle nutrients from agro-industrial wastewater and incorporate them into their metabolic processes to synthesize high-value compounds. In our research, promising results were obtained regarding the protein concentration of *L. platensis*. The protein content increased by 14.61% in cultures grown in pig slurry compared to those grown in the standard Zarrouk medium, reaching 402.94 ± 48.67 mg g^{-1} and 461.80 ± 10.66 mg g^{-1} in

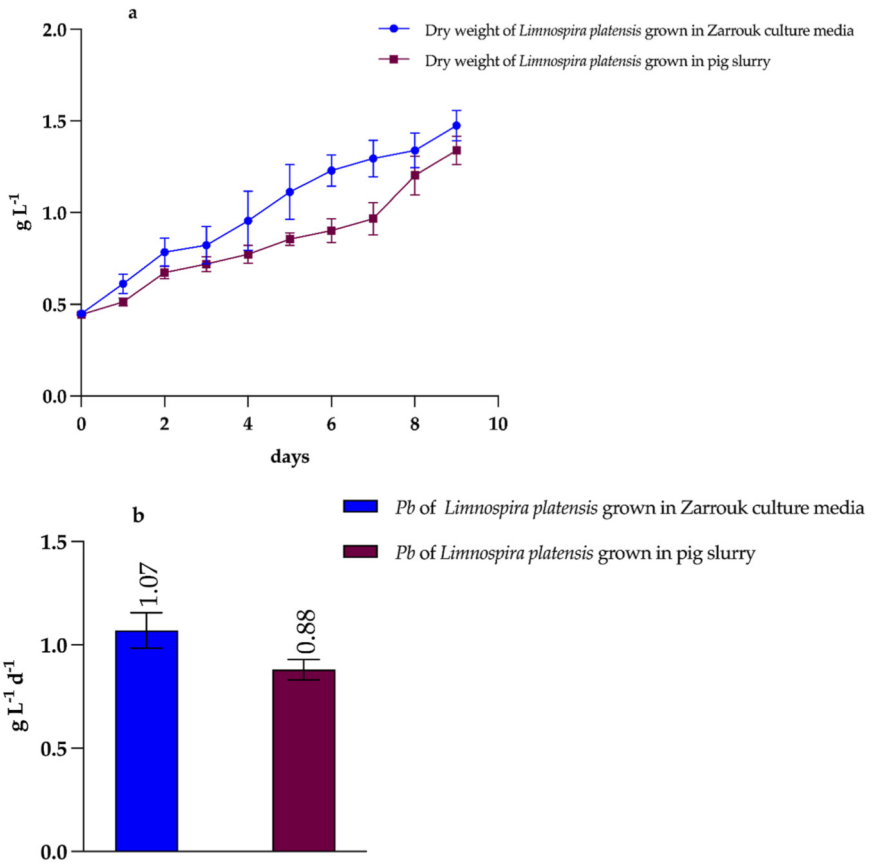


FIGURE 2
Growth of *Limnospira platensis*, measured by **(a)** dry weight, and **(b)** *Pb* (biomass productivity), grown in standard Zarrouk media and swine slurry. Data are presented as mean ± standard deviation (error bars), derived from four photobioreactors per treatment.

TABLE 5 Physicochemical and microbiological parameters of pig slurry with and without treatment by *Limnospira platensis*.

Parameter	Raw swine slurry	Swine slurry with culture conditions without *Limnospira platensis*	Swine slurry with *Limnospira platensis*
COD (mg L^{-1})	16,266.67 ± 450.90	552.33 ± 0.58	11.00 ± 2.00
TN (mg L^{-1})	1000.63 ± 0.30	103.67 ± 1.53	10.67 ± 1.15
TP (mg L^{-1})	60.56 ± 0.31	36.33 ± 1.15	3.13 ± 0.68
NH_4^+ (mg L^{-1})	134.54 ± 0.15	126.14 ± 2.06	-
pH	8.46 ± 0.15	8.22 ± 0.22	8.22 ± 0.01
Total coliforms (UFC mL^{-1})	1.17 ± 0.15 × 10^7	1.57 ± 0.04 × 10^6	-

the control and treated samples, respectively (see Figure 3). Conversely, carbohydrate levels decreased by 34.51% in cultures grown in pig slurry relative to those in the standard Zarrouk medium, with values of 337.77 ± 20.40 mg g^{-1} and 221.22 ± 16.76 mg g^{-1} for the control and treated samples, respectively (see Figure 3). Lipid concentrations did not differ significantly; consequently, the lipid fractions were measured at 184.53 ± 40.19 mg g^{-1} and 181.60 ± 5.71 mg g^{-1} for the control and treatment groups, respectively (see Figure 3).

In the electropherogram of the standard amino acid solution, the retention time for each amino acid is indicated by its position. The retention process is closely related to various factors, including the physicochemical properties of each amino acid (such as total charge and molecular weight), the pH of the assay buffer, the surfactant concentration, the temperature, the capillary dimensions (length and diameter), and, as previously noted, the derivatization parameters (Figure 4a). The identification of free amino acids present in the biomass of *L. platensis* was conducted by adding

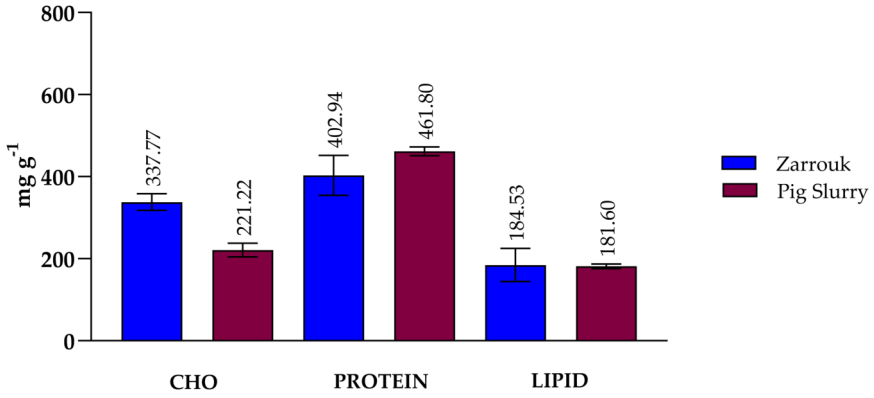


FIGURE 3
Biochemical composition of *Limnospira platensis* biomass with respect to carbohydrates (CHO), proteins, and lipids. Results are presented for cultures grown in Zarrouk standard medium and in pig slurry. Data are presented as mean ± standard deviation (error bars), derived from four photobioreactors per treatment.

