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Effect of Non-Microbial Biostimulants on Metabolome, Quality Traits, and Shelf-Life of Lettuce Under Field Conditions

Alejandro Castro-Cegrí[1] · Maria Chiara Fontanella[1] · Giulia Fappani[1] · Manuela Delpero[2] · Stefano Bergaglio[2] · Tito Caffi[1,3] · Luigi Lucini[1,4]

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Abstract
Plant-based biostimulants are sustainable tools widely applied in agriculture to enhance plant physiology and stress resilience. Shelf-life extension has a pivotal role in postharvest as low-temperature storage of lettuce (*Lactuca sativa* L.) often results in weight loss, surface damage, and a decline in quality and nutritional value. This study investigated the metabolic responses of lettuce to three commercial biostimulants, *Ascophyllum nodosum* extracts (Algae), plant-derived protein hydrolysate (PHs), and humic substances (Hum), applied via foliar spray (-S) and drip irrigation (-D). All treatments improved agronomic traits, with foliar-applied PHs (PHs-S) showing the most pronounced effects. Metabolomic profiling revealed treatment-specific shifts, particularly in secondary metabolites such as alkylphenols, flavonoids, and flavonoid glucosides, along with increased levels of sugars, polyols, vitamins, and phytohormones. In parallel, biostimulants positively influenced key agronomic parameters during lettuce growth, including plant diameter, chlorophyll content, and nitrogen balance index, with PHs applied via foliar spray showing the most pronounced effects. At harvest, these metabolic changes reflected a broader metabolic reprogramming, which appeared to contribute to improved postharvest traits, including reduced weight loss, enhanced leaf firmness, increased antioxidant capacity, and higher total phenolic content, particularly under Algae-D and PHs-S treatments. These biochemical changes suggest that biostimulants, particularly via foliar application, enhance antioxidant capacity, improve nutritional quality, and may contribute to extended shelf life in lettuce.

Keywords *Lactuca sativa* · Food quality · Untargeted metabolomics · Texture · Phenolic compounds.

Introduction

Lettuce (*Lactuca sativa* L.), one of the most widely cultivated leafy vegetables worldwide, is appreciated for its nutritional value, low-calorie content, and versatility in human diets. It belongs to the Asteraceae family and is commonly consumed fresh. Lettuce is a source of health-promoting compounds, including fiber, phenolics, flavonoids, carotenoids, and essential vitamins (A, C, E) and minerals like iron and calcium (Ahmed et al. 2021; Kim et al. 2016). In recent years, there has been a significant increase in the availability of products with a biostimulant effect on plants in the market. In the European Union, legislation has already been established to define and regulate the use of plant biostimulants in agriculture (Regulation (EU) 2019/1009).

Biostimulants derived from the brown seaweed *Ascophyllum nodosum* (Algae) have shown promising performance. They contain high amounts of polysaccharides and

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Alejandro Castro-Cegrí and Maria Chiara Fontanella have contributed equally.

✉ Alejandro Castro-Cegrí
alejandro.castrocegri@unicatt.it

[1] Department for Sustainable Food Process, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, Piacenza 29122, Italy

[2] ANADIAG Italia, Strada Comunale Savonesa, 9, Tortona 15097, AL, Italy

[3] Research Center on Plant Health Modelling (PHeM), Università Cattolica del Sacro Cuore, Piacenza 29122, Italy

[4] Institute of Bioimaging and Biological Complex Systems (IBSBC), National Research Council (CNR), Milan 20054, Italy

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other substances, such as phenolics, amino acids, phytohormones, alginates, minerals, and trace elements, having biostimulant properties that support plant physiological processes and improve stress tolerance (Chrysargyris et al. 2018; Drygaś et al. 2024).

Another sustainable and effective strategy to enhance the growth, nutritional quality, and stress resilience of lettuce is the use of protein hydrolysates (PHs) derived from plant sources. Plant-based PHs are composed of amino acids and small peptides that function as signaling molecules, stimulating hormonal responses and enhancing key physiological processes such as nutrient uptake, photosynthesis, and the synthesis of secondary metabolites. Several investigations have shown that PHs improve biomass accumulation, leaf number, and antioxidant activity in various lettuce cultivars, especially when applied via foliar spray or root fertigation (Cristofano et al. 2023; Harakotr et al. 2025; Rouphael et al. 2017).

Similarly, leonardite, a highly oxidized form of lignite rich in humic and fulvic acids, has emerged as a biostimulant in sustainable agriculture. Humic substances (Hum) are known to improve soil fertility, enhance nutrient uptake, and promote plant resilience under stress conditions (du Jardin 2015). When applied to *Lactuca sativa*, leonardite-based biostimulants have demonstrated significant effects on growth and physiological responses (Atero-Calvo et al. 2024a, b).

Metabolomics is a powerful tool for conducting studies on compositional changes during plant growth and development, providing insights into mechanisms of tolerance against biotic and abiotic stress in lettuce (Secomandi et al. 2025; Yang et al. 2024a, b). Despite the increasing availability of biostimulants, their mechanisms of action and the impact of different application strategies remain poorly understood in lettuce. Thus, this study aims to unravel the metabolic mechanisms modulated by biostimulants derived from *Ascophyllum nodosum*, a physiological activator based on plant-derived protein hydrolysate, and a humic and fulvic acids solution extracted from leonardite, applied *via* drip irrigation or foliar spray to *Lactuca sativa* cultivated in open field conditions. With this aim, an untargeted metabolic approach based on ultra-high-performance liquid chromatography coupled to quadrupole-time-of-flight high-resolution mass spectrometry (UHPLC/QTOF-HRMS) was applied, searching for the differential accumulation of metabolites in lettuce by biostimulants that can affect the increase the quality maintenance after the postharvest period.

Materials and methods

Plant Material and Experimental Design

Crop Description

The crop used in the experiment was lettuce (*Lactuca sativa* L. cv. Cappuccio), a leafy vegetable belonging to the Asteraceae family. This cultivar was selected due to its widespread use in Mediterranean open-field cultivation, its compact head morphology, and its moderate sensitivity to postharvest dehydration. These traits make it particularly suitable for evaluating the effects of biostimulants on growth, tissue consistency, and cold storage performance. Transplanting took place on 7 September 2024, and harvest was carried out on 11 November 2024, resulting in a 65-day growing cycle. Plants were established at a density of 94,696 plants per hectare, with a row spacing of 0.33 m and an intra-row spacing of 0.32 m. All cultural practices and fertilization management followed the Regione Piemonte Integrated Production Technical Standards (Regione Piemonte, 2025).

Trial Location

The field trial was conducted in Sale, Province of Alessandria, Italy (coordinates: 45.001565° N, 8.819025° E), at an elevation of 84 m above sea level. The soil was of medium texture, and the area is classified within the EPPO Mediterranean climate zone. Climatic data during the experimental period, including daily minimum, mean, and maximum temperatures, precipitation, and wind speed, are provided in Table S1 and Figure S1.

Experimental Design

The trial followed a randomized complete block design (RCBD) with four biological replicates (4 plants per replicate) and seven treatments, including an untreated control. The aim was to evaluate the efficacy of different biostimulants applied via foliar spraying or drip irrigation to improve the final quality of the produce. Treatments varied in product type, application method, and dosage. The biostimulants used were ABYSS (algae *Ascophyllum nodosum* extract), PERFECTOSE PLUS (protein hydrolysates (PHs)), and Blackjak bio (humic acids extract). All biostimulants were commercial formulations manufactured by Sipcam Italia SpA. The treatments applied to perform the study were Control, Algae-S (ABYSS – 1.5 L/ha, foliar spray), Algae-D (ABYSS – 3.0 L/ha, drip irrigation), PHs-S (PERFECTOSE PLUS – 4.0 L/ha, foliar spray), PHs-D (PERFECTOSE PLUS – 6.0 L/ha, drip irrigation), Hum-S (BLACKJAK BIO – 1.0 L/ha, foliar spray), and Hum-D (BLACKJAK

BIO – 5.0 L/ha, drip irrigation). Applications were conducted according to label recommendations at three phenological stages: 4, 13, and 41 days post-transplant. Sprayed biostimulants were applied using a HONDA WJR 25 motorized sprayer. Flat fan nozzles (FLAFAN 11003 AFC) or cone nozzles were used depending on the treatment. Nozzle spacing was 50 cm for flat fan nozzles and 20 cm for cone nozzles. Operating pressure was set at 1, 3, or 5 bar, depending on the application type. The spray volume was 500 L/ha for foliar applications and 10,000 L/ha for drip applications. Treatments were applied uniformly across each plot to ensure consistent coverage.

Field Measurements

These measurements were taken after 9 days of first application consisting in plant diameter (cm), chlorophyll (ChlM), flavonol (FlvM), and the Nitrogen Balance Index (NBI=ChlM/FlvM). These were assessed using a Multiple Pigment Meter (MPM-100, Opti-Sciences, USA).

