



Research article

Biofertilizer alters primary and secondary metabolism in winter-grown lettuce in a cultivar-dependent manner

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Abstract: In this study, we evaluated the effects of biofertilizer on primary and secondary metabolites in lettuce. Green 'Aleppo' and red 'Murai' cultivars were grown in an unheated greenhouse during winter production under biofertilizer and control (no fertilization) conditions. Main effects of cultivar showed that red 'Murai' exhibited higher total antioxidant capacity (TAC), total phenolic content (TPC), and total carotenoid content, myo-inositol, malic, propionic, and citric acids, and higher concentrations of chicoric and chlorogenic acids, caffeoylquinic acid glucoside, along with all quantified flavonoids except kaempferol. In contrast, green 'Aleppo' showed higher fructose, sucrose, total sweetness index (TSI), total soluble solids (TSS), shikimic acid, fumaric acid, lactucopiricin, and lactucopiricin-15-oxalate. Biofertilizer application significantly increased glucose by 42.9%, fructose by 39.1%, and sucrose by 57.8%, resulting in a 40.5% increase in TSI. Furthermore, malic acid increased significantly by 23.2% and fumaric acid by 36.0%, whereas myo-inositol and propionic acid significantly decreased by 23.3% and 21.6%, respectively. Biofertilizer also reduced major phenolic compounds, including chicoric acid (66.9%), chlorogenic acid (81.2%), and caffeoylquinic acid glucoside (32.6%), along with TPC (19.4%) and TAC (54.0%). All principal flavonoids were significantly reduced (30.1–70.8%) when averaged across cultivars, whereas sesquiterpene lactones showed no significant biofertilizer main effect, and lactucin was the predominant lactone. Significant cultivar × biofertilizer interactions revealed contrasting metabolic responses between cultivars. EM Aktiv reduced phenolic acids and flavonoids in 'Murai', whereas in 'Aleppo', these compounds were generally maintained or enhanced in a compound-dependent responses, alongside increases in sugars and selected organic acids. Lactones showed a crossover response, increasing under EM treatment in 'Aleppo' but decreasing in 'Murai'. These results demonstrate that lettuce responses to biofertilizer treatment are strongly cultivar-dependent, highlighting the importance of genotype selection in determining metabolic responses

under biofertilizer application. Overall, these changes may indicate altered allocation between primary and secondary metabolism under winter conditions.

Keywords: *Lactuca sativa*; effective microorganisms; winter season; spectrophotometric assays; sugars; organic acids; phenolic acids; flavonoids; sesquiterpene lactones; HPLC/LC/MS/DAD

1. Introduction

According to FAO data [1], global lettuce and chicory production in 2024 reached more than 28 million tons, with leading producers, China, the USA, and India, accounting for 71.8% of world output, while in Europe, the main countries, Spain, Italy, and Belgium, collectively contributed 7.5% to the global total. Lettuce (*Lactuca sativa* L.) ranks among the most widely consumed vegetable worldwide as fresh produce and in popular ready-to-eat products. This aligns with modern urban lifestyles, boosting demand for minimally processed foods, where operations like peeling, cutting, and dipping do not compromise nutritional properties compared to intense thermal processing [2]. Nevertheless, its nutritional value has been underestimated and considered low due to its high-water content (close to 95%) [3,4]. Depending on type, lettuce challenges "nutritious" vegetables as a source of health-promoting compounds like phenolics, carotenoids, chlorophylls, vitamins, and sesquiterpene lactones, as well as dietary fiber and minerals [5,6]. These compounds' antioxidant properties offer protection against metabolic syndrome, cancer, osteoporosis, and neurodegenerative diseases, as supported by epidemiological studies and meta-analyses on plant polyphenol-rich diets [7]. Since lettuce is generally eaten raw, more nutrients are retained compared to other vegetables that are cooked or thermally processed [4].

The total antioxidant capacity (TAC) and total phenolic content (TPC) are significantly affected by cultivar, with red lettuce cultivars consistently exhibiting higher levels than green counterparts [6,8]. Moreover, genotype and growing conditions alter antioxidant composition and properties in different crops [9]. Significant phytochemical variability across cultivars emphasizes selecting specific types of cultivars for optimal results [10]. In lettuce, key phenolic compounds include hydroxycinnamic acid, caffeic acid derivatives, caffeoylquinic acid isomers, chicoric acid, and flavonols such as quercetin-3-glucuronide and quercetin-hexoside [11,12]; phenolic acids are usually present in bound forms as glycosylated derivatives or esters [13]. The glycosylated derivatives of quercetin and kaempferol are the major flavonoids and flavonols found in lettuce [14,15].

Phenolic compounds are synthesized via the phenylpropanoid pathway and may accumulate under abiotic stresses such as nitrogen limitation, salinity, and drought in lettuce [11,16,17]. Lettuce prefers temperate climates with an optimal temperature range of 15–20 °C, and heat stress above 30 °C impairs development and causes premature bolting [18]. In continental regions, lettuce is commonly cultivated in unheated greenhouses without supplemental lighting, typically in soil-based systems for large-scale production. Covering plants with agrotexile during late autumn-winter production increases temperatures up to 10 °C compared to open-field cultivation [19]. On the other hand, chilling injury occurs at 0–8 °C and freezing injury below 0 °C, causing blisters, peeling, decay, rot, and pathogen entry through damaged leaves [20]. Abiotic stress conditions, including low temperatures, can trigger physiological and biochemical responses in plants, such as increased production of reactive oxygen species (ROS), activation of antioxidant systems, and changes in membrane lipid composition [21].

Seasonal variation has been shown to influence phenolic accumulation in lettuce, with one study reporting higher levels in winter compared to summer conditions [22], and flavonoid biosynthesis increases with lower temperatures in *Arabidopsis* and kale [23,24]. However, fewer researchers have explored seasonal environmental effects on lettuce phenolic composition [25,26]. Additionally, sesquiterpene lactones in lettuce exhibit anti-herbivory, antimicrobial, and allelopathic effects contributing to cold stress and wound defense [27], as cold compromises tissue integrity, creating infection sites for fungi and bacteria.

Climate vulnerabilities are increasingly intensified by global change, which unpredictably disrupts ecosystems and agriculture, compelling plants to metabolically adapt via secondary metabolites central to plant-environment interactions [28]. This highlights the urgent need for sustainable solutions like biofertilizers and beneficial microbes to mitigate plant stress responses amid intensifying soil degradation and rising production costs [29].

To address these stressors and soil challenges, biofertilizers containing bacteria, fungi, and algae provide a sustainable alternative to mineral fertilizers and pesticides [29]. They enrich soil organic matter, solubilize phosphates, potassium, and micronutrients, fix nitrogen in legumes, produce plant growth-promoting hormones, enhance root architecture, nutrient absorption, and photosynthesis, and contribute to improved crop quality and yield while reducing dependence on chemical inputs [30–32]. Additionally, plant growth-promoting rhizobacteria (PGPR) enable biological pathogen control [33]. Climate-controlled studies isolate variables but poorly predict field responses in plants, where wind, radiation, humidity, and temperature oscillations interact [34]. Given that 95% of food is still produced in soil [35], winter biofertilizer-field research remains scarce. Therefore, there is limited information on how biofertilizer application modulates primary and secondary metabolite profiles in lettuce cultivated under winter, unheated greenhouse conditions with natural photoperiods, particularly when cultivar-dependent responses and multiple metabolite classes are considered within the same experimental framework. Addressing this gap may provide a more integrated understanding of how biofertilizer application influences lettuce nutritional and phytochemical quality under commercially relevant growing conditions.

Phenylpropanoid pathways respond to environmental cues and are involved in the accumulation of phenolic compounds in lettuce [22,36,37]. Thus, we hypothesized that biofertilizer application during winter lettuce production would enhance primary (sugars, organic acids) and secondary (phenolic acids, flavonoids, sesquiterpene lactones) metabolites, thereby improving nutritional value and potential health benefits. We aimed to assess the impact of biofertilizer application on the composition and quantitative changes of primary and secondary metabolites in green and red lettuce grown under winter conditions in an unheated greenhouse, compared with non-treated control plants.

2. Materials and methods

2.1. Plant material and biofertilizers

Two lettuce cultivars, 'Aleppo' and 'Murai' (both belonging to *Lactuca sativa* var. *crispa*), were examined in this study. Lettuce seeds were obtained from the local company RZ Agro (Novi Banovci, Serbia), which sources them from the vegetable seed company Rijk Zwaan (De Lier, the Netherlands). Lettuce seeds were sown in 4-cm-sized peat cubes (Potgrond H, Klasman-Deilmann GmbH, Geeste, Germany) and cultivated under glasshouse conditions for 39 days. At the four-leaf stage, lettuce

transplants were transferred to their final positions in the greenhouse.