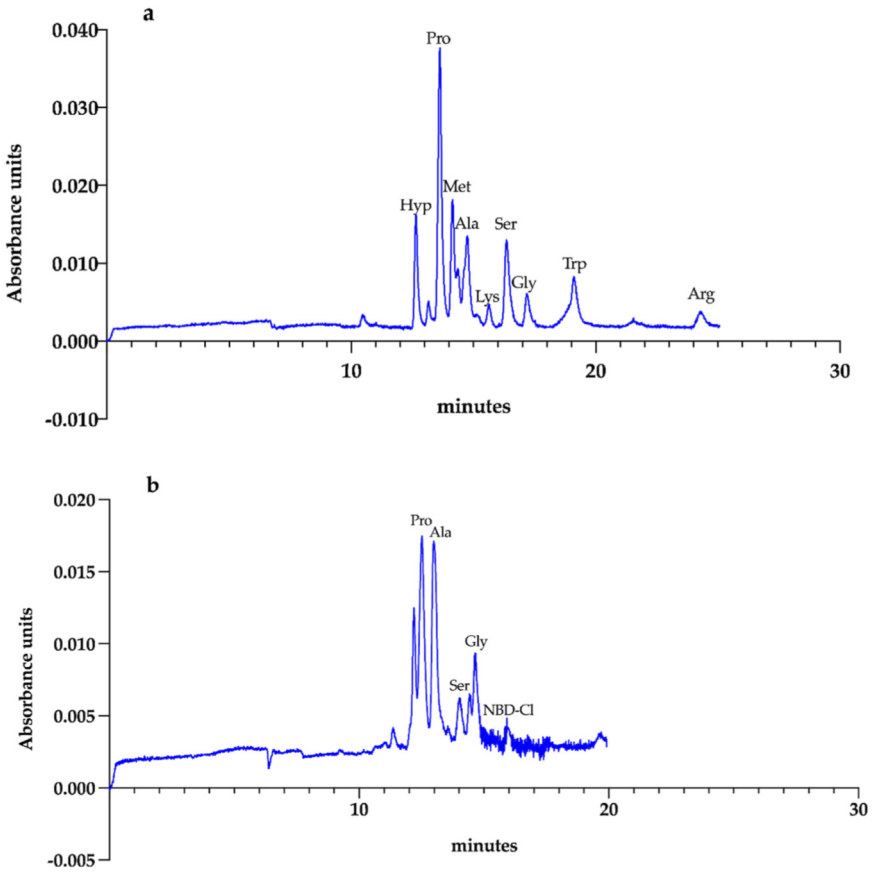


FIGURE 4
Amino acid profile electrophorograms obtained at pH 9.28. Derivatization conditions: NBD-Cl concentration: 20 mg mL, reaction time: 30 min, borate buffer 30 mM, reaction temperature: 60 °C. Peaks: **(a)** mix standard: Hyp, hydroxyproline; Pro, proline; Met, methionine; Ala, alanine; Lys, lysine; Ser, serine; Gly, glycine; Trp, tryptophan; Arg, arginine, **(b)** microalgae biomass: Pro, proline; Ala, alanine; Ser, serine; Gly, glycine.

peaks (free amino acid standards of known concentration) to each sample (Figure 4b).

Using the calibration curves previously prepared for each amino acid, the concentration of each amino acid in the biomass of *L. platensis* was determined. This was accomplished by adding spikes (standard free amino acids of known concentration) to each sample (Figure 5).

Regarding total nitrogen and phosphorus content (TN, TP), the results showed that *Limnospira platensis* biomass cultivated with pig

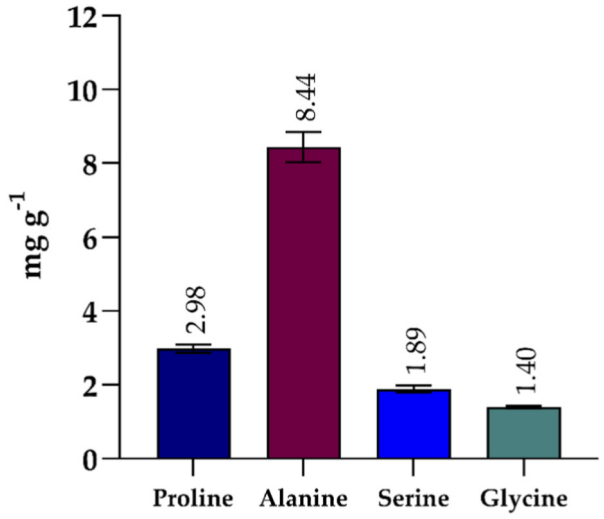


FIGURE 5
Concentration of free amino acids: Pro, proline; Ala, alanine; Ser, serine; Gly, glycine, determined by capillary electrophoresis in the biomass of *Limnospira platensis*.

slurry has a composition rich in essential macronutrients, with TN and TP concentrations of 87.64 ± 2.25 mg g^{-1} and 10.66 ± 0.62 mg g^{-1}, respectively (Table 6). Regarding total coliforms, the *L. platensis* biomass did not contain these pathogens.

3.5 Biostimulant effect of *Limnospira platensis* biomass cultivated on swine manure in *Cucumis sativus*

Evaluating different treatments and concentrations of *L. platensis* biomass grown in pig slurry as a biostimulant showed its potential to improve germination and morphological features in *Cucumis sativus* seedlings (Figure 6).