Nondestructive reflectance-based instrumentation was employed to assess photosynthetic performance and related physiological parameters on intact, fully expanded leaves. Measurements were conducted using the MultispeQ device (PHOTOSYNQ INC., East Lansing, MI, USA) with the Photosynthesis RIDES 2.0 protocol. Data acquisition and analysis were performed via the PhotosynQ web platform (http://www.photosynq.org) (Kuhlgert et al. 2016). The fol lowing variables were recorded to evaluate photosynthetic efficiency and physiological responses: leaf temperature, leaf thickness, the effective quantum yield of photosystem II (ΦII or Phi2), non-regulated energy dissipation (ΦNO or PhiNO), regulated non-photochemical quenching (ΦNPQ or PhiNPQ), linear electron flow (LEF), and relative chlorophyll content, expressed as SPAD (Soil Plant Analysis Development) values.

Postharvest Performance

Weight loss

Harvested lettuce were maintained for 9 days at 4 °C to evaluate the postharvest cold tolerance. To evaluate postharvest weight loss, plants were weighed immediately after harvest and again after nine days of cold storage at 4 °C.

Changes in the percentage of weight loss along cold storing per replicate were calculated following the Eq. 1, being Wi the initial weight and Wf the final weight.

$$\%\ Weight\ loss\ = \frac{(Wi - Wf)}{Wi} \times\ 100 \quad (1)$$

Texture Analysis

Leaf firmness was assessed using a texture analyzer (TVT 6700, Perten, Sweden) equipped with a knife blade probe. Prior to testing, the outermost leaves were removed, and a total of eight outer and eight inner leaves per replicate were selected. The instrument was set to the following parameters: 100% compression, initial speed of 1.5 mm/s, test speed of 2 mm/s, retraction speed of 10 mm/s, and a trigger force of 0.05 N. The pressure applied allowed the measurement of the "hardness" attribute, defined as the peak force required to rupture the tissue, calculated from the force-time curves.

Dry Substances

Dry matter content was evaluated on the same leaf samples used for texture analysis to further investigate tissue consistency and water retention characteristics. The analysis was conducted using a Radwag moisture analyzer based on thermogravimetric principles. Approximately 2 g of homogenized sample was weighed directly on the instrument's built-in balance. Three technical replicates per sample underwent a drying cycle at 105 °C until constant weight was achieved, typically within 12 min. The analyzer automatically calculated:

Moisture content (%): representing the evaporated water.

Dry matter (%): calculated as 100% minus the moisture content.

Total Phenolic Content (TPC) and Ferric Reducing Antioxidant Power (FRAP) Assay

Fresh lettuce tissue was finely ground in a mortar under liquid nitrogen. 1 g of the powdered material was extracted with 10 mL of 80% methanol (*v/v*). The mixture was subjected to ultrasonic bath treatment for 30 min at 10 °C. The resulting extract was then filtered and used for the determination of total phenolic content (TPC) and antioxidant capacity through the FRAP assay.

The total phenolic content was determined using a modified Folin–Ciocalteu colorimetric method, originally described by Singleton et al. (1999). Briefly, 0.33 mL of extract was mixed with 0.66 mL of distilled water and 0.66 mL of diluted Folin–Ciocalteu reagent (1:9, v/v). Subsequently, 1 mL of sodium carbonate 7% (*w/v*) was added. After 40 min of incubation at room temperature in permanent darkness, absorbance was measured at 760 nm. Quantification was achieved using a gallic acid standard curve, and the results were expressed as g gallic acid equivalents (GAE) per Kg of fresh weight (FW).

The antioxidant capacity of the samples was evaluated by the Ferric Reducing Antioxidant Power (FRAP) assay (Benzie and Strain 1996), with some modifications. The reaction mixture consisted of 0.1 mL of extract and 2.5 mL of FRAP reagent [0.3 M acetate buffer (pH 3.6), 0.01 M TPTZ (2,4,6-tripyridyl-s-triazine) in 0.04 M HCl, and 0.02 M $FeCl_3$ $6H_2O$ (10:1:1, v/v/v)], incubated for 30 min at 37 °C in permanent darkness. Absorbance was measured at 593 nm, and results were reported as g Trolox equivalents (TE) per Kg of fresh weight.

Sample Extraction and Metabolomics Analysis of Lettuce at Harvest

For metabolomic analysis, 5 biological replicates of three lettuces each were used. 1 g of frozen sample was extracted with 10 mL of methanol/H_2O/formic acid solution (80.0/19.9/0.1; *v/v/v*). Extraction was done using a homogenizer (PT 1200 E Polytron, Malters, Switzerland) for 1 min at maximum speed, and extracts were then centrifuged at 8,000 × *g* for 15 min at 4 °C. The supernatants were incubated overnight at -18 °C, collected and syringe-filtered (cellulose membrane, 0.22 µm pore size) into analytical vials. The untargeted metabolomics analysis was done using a Q-Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (high-resolution mass spectrometry (HRMS) (Thermo Scientific, Waltham, MA, USA) coupled to a Vanquish ultra-high-pressure liquid chromatography (UHPLC). The metabolic profiling of samples was performed as proposed by Becchi et al. 2023. Briefly, 3 µL of each sample were injected into an Agilent® ZORBAX RRHD Eclipse Plus C18 column (2.1 × 50 mm, 1.8 µm), the solvent flow rate was set at 0.2 mL/min with gradient elution (6–94% of eluent B in 35 min), prepared with eluent A (water) and eluent B (acetonitrile), both containing 0.1% (*v/v*) formic acid. The mass spectrometer was operated in SCAN and positive ionization mode (ESI+). The mass acquisition of samples was performed in MS-only mode set in the *m/z* 80–1200 range (1 spectra/s) with a mass resolution of 70,000 full width at half maximum (FWHM) at 200 *m/z*. The automatic gain control target (AGC target) and the maximum injection time (IT) were $1e^6$ and 200 ms, respectively. Randomized injections of pooled quality control (QC) samples were acquired in a data-dependent (Top $N = 3$) MS/MS mode with full scan mass resolution reduced to 17,500 at m/z 200, with an AGC target value of $1e^5$, maximum IT of 100 ms, and isolation window of 1.0 m/z, respectively. The Top N ions were selected for fragmentation under stepped Normalized Collisional Energy (i.e., 10, 20, 40 eV). The HESI parameters for both MS and MS/MS were as follows: sheath gas flow 40 arb (arbitrary units), auxiliary gas flow 20 arb, spray voltage 3.5 kV, capillary temperature 320 °C. Prior to data collection, the mass spectrometer was calibrated using Pierce™ positive ion calibration solution (Thermo Fisher Scientific, San Jose CA, USA). To avoid possible bias, the sequence of injections was randomized.

Data Processing

The data processing was done according to Castro-Cegrí et al. (2025). Once the detection of features was completed, the acquired raw data were processed by MS-DIAL software (v. 4.90), including peak finding, feature identification by library matching, gap filling, and alignment steps. Firstly, the raw '.d' format files were converted into '.abf' files by the Reifycs Abf Converter. These '.abf' data files were imported into MS-DIAL, searching the features in the retention time range of 1–32 min, *m/z* range 100–1200 for MS-only data and 80–1200 for MS/MS data, setting a minimum of 3,000 counts for peak detection. The FooDB database (https://foodb.ca/) was chosen to identify features with tolerances of 0.01 Da and 0.05 Da for MS and MS/MS, respectively, and with an identification score cut-off > 60%. Feature identification was based on mass accuracy data, isotopic patterns and spectral matching. For isotope recognition, only the maximum number of isotopes was set to 1, and the maximum charged number was set to 2, as the mass range for metabolite annotation was < 2000 Da. The adducts considered for annotation were selected based on the experimental matrix and the analytical procedure, i.e. $[M+H]^+$, $[M+H_2O]^+$, $[M+H\text{-}H2O]^+$ (neutral loss), $[M+ACN]^+$, $[M+Na]^+$, $[M+K]^+$, and all the possible combinations among them (Risoli et al. 2025; Tsugawa et al. 2015). All features that were not detected in at least 83.33% of replications per group were removed. According to the Metabolomics Standards Initiative (MSI) (Salek et al. 2013), a level 2 of confidence in features' annotation was achieved, conferring the assessment of putatively annotated compounds further involved in multivariate statistical analysis.