The biofertilizer EM Aktiv (Candor, Valpovo, Croatia) is a liquid formulation produced from the microbiological fermentation of sugarcane and molasses. This formulation is based on different beneficial microorganisms (*Lactobacillus plantarum*, *Lactobacillus casei*, *Lactococcus lactis*, *Rhodopseudomonas palustris*, *Rhodobacter sphaeroides*, *Saccharomyces cerevisiae*, *Streptomyces albus*, *Streptomyces griseus*, *Aspergillus oryzae*, *Bacillus subtilis*, *Bacillus megaterium*, and *Azotobacter chroococcum*), at 11.6×10^3 – 12×10^8 CFU (colony-forming units)/mL. Additionally, it contains a minimum of 56% total organic matter, a minimum of 33% organic C, 1.6%–1.9% total N, 0.06%–0.075% total P₂O₅, 1.75%–1.99% total K₂O, and 0.57%–0.68% Ca (on dry weight basis). The fertilization treatments were carried out via soil and foliar applications during the vegetation period.

2.2. Experimental design and climatic conditions

The experiment was carried out in an unheated greenhouse (144 m²) in the municipality of Surčin, Belgrade, Serbia (44°47'39"N, 20°20'12"E; 73 m above sea level) under natural light conditions. Plants were cultivated during a winter experiment from December to April. Soil analysis revealed sandy clay loam texture with moderately alkaline pH (8.1). It contained total nitrogen (0.14%), readily available phosphorus (33.92 mg/100 g), readily available potassium (18.69 mg/100 g), and organic matter content (2.83%). The experiment was arranged in a randomized complete block design with a 2 × 2 factorial structure, consisting of two lettuce cultivars and two fertilization treatments. The treatments included a control (without fertilization) and application of biofertilizer EM Aktiv, with three replicates. Experimental plots measured 0.75 m × 1 m, containing 12 plants in each plot, cultivated at a spacing of 25 cm × 25 cm. The spacing between plots within each block was 50 cm, while the spacing between blocks was 100 cm. After soil preparation (including tractor and tiller operations), soil treatment was applied at a concentration of 15 mL of EM Aktiv. Black mulch film was then positioned, and lettuce transplants were planted in the soil. Foliar application consisted of 5 mL of EM Aktiv using a battery sprayer, applied four times during the growing cycle. Standard agronomic practices were applied during the vegetation period to maintain optimal growth conditions (irrigation, ventilation, hoeing, manual weeding, and preventive protection against diseases and pests).

Climatic data were measured using an RC-4HC Data Logger (Elitech Technology Inc., San Jose, CA, USA), which continuously collected air temperature and humidity data over 24 hours (Figure 1). During the experimental period, average air temperature ranged from −1.5 °C to 20.4 °C, while maximum and minimum temperatures reached 39.8 °C and −14.3 °C, respectively. Average relative air humidity ranged from 51.7% to 84.4% (Figure 1). Photoperiod varied between 9 and 13 h under natural light conditions, with no supplemental lighting, and light intensity was not measured.

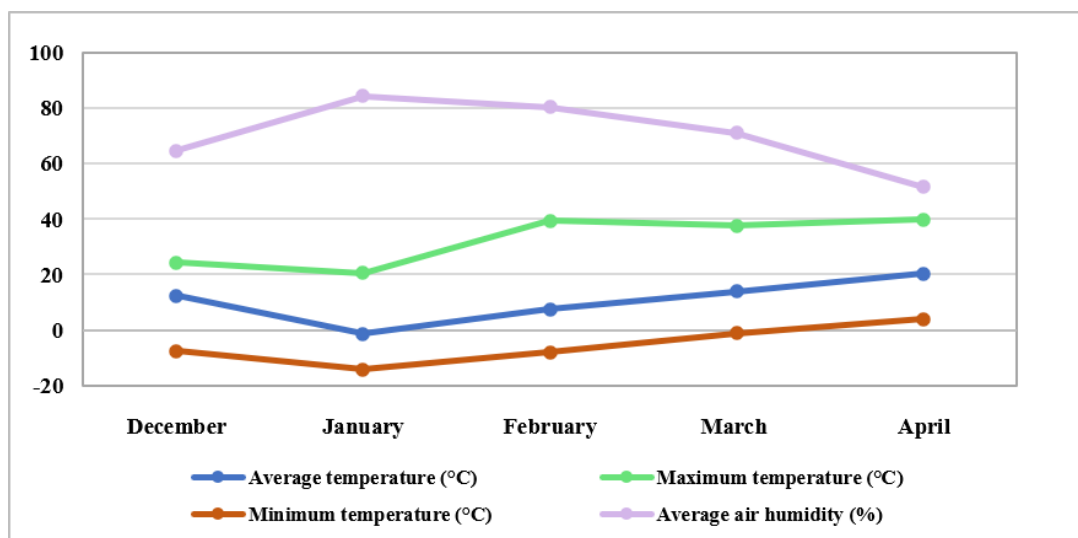


Figure 1. Climatic conditions during the lettuce vegetation period.

2.3. Data collection and sample preparation for spectrophotometric assays

Lettuce plants were hand-harvested 100 days after transplanting, when they reached marketable size, adequate compactness, and physiological maturity. Immediately after harvest, lettuce leaves were packed in plastic bags and stored at $-20\text{ }^{\circ}\text{C}$ until further analysis. Frozen samples were ground in liquid nitrogen, homogenized, and extracted ($w:v = 1:3$) in 80% methanol, then centrifuged at $13.000 \times g$ for 10 min to obtain supernatant for TAC and TPC assays. For chlorophyll and carotenoid assays, samples were extracted with 80% acetone ($w:v = 1:2$) and centrifuged at $13.000 \times g$ for 10 min.

2.3.1. Spectrophotometric determination of chlorophyll, carotenoids, TAC, and TPC

Chlorophyll and carotenoid contents were measured spectrophotometrically from 80% acetone extracts using Lichtenthaler's method [38]. Absorbance was measured at 663, 647, and 470 nm (Multiskan Spectrum, Thermo Electron Corporation, Vantaa, Finland), and results were expressed as microgram per gram of fresh weight ($\mu\text{g/g FW}$), calculated using the following equations:

$$\text{Chl}_a = 12.25 \times A_{663} - 2.79 \times A_{647} \text{ (}\mu\text{g/mL)} \quad (1)$$

$$\text{Chl}_b = 21.50 \times A_{647} - 5.10 \times A_{663} \text{ (}\mu\text{g/mL)} \quad (2)$$

$$\text{Chl}_{(a+b)} = 7.15 \times A_{663} + 18.71 \times A_{647} \text{ (}\mu\text{g/mL)} \quad (3)$$

$$\text{Chl}_{x+c} = (1000 \times A_{470} - 1.82 \times \text{Chl}_a - 85.02 \times \text{Chl}_b)/198 \text{ (}\mu\text{g/mL)} \quad (4)$$

TAC was determined using the ABTS assay [39]. The reaction mixture contained 2 mM ABTS, 15 $\mu\text{M H}_2\text{O}_2$, and 0.25 μM horseradish peroxidase (HRP) in 50 mM phosphate buffer (pH 7.5). Absorbance was recorded at 730 nm (Multiskan Spectrum, Thermo Electron Corporation, Vantaa, Finland) at $25\text{ }^{\circ}\text{C}$ until stabilization, indicating $\text{ABTS}^{\bullet+}$ radical formation. Different concentrations of ascorbic acid (AA, 0.1–0.8 mM) used as standard and sample extracts were added to the reaction mixture, causing a decrease in absorbance due to $\text{ABTS}^{\bullet+}$ radical scavenging. TAC was quantified using the linear regression equation from the ascorbic acid standard curve and expressed as milligrams

of ascorbic acid equivalents per g of fresh weight (mg AA eq/g FW).

TPC was measured using the Folin-Ciocalteu spectrophotometric method with gallic acid (GA) for the standard curve calibration [40]. Samples and standards were mixed with 0.25 N Folin-Ciocalteu reagent and incubated for 3 min at room temperature. Following the addition of 0.2 M Na₂CO₃, mixtures were incubated for 60 min at room temperature. Absorbance was measured at 724 nm using a Multiskan Spectrum spectrophotometer (Thermo Electron Corporation, Vantaa, Finland), with results expressed as microgram of gallic acid equivalents per g of fresh weight (µg GA eq/g FW).

2.4. Preparation of samples for high-performance liquid chromatography (HPLC) analysis

For the analysis of sugars and organic acids, frozen samples were homogenized in liquid nitrogen and extracted with 80% methanol (*w:v* = 1:3). Following homogenization, the mixture was centrifuged at $13.000 \times g$ for 10 min at 4°C, and the supernatant was collected for HPLC analysis.

For phenolic and sesquiterpene lactone analysis, samples were freeze-dried for 48 h. The lyophilized material was then sealed in plastic bags and kept at -20 °C until further analysis. Dried samples underwent extraction using an ultrasonic processor (USCG-300, Infitek Co., Ltd., Jinan, China) in 80% methanol (*w:v* = 1:25) for 5 min. Next, samples were shaken at 1.000 rpm (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) for 17 h at 25°C. The mixture was centrifuged at $11.000 \times g$ (Sigma Centrifuge, Sigma Laboratory Centrifuges, Osterode, Germany) for 30 min at 25 °C, and the supernatant was used for further HPLC analysis of phenolics and sesquiterpene lactones.