Regarding the germination index (GI), statistically significant differences ($p < 0.05$) were observed among all *L. platensis* treatments and the control. Notably, the most prominent effect was recorded in treatment T_2 (515.34% ± 81.81%), followed by T_6 (419.08% ± 69.75%), T_4 (249.25% ± 39.73%), T_1 (247.49% ± 65.07%), and T_8 (209.81% ± 27.17%). All these treatments employed a biomass concentration of 0.5 g L^{-1} (2 mg final concentration), except T_1. Conversely, treatment T_7 (169.07% ± 30.95%) at a concentration of 0.1 g L^{-1} (0.4 mg final concentration) displayed the most modest significant improvement, thereby illustrating the range of efficacy of the applications (Figure 6a).

Similar to the observations regarding germination, the number of shoots differed significantly ($p < 0.05$) across all treatments relative to the control. Treatments T_2 (2.20 ± 0.04), T_1 (1.83 ± 0.05), T_6 (1.77 ± 0.07), and T_5 (1.69 ± 0.04) demonstrated the highest rates of shoot production, whereas treatment T_7 again exhibited the lowest significant enhancement (1.35 ± 0.02). This consistency in results across multiple parameters further substantiates the biostimulant's efficacy (Figure 6b).

The biomass of *Limnospira platensis* fundamentally altered root development in *C. sativus*. The primary treatments, T_8, T_1, T_4, T_6, and T_2, served as potent stimulators of rhizogenesis, resulting in root systems with root densities 1.64, 1.69, 1.91, 2.00, and 3.50 times those of the control, respectively. This notable effect in the elite treatments was significantly greater than the moderate enhancements observed in other treatments, suggesting that signaling compounds in *L. platensis* substantially influence pathways regulating root growth. The pronounced response elicited by T_2 renders it an excellent candidate for elucidating the molecular mechanisms underlying this phenomenon (see Figure 6c).

4 Discussion

Molecular and ultrastructural analyses conducted by Nowicka-Krawczyk et al. (2019) distinguished the genus *Arthrospira* into two separate lineages: *Arthrospira jenneri*, initially described in 1892 by Stizenberger and Gomont (Gomont, 1893), and the genus *Limnospira*, encompassing the species formerly known as *A. fusiformis*, *A. indica*, and *A. maxima*, now designated as *L. fusiformis*, *L. indica*, and *L. maxima* (Nowicka-Krawczyk et al., 2019). Nevertheless, the authors did not classify *A. platensis* within the new genus *Limnospira* because of insufficient data for the type strain. In contrast, Rousell et al. (2023) developed a comprehensive database on all *Limnospira* strains, including newly isolated strains from 72 French sources. Employing a polyphasic approach—covering phylogenetic, phylogenomic, presence or absence of coding DNA sequences, morphological, and ultrastructural analyses—they confirmed that *A. platensis* is part of the genus *Limnospira* (*L. platensis* Gomont comb. nov. Basionym: *Arthrospira platensis* Gomont, 1893), and asserted that *Limnospira* is monospecific, represented solely by *L. platensis* (Rousell et al., 2023). In this study, molecular analysis of the BioMicro-001 strain was conducted using PCR and Sanger sequencing of the 16S ribosomal RNA gene. Morin et al. (2010) established an efficient protocol for isolating DNA from the genus *Arthrospira*. They compared it with other extraction protocols and determined that this optimized method enables greater DNA recovery. Furthermore, the extracted DNA exhibits a high molecular weight, reduced degradation, and excellent overall quality. Rousell et al. (2023) analyzed 92 new *Limnospira strains*.

TABLE 6 Composition of total nitrogen (TN), total phosphorus (TP), and total coliforms in the dry biomass of *Limnospira platensis* grown with pig slurry.

Parameter	Dry biomass of *Limnospira platensis* grown in pig slurry
TN (mg g^{-1})	87.64 ± 2.25
TP (mg g^{-1})	10.66 ± 0.62
Total coliforms (UFC g^{-1})	0.00