Statistical Analysis

Experiments followed a randomized design. Each treatment consisted of four independent biological replicates, with four plants per replicate (16 plants in total per treatment). Plant-level measurements were averaged within each biological replicate prior to statistical analysis, and the replicate mean ($n = 4$) was used as the unit of analysis in the ANOVA. Quality parameters were analyzed by one-way ANOVA considering treatment as a fixed factor using SPSS 28.0 (IBM Corp., Armonk, NY, USA). Prior to analysis, normality and homogeneity of variances were verified using Shapiro–Wilk and Levene's tests, respectively. When significant effects were detected ($p < 0.05$), mean separation was performed using

Tukey's honestly significant difference (HSD) test. Detailed ANOVA results and assumption tests (Shapiro–Wilk and Levene) are presented in Supplementary Tables S2–S4. Raw metabolomics data were first subjected to $\log_2$ transformation and normalization at the 75th percentile by the software Mass Profiler Professional 15.1 (Agilent Technologies®), before applying multivariate statistical analysis. An unsupervised hierarchical cluster analysis (HCA; Euclidean distance, Ward's linkage method was performed to assess global metabolomic patterns according to biostimulant type and application method. In parallel, supervised multivariate data analysis was carried out in terms of orthogonal projection to latent structures discriminant analysis (OPLS-DA) by SIMCA® 16 software (Sartorius®, Umeå, Sweden), generating independent models to discriminate treatment effects relative to the control. The goodness-of-fit (R^2Y) and the goodness-of-prediction (Q^2) parameters were considered to evaluate the quality of the models, setting a Q^2 predictive ability > 0.5 as a threshold for good predictability. Finally, the models were combined with the variable importance in the projection (VIP) approach to identify the compounds showing the highest contribution to the discriminating the treatment effect. Common VIP markers showing the highest discrimination power (VIP score > 1.4), as in Rivera-Pérez et al. (2022), over time were investigated through a Venn diagram by jvenn tool (https://jvenn.toulouse.inrae.fr/app/index.html). Differentially accumulated metabolites (DAMs) were identified using volcano analysis based on fold change (FC) thresholds (cut-off = ± 2), in combination with one-way ANOVA followed by Bonferroni family-wise error rate (FWER) *post hoc* test ($p < 0.01$). Comparisons were made for each treatment against the control. Identified DAMs were further subjected to pathway-level analysis by summing fold changes within metabolomic classes.

Pearson's correlation analysis was conducted using the online platform MetaboAnalyst 5.0 (www.metaboanalyst.ca). Data were normalized by the median and auto-scaled to ensure comparison across variables.

Results

Effect of Biostimulant Application on Lettuce Growth and Development

Phenotypical State of Lettuce Plants

Overall, the application of biostimulants did not significantly influence the photosynthetic performance of lettuce plants, with just Algae-S and PHs-S showing a significant increment in PhiNO compared to control (Table S5). However, notable effects were observed in plant growth and development. Specifically, foliar application of the protein hydrolysate-based biostimulant (PHs-S) led to a 30% increase in plant diameter compared to control plants (Fig. 1A). Although no statistically significant differences were observed in flavonol content among treatments (Fig. 1C), almost all treatments resulted in a substantial increase in leaf chlorophyll content. In particular, the PHs-S and PHs-D led to increases in chlorophyll content of 510% and 550%, respectively, compared to the control (Fig. 1B). The pronounced increase in chlorophyll content was accompanied by a rise in the Nitrogen Balance Index (NBI), with all treatments exhibiting higher values than the control. Interestingly, a shift was observed between foliar spray and drip applications of the protein hydrolysate-based biostimulant, suggesting an enhanced nitrogen status in lettuce plants treated with PHs-S (Fig. 1D).

Antioxidant Capacity at Harvest

The effects of algae-, protein hydrolysate-, and humic acid-based biostimulants on lettuce antioxidant capacity at harvest were assessed through total phenolic content and FRAP assays. Among the treatments, the algae-based biostimulant applied via drip irrigation (Algae-D) and the protein hydrolysate-based treatment applied via foliar spray (PHs-S) emerged as the most effective in enhancing antioxidant capacity. Specifically, FRAP assay results showed increases of 3.0- and 2.7-fold in Algae-D and PHs-S treated plants, respectively, compared to the control (Fig. 2A). Similarly, total phenolic content increased by 58% and 52% for Algae-D and PHs-S treatments, respectively, relative to control plants (Fig. 2B). In contrast, Hum-D treatment showed a diminution of antioxidant capacity and total phenolic content compared to the control.

Biostimulant Role in the Postharvest Period

To assess postharvest behavior of biostimulant-treated lettuces, parameters such as weight loss, dry matter content, and leaf firmness (evaluated separately in the lower and upper parts of the plant) were measured.

Weight loss was significantly reduced in Algae-D and Hum-D treatments compared to the control, with reductions of up to 34% observed in Algae-D (Table 1). Regarding dry matter content, PHs-D showed the highest values both at harvest and after cold storage, being the treatment that exhibited the most pronounced differences compared to the control. In terms of leaf firmness, PHs-S was the only treatment to significantly increase firmness in the lower leaves at both harvest and after cold storage compared to the control. For the top leaves, Algae-S and Algae-D showed the highest firmness values at harvest. However, no statistically

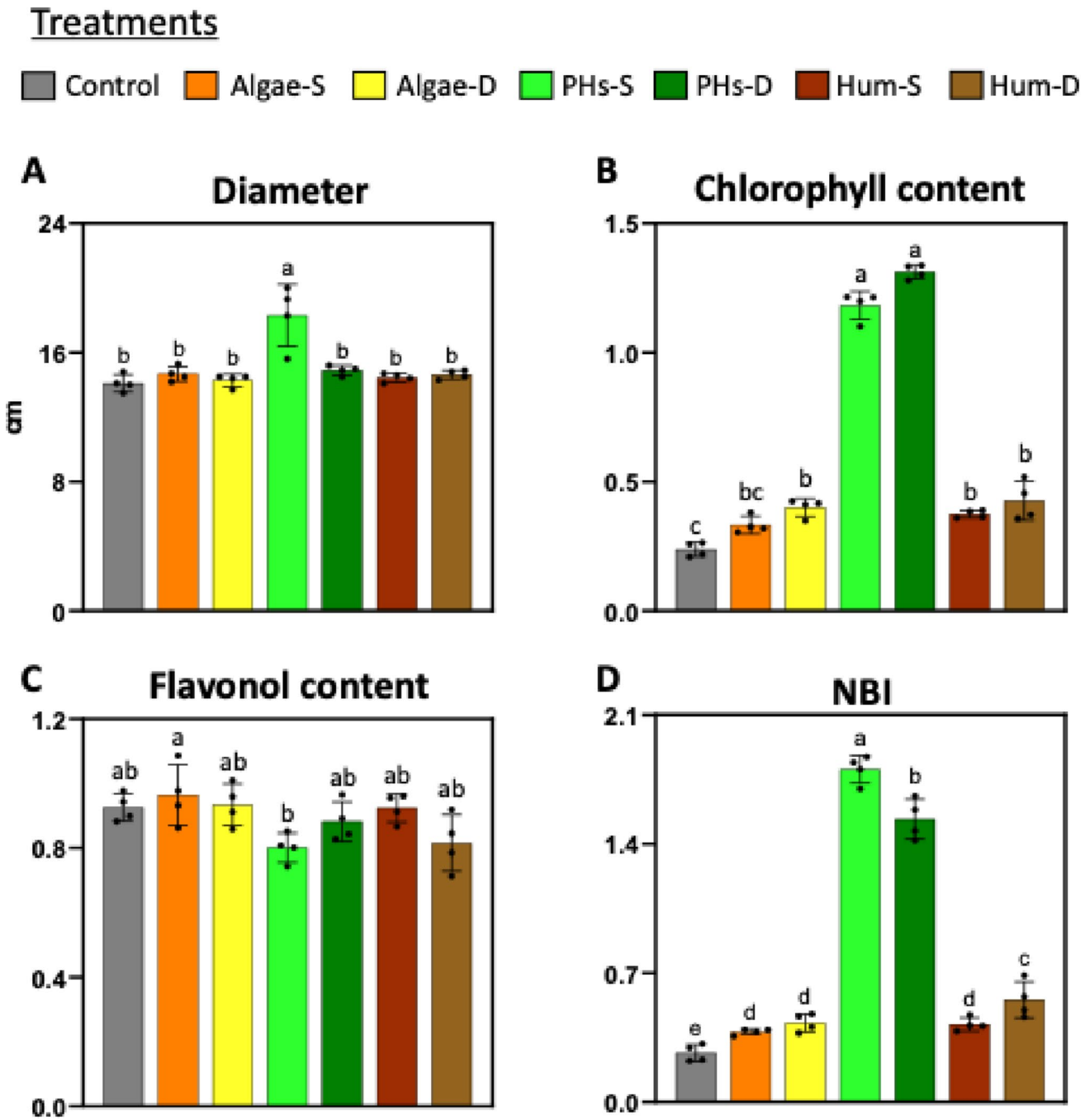

Fig. 1 Determination of plant diameter (**A**), chlorophyll content (**B**), flavonol content (**C**), and nitrogen balance index (NBI) (**D**) in lettuce plants after 9 days of biostimulant application. Data represent means ± SE of four independent biological replicates (four plants per replicate). Different letters indicate significant differences among treatments according to one-way ANOVA followed by Tukey's HSD test ($p < 0.05$)

significant increases in firmness were observed in the upper leaves after cold storage across treatments, and in some cases (Hum-S and Hum-D), firmness values were even lower than the control (Table 1).