2.4.1. HPLC analysis of sugars

Analysis of sugars was performed using a CarboPac PA1 column (250 × 4 mm, Dionex, Thermo Fisher Scientific Inc., Waltham, MA, USA) together with its matching guard column. The column was maintained at a constant temperature of 30 °C and a 1.0 mL/min flow rate to ensure optimal separation. A Waters 2465 electrochemical detector, equipped with a 3 mm gold working electrode and hydrogen reference electrode, provided highly sensitive and selective sugar detection under these conditions. The eluent was 200 mM sodium hydroxide, obtained by adding 10.5 mL of 50% (*w/w*) low-carbonate NaOH solution (J.T. Baker, Deventer, Netherlands) to vacuum-degassed deionized water up to 1 L of final volume. Injection volume was 20 µL. The 2465 detector operated in pulsed amperometric mode, capturing signals across 150 ms via waveform stages: E1 = +0.15 V for 400 ms, E2 = +0.75 V for 200 ms, E3 = -0.8 V for 200 ms. Settings also featured a 0.2 s filter time and 1 µA range. Waters Empower 2 software (Waters, Milford, MA, USA) was used for data recording and processing. Peaks corresponding to myo-inositol, glucose, fructose, and sucrose were confirmed and quantified using an external calibration method, expressed in milligrams per gram of fresh weight (mg/g FW). An unidentified peak (Compound 1) was quantified using arabinose as an external standard and expressed as milligram arabinose equivalents per g of fresh weight.

2.4.2. HPLC analysis of organic acids

Organic acids were quantified on the HPLC system (Waters, Milford, MA, USA), which included a 1525 binary pump, column thermostat, 717+ autosampler, and a Waters 2996 photodiode array detector set up to 210 nm. The Supelco C-610H column (300 × 7.8 mm, Sigma-Aldrich, Barcelona,

Spain), along with its guard column, was used in isocratic elution with 0.1% phosphoric acid as eluent at 0.5 mL/min flow, and at 40 °C column temperature. The injection volume was 20 µL. Waters Empower 2 software (Waters, Milford, MA, USA) was used for data recording and evaluation. Peaks of malic, citric, tartaric, and propionic acid were confirmed and quantified via the external standard method, expressed as milligrams per gram of fresh weight (mg/g FW). Shikimic and fumaric acid were expressed in microgram per gram of fresh weight (µg/g FW). Compounds 2 and 3 were quantified using oxalic acid and formic acid as external standards, respectively, and expressed as equivalent concentrations (mg/g FW). All reference standards originated from Sigma-Aldrich (St. Louis, MO, USA).

2.4.3. HPLC/DAD/LC/MS analysis of phenolic compounds and sesquiterpene lactones

Samples underwent analysis on a Waters HPLC system featuring a 1525 binary pump, column thermostat, 717+ autosampler, 2996 diode array detector (DAD), and EMD 1000 quadrupole mass spectrometer with electrospray ionization (ESI) interface (Waters, Milford, MA, USA). Phenolics were separated on a Symmetry C-18 reversed-phase column (150 × 4 mm, 5 µm particle size) using a matching Symmetry guard column. Eluents consisted of 0.1% formic acid (A) and acetonitrile (B), pumped at 1 mL/min via this gradient: initial 10% B, ramping linearly to 50% B in 35 min, then back to 10% B over 10 min, with 5 min re-equilibration. A 5:1 post-column splitter (ASI, Richmond, CA, USA) reduced flow to 0.2 mL/min for MS. Injection volume for phenolics and sesquiterpene lactones analysis was 50 µL. DAD recorded peaks at 254 nm (sesquiterpene lactones), 330 nm (phenolic acids), and 380 nm (flavonoids). MS scans were recorded using LC/MS/ESI in negative ion scanning mode (m/z 100–900). ESI source parameters were: capillary voltage 3.0 kV, cone voltage –35 V, extractor voltage 3.0 V, RF lens 0.2 V, source temperature 130 °C, desolvation temperature 400 °C, and nitrogen flow at 500 L/h. All recorded peaks were validated and quantified with Empower 2 software (Waters, Milford, MA, USA) via the external standard method. Protocatechuic, chlorogenic, caffeic acids, and kaempferol were quantified using commercial standards (Sigma-Aldrich, St. Louis, MO, USA) and expressed as microgram per g of fresh weight (µg/g FW). Chicoric acid, other caffeic derivatives, and caffeoylquinic acid glucoside were expressed as µg caffeic acid equivalents per g FW. Quercetin and kaempferol derivatives were quantified as µg quercetin and µg kaempferol equivalents per g FW, respectively. Lactucin and lactucopicrin were quantified via the external standard method using available standards (Extrasynthese, Genay, France) and expressed as µg/g fresh weight (FW). Lactucopicrin-15-oxalate was reported as µg lactucopicrin equivalents per g FW.

2.5. Total soluble solids and total sweetness index

Total soluble solids (TSS) were measured at room temperature using a digital hand refractometer (ORA20BA, Kern & Sohn GmbH, Balingen, Germany) from the leaf sap following the procedure of Ntsoane and coauthors [41], and expressed as degrees Brix (°Brix).

The total sweetness index (TSI) was calculated from the results obtained from the HPLC analysis of sugars (glucose, fructose, and sucrose) based on the concentration and sweetness coefficient of each soluble sugar using the equation [42]:

$$\text{TSI} = 1.50 \times \text{fructose} + 0.76 \times \text{glucose} + 1.00 \times \text{sucrose} \quad (5)$$

Equation 5 shows that each sugar is multiplied by a specific factor reflecting its perceived sweetness compared to sucrose.

2.6. Statistical analysis

Statistical analysis was performed using a two-way ANOVA to evaluate the effects of two main factors, cultivar and biofertilizer treatment, and their interaction (cultivar $\times$ biofertilizer). Tukey's HSD post hoc test ($\alpha = 0.05$) was applied for pairwise comparisons of significant main effects. Pearson's correlation coefficient was used to examine relationships between parameters. As the analysis was exploratory, no correction for multiple comparisons was applied, and the results were interpreted accordingly. Analyses were conducted using SPSS Statistics (Version 25.0; IBM Corp., Armonk, NY, USA) and Microsoft Office Excel 2019 (Microsoft Corp., Redmond, WA, USA).

3. Results

3.1. Results from spectrophotometric assays

Cultivar and biofertilizer showed no significant main effects on chlorophyll a, chlorophyll b, or total chlorophyll (a + b) levels (Table 1). In contrast, cultivar significantly affected total carotenoid content, with the red cultivar 'Murai' achieving 1.94-fold higher levels ($4.05 \mu\text{g/g FW}$) than the green cultivar 'Aleppo' ($2.09 \mu\text{g/g FW}$). The EM Aktiv fertilizer resulted in slightly elevated carotenoid levels, but this was not significant as a main effect of treatment. However, the significant cultivar $\times$ fertilizer interaction indicated that responses differed between cultivars.

Table 1. Effects of cultivar, fertilizer, and their interaction on lettuce spectrophotometric assays.

Main factors		Chl _a ($\mu\text{g/g FW}$)	Chl _b ($\mu\text{g/g FW}$)	Chl _(a+b) ($\mu\text{g/g FW}$)	Total Car ($\mu\text{g/g FW}$)	TAC (mg AA eq/g FW)	TPC ($\mu\text{g GA eq/g FW}$)
Cultivar	Murai	2.26 ± 0.12	2.33 ± 0.04	4.78 ± 0.32	4.05 ± 0.19^b	0.91 ± 0.08^b	265.23 ± 18.79^b
	Aleppo	2.28 ± 0.16	2.23 ± 0.10	4.51 ± 0.25	2.09 ± 0.07^a	0.74 ± 0.01^a	168.14 ± 13.00^a
Fertilizer	Control	2.35 ± 0.16	2.35 ± 0.06	4.90 ± 0.38	2.95 ± 0.12	1.13 ± 0.04^b	239.93 ± 17.90^b
	EM Aktiv	2.18 ± 0.12	2.21 ± 0.08	4.39 ± 0.20	3.19 ± 0.14	0.52 ± 0.04^a	193.44 ± 13.88^a
Significance	Cultivar (C)	ns	ns	ns	***	*	***
	Fertilizer (F)	ns	ns	ns	ns	***	*
Interaction factors	C $\times$ F	*	ns	ns	*	***	***

Note: The data show the means ($n = 3$) $\pm$ SE. Different letters within each column indicate significant differences according to Tukey's HSD test ($p \leq 0.05$). Asterisks indicate significant differences at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns, non-significant. Chl_a: chlorophyll a, Chl_b: chlorophyll b, Chl (a+b): chlorophyll (a+b), Total Car: total carotenoids, TAC: total antioxidant capacity, TPC: total phenolic content.