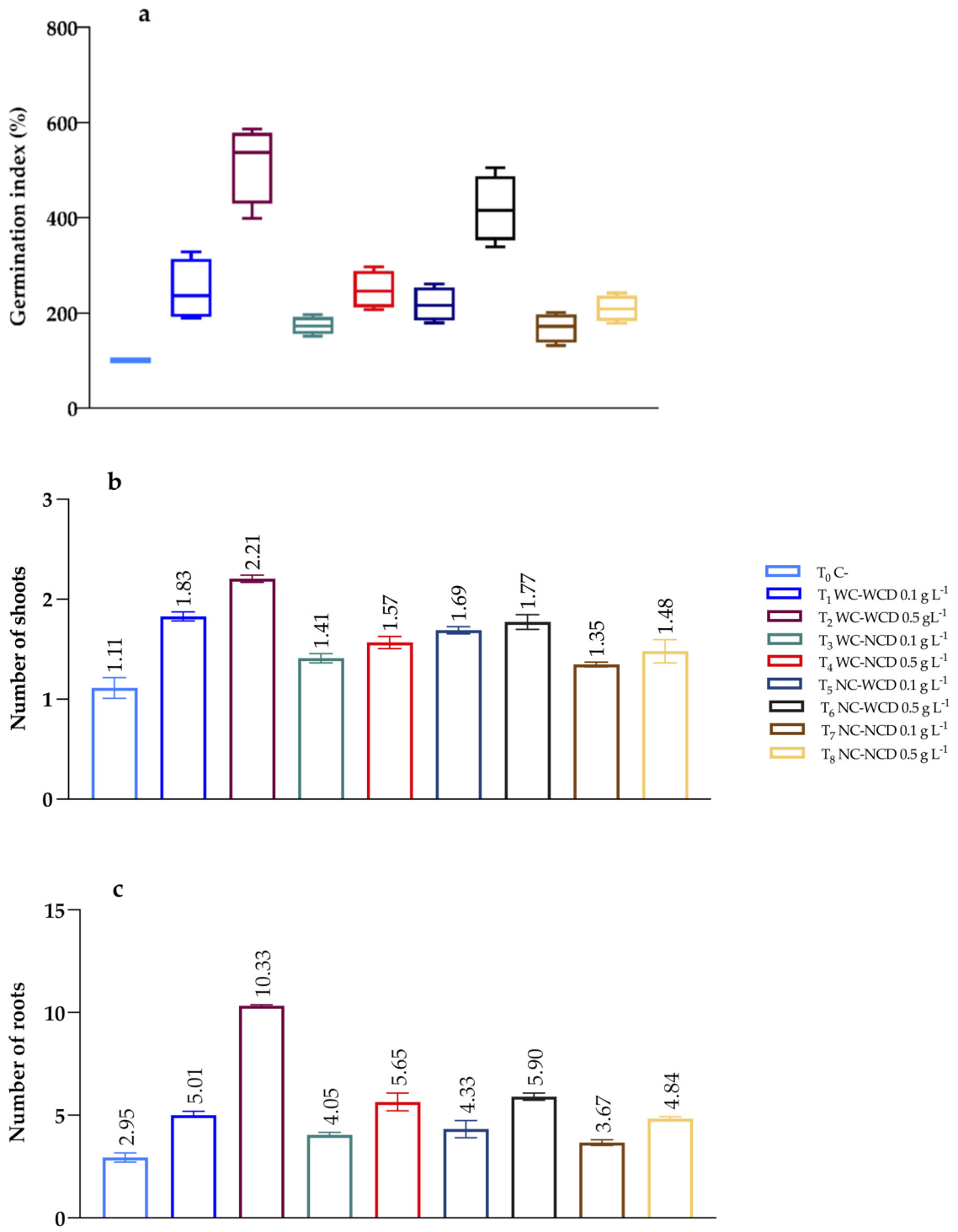


FIGURE 6
Germination index **(a)**, number of shoots **(b)**, and number of roots **(c)** of cucumber seeds (*Cucumis sativus*) with the application of fresh *Limnospira platensis* biomass under different post-treatments: T_0 C-: distilled water; T_1 WC-WCD: 0.1 g L^{-1}; T_2 WC-WCD: 0.5 g L^{-1}; T_3 WC-NCD: 0.1 g L^{-1}; T_4 WC-NCD: 0.5 g L^{-1}; T_5 NC-WCD: 0.1 g L^{-1}; T_6: NC-WCD 0.5 g L^{-1}; T_7 NC-NCD: 0.1 g L^{-1}; T8 NC-NCD: 0.5 g L^{-1}. Data are presented as the mean ± standard deviation (error bars) of four Petri dishes per treatment, each with 25 seeds. Key abbreviations (WC = with centrifugation; NC = no centrifugation; WCD = with cell disruption; NCD = no cell disruption).

PCR amplification of the 16S rRNA gene was performed using primers 8F (Lane, 1991) and 920R (Gugger and Hoffmann, 2004); 861F (Gugger and Hoffmann, 2004) and 1492R (Lane, 1991). They confirmed that the species *A. platensis* belonged to the genus *Limnospira* and that *Limnospira* was monospecific, represented only by *L. platensis* (Rousell et al., 2023).

The growth of *Limnospira platensis*-BioMicro-001 in pig slurry demonstrated its ability to thrive in this wastewater type, highlighting its potential for wastewater treatment and cost-effective biomass production. Although growth is comparatively slower and the yield is lower than that obtained in cultures on the standard Zarrouk medium, this medium remains optimal for growth. Previous studies have examined the utilization of microalgae to treat pig manure. For example, Ferreira et al. (2018) used *Scenedesmus obliquus* to remove nutrients from various wastewaters, including effluents from poultry, swine, and livestock industries, as well as from brewing and dairy industries, and from urban sources, to generate biomass suitable for bioenergy production. In their investigation, microalgae were cultured in 0.8 L bubble-column photobioreactors, with wastewater diluted to 5% (v/v) with tap water. The observed productivity was 0.3 g L^{-1} day^{-1} (Ferreira et al., 2018). Kwon et al. (2020) cultivated *Chlorella vulgaris* in raw pig slurry (PW), and its digestion was followed by anaerobic digestion (AD). Both wastewaters were filtered through a 2 mm mesh and subjected to pretreatment, including: PW with air stripping (PW_S), AD with sodium hypochlorite (AD_C), AD with air stripping (AD_S), and AD with combined air stripping and NaOCl (AD_M). All samples were diluted by a factor of ten before cultivation. The most favorable growth outcomes were observed with PW_S, which achieved a biomass concentration of 3.98 g L^{-1}, a specific growth rate of 0.204 $days^{-1}$, and a productivity of 0.57 g L^{-1} day^{-1} (Kwon et al., 2020). Huang et al. (2024) cultivated *Chlorella* sp. YSD-2 in various dilutions of digestate derived from anaerobic digestion of swine manure (biogas slurry), specifically at dilutions of 1:1, 1:2, 1:3, and 1:4. The maximum growth was recorded at a 1:2 dilution (1.84 g L^{-1}). In comparison, the minimum was observed at 1:4 (0.91 g L^{-1}) (Huang et al., 2024). The present study demonstrates that *Limnospira platensis*-BioMicro-001 can be cultivated directly in raw, undiluted pig slurry with only simple filtration as pretreatment, achieving a substantial biomass productivity of 0.88 g L^{-1} day^{-1}. This performance exceeds the yields reported in similar systems, nearly tripling the 0.3 g L^{-1} day^{-1} obtained by *S. obliquus* cultivated in highly diluted (5% v/v) pig manure. It surpasses the 0.57 g L^{-1} day^{-1} achieved by *C. vulgaris*, even after extensive pretreatment and a 1:10 dilution. Although the maximum biomass concentration (1.34 g L^{-1}) was lower than reported in some studies, it was achieved in a notably more concentrated and challenging medium than those used in other studies, such as the 1:2 dilution required for optimal growth of *Chlorella* species. These findings underscore a significant advantage of the proposed methodology: by avoiding energy- and water-intensive pretreatments and dilution steps, cultivating *L. platensis*-BioMicro-001 directly in raw pig slurry presents a more straightforward, resource-efficient, and economically beneficial strategy for simultaneous wastewater bioremediation and high-value biomass production. Martínez-Hernando et al. (2026) used digestate derived from the anaerobic digestion of pig slurry to cultivate microalgal biomass. The digestate incorporated into the photobioreactor is diluted with water before being introduced as a nutrient source for the microalgae. The relatively small volume of digestate introduced into the raceway is determined based on the nutritional requirements—specifically nitrogen and phosphorus—necessary to sustain microalgal productivity. The raceway, a shallow pond spanning 1500 square meters, serves as the cultivation platform for microalgae, which are subsequently employed in the absorption column for biogas conditioning. Within this column, a counterflow process involving biogas containing approximately 70% methane and circulating microalgae facilitates carbon dioxide absorption, thereby producing a biogas stream enriched in methane and an algal biomass stream used as fertilizer (Martínez-Hernando et al., 2026).