Unravelling the Hierarchical Biostimulant Effects on the Metabolic Profile of Lettuce by Multivariate Statistics

Lettuce leaf samples collected at harvest from control plants and those treated with algae-, protein hydrolysate-, and humic acid-based biostimulants, applied via foliar spray or

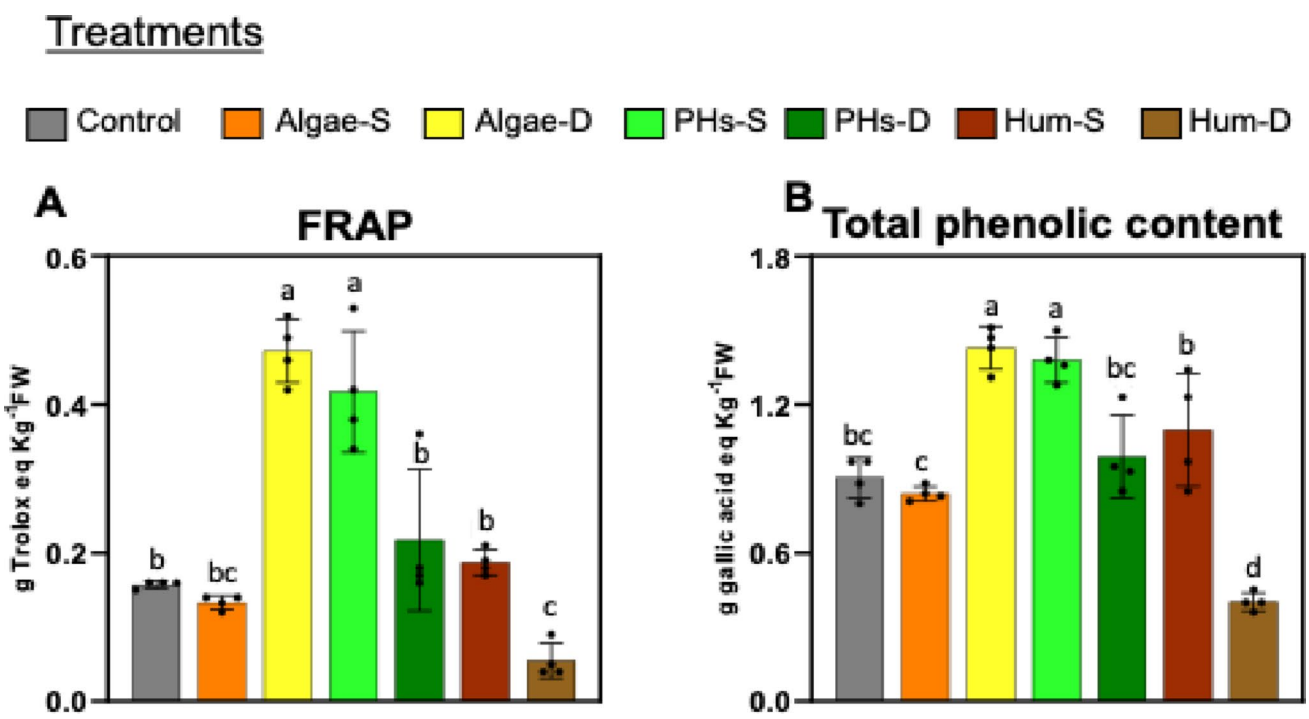

Fig. 2 Determination of antioxidant capacity by FRAP assay (**A**) and total phenolic content (**B**) after 9 days of postharvest cold storage of lettuces. Data represent means ± SE of four independent biological replicates (four plants per replicate). Different letters indicate significant differences among treatments according to one-way ANOVA followed by Tukey's HSD test ($p < 0.05$)

Table 1 Effect of cold storage and biostimulant treatments on postharvest quality parameters of lettuce, measured by changes in weight loss (%), dry matter (%), and firmness (N) in low and top leaves, both at harvest and after 9 days of cold storage

		Control	Algae-S	Algae-D	PHs-S	PHs-D	Hum-S	Hum-D
Weight loss (%)		9.6 ± 0.1^{a}	6.4 ± 1.5^{ab}	5.5 ± 0.8^{b}	5.8 ± 1^{ab}	7.5 ± 1.1^{ab}	6.2 ± 1.4^{ab}	5.5±0.5b
Dry matter (%)	At harvest	7.6 ± 0.2^{b}	10.6 ± 0.3^{a}	10 ± 0.3^{a}	10.1 ± 0.1^{a}	10.5 ± 0.1^{a}	9.5 ± 0.2^{b}	8.1 ± 0.3^{b}
	9 days at 4 °C	11.7 ± 0.3^{bc}	11.7 ± 0.3^{c}	14.2 ± 0.9^{ab}	12.4 ± 0.6^{bc}	15.7 ± 0.3^{a}	12.5 ± 0.3^{bc}	14.8 ± 0.9^{ab}
Firmness low (N)	At harvest	24.6 ± 0.3^{b}	25.4 ± 0.9^{ab}	26.7 ± 0.7^{ab}	28.3 ± 1.2^{a}	26.1 ± 0.6^{ab}	22.3 ± 1.5^{bc}	19 ± 1.5^{c}
	9 days at 4 °C	24.7 ± 0.6^{b}	27.7 ± 1^{ab}	25.9 ± 0.4^{b}	30.4 ± 1^{a}	27 ± 1.5^{ab}	25.8 ± 0.7^{b}	24.1 ± 0.8^{b}
Firmness top (N)	At harvest	13.6 ± 0.4^{c}	20.1 ± 1^{a}	19.4 ± 0.7^{ab}	17.9 ± 1^{ab}	16 ± 1^{bc}	13 ± 0.3^{c}	13.1 ± 1.3^{c}
	9 days at 4 °C	22.3 ± 0.8^{a}	20.5 ± 0.9^{ab}	22.2 ± 0.5^{a}	21.7 ± 0.8^{a}	23.1 ± 0.9^{a}	17.2 ± 0.7^{bc}	16.2 ± 1.9^{c}

Data represent means ± SE of four independent biological replicates (four plants per replicate). Different letters indicate significant differences among treatments according to one-way ANOVA followed by Tukey's HSD test ($p < 0.05$).

drip irrigation, were analyzed using an untargeted metabolomics approach. A total of 2,216 chemical entities were putatively annotated against FooDB database: 28 entities were annotated with MS^2 fidelity, whereas 2188 entities were reported at the MS-only level. Comprehensive information for each feature, including metabolite name, retention time (Rt, min), mass (m/z), molecular formula, ontology, MS and MS/MS spectra, annotation, and total score, is provided in Table S6. To assess the impact of biostimulant type and application method on lettuce metabolite composition, unsupervised hierarchical cluster analysis (HCA) was performed (Fig. 3). The results indicated that foliar application of protein hydrolysate and humic substance treatments induced the most substantial changes in the metabolic profile. Furthermore, algae-based treatments (via both foliar and drip methods), together with PHs-D, formed a distinct subcluster. In contrast, the metabolic profile of Hum-D lettuce closely resembled that of the control group (Fig. 3).

To further investigate the biostimulant-induced accumulation of metabolites, a supervised orthogonal projections to latent structures discriminant analysis (OPLS-DA) was performed. All models demonstrated robust performance in terms of goodness-of-fit ($R^2 > 0.997$) and goodness-of-prediction ($Q^2 = 0.8–0.888$). Variable importance in projection (VIP) analysis was then conducted to identify the metabolites with the highest discriminative power (VIP score > 1.40) (Table S7). The results highlighted a distinct metabolic fingerprint associated with each biostimulant treatment. Notably, only approximately 10% of VIP markers were shared across treatments when comparing spray