Cultivar and biofertilizer showed significant main effects on TAC and TPC values (Table 1). The red cultivar 'Murai' exhibited significantly higher TAC ($0.91 \text{ mg AA eq/g FW}$) and TPC ($265.23 \mu\text{g GA eq/g FW}$) compared to the green cultivar 'Aleppo'. Control plants exhibited significantly higher

TAC and TPC contents than those treated with EM Aktiv, which reduced TAC by 54.0% and TPC by 19.4% relative to the control (main effect of fertilizer across cultivars).

In addition to the main factor effects, significant cultivar $\times$ treatment interactions were observed for chlorophyll a, total carotenoids, TAC, and TPC levels (Figure 2).

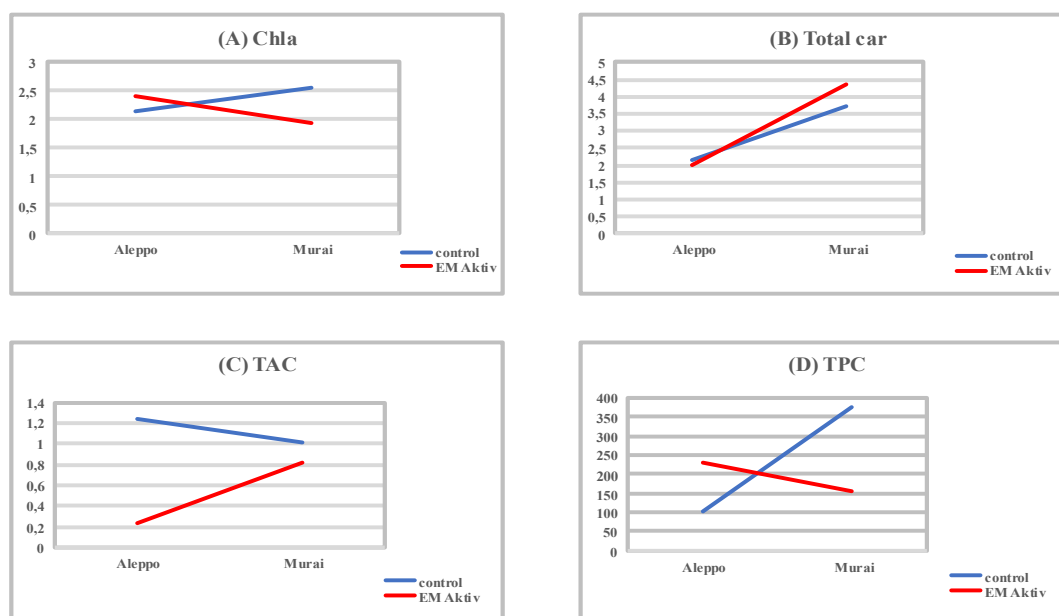


Figure 2. Cultivar $\times$ biofertilizer interaction effects on spectrophotometric assays: (A) chlorophyll a (Chl a, $\mu\text{g/g FW}$), (B) total carotenoids (Total car, $\mu\text{g/g FW}$), (C) total antioxidant capacity (TAC, mg AA eq/g FW), and (D) total phenolic content (TPC, $\mu\text{g GA eq/g FW}$).

Chlorophyll a, total carotenoids, and TPC exhibited crossover interaction patterns, indicating that the response to EM Aktiv differed between cultivars. In 'Aleppo', EM Aktiv increased chlorophyll a and TPC, whereas in 'Murai', higher values of both parameters were observed under the control treatment. A cultivar-dependent response was also observed for total carotenoids, with slightly higher values under the control treatment in 'Aleppo' and higher values under EM Aktiv in 'Murai'. In contrast, TAC displayed a non-crossover interaction, as control plants exhibited higher TAC values than EM Aktiv-treated plants in both cultivars, although the magnitude of this reduction differed between cultivars. Overall, the interaction analysis showed that the direction and magnitude of the response to EM Aktiv varied among cultivars depending on the measured trait.

3.2. Results of sugar quantification by HPLC, TSI, and TSS

Cultivar and biofertilizer showed significant effects on sugar content in lettuce samples, except for the cultivar effect on glucose content (Table 2). The most represented sugars were fructose and glucose, followed by myo-inositol (sugar alcohol), while sucrose was detected only in the green cultivar 'Aleppo'. The green cultivar 'Aleppo' showed higher fructose (20.07 mg/g FW) and sucrose (1.65 mg/g FW) levels than the red cultivar 'Murai', which in turn exhibited greater myo-inositol (3.15

mg/g FW) and unidentified sugar-compound 1 content (5.19 mg arabinose eq/g FW). Application of EM Aktiv biofertilizer resulted in significantly higher levels of glucose (increased by 42.9%), fructose (increased by 39.1%), and sucrose (increased by 57.8%) compared to the control treatment when averaged across cultivars. In contrast, application of EM Aktiv decreased myo-inositol content by 23.3% relative to the control, while it had no significant effect on unidentified sugar-compound 1.

Cultivar significantly affected TSI and TSS, whereas biofertilizer influenced only TSI (Table 2). The green cultivar 'Aleppo' exhibited significantly higher TSI (44.15) and TSS (4.04 °Brix) values than the red cultivar 'Murai'. EM Aktiv significantly increased TSI by 40.5% compared with control (main effect of the fertilizer across cultivars).

Significant cultivar $\times$ treatment interactions were observed for myo-inositol, sucrose, and TSS (Figure 3). For myo-inositol and TSS, the interactions exhibited crossover patterns, with EM Aktiv increasing concentrations in 'Aleppo' but decreasing them in 'Murai', indicating contrasting responses between cultivars. For sucrose, EM Aktiv increased sucrose level in 'Aleppo', whereas sucrose was not detected in 'Murai' under either treatment, indicating a strong genotype-dependent difference in sucrose accumulation driven by presence/absence rather than a simple treatment effect. These results indicate that responses to EM Aktiv were metabolite- and genotype-dependent, rather than uniformly expressed across cultivars.

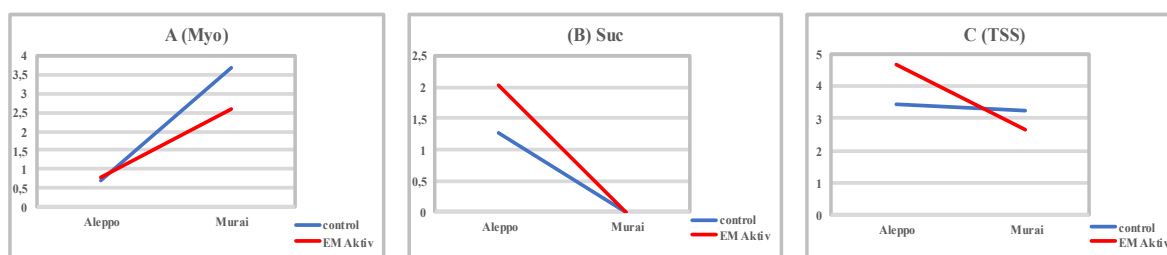


Figure 3. Cultivar $\times$ biofertilizer interaction effects on sugars and total soluble solids: (A) myo-inositol (Myo, mg/g FW), (B) sucrose (Suc, mg/g FW), and (C) total soluble solids (TSS, °Brix).

3.3. Organic acid profiles from HPLC analysis

The major organic acids detected in lettuce samples were malic, propionic, citric, and tartaric acids (Table 3). Cultivar significantly affected the content of most organic acids, except for tartaric acid and unidentified organic acid-compound 2. The red cultivar 'Murai' generally exhibited higher levels of most organic acids, including malic acid (3.93 mg/g FW), propionic acid (1.10 mg/g FW), citric acid (0.43 mg/g FW), and unidentified organic acid-compound 3 (0.08 mg formic acid eq/g FW). The biofertilizer significantly influenced organic acid contents, except for citric, tartaric, shikimic, and unidentified organic acid compounds. EM Aktiv application significantly increased malic acid by 23.2% and fumaric acid by 36.0%, while significantly decreasing propionic acid by 21.6% when averaged across cultivars. Tartaric acid content was unaffected by cultivar, biofertilizer, or their interaction.

Table 2. Effects of cultivar, fertilizer, and their interaction on lettuce sugar content, total sweetness index, and total soluble solids.