The utilization of microalgae to recycle nutrients from nutrient-rich agro-industrial wastewater constitutes a significant paradigm shift in environmental management, transitioning from costly wastewater treatment processes to a circular bioeconomy model. Microalgae, such as *Chlorella*, *Limnospira*, and *Scenedesmus*, flourish in nutrient-rich wastewater environments, efficiently assimilating nitrogen, phosphorus, and organic pollutants while concurrently sequestering carbon dioxide. This process mitigates risks associated with water pollution and eutrophication, yielding valuable biomass suitable for applications such as biofertilizers, animal feed, and biofuels. Advances in photobioreactor technology and strain optimization have rendered microalgae-based systems scalable and solar-powered, providing innovative solutions to challenges in water security and agricultural sustainability. In regions such as Latin America, characterized by expanding agribusiness sectors, this technology offers potential benefits, including reduced operational costs, lowered carbon footprints, and the development of new value chains—transforming wastewater from an environmental liability into a vital resource for green growth (Gupta et al., 2025).

Correspondingly, nutrient recycling employing *L. platensis* in pig slurry has demonstrated effectiveness. At the same time, culture conditions facilitated the removal of chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP), ammonium (NH_4^+), and total coliforms, the microalgal contribution significantly enhanced nutrient recycling and bioremediation efficiency, achieving removal rates of 99.93%, 98.93%, 94.83%, 100.00%, and 100.00%, respectively. *Limnospira* is an alkaliphilic cyanobacterium distinguished by its capacity to tolerate and utilize ammonium at elevated concentrations (NH4+/NH3), a feat uncommon among many other photosynthetic microorganisms. This characteristic is attributable to its elevated internal pH, which restricts intracellular ammonium accumulation and thereby averts the uncoupling of photosynthesis (Abeliovich and Azov, 1976). Two factors influenced the cultivation of *L. platensis* in undiluted pig slurry: the first was the strain's adaptation period to increasing slurry concentrations, and the second was its ability to utilize ammonia as the sole nitrogen source even at pH 10 and above (Boussiba, 1989), in contrast to the general idea that ammonia is toxic to photosynthesis at high pH values (Abeliovich and Azov, 1976). Ammonia uptake into cells depends on △pH and is limited by a relatively high average internal pH. This high pH appears to be maintained primarily by an elevated intrathylakoid pH (Belkin and Boussiba, 1991). Various microalgae species have been utilized in pig slurry treatment; for example, Wang et al. (2017) employed pig slurry pretreated via centrifugation (9000 rpm for 5 minutes), sterilization (121 ˚C for 15 minutes), and pH adjustment (7.5). They cultivated *Neochloris aquatica* CL-M within 200 mL glass photobioreactors containing the pretreated slurry under light intensities ranging from 50 to 200 μmol photon m^2 s^{-1}. Optimal removal efficiencies were observed at 150 μmol photon m^2 s^{-1}, with COD removal at 66.5%, nitrate nitrogen (NO_3-N) at 99.8%,

phosphate (PO_4-P) at 100%, and ammonium (NH_4^+) at 79.2%. Subsequently, the biomass was hydrolyzed and fermented with *Clostridium acetobutylicum* ATCC 82 to produce butanol (Wang et al., 2017).

Morillas-España et al. (2021) evaluated scale-up processes for pig slurry treatment employing cyanobacteria species *Anabaena* sp. (BEA-0912B) and *Dolichospermum* sp. (BEA-0866B). Initially, both cyanobacteria were cultivated in 0.3 L bubble column photobioreactors. Later, an 80 L pH-controlled bubble column photobioreactor located within a greenhouse served as an inoculum source for two raceway reactors, one of 1.04 m^3 and the other of 7.13 m^3. These reactors treated the pig slurry with a mixture of 5% filtered slurry and 95% tap water, supplemented with 30 g L^{-1} NaCl. The experiments were conducted semi-continuously at dilution rates of 0.1, 0.2, or 0.3 $days^{-1}$. Findings indicated that *Anabaena* sp. and *Dolichospermum* sp. could be successfully cultivated outdoors using pig slurry as the sole nutrient source, with nitrogen and phosphorus removal rates comparable to other microalgal strains, and biomass productivity aligned with expectations for raceway reactor systems. Remarkably, the cultivation of *Limnospira platensis*-BioMicro-001 in raw, undiluted pig slurry with minimal filtration achieved excellent nutrient recycling—surpassing the performance of more complex treatment approaches—with removal efficiencies of 99.93% for COD, 98.93% for TN, 94.83% for TP, and complete removal (100%) of NH_4^+ and total coliforms. These results underscore a strong bioremediation capacity, in contrast to other methodologies. For instance, Wang et al. (2017) employed energy-intensive pretreatments, such as centrifugation and sterilization, but observed lower removal rates of 66.5% for COD and 79.2% for NH_4^+ in undiluted slurry. Additionally, Morillas-España et al. (2021) successfully scaled cyanobacterial cultivation, which requires high dilution (5% suspension), salt addition, and advanced control of the semi-continuous reactor processes. The current study demonstrates that comparable high levels of nutrient removal, especially the complete recycling of NH_4^+, can be achieved using a considerably more straightforward, more resource-efficient method. Consequently, cultivating *L. platensis*-BioMicro-001 in undiluted crude pig slurry offers a highly effective bioremediation approach and a more direct, economically viable pathway for valorizing swine wastewater.