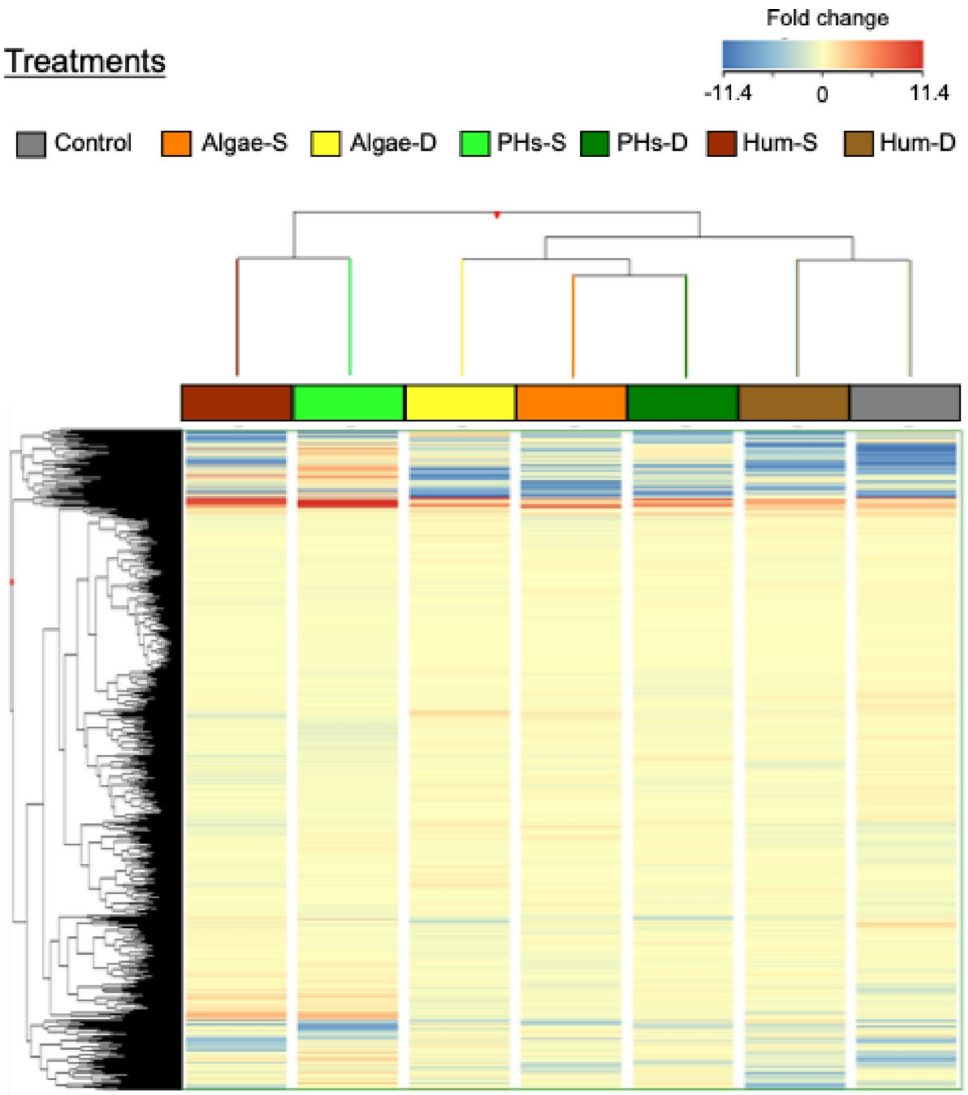

Fig. 3 Hierarchical cluster analysis (HCA) of the untargeted metabolic profile of lettuce leaves treated with different biostimulant tested, including control, Algae-S, Algae-D, PHs-S, PHs-D, Hum-S, Hum-D. Clustering was performed according to the fold change-based heatmap, baselined to the median values of all samples (Euclidean distance, Ward's algorithm)

and drip application methods, underscoring the influence of application strategy on metabolic responses (Fig. S2A-B). Moreover, even within the same biostimulant treatment, only 20–25% of the VIP markers were common between foliar spray and drip irrigation, suggesting that the route of application significantly modulates the accumulation of metabolites (Fig. S2C-E).

Among the VIP markers with the highest discriminative power comparing all treatments (Table S7; Fig. 4), several compounds related to proline metabolism were specifically associated with algae-based treatments. Galactosyl 4-hydroxyproline (LogFC = 7.6) was uniquely detected in the foliar spray treatment (Algae-S), while Val-Pro-Pro (LogFC = 7.5) was exclusive to drip irrigation (Algae-D). Additionally, Leu-Pro-Ile was found in both application modes, with fold changes of 0.79 and 1.09 for Algae-S and Algae-D, respectively. In the case of protein hydrolysate-based foliar spray application (PHs-S), the metabolic response was predominantly characterized by up-accumulation of compounds. Notably, N-hydroxycadaverine (LogFC = 13.7) and methyl dihydrojasmonate (LogFC = 2.0) were among the most strongly induced, suggesting potential roles in modulating lettuce metabolic pathways under this treatment. Conversely, the drip-applied protein hydrolysate treatment (PHs-D) also led to a diverse metabolic activation, prominently increasing the levels of Salvianolic acid C (LogFC = 3.2), 3-(1-hydroxymethyl-1-propenyl)pentanedioic acid (LogFC = 7.9), and riboflavin (LogFC = 2.8). As observed for PHs-S, a general up-accumulation of compounds was noted in the VIPs compounds of Hum-S, pointing xi-7-Hydroxyhexadecanedioic acid, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol, and Aspartyl-Tryptophan as the most induced due to this treatment (LogFC = 3.09, 2.73, and 7.7, respectively). In contrast, the humic acid drip treatment (Hum-D) exhibited minimal impact on the metabolic profile, consistent with hierarchical

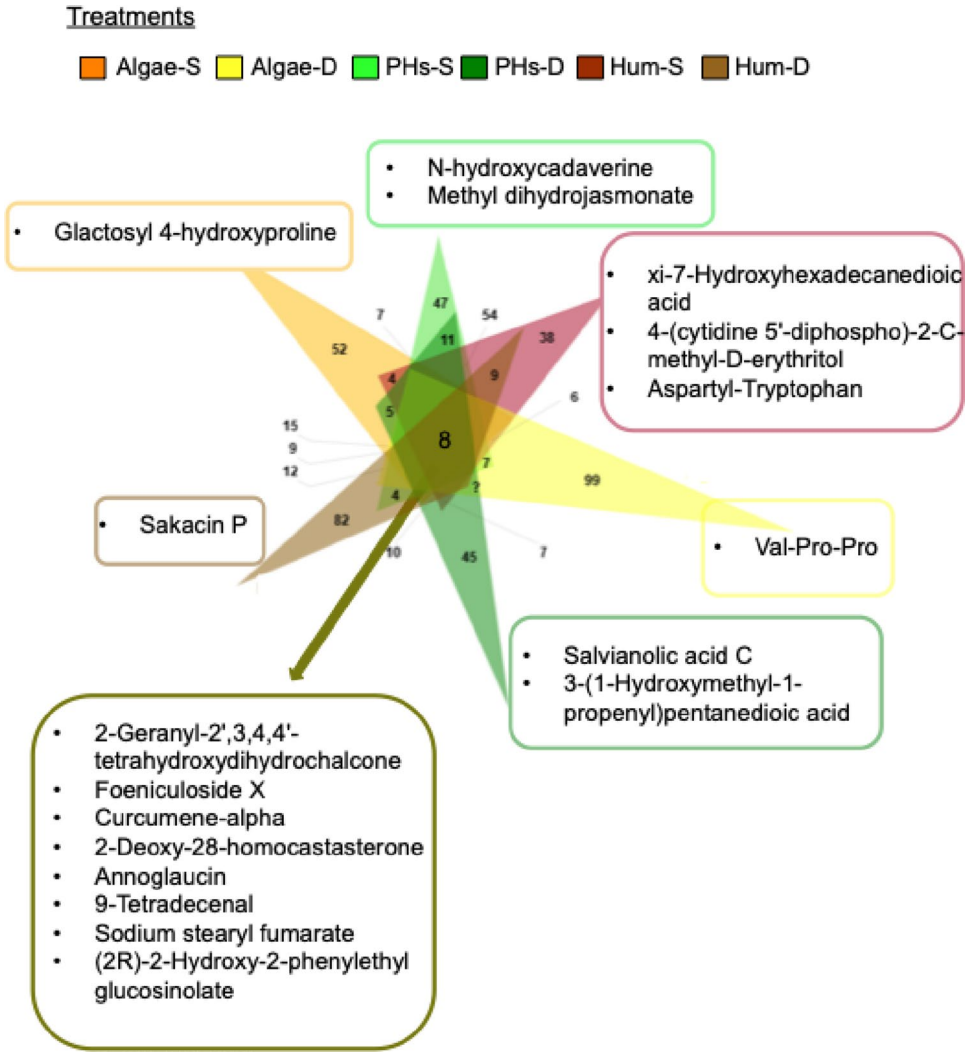

Fig. 4 Venn diagram for the VIP markers found as discriminant for each OPLS model (VIP score > 1.4). Some of the most influential in each group were noted, as well as all VIP markers commonly induced

cluster analysis (HCA) results. The most notable change under Hum-D was the down-accumulation of Sakacin P (LogFC = −7.1), serving as the principal discriminant metabolite for this treatment. After examining the compounds distinctly accumulated in response to individual treatments, attention was directed toward identifying commonly regulated metabolites across all biostimulant applications (Table S7; Fig. 4). This analysis revealed a limited set of eight metabolites that were consistently modulated, although with relatively mild fold changes. Of these, seven compounds, 2-Geranyl-2′,3,4,4′-tetrahydroxydihydrochalcone, Foeniculoside X, Curcumene-alpha, 2-Deoxy-28-homocastasterone, Annoglaucin, 9-Tetradecenal, and Sodium stearyl fumarate, were uniformly down-accumulated across treatments, with LogFC values ranging from −1.74 to −0.26. In contrast, only one compound, (2R)-2-Hydroxy-2-phenylethyl glucosinolate, exhibited consistent up-accumulation in all treatment groups, with LogFC values ranging from 0.46 to 0.82.