Main factors		Myo (mg/g FW)	Glu (mg/g FW)	Fru (mg/g FW)	Suc (mg/g FW)	Compound 1 (mg arabinose eq/g FW)	TSI	TSS (°Brix)
Cultivar	Murai	3.15 ± 0.23 ^b	14.88 ± 0.43	17.87 ± 1.10 ^a	0.0 ± 0.0 ^a	5.19 ± 0.19 ^b	38.11 ± 1.98 ^a	2.96 ± 0.36 ^a
	Aleppo	0.73 ± 0.01 ^a	16.32 ± 0.71	20.07 ± 0.63 ^b	1.65 ± 0.08 ^b	4.32 ± 0.30 ^a	44.15 ± 1.45 ^b	4.04 ± 0.28 ^b
Fertilizer	Control	2.19 ± 0.14 ^b	12.85 ± 0.96 ^a	15.87 ± 1.01 ^a	0.64 ± 0.002 ^a	4.95 ± 0.31	34.21 ± 2.25 ^a	3.33 ± 0.30
	EM Aktiv	1.68 ± 0.11 ^a	18.36 ± 0.18 ^b	22.07 ± 0.71 ^b	1.01 ± 0.08 ^b	4.56 ± 0.18	48.06 ± 1.18 ^b	3.67 ± 0.33
Significance	Cultivar (C)	***	ns	*	***	**	*	**
	Fertilizer (F)	*	***	***	**	ns	***	ns
Interaction factors	C × F	**	ns	ns	**	ns	ns	*

Note: The data show the means ($n = 3$) ± SE. Different letters within each column indicate significant differences according to Tukey's HSD test ($p \leq 0.05$). Asterisks indicate significant differences at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns, non-significant. Myo: myo-inositol, Glu: glucose, Fru: fructose, Suc: sucrose, Compound 1: unidentified sugar peak, TSI: total sweetness index, TSS: total soluble solids.

Table 3. Effects of cultivar, fertilizer, and their interaction on lettuce organic acid content.

Main factors		Mal (mg/g FW)	Prop (mg/g FW)	Cit (mg/g FW)	Tar (mg/g FW)	Shik (μg/g FW)	Fum (μg/g FW)	Compound 2 (mg oxalic acid eq/g FW)	Compound 3 (mg formic acid eq/g FW)
Cultivar	Murai	3.93 ± 0.32 ^b	1.10 ± 0.04 ^b	0.43 ± 0.03 ^b	0.08 ± 0.01	19.76 ± 1.52 ^a	32.54 ± 2.84 ^a	0.006 ± 0.0004	0.08 ± 0.003 ^b
	Aleppo	2.99 ± 0.16 ^a	0.72 ± 0.03 ^a	0.23 ± 0.01 ^a	0.09 ± 0.01	25.02 ± 2.14 ^b	41.38 ± 2.74 ^b	0.005 ± 0.0004	0.06 ± 0.005 ^a
Fertilizer	Control	3.10 ± 0.29 ^a	1.02 ± 0.06 ^b	0.34 ± 0.02	0.08 ± 0.01	20.95 ± 1.77	31.32 ± 2.91 ^a	0.005 ± 0.001	0.07 ± 0.004
	EM Aktiv	3.82 ± 0.19 ^b	0.80 ± 0.01 ^a	0.32 ± 0.02	0.09 ± 0.01	23.82 ± 1.88	42.60 ± 2.67 ^b	0.006 ± 0.0003	0.07 ± 0.004
Significance	Cultivar (C)	**	***	***	ns	*	*	ns	**
	Fertilizer (F)	*	***	ns	ns	ns	**	ns	ns
Interaction factors	C × F	***	*	***	ns	ns	***	*	ns

Note: The data show the means ($n = 3$) ± SE. Different letters within each column indicate significant differences according to Tukey's HSD test ($p \leq 0.05$). Asterisks indicate significant differences at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns, non-significant. Mal: malic acid, Prop: propionic acid, Cit: citric acid, Tar: tartaric acid, Shik: shikimic acid, Fum: fumaric acid, Compound 2: unidentified organic acid peak, Compound 3: unidentified organic acid peak.

Significant cultivar $\times$ biofertilizer interactions were observed for malic, citric, propionic, fumaric acids, and unidentified compound 2 (Figure 4), indicating cultivar-dependent responses to the treatment. Malic, citric, and fumaric acids exhibited crossover interaction patterns, with EM Aktiv increasing concentrations in 'Aleppo' but decreasing them in 'Murai'. Propionic acid showed a non-crossover interaction, with EM Aktiv reducing concentrations in both cultivars, although the decrease was more pronounced in 'Murai'. Compound 2 also showed a significant interaction, driven by an increase under EM Aktiv application in 'Murai', while no treatment effect was observed in 'Aleppo'.

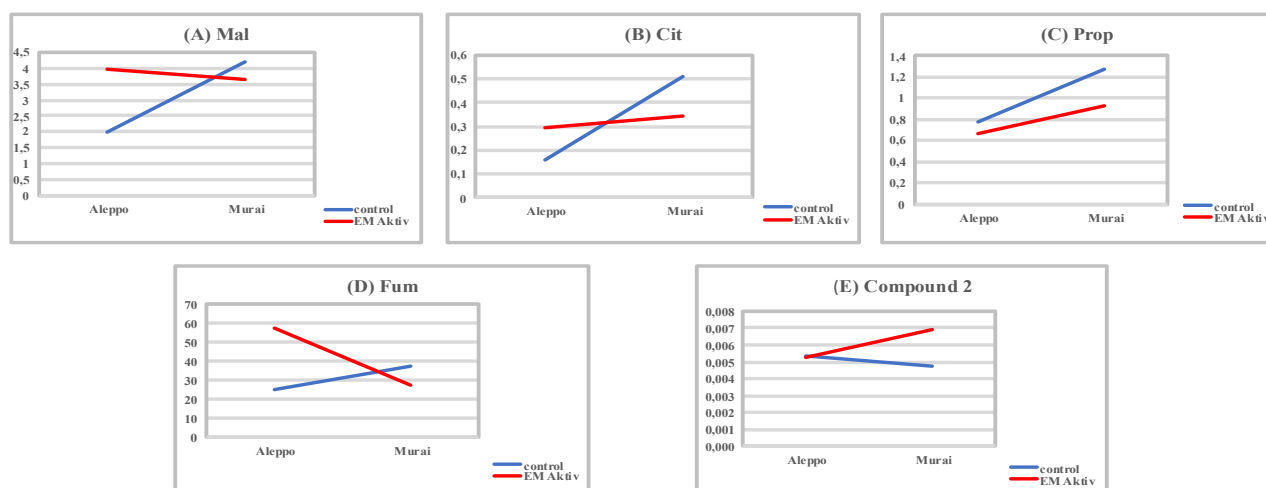


Figure 4. Cultivar $\times$ biofertilizer interaction effects on organic acids: (A) malic acid (Mal, mg/g FW), (B) citric acid (Cit, mg/g FW), (C) propionic acid (Prop, mg/g FW), (D) fumaric acid (Fum, μ g/g FW), and (E) unidentified organic acid peak-compound 2 (Compound 2, mg oxalic acid eq/g FW).

3.4. Phenolic acid profiles from HPLC analysis

Cultivar and biofertilizer significantly affected phenolic acid content, particularly chlorogenic, chicoric, caffeoylquinic acid glucoside, and caffeic acid glucuronide A (Table 4), with no effects on protocatechuic acid, caffeic acid, caffeic acid-3-glucoside, or caffeic acid glucuronide B. The red cultivar 'Murai' generally exhibited higher phenolic acid levels, while 'Aleppo' showed elevated concentration of caffeic acid glucuronide A. EM Aktiv application decreased major phenolic acids: chlorogenic acid by 81.2%, caffeoylquinic acid glucoside by 32.6%, chicoric acid by 66.9%, and caffeic acid glucuronide A by 21.8%, when averaged across cultivars.

Table 4. Effects of cultivar, fertilizer, and their interaction on lettuce phenolic acid content.

Main factors		PCA ($\mu\text{g/g FW}$)	CGA ($\mu\text{g/g FW}$)	CA ($\mu\text{g/g FW}$)	CQAG (μg caffeic acid eq/g FW)	CCA (μg caffeic acid eq/g FW)	CAG (μg caffeic acid eq/g FW)	CAGrA (μg caffeic acid eq/g FW)	CAGrB (μg caffeic acid eq/g FW)
Cultivar	Murai	4.38 $\pm$ 0.38	16.80 $\pm$ 1.46 ^b	1.99 $\pm$ 0.11	5.87 $\pm$ 0.48 ^b	32.11 $\pm$ 2.18 ^b	2.68 $\pm$ 0.08	3.55 $\pm$ 0.19 ^a	2.63 $\pm$ 0.20
	Aleppo	4.25 $\pm$ 0.22	3.19 $\pm$ 0.06 ^a	2.25 $\pm$ 0.14	4.56 $\pm$ 0.32 ^a	19.79 $\pm$ 1.68 ^a	3.00 $\pm$ 0.28	4.30 $\pm$ 0.28 ^b	2.66 $\pm$ 0.13
Fertilizer	Control	4.67 $\pm$ 0.46	16.82 $\pm$ 1.45 ^b	2.20 $\pm$ 0.14	6.23 $\pm$ 0.70 ^b	39.00 $\pm$ 2.84 ^b	2.86 $\pm$ 0.21	4.41 $\pm$ 0.34 ^b	2.77 $\pm$ 0.25
	EM Aktiv	3.95 $\pm$ 0.13	3.16 $\pm$ 0.07 ^a	2.04 $\pm$ 0.10	4.20 $\pm$ 0.10 ^a	12.91 $\pm$ 1.01 ^a	2.81 $\pm$ 0.15	3.45 $\pm$ 0.13 ^a	2.52 $\pm$ 0.09
Significance	Cultivar (C)	ns	***	ns	*	***	ns	*	ns
	Fertilizer (F)	ns	***	ns	**	***	ns	**	ns
Interaction factors	C $\times$ F	*	***	*	**	***	ns	*	**

Note: The data show the means ($n = 3$) $\pm$ SE. Different letters within each column indicate significant differences according to Tukey's HSD test ($p \leq 0.05$). Asterisks indicate significant differences at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns, non-significant. PCA: protocatechuic acid, CGA: chlorogenic acid, CA: caffeic acid, CQAG: caffeoylquinic acid glucoside, CCA: chicoric acid, CAG: caffeic acid-3-glucoside, CAGrA: caffeic acid glucuronide A, CAGrB: caffeic acid glucuronide B.