The biochemical composition of microalgae is variable and not fixed. Modifying the nutrient profile of the culture medium is an effective way to manipulate it. This approach constitutes a valuable tool for "biomass adaptation" to particular markets. The study distinctly demonstrates the inverse relationship between proteins and carbohydrates. Future research may consider a two-phase approach: initially, a nitrogen-rich stage in wastewater to produce biomass and proteins, followed by a nitrogen-deficient stage to enhance lipids or carbohydrates, if required, for a targeted product profile. Alvarez and Otero (2020) observed a comparable deviation in carbon flow during the cultivation of *Arthrospira* sp. within the central region of anaerobic digestion of agro-industrial effluents. They reported a protein concentration of 49.0%, compared to 33.3% obtained using the standard Zarrouk culture medium (Alvarez and Otero, 2020). In summary, these results are not merely a collection of data, but a coherent narrative. They offer substantial evidence that *L. platensis*-BioMicro-001 efficiently bioremediates nitrogen-rich pig slurry wastewater by assimilating surplus nitrogen into its cellular proteins. This results in high-quality, protein-enriched biomass, rendering the process environmentally sustainable and economically viable. The notable shift in composition from carbohydrates to proteins represents a well-established and anticipated metabolic response, thus affirming the scientific principles underlying the experiment.

Using microalgal biomass as a plant biostimulant cultivated in agro-industrial wastewater offers a transformative approach to sustainable agriculture and the circular economy. By employing wastewater as a growth medium, microalgae such as *Chlorella*, *Limnospira*, and *Scenedesmus* effectively recycle nutrients, including nitrogen and phosphorus, while producing biomass rich in bioactive compounds, including phytohormones, free amino acids, and antioxidants. These compounds enhance crop seed germination, root development, and nutrient uptake, thereby reducing reliance on chemically synthesized fertilizers and improving soil health and plant resilience to abiotic stress. Given the increasing global demand for food and the scarcity of water, this strategy mitigates wastewater treatment costs and provides a sustainable source of high-value bioproducts. Expanding the use of microalgae-based biostimulants could revolutionize farming practices by integrating environmental management with agricultural productivity in regions such as Latin America, where agriculture and agroindustry coexist. The biostimulant effect of *L. platensis*-BioMicro-001 in enhancing germination and morphological characteristics of *Cucumis sativus* seedlings is attributed to the presence of four identified free amino acids: proline (Pro), alanine (Ala), serine (Ser), and glycine (Gly). Amino acids are recognized as biostimulants-substances that foster plant growth, augment nutrient availability, and enhance plant quality (Rouphael and Colla, 2018; Rouphael et al., 2018). Their importance is increasingly recognized, not only for alleviating damage caused by abiotic stress (Kowalczyk et al., 2008), but also because they serve as precursors to hormones (Zhao, 2010; Maeda and Dudareva, 2012; Calvo et al., 2014; Rouphael and Colla, 2018), signaling molecules involved in various physiological processes, such as glutamate receptors (GRLs) (Maeda and Dudareva, 2012; Calvo et al., 2014; Forder and Roberts, 2014), as well as regulators of nitrogen uptake (Miller et al., 2007), root development (Walch-Liu and Forde, 2007; Calvo et al., 2014; Halpern et al., 2015; Weiland et al., 2016), and antioxidant metabolism (Ertani et al., 1956; Calvo et al., 2014; Halpern et al., 2015; Teixeira et al., 2017). Enhanced root development, facilitated by amino acid supplementation, can increase nitrogen fixation, thereby expanding the root surface area available for nutrient absorption (Hildebrandt et al., 2015; Weiland et al., 2016). Derrien et al. (1998), using HPLC separation of fluorescent *o*-phthalaldehyde (OPA) derivatives, applied this method to the analysis of free amino acids from five microalgae commonly used in aquaculture: *Tetraselmis suecica*, *Skeletonema costatum*, *Chaetoceros calcitrans*, *Thalassiosira* sp., and *Isochrysis galbana*. As part of an evaluation of their potential use in cosmetic products, they determined the following free amino acids: aspartic acid, glutamic acid, asparagine, serine, arginine, tyrosine, valine, phenylalanine, isoleucine, leucine, and lysine (Derrien et al., 1998). Araya et al. (2021) incorporated *Arthrospira* sp. Into the analysis of free amino acids, which were

also detected in the subsequent species: *Haematococcus pluvialis*, *S. costatum*, *Acutodesmus acuminatus*, and *Botryococcus braunii*, employing an automated reverse-phase HPLC-DAD precolumn analytical technique. They established that *Arthrospira* sp. exhibits the most comprehensive free amino acid profile (Araya et al., 2021). Therefore, Oancea et al. (2013) evaluated the biostimulant effect of *Nannochloris* sp. 424-1. The biomass concentrate was homogenized using a high-pressure piston homogenizer and hydrolyzed with lytic enzymes, including β-glucanase, cellulases, and proteases. Following this, the mixture was sonicated for 45 min at 400 W and 45 °C. Ultimately, the cell lysate was centrifuged. Two foliar applications, each containing 10 mg of the supernatant (on the 2nd and 29th days after repotting), were applied to each tomato plant, some under water stress and others without (20 individuals per treatment). In plants without water stress, the biostimulant promoted greater root length development (+108.08%), a greater number (+120.31%), and area of leaves (+105,16%) than the control. Furthermore, applying the biostimulant mitigated the effects of water stress on the tomato plants, increasing their height by more than 10% compared to the control (Oancea et al., 2013).