Overall, these findings demonstrate that both biostimulant type and application method strongly shape lettuce metabolic reprogramming, generating largely treatment-specific metabolomic signatures with limited overlap across delivery strategies.

Pathway Analysis Provides the Functional Signature of Biostimulant Effect on Lettuce

The pathway analysis was performed considering the differentially accumulated metabolites (DAM) provided by Volcano analysis ($p < 0.05$ and FC cut-off = ±2). A total of 1766 compounds satisfied both criteria and were subjected to pathway analysis. The full list of DAMs is provided in Table S8, together with their logFC values and the chemical ontology. The results revealed distinct metabolic signatures depending on both biostimulant type and application method (Fig. 5A). Regarding general biosynthesis, secondary metabolism showed the highest positive modulation among treatments, with functional and primary metabolites also highly up-accumulated, and being lipids, terpenoids, and nitrogen-containing compounds (NCCs) in a third level.

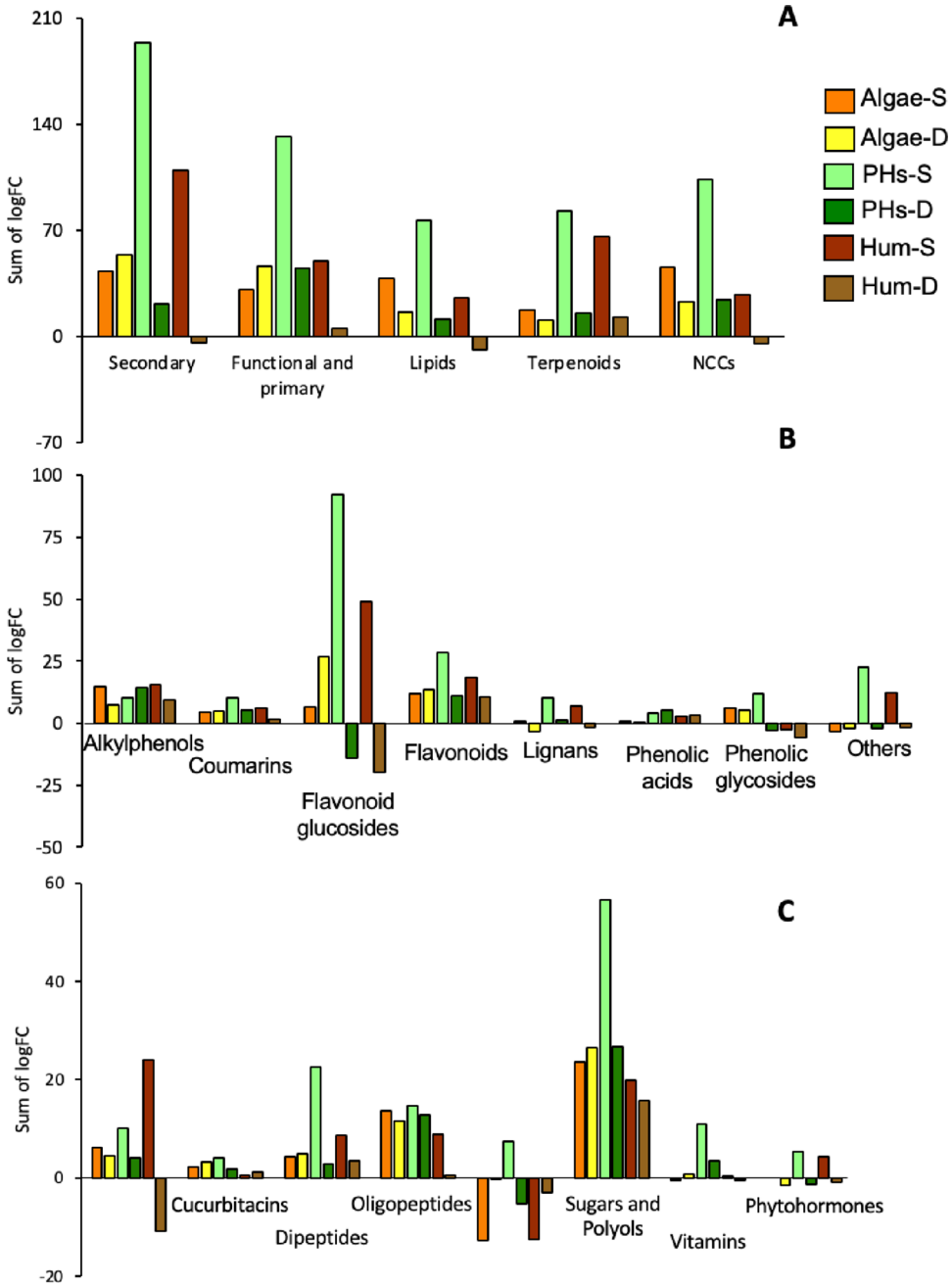

Fig. 5 Pathway analysis on the metabolic profile of lettuce samples. **A**. Modulation of biosynthetic pathways. **B**. Modulation of secondary metabolism. **C**. Modulation of functional and primary metabolism. Results are expressed as the sum of the logarithm of fold change (logFC) values of each category with respect control. Only the compounds showing a differential abundance (FC > 2 or FC < -2) and statistically significant differences according to one-way through one-way ANOVA and Bonferroni FWER post hoc test ($p < 0.01$) with respect to control were considered

Algae-based biostimulant provoked a similar metabolic modulation applied via foliar spray or drip, increasing the accumulation of compounds at a wide level. In contrast, an important effect was noted in the way of application for PHs and Hum, being in both of them the application by foliar spray more effective to promote the accumulation of metabolites. PHs-S was the treatment with the highest metabolic induction, accumulating secondary metabolites, but also showing an important elicitation of all metabolic classes evaluated. In contrast, the accumulation of metabolites in lettuce plants after Hum-D application remained as control (Fig. 5A). To go more in dept secondary metabolism, and functional and primary metabolites were further investigated. Flavonoid glucosides resulted as the most interesting metabolic class of secondary metabolites, as was the one more induced by PHs-S and Hum-S applications, with sum of logFC of 92.2 and 48.9, respectively. However, an important down-accumulation of flavonoid glucosides was noted with the same treatments applied via drip irrigation (PHs-D and Hum-D) with sum of logFC of -13.9 and − 19.7, respectively (Fig. 5B). Interestingly, a contrasting effect was also noted for amino acid accumulation, with Hum-S showing the highest induction (sum logFC = 23.9), while Hum-D down-accumulated this class of compounds (sum logFC=

-10.9) (Fig. 5C). Also, a general accumulation of dipeptides and oligopeptides was noted among treatments. Sugars and polyols represented the only metabolic class consistently induced across all treatments, with PHs-S showing the highest response. By other side PHs was the only one enhancing the accumulation of vitamins in lettuce, noting an important accumulation of folic acid and some derivatives (Table S8). Also, an accumulation of riboflavin was noted among all treatments, with a shared accumulation of riboflavin (log FC = 2.3 and 2.8), and riboflavin cyclic-4',5'-phosphate (log FC = 2.3 and 1.5) by PHs-S and PHs-D, respectively. Noteworthy, PHs-S and Hum-S increased the accumulation of phytohormones (Fig. 5C), with methyl dihydrojasmonate (log FC = 2.02 and 2.18, respectively) and 16,17-Dihydro-16a,17-dihydroxygibberellin A4 17-glucoside (log FC = 3.1 and 1.0, respectively) showing the highest accumulation. Altogether, the data reveal that biostimulant effects are strongly modulated by application method, with foliar treatments, especially PHs-S, eliciting a coordinated activation of secondary metabolism, nitrogen-related pathways, sugars, and hormone-related compounds, thereby defining treatment-specific functional metabolic signatures.