Table 5. Effects of cultivar, fertilizer, and their interaction on lettuce flavonoid content.

Main factors		QMDG (μg quercetin eq/g FW)	QG (μg quercetin eq/g FW)	QMG (μg quercetin eq/g FW)	QacG (μg quercetin eq/g FW)	KMalG (μg kaempferol eq/g FW)	K ($\mu\text{g/g FW}$)
Cultivar	Murai	1.91 $\pm$ 0.15 ^b	73.23 $\pm$ 5.37 ^b	51.91 $\pm$ 5.28 ^b	3.06 $\pm$ 0.30 ^b	0.80 $\pm$ 0.05 ^b	4.78 $\pm$ 0.09
	Aleppo	1.20 $\pm$ 0.12 ^a	25.96 $\pm$ 2.21 ^a	11.28 $\pm$ 0.93 ^a	0.69 $\pm$ 0.05 ^a	0.32 $\pm$ 0.02 ^a	5.35 $\pm$ 0.37
Fertilizer	Control	1.86 $\pm$ 0.16 ^b	58.37 $\pm$ 5.77 ^b	47.66 $\pm$ 5.61 ^b	2.91 $\pm$ 0.29 ^b	0.77 $\pm$ 0.06 ^b	5.16 $\pm$ 0.23
	EM Aktiv	1.24 $\pm$ 0.11 ^a	40.82 $\pm$ 1.81 ^a	15.52 $\pm$ 0.60 ^a	0.85 $\pm$ 0.06 ^a	0.35 $\pm$ 0.01 ^a	4.97 $\pm$ 0.23
Significance	Cultivar (C)	**	***	***	***	***	ns
	Fertilizer (F)	**	**	***	***	***	ns
Interaction factors	C $\times$ F	***	***	***	***	***	ns

Note: The data show the means ($n = 3$) $\pm$ SE. Different letters within each column indicate significant differences according to Tukey's HSD test ($p \leq 0.05$). Asterisks indicate significant differences at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns, non-significant. QMDG: quercetin 3-O-(6"-O-malonyl)- β -D-glucoside 7-O- β -D-glucoside, QG: quercetin-3-glucoside, QMG: quercetin-3-O-(6"-O-malonyl)-glucoside, QAcG: quercetin 3-O-(6"-acetyl-glucoside), KMalG: kaempferol(6-O-malonyl) glucoside, K: kaempferol.

Significant cultivar $\times$ biofertilizer interactions were observed for all tested phenolic acids except caffeic acid-3-glucoside (Figure 5), indicating cultivar-dependent responses to the EM Aktiv treatment. Application of EM Aktiv reduced phenolic concentrations in the red cultivar 'Murai', whereas in the green cultivar 'Aleppo', responses were more variable, ranging from stable to slightly increased levels depending on the compound. Caffeic acid glucuronide B, caffeoylquinic acid glucoside, protocatechuic, chlorogenic, chicoric, and caffeic acids exhibited crossover-like interaction patterns, with EM Aktiv increasing or maintaining concentrations in 'Aleppo' but decreasing them in 'Murai'. A particularly strong response was observed for chlorogenic acid, where EM Aktiv caused a pronounced reduction in 'Murai'. Caffeic acid glucuronide A also showed significant cultivar-dependent interactions, with EM Aktiv slightly reducing levels in 'Aleppo' but causing strong decreases in 'Murai'. Overall, EM Aktiv altered phenolic acid profiles in a cultivar-dependent manner, with consistently stronger reductions observed in 'Murai' compared with 'Aleppo'.

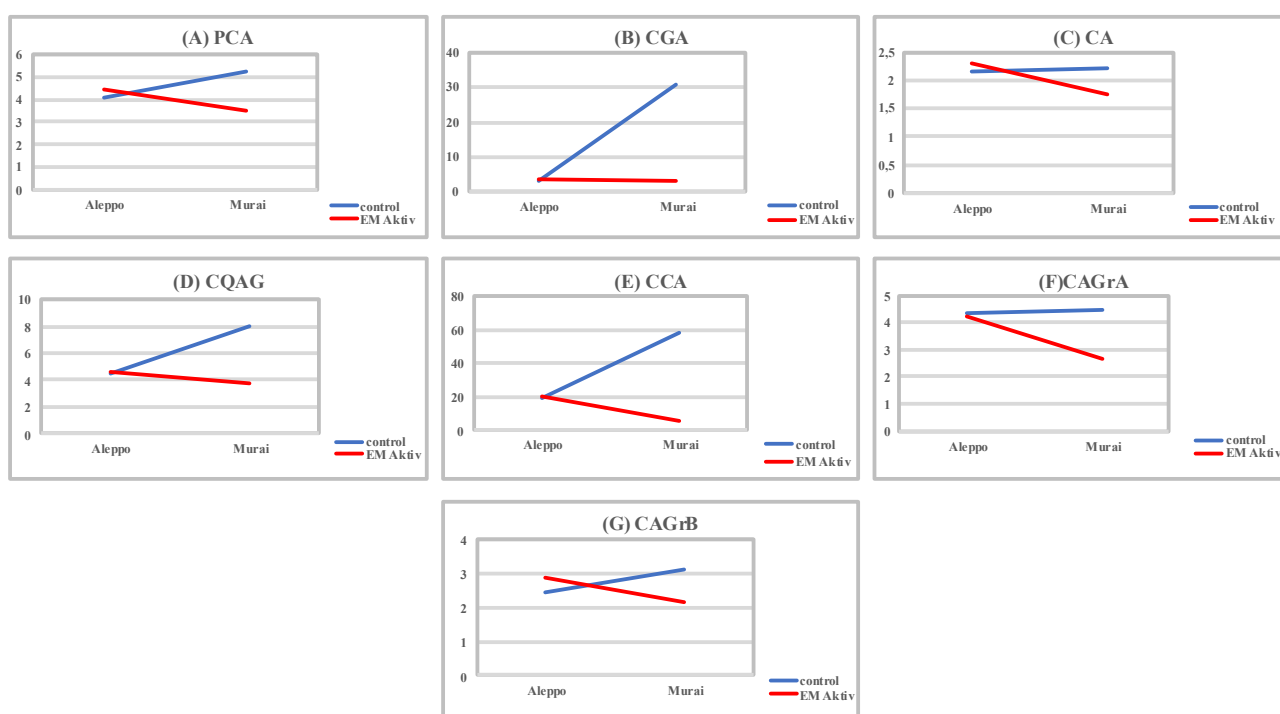


Figure 5. Cultivar $\times$ biofertilizer interaction effects on phenolic acids: (A) protocatechuic acid (PCA, $\mu\text{g/g}$ FW), (B) chlorogenic acid (CGA, $\mu\text{g/g}$ FW), (C) caffeic acid (CA, $\mu\text{g/g}$ FW), (D) caffeoylquinic acid glucoside (CQAG, μg caffeic acid eq/g FW), (E) chicoric acid (CCA, μg caffeic acid eq/g FW), (F) caffeic acid glucuronide A (CAGrA, μg caffeic acid eq/g FW), and (G) caffeic acid glucuronide B (CAGrB, μg caffeic acid eq/g FW).

3.5. Flavonoid profiles from HPLC analysis

Cultivar and biofertilizer significantly affected most flavonoids, except for kaempferol (Table 5). The red cultivar 'Murai' showed 1.6-fold higher quercetin 3-O-(6"-O-malonyl)- β -D-glucoside 7-O- β -D-glucoside, 2.8-fold higher quercetin-3-glucoside, 4.6-fold higher quercetin-3-O-(6"-O-malonyl)-glucoside, 4.4-fold higher quercetin 3-O-acetyl-glucoside, and 2.5-fold higher kaempferol (6-O-

malonyl) glucoside than the green cultivar 'Aleppo'. EM Aktiv application decreased most flavonoids by 30.1–70.8% when averaged across cultivars.