Ronga et al. (2019) employed freeze-dried biomass extracts from various microalgae, including *Arthrospira platensis* cultured on Zarrouk standard medium, *Chlorella vulgaris* and *Scenedesmus acuminata* cultivated on MBBM standard medium (Bold, 1949; Bischoff and Bold, 1963), as well as *Isochrysis galbana*, *Nannochloropsis gaditana*, *Porphyridium cruentum*, and *Tetraselmis suecica* grown on Guillard standard medium (F/2) (Guillard and Ryther, 1962; Guillard, 1975). The investigation focused on assessing the biostimulant effects of the treatment on *Nasturtium officinale* seeds. The experiment was conducted under conditions comparable to our study; however, a concentration of 50 mg L^{-1} of freeze-dried biomass was used, with 4 mL of this solution administered, resulting in a final concentration of 0.2 mg per individual unit evaluated. The highest germination index (GI%) values were recorded for the *A. platensis* and *P. cruentum* extracts, at 152.99 ± 2.00 and 147.98 ± 3.00, respectively. Conversely, the lowest GI% values were observed with *S. acuminata* and *T. suecica* extracts, at 97.30% ± 7.2% and 90.56% ± 13.32%, respectively (Ronga et al., 2019). Álvarez-Montero et al. (2025) assessed extracts and cultures of *Scenedesmus* sp. on *Phaseolus vulgaris*. The microalga was cultivated in dairy wastewater; both treatments used a cell concentration of 4.4 ± 0.1 × 10^7 cells mL^{-1}. For treatments involving *Scenedesmus* sp. cell extract, ultrasound pulses (Portable Ultrasonic Cell Disruptor, UCD-P01, Biobase©) operating at 20 kHz were applied for 20 min at 4 °C to rupture cells and release intracellular components of the microalga. In the culture treatment, the microalgae were cultivated in the exponential growth phase without any preliminary treatment. Both experimental procedures followed methodologies similar to those of our study.

The highest germination index (GI%) was recorded in the extracts (305.81 ± 53.62), whereas the culture of *Scenedesmus* sp. exhibited a GI% of 219.07 ± 52.58 (Álvarez-Montero et al., 2025). Our research findings unequivocally establish that aqueous extracts of *L. platensis*-BioMicro-001 biomass serve as a potent biostimulant for *C. sativus*, substantially enhancing key physiological parameters associated with germination and early seedling growth. The effectiveness of the treatments varied and was markedly influenced by the concentration and potentially by the algal biomass processing method, thereby demonstrating a well-defined dose-response relationship. The most substantial evidence of the biostimulant's effectiveness is the notable rise in the Germination Index (GI). All treatments with *L. platensis*-BioMicro-001 significantly outperformed the control, highlighting the extract's potent bioactivity. The outstanding performance of treatments T_2 (515.34%) and T_6 (419.08%), both at a concentration of 0.5 g L^{-1}, suggests this concentration is particularly effective at activating physiological processes that accelerate and coordinate germination. This effect is much more significant than reported in a similar study by Ronga et al., in 2019, in which the highest germination percentage (GI%) for *Nasturtium officinale* seeds treated with *A. platensis* (a synonym for *L. platensis*) was 152.99%. The GI values in our study, which exceeded 500% in the most effective treatment, suggest that our extraction or application method may have successfully mobilized or concentrated compounds in the cyanobacterial biomass that promote germination. This positive influence extended beyond germination, encompassing post-germination growth, as evidenced by the significant increase in shoots. The consistency in treatment performance ranking between germination index (*GI*) and shoot number—with T_2, T_1, and T_6 consistently among the highest performers—strengthens the reliability of the observed biostimulant effect. This correlation suggests that compounds that stimulate germination also contribute to vigorous early vegetative development, possibly by enhancing meristematic activity or providing essential growth precursors. We now emphasize that free amino acids are recognized not only as fundamental building blocks but also as crucial signaling molecules and osmolytes. Proline serves as a prototypical osmoprotectant and stress indicator (hydric and salinity stress), also have a positive impact on the plant's growth and development (Yao et al., 2020; Trovato et al., 2021; Alfosea-Simon et al., 2021); glycine functions as a precursor to chlorophyll and a constituent of glutathione (a major antioxidant), also increase the activity of the antioxidant enzymes (peroxidase, phenylalanine ammonia-lyase, polyphenol oxidase) (Teixeira et al., 2017); and serine participates in photorespiration, stress signaling pathways, stem elongation, synthesis of specialized metabolites for plant defense, responses to biotic and abiotic stress, and precursor of amino acid biosynthesis (tryptophan); phospholipids; sphingolipids (Yao et al., 2020; Kishor et al., 2020; Trovato et al., 2021; Kawade et al., 2023). Alanine is involved in the development of seeds and roots (Yao et al., 2020; Trovato et al., 2021; Alfosea-Simon et al., 2021). Elevated levels of these amino acids can directly account for the improved germination vigor and root development observed under abiotic stress, potentially induced by the extract medium. The synergistic effect of this particular amino acid mixture, released in high concentration during the cell disruption step in T_2, provides a comprehensive theoretical explanation for the observed bioactivity. This aligns with the mechanism of action of commercial plant biostimulants derived from protein hydrolysates, whose effectiveness is primarily attributed to their specific amino acid and peptide composition (Ertani et al., 1956; Colla et al., 2017). Furthermore, the substantial increase in total protein content in biomass cultivated in wastewater (Figure 3) indicates a reservoir of potential bioactive peptides. Ultrasonic

disruption—a defining feature of the high-performing T_2 treatment—efficiently hydrolyzes these proteins, thereby enhancing the bioavailability of free amino acids and short-chain peptides that function as signaling molecules (Guo et al., 2020). Consequently, integrating protein-enriched biomass with a processing step that releases its intracellular constituents yields a highly effective biostimulant formulation.