Correlation Analysis Among Metabolic Class Induction and Phenotypic Performance

To gain deeper insight into the involvement of the accumulation of metabolites noted due to the treatment applied in the growth and postharvest of lettuce, a Pearson's correlation analysis was conducted. The corresponding r-values ($p < 0.05$) are provided in Table S9 and visually depicted in the heatmap shown in Fig. 6. This heatmap illustrates a positive correlation between antioxidant capacity (FRAP) and the accumulation of total phenolic compounds and vitamins (r-value 0.998 and 0.991). A differential subcluster was noted correlating together the increase of glucosinolates, oligopeptides, polyamines, alkaloids, monoterpenoids, amino acids, and the dry matter %, with the changes noted in firmness on low leaves of lettuce after postharvest period (r-values 0.98–1), being also correlated with the content of coumarins and flavonoid glucosides (r-value 0.9 and 0.63, respectively). By other side, increase of firmness at harvest in low and top leaves was correlated with the accumulation of phenolic compounds and cucurbitacins (r-value 0.72 and 0.89, respectively). In summary, these correlations indicate that biostimulant-induced modulation of specific metabolic classes, such as phenolics, glucosinolates, and nitrogen-related compounds, is closely linked to enhanced antioxidant capacity and improved postharvest firmness in lettuce.

Discussion

Phenotypical Effect

Foliar and drip applications of the evaluated biostimulants induced distinct physiological responses in lettuce, indicating treatment-specific modes of action rather than a generalized growth-promoting effect. Among them, only the foliar application of protein hydrolysates (PHs-S) enhanced plant diameter, suggesting a direct stimulation of vegetative growth processes. This response was accompanied by a marked increase in chlorophyll content and nitrogen balance index (NBI), particularly under PHs-S and PHs-D treatments, pointing to an improvement in nitrogen assimilation efficiency and photosynthetic performance. These findings align with previous studies demonstrating that foliar application of protein hydrolysates derived from hempseed and soy substantially improves yield-related traits and leaf nutritional quality (Harakotr et al. 2025; Sabatino et al. 2021). Under varying nitrogen fertilization regimes, PHs also enhanced nitrogen use efficiency and modulated nitrate assimilation (Choi et al. 2022; Cristofano et al. 2023). These effects have been linked to improved nitrogen uptake and internal translocation, especially under suboptimal nutrient conditions, ultimately contributing to enhanced plant vigor and productivity (Di Mola et al. 2020). For instance, legume-derived protein hydrolysates have been shown not only to improve nitrogen use efficiency and growth in tomato and other crops but also to modulate expression of genes involved in nitrogen uptake and assimilation, including nitrate reductase (NR) and nitrogen transporters, under varying nitrogen regimes (Sestili et al. 2018). This suggests that PHs can trigger transcriptional reprogramming of key nitrogen metabolism pathways, thereby enhancing N incorporation into primary metabolism. Similarly, algae- and humic-based biostimulants significantly increased both chlorophyll content and NBI compared to the untreated control suggesting activation of photosynthetic machinery and redox-related pathways. Specifically, *Ascophyllum nodosum*-derived formulations have been reported to mitigate potassium deficiency symptoms, enhancing photosynthetic capacity, antioxidant enzyme activities, and chlorophyll levels, while also prolonging the postharvest shelf life of fresh-cut lettuce (Chrysargyris et al. 2018; Drygaś et al. 2024). Similarly, the application of leonardite has been associated with increased chlorophyll accumulation in lettuce (Atero-Calvo et al. 2024a).

Preventing oxidative damage is essential to preserve both the commercial value and nutritional quality of fresh produce during postharvest storage. In this context, the observed increase in total phenolic content and associated antioxidant capacity induced by Algae-D and PHs-S

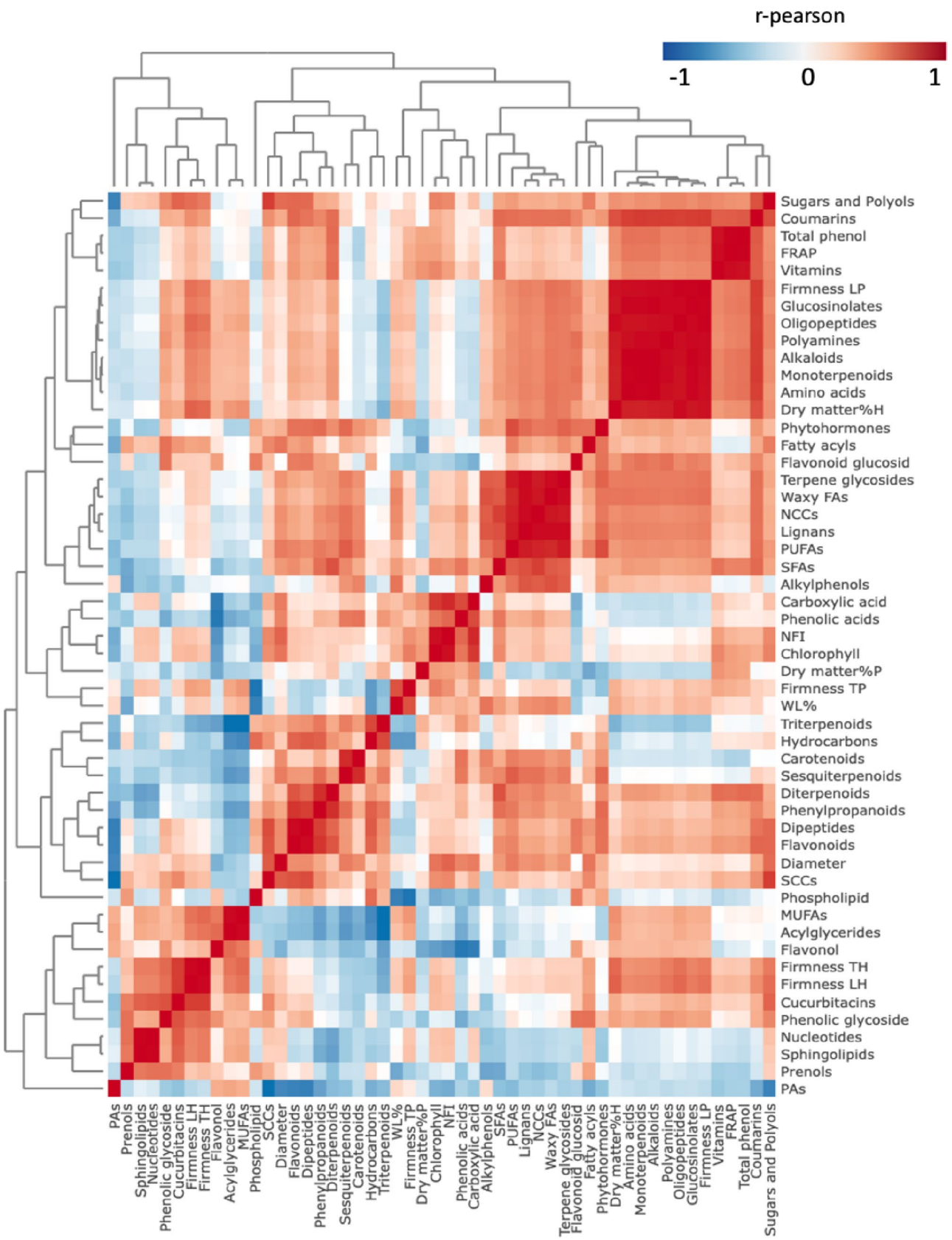

Fig. 6 Correlation heatmap performed using the significant metabolic classes annotated as well as diameter, chlorophyll, flavonol, NFI, antioxidant capacity (FRAP), total phenol content, weight loss (WL), firmness at top leaves at harvest (Firmness.TH), and after postharvest (Firmness.TP), firmness at low leaves at harvest (Firmness.LH) and after postharvest (Firmness.LP)

treatments may contribute to extending the shelf life of lettuce. The stimulation of phenylpropanoid metabolism by biostimulants has been previously associated with the transcriptional activation of PAL and downstream flavonoid biosynthetic genes, leading to enhanced accumulation of antioxidant compounds (Villa et al. 2025; Zuluaga et al. 2023). Moreover, the increase in dry matter content and firmness at harvest, together with reduced weight loss during cold storage, suggests structural and osmotic adjustments occurring during growth. These responses may result from enhanced carbohydrate accumulation and modulation of cell wall metabolism, processes frequently associated with biostimulant-induced metabolic reprogramming (Bodin et al. 2020; Ebinezer et al. 2020). Notably, protein hydrolysates exerted the strongest effect in maintaining leaf firmness, pointing toward a possible involvement of polyamine metabolism and cell wall-associated pathways, which will be further discussed in the metabolomic section. Collectively, these findings indicate that the physiological improvements observed are not isolated traits but rather the phenotypic expression of a deeper metabolic reconfiguration induced by biostimulant application.

Metabolic Induction Affecting Growth and Postharvest of Lettuce Plant

Untargeted metabolomic profiling revealed that biostimulant application triggered a coordinated metabolic reprogramming rather than isolated metabolite fluctuations. Foliar-applied protein hydrolysates and humic substances elicited the strongest metabolic divergence from the control, whereas Hum-D clustered closely with untreated plants, suggesting limited systemic metabolic activation. These differences indicate that the mode of application critically influences the intensity and specificity of metabolic responses.