Significant cultivar $\times$ biofertilizer interactions were observed for all flavonoids except kaempferol (Figure 6). Under control conditions, the cultivar 'Murai' consistently showed higher flavonoid concentrations than 'Aleppo' across all measured metabolites. EM Aktiv application induced two distinct response patterns depending on the compound. All quercetin derivatives showed cultivar-dependent crossover pattern interactions. In these metabolites, EM Aktiv generally increased concentrations in 'Aleppo' while decreasing them in 'Murai', indicating a clear reversal of treatment effects between the two cultivars. In contrast, kaempferol(6-O-malonyl) glucoside exhibited a non-crossover interaction pattern. EM Aktiv reduced its concentration in both cultivars, although the decrease was more pronounced in 'Murai', indicating a consistent directional response with different magnitude between genotypes. Overall, EM Aktiv treatment promoted accumulation of quercetin-based flavonoids in 'Aleppo' while reducing their levels in 'Murai', indicating strong cultivar-dependent responses.

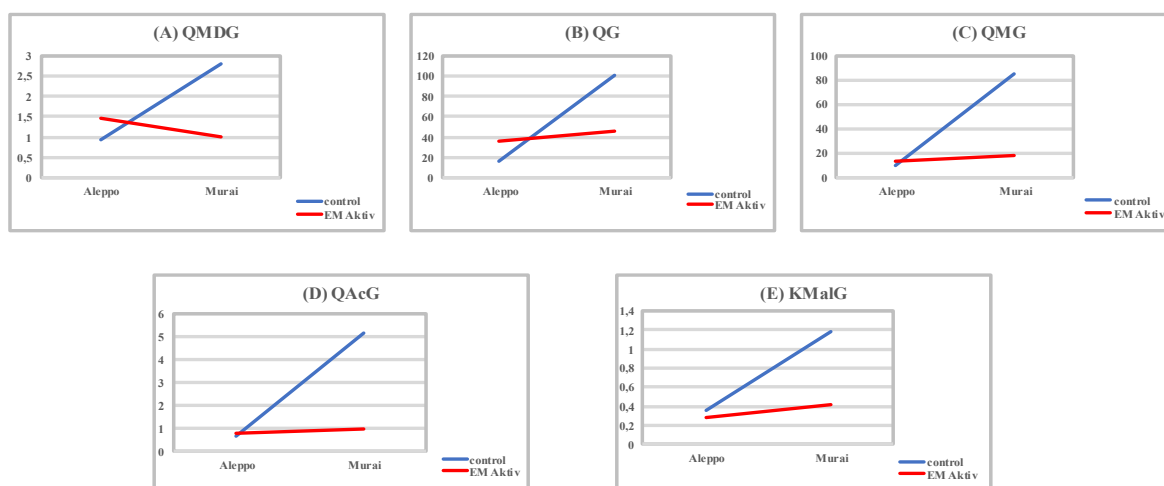


Figure 6. Cultivar $\times$ biofertilizer interaction effects on flavonoids: (A) quercetin 3-O-(6''-O-malonyl)- β -D-glucoside 7-O- β -D-glucoside (QMDG, μ g quercetin eq/g FW), (B) quercetin-3-glucoside (QG, μ g quercetin eq/g FW), (C) quercetin-3-O-(6''-O-malonyl)-glucoside (QMG, μ g quercetin eq/g FW), (D) quercetin 3-O-(6''-acetyl)-glucoside (QAcG, μ g quercetin eq/g FW), and (E) kaempferol(6-O-malonyl) glucoside (KMalG, μ g kaempferol eq/g FW).

3.6. Sesquiterpene lactone profiles from HPLC analysis

Cultivar significantly affected lactucopicrin and lactucopicrin-15-oxalate contents, whereas biofertilizer had no significant effect on any sesquiterpene lactone content (Table 6). The green cultivar 'Aleppo' exhibited significantly higher lactone levels than the red cultivar 'Murai'. Despite the lack of biofertilizer's main effects, cultivar $\times$ biofertilizer interactions for all lactones were significant.

Table 6. Effects of cultivar, fertilizer, and their interaction on lettuce sesquiterpene lactone content.

Main factors		Lc ($\mu\text{g/g FW}$)	Lp ($\mu\text{g/g FW}$)	Lp-ox ($\mu\text{g lactucopiricin eq/g FW}$)
Cultivar	Murai	4.00 ± 0.17	2.77 ± 0.14^a	2.41 ± 0.21^a
	Aleppo	3.61 ± 0.27	3.82 ± 0.17^b	3.28 ± 0.17^b
Fertilizer	Control	3.93 ± 0.31	3.47 ± 0.19	2.96 ± 0.21
	EM Aktiv	3.68 ± 0.12	3.12 ± 0.13	2.73 ± 0.17
Significance	Cultivar (C)	ns	***	**
	Fertilizer (F)	ns	ns	ns
Interaction factors	C $\times$ F	*	***	**

Note: The data show the means ($n = 3$) $\pm$ SE. Different letters within each column indicate significant differences according to Tukey's HSD test ($p \leq 0.05$). Asterisks indicate significant differences at * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns, non-significant. Lc: lactucin, Lp: lactucopiricin, Lp-ox: lactucopiricin-15-oxalate.

Significant cultivar $\times$ fertilizer interactions were observed for the three sesquiterpene lactones (Figure 7). All parameters exhibited crossover patterns, indicating contrasting responses to EM Aktiv between cultivars. In 'Aleppo', EM Aktiv treatment increased lactucin, lactucopiricin, and lactucopiricin-15-oxalate concentrations compared with the control, whereas the 'Murai' EM Aktiv treatment resulted in lower values for all compounds.

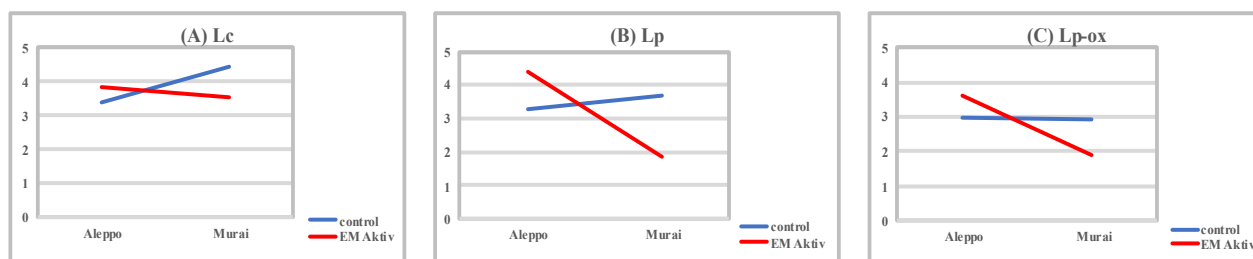


Figure 7. Cultivar $\times$ biofertilizer interaction effects on sesquiterpene lactones: (A) lactucin (Lc, $\mu\text{g/g FW}$), (B) lactucopiricin (Lp, $\mu\text{g/g FW}$), and (C) lactucopiricin-15-oxalate (Lp-ox, $\mu\text{g lactucopiricin eq/g FW}$).

3.7. Correlations between key metabolite compounds

Pearson correlations for all parameters are presented in Supplementary Table 1 and visualized as a heatmap in Supplementary Figure 1. These correlations were analyzed to identify potential associations among metabolites and physiological traits.

Fructose, as the major sugar, showed very strong positive correlations with TSI ($r = 0.99^{**}$) and glucose ($r = 0.97^{**}$) and strong negative correlations with chicoric acid ($r = -0.76^{**}$) and chlorogenic acid ($r = -0.74^{**}$), as well as with flavonoids kaempferol (6-O-malonyl) glucoside ($r = -0.74^{**}$), quercetin 3-O-(6"-acetyl-glucoside) ($r = -0.70^*$), and quercetin-3-O-(6"-O-malonyl)-glucoside ($r = -0.67^*$).

Malic acid exhibited broad positive correlations with TPC ($r = 0.67^*$), TCA intermediate citric

acid ($r = 0.82^{**}$), fumaric acid ($r = 0.62^*$), lactucin ($r = 0.64^*$), quercetin 3-O-(6"-O-malonyl)- β -D-glucoside 7-O- β -D-glucoside ($r = 0.63^*$), and quercetin-3-glucoside ($r = 0.69^*$).

Chicoric acid showed very strong positive correlations with quercetin derivatives ($r = 0.81-0.92^{**}$), kaempferol (6-O-malonyl) glucoside ($r = 0.91^{**}$), TPC ($r = 0.82^{**}$), other phenolic acid protocatechuic ($r = 0.82^{**}$), chlorogenic ($r = 0.96^{**}$), caffeoylquinic acid glucoside ($r = 0.93^{**}$), and strong correlations with lactucin ($r = 0.71^{**}$), propionic acid ($r = 0.73^{**}$), and citric acid ($r = 0.65^*$), and negative correlations with fructose ($r = -0.76^{**}$) and glucose ($r = -0.78^{**}$).

Quercetin-3-glucoside showed a similar pattern, exhibiting very strong positive correlations with other flavonoids ($r = 0.93-0.97^{**}$), chlorogenic acid ($r = 0.92^{**}$), caffeoylquinic acid glucoside ($r = 0.86^{**}$), chicoric acid ($r = 0.81^{**}$), citric acid ($r = 0.92^{**}$), propionic acid ($r = 0.90^{**}$), TPC ($r = 0.83^{**}$), and a strong positive correlation with lactucin ($r = 0.76^{**}$). It showed negative correlations with sucrose ($r = -0.63^*$) and tartaric acid ($r = -0.64^*$).