The most significant effect of the *L. platensis*-BioMicro-001 extract is observed in the development of the root system. The treatments, particularly T_2, which facilitated a root system 3.5 times denser than the control, served as potent stimulators of rhizogenesis. This considerable increase in root density is essential for plant establishment, as it enhances water and nutrient uptake, thereby increasing resilience to abiotic stressors. This observation aligns with, and in some cases exceeds, the findings reported by Oancea et al. (2013), who documented a 108% increase in root length in tomato plants treated with a biostimulant derived from *Nannochloris* sp. The notable root proliferation observed in our elite treatments suggests that *L. platensis*-BioMicro-001 contains signaling compounds, such as phytohormones (e.g., auxins, cytokinins) or betaines, that significantly influence molecular pathways regulating root growth and architecture. The pronounced response elicited by T_2 makes it an excellent candidate for future investigations aimed at elucidating these specific molecular mechanisms. The dose-dependent efficacy of the biostimulant constitutes a significant finding. Treatment T_7, administered at the minimal concentration of 0.1 g L^{-1}, corresponding to a final concentration of 0.4 mg, consistently demonstrated the most modest enhancements across all evaluated parameters. This indicates that a threshold concentration of bioactive compounds is necessary to elicit a substantial physiological response. The superior performance observed at 0.5 g L^{-1}, with a final concentration of 2.0 mg (T_2, T_4, T_6, T_8), compared with other treatments, suggests that this concentration may be optimal for maximizing the biostimulant effect under the specific conditions of this study. Most notably, our findings demonstrate a significant advancement over the recent research by Álvarez-Montero et al. (2025), who reported a germination index (GI) of 305.81% using *Scenedesmus* sp. extracts on *Phaseolus vulgaris*. While their results are commendable and substantiate the potential application of microalgae extracts derived from wastewater—specifically from the dairy industry—the superior performance observed in our T_2 treatment (515.34%) underscores the enhanced biostimulatory efficacy of *L. platensis*-BioMicro-001 biomass cultivated on pig slurry. This direct comparison suggests that the specific combination of cyanobacterial species (*Limnospira*) and the nutritional composition of pig slurry may be particularly effective in producing a diverse array of compounds conducive to germination. The notable improvement can primarily be attributed to two critical factors: the precise final concentration used and, more significantly, the potential enrichment of *L. platensis*-BioMicro-001 biomass with biostimulants through cultivation in nutrient-dense pig slurry, which may have facilitated the accumulation of stress-related bioactive compounds.

The most effective biostimulant treatment identified in this study, T_2, which combined a biomass concentration of 0.5 g L^{-1} with centrifugation and ultrasonic cell disruption, achieved a remarkable germination index (*GI*) of 515.34% in *C. sativus*. While this laboratory-scale protocol demonstrates exceptional efficacy, its economic viability and scalability for large-volume applications remain valid considerations, given the additional energy and equipment required for the disruption step. A holistic cost-benefit analysis must, therefore, extend beyond simple operational costs to encompass the value created by the superior product and the realities of industrial bioprocessing. The fundamental operational units in T_2—centrifugation and cell disruption—are not innovative laboratory techniques (Navarro-López et al., 2020) but are well-established in industrial biotechnology. High-throughput disc-stack centrifuges or filtration equipment are routinely employed for large-scale microalgal biomass harvesting in wastewater treatment and biofuel sectors, capable of processing hundreds of liters per hour (Show et al., 2017). Similarly, cell disruption via high-pressure homogenization (HPH) is a standard, scalable technology for extracting intracellular compounds from microbial and algal biomass across the food, pharmaceutical, and bioproduct industries (Chisti and Moo-Young, 1986; Harrison, 1991). For example, industrial bead mills and high-pressure homogenizers are frequently utilized for the commercial production of bioactive extracts from yeast and microalgae (Gupta et al., 2025). Consequently, the primary technical obstacle is not feasibility but optimizing these processes to accommodate the specific rheological properties of *Limnospira platensis* biomass. A pragmatic scale-up pathway would involve integrated processing: continuous centrifugation of the algal culture, followed by high-pressure homogenization of the resulting paste or concentrated slurry. This approach minimizes the volume subjected to the most energy-intensive step (homogenization). Future techno-economic analysis (TEA) based on pilot-scale data is the essential next step to quantify these parameters precisely. However, the foundational principle is clear: the exceptional biostimulant activity (*GI* > 500%, root proliferation increase of 3.5x) generated by T_2 establishes a strong value foundation. This high-value output can economically sustain the integration of scalable disruption technologies, aligning with the circular bioeconomy model in which waste treatment is synergistically coupled with the production of high-value agri-inputs (Rouphael and Colla, 2018; Gupta et al., 2025).

5 Conclusion

This study thoroughly and convincingly demonstrates the viability of a circular economy model for swine waste management utilizing the cyanobacterium *Limnospira platensis* BioMicro-001. We have not only confirmed the molecular identity of the strain but also demonstrated its remarkable resilience and efficacy in bioremediating raw, undiluted swine manure, achieving near-complete removal of nutrients and pathogens while producing substantial biomass. Beyond mere decontamination, this research enhances the value of the resulting biomass and converts it into a potent biostimulant. Aqueous extracts of *L. platensis*, particularly treatment T_2, induced an unprecedented biostimulant effect in *C. sativus*, characterized by a germination rate exceeding 500% and

significantly increased root proliferation. A rich composition of free amino acids and other bioactive compounds likely mediates this effect. Collectively, our findings present not merely an alternative wastewater treatment method but a synergistic solution that simultaneously addresses three critical global challenges: environmental pollution from agro-industrial waste, the increasing scarcity of water through nutrient recycling, and the demand for sustainable agricultural inputs. The capacity to cultivate a highly effective biostimulant from waste, without the need for costly pretreatments or dilutions, positions this technology as a scalable, economically viable, and environmentally sustainable strategy. Consequently, this work establishes the foundation for a new generation of rural biorefineries, where waste is no longer an issue to be managed but a fundamental resource for regenerative agriculture and a circular bioeconomy.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, PP700501.

Author contributions

XÁ-M: Conceptualization, Writing – review & editing, Methodology, Investigation, Writing – original draft, Data curation, Formal Analysis. IM-R: Writing – review and editing, Methodology, Writing – original draft, Data curation, Investigation. LJ-S: Methodology, Investigation, Writing – review and editing, Writing – original draft. LJ-G: Methodology, Writing – review and editing, Writing – original draft, Investigation. WC-C: Investigation, Writing – review and editing, Writing – original draft, Methodology. GU-M: Writing – original draft, Investigation, Writing – review and editing, Methodology. AR-M: Writing – review and editing, Methodology, Writing – original draft, Investigation. ES-O: Data curation, Writing – original draft, Methodology, Investigation, Conceptualization, Writing – review and editing.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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