One of the most consistent signatures across treatments was the modulation of osmolyte-related compounds, particularly proline derivatives under algae-based applications, as observed in various herbaceous species following seaweed extract application (Sales et al. 2024). Proline and its derivatives are central mediators of osmotic adjustment and redox buffering, functioning as ROS scavengers and stabilizers of proteins and membranes under stress conditions. The accumulation of galactosyl 4-hydroxyproline and related dipeptides suggests activation of osmoprotective and cell wall-associated pathways, potentially contributing to enhanced structural stability and improved postharvest resilience, as also observed in lettuce treated with exogenous proline (Zhang et al. 2020) and in cassava (Tang et al. 2024). Beyond osmolyte modulation, protein hydrolysate treatments were characterized by the marked accumulation of N-hydroxycadaverine, which has been studied for its role in enhancing lettuce growth (Zhou et al. 2024), and is associated with putrescine accumulation and quality maintenance in zucchini and strawberry fruits during postharvest cold storage (Khosroshahi et al. 2007; Palma et al. 2015). The induction of N-hydroxycadaverine therefore suggests that protein hydrolysates may influence nitrogen-derived secondary metabolism beyond simple nutrient supply, potentially through transcriptional regulation of polyamine biosynthetic genes. This metabolic adjustment could contribute to the improved firmness and reduced weight loss observed during storage. The concurrent increase in methyl dihydrojasmonate further reinforces the hypothesis of biostimulant-driven signaling activation. Jasmonates are central coordinators of defense-related metabolic pathways, particularly the phenylpropanoid cascade (Liu et al. 2023). Its application has been linked to enhanced antioxidant capacity through increased accumulation of phenolic compounds and carotenoids in lettuce (H.-J. Kim et al. 2007). A wide diversity of compounds was identified as VIP markers that were generally up-accumulated across the remaining treatments. These findings underscore the nuanced, treatment-specific nature of the metabolic responses in lettuce, while also revealing a small subset of metabolites that may serve as general markers of biostimulant-induced modulation. In contrast, most commonly induced VIP markers were down-accumulated. This shared response suggests the presence of a generalized metabolic suppression, potentially linked to stress mitigation or homeostatic adjustments triggered by biostimulant exposure. To further evaluate the extent of metabolic modulation, a pathway analysis was conducted. The metabolic changes induced by each biostimulant revealed a similar response pattern between foliar and drip applications of algae extract. Conversely, protein hydrolysates and humic substances displayed more heterogeneous effects, with foliar treatments in both cases resulting in the highest accumulation of metabolites. A comparable outcome was observed with *Ascophyllum nodosum* extract applied to tomato, where significant metabolic activation was also noted (Baghdadi et al. 2022), and in basil metabolism boosted by protein hydrolysate (Rouphael et al. 2023), both via foliar spray. A significant modulation of secondary metabolism was observed, with each treatment inducing a distinct pattern across different metabolite subclasses. A consistent response was noted in the up-accumulation of alkylphenols, a class of phenolic compounds known for their amphipathic structure, comprising a phenolic ring and a long hydrophobic alkyl chain, which enables their integration into waxy tissues, where they may play a functional role in structural integrity and protective barriers (Boulebd and Spiegel 2023). The accumulation of these compounds influences membrane fluidity and stability, while also

facilitating interactions with membrane-associated proteins, likely modulating enzymatic functions (Sampietro et al. 2013). In fact, an increased accumulation of alkylphenols has been observed following riboflavin treatment, which may contribute to the enhanced cold tolerance of zucchini fruit (Castro-Cegrí et al. 2025). A consistent increase in total flavonoid content was also detected; however, this trend did not extend to flavonoid glucosides, which exhibited a differential response depending on the treatment. Among the treatments, PHs-S resulted in the highest accumulation of flavonoid glucosides, followed by Hum-S. In contrast, when these biostimulants were applied via drip irrigation, a down-accumulation was noted. Flavonoids are commonly glycosylated to enhance their stability and facilitate their storage in the vacuole until required for plant defense responses. This mechanism has been demonstrated in tomato plants under abiotic stress conditions (Martinez et al. 2016). However, during the postharvest period, flavonoids often undergo significant degradation, which can affect shelf life in a wide range of plant species (Bozzo and Unterlander 2021). Given the crucial role of flavonoid glucosides in plant defense, their notable accumulation in PHs-S and Hum-S treatments may improve the protective capacity of lettuce at harvest, potentially enhancing tolerance to chilling and other postharvest stresses.

A wide range of metabolic classes were included into the functional and primary group, with a heterogeneous metabolic modulation among treatments. An important down-accumulation of sulfur-containing compounds (SCCs) was induced by both Algae-S and Hum-S treatments. This reduction may be attributed to enhanced catabolism and the redirection of SCCs toward other metabolic pathways associated with defense responses and stress mitigation (Abdullah-Zawawi et al. 2022). In contrast, all treatments led to a marked up-accumulation of sugars and polyols, with PHs-S displaying the highest levels. This accumulation likely contributes to improved abiotic stress tolerance, as previously observed with protein hydrolysates applied to lettuce under salt stress (Monterisi et al. 2024). The increase in sugars is particularly relevant for extending the postharvest shelf life of lettuce by reducing water loss and chlorophyll degradation (J. Yang et al. 2024a, b), which aligns with the findings of the present study. One of the most intriguing findings was the consistent modulation of vitamin-related metabolites. Protein hydrolysates uniquely enhanced folate accumulation, while riboflavin and its phosphorylated derivatives increased across all treatments. Folic acid (vitamin B9) applications have been shown to delay postharvest quality deterioration by increasing the antioxidant capacity and regulating cell wall metabolism (Ghorbani et al. 2025; Pei et al. 2023; Xu et al. 2021). Moreover, folic acid is an essential vitamin in the human diet, as animals, including humans, are unable to synthesize it de novo and must obtain it from dietary sources such as green leafy vegetables (O'Hare et al. 2012). Deficiencies in folic acid can lead to serious health conditions, including spina bifida, anencephaly, and anemia (Lucock 2000). Therefore, fortifying lettuce through foliar application of protein hydrolysates to increase folate levels could not only extend postharvest shelf life but also improve the nutritional value of the crop, contributing to human health. Riboflavin (vitamin B2) has also demonstrated this dual functionality. It plays a critical role in human health (Thakur et al. 2017) and enhances antioxidant defenses by activating the phenylpropanoid pathway (Castro-Cegrí et al. 2024; Zhang et al. 2023). Beyond individual metabolite fluctuations, the coordinated modulation of phenylpropanoids, vitamins, phytohormones, and secondary metabolites suggests that biostimulant application induces a systemic metabolic reprogramming rather than isolated biochemical adjustments. The consistent enhancement of antioxidant-related metabolites and their strong correlation with FRAP values indicate reinforcement of cellular redox homeostasis as a central outcome of biostimulant treatment. capacity. Additionally, the observed increase in cucurbitacins, positively correlated with leaf firmness, suggests that biostimulants may also influence structural and defense-related secondary metabolism. Although cucurbitacins are often studied for their pharmacological relevance, in planta their accumulation may reflect enhanced defensive capacity and stress adaptation mechanisms (Mkhize et al. 2023), which could indirectly support improved tissue integrity during storage.

Taken together, the integration of physiological, biochemical, and metabolomic data indicates that these commercial biostimulants act through coordinated regulation of redox balance, hormonal signaling, and carbon–nitrogen metabolic networks. Rather than functioning solely as nutrient supplements, they appear to trigger metabolic priming and signaling cascades that enhance growth performance and confer improved postharvest stability. This study therefore advances current understanding of biostimulant mode of action by providing integrative evidence that distinct formulations and application methods induce specific metabolic fingerprints linked to functional outcomes in lettuce. Such mechanistic insights contribute to refining biostimulant use strategies and support their rational deployment in sustainable horticultural systems.

Conclusions

This study evaluated the effects of three commercial non-microbial biostimulants applied via foliar spray or drip irrigation on lettuce growth, metabolic profile, and postharvest

performance. Protein hydrolysates applied as foliar spray produced the greatest improvements in plant diameter, chlorophyll content, and nitrogen balance index. Metabolomic analysis revealed treatment- and application-dependent metabolic reprogramming, with foliar applications generally inducing stronger modulation of secondary metabolism.

Enhanced accumulation of phenolic compounds, flavonoid glucosides, vitamins, and specific secondary metabolites was associated with increased antioxidant capacity, improved firmness, and reduced postharvest weight loss. The data suggest that these biostimulants act through coordinated modulation of redox-related pathways, hormonal signaling, and carbon–nitrogen metabolism rather than through simple nutritional supplementation. Overall, the results demonstrate that biostimulant type and application method determine distinct metabolic signatures that translate into measurable agronomic and postharvest outcomes in lettuce.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00344-026-12277-x.

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Declarations

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

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