Lactucin showed strong positive correlations with flavonoids ($r = 0.74-0.78^{**}$), a very strong correlation with caffeoylquinic acid glucoside ($r = 0.86^{**}$), a strong positive correlation with chlorogenic acid ($r = 0.72^{**}$), chicoric acid ($r = 0.71^{**}$), TPC ($r = 0.71^{**}$), and organic acids ($r = 0.63-0.69^*$).

Correlation analysis revealed negative associations between sugar and phenolic compounds, positive associations among phenolic compounds, flavonoids, and lactones, and multiple correlations involving malic acid (Supplementary Table 1, Supplementary Figure 1).

4. Discussion

Low temperature frequently stresses spring crops like lettuce, the most cultivated leafy vegetable in temperate regions, slowing growth, organogenesis, and metabolism [43]. Iceberg lettuce at 4 °C shows elevated levels of proline, carbohydrates, ascorbic acid, and carotenoids, which are essential for acclimatization [44]. Moreover, a balance between primary and secondary metabolism is essential for plant adaptation to changing environmental conditions and optimal growth. Secondary metabolites play key roles in plant defense and plant-microbe interactions, and phenolics contribute to the cell wall strengthening against freezing [45,46]. Cold stress has been reported to stimulate phenolic biosynthesis, potentially through increased activity or expression of enzymes such as phenylalanine ammonia-lyase (PAL), cinnamyl alcohol dehydrogenase (CAD), and hydroxycinnamoyl transferase (HCT), while polyphenols contribute to ROS scavenging and chilling protection [47]. These stress-induced metabolic responses are particularly relevant under fluctuating winter greenhouse conditions, where plants experience prolonged exposure to low temperatures.

Climate change profoundly impacts agriculture through severe, unpredictable weather fluctuations, including prolonged extreme low and high temperatures in temperate regions. Although relatively cold-tolerant, lettuce remains vulnerable to freezing temperatures, causing chilling injury to leaves, leading to yield losses [48]. In this study, cold stress started 7 days after transplanting and continued for the next 51 days, with average night temperatures below 0 °C (Figure 1). Thus, maintaining yield and quality under these conditions is of high agronomic importance. Although plants were exposed to winter low temperatures in an unheated greenhouse (Figure 1), these conditions were consistent across all treatments; therefore, observed differences in metabolite profiles are mostly attributable to cultivar and biofertilizer effects, while temperature represents a shared environmental factor. In this study, we applied EM Aktiv biofertilizer containing beneficial microorganisms, including *L. plantarum* and *B. subtilis*, both demonstrating documented cold tolerance through

producing cold-shock proteins and stress response mechanisms [49,50]. Furthermore, psychrophilic and psychrotolerant phosphate-solubilizing bacteria are known to ameliorate low-temperature conditions through antifreeze proteins production and stress-induced gene expression, enhancing survival during cold stress [50]. In contrast, *Vitis vinifera* plantlets inoculated with cold-tolerant *Burkholderia phytofirmans* accumulated starch, proline, and phenolics, correlating with enhanced cold tolerance [51]. These variable responses among cold-tolerant microbes highlight the need for further research into their interactions with lettuce under field and semi-controlled conditions. Literature on biofertilizer application in lettuce has entailed mostly salinity, drought stress, or optimal growing conditions; therefore, further studies are needed to clarify their effects under low-temperature conditions.

Leaf chlorophyll levels are commonly used as an indicator of photosynthetic status, while biostimulants are often reported to enhance chlorophyll a and b, as well as carotenoids involved in photosynthetic processes [52]. Our study revealed no significant effects of cultivar or treatment on chlorophyll a, b, or total chlorophyll levels; however, total carotenoids varied by cultivar, with red lettuce exhibiting higher concentrations (Table 1). A crossover interaction pattern between cultivar and EM Aktiv application was observed for chlorophyll a and total carotenoids (Figure 2). In 'Aleppo', EM Aktiv increased chlorophyll a, whereas in 'Murai', higher values were recorded under the control treatment. A similar cultivar-dependent response was observed for total carotenoids, with slightly higher values under the control in 'Aleppo' and higher values under EM Aktiv in 'Murai'. This crossover response suggests a cultivar-dependent modulation of pigment stability or allocation under EM Aktiv application, potentially reflecting differences in physiological plasticity between genotypes under low-temperature conditions.

This pattern in total carotenoid content between green and red lettuce cultivars aligns with findings on these cultivars grown in organic matter-rich soil [37]. Chlorophyll and carotenoid values were consistent with those from the autumn growing cycle for these cultivars [6], while total carotenoids showed an increase in the winter study. Chlorophyll and carotenoid levels in vegetables vary depending on growing conditions [53,54]. Besides their light-harvesting role, carotenoids protect cells against oxygen free radicals when light energy exceeds photosynthesis needs [55]. No significant changes were observed with EM Aktiv application under low temperature, indicating stable chlorophyll b and total chlorophyll levels under the tested conditions. A similar pattern was detected with applications of *Bacillus velezensis* RI3 and *Pseudomonas psychrophila* P10 in pot and open-field lettuce experiments [56]. Furthermore, *Trichoderma asperellum* strains TaMFP1 and TaMFP2 and *Trichoderma harzianum* in hydroponic greenhouse systems for lettuce and spinach showed no significant effect on pigments [57,58], as well as PGPR application in curly lettuce field cultivation compared to the control [59]. Application of microalgal biofertilizers showed no chlorophyll b increase in lettuce, possibly because chlorophyll b acts as an accessory pigment transferring energy to chlorophyll a [60] or to a limited responsiveness of pigment synthesis to the applied nutrient conditions. However, contrasting studies report elevated total chlorophyll and carotenoid levels with application of *Azospirillum brasilense* strains DSM 1690, DSM 2298, and *Pseudomonas* sp. DSM 25356 compared to non-inoculated controls [61], and higher *Bacillus* sp. VWC18 doses (10^9 CFU/mL versus 10^3 CFU/mL) significantly raised chlorophyll a, b, and carotenoids [62]. Given the winter temperature conditions, no significant pigment increases were observed under the applied treatment; these results may reflect a stable chlorophyll status rather than changes in pigment accumulation, which could be better clarified by additional photosynthetic efficiency and gas exchange analyses. Cold and chilling

stress disrupts chloroplast ultrastructure, photosynthetic processes, carbon assimilation, and stomatal conductance, jeopardizing crop production [63]. Within the same experimental system, morphological analyses showed that application of EM Aktiv decreased rosette fresh weight, stem fresh weight, rosette height, and stem length in both cultivars compared with the control [64]. Overall, pigment accumulation depends on physiological, biochemical, and genetic traits, as well as environmental factors such as light, temperature, and fertilization [60].

TPC and TAC values were affected by cultivar and treatment, as well as their interaction (Table 1). The red cultivar showed higher values for TPC and TAC parameters. This is consistent with other studies [6,65,66]. These studies indicate that red cultivars accumulate more phenolics compared to green cultivars and highlight the influence of genotype on TPC [8,9]. Plants treated with EM Aktiv showed a decrease of 19.4% in TPC and 54.0% in TAC across the cultivars (Table 1). Similar trends were observed with the application of two arbuscular mycorrhizal fungi (AMF) inocula, *Rhizophagus intraradices* and *Diversispora* spp., in lettuce, where their application decreased antioxidant activity by 20.0%, with no significant effect on TPC [67]. Differences among studies may be related to variations in cultivar genotype, microbial strain specificity, and environmental conditions during cultivation. The lower antioxidant capacity in EM Aktiv-treated plants coincided with elevated glucose, fructose, sucrose, and malic acid levels (Tables 2 and 3), indicating an inverse relationship between antioxidant-related metabolites and primary metabolite accumulation under winter conditions.

A significant cultivar $\times$ treatment interaction was observed for TPC and TAC, indicating differential metabolic responses between cultivars (Figure 2). In 'Aleppo', EM Aktiv increased TPC, whereas in 'Murai', higher values were observed under the control treatment. This represents a crossover cultivar $\times$ treatment interaction, indicating a genotype-dependent reversal in phenolic accumulation in response to EM Aktiv application. Similarly, lettuce plants exposed to salinity stress and treated with the biostimulant BALOX[®] also exhibited lower TPC values compared to the control [68]. In contrast, TAC also showed a significant cultivar $\times$ treatment interaction, as control plants exhibited higher TAC values than EM Aktiv-treated plants in both cultivars, although the magnitude of reduction differed between cultivars. This indicated a non-crossover interaction, where EM Aktiv consistently reduced TAC, but the strength of the response was cultivar-dependent. Overall, these results demonstrate that antioxidant-related metabolism is strongly modulated by genotype and biofertilizer application under low-temperature conditions.

Carbohydrate content and composition play major roles in determining vegetable flavor and overall quality, whereas soluble solids serve as a quality trait by directly influencing taste [69,70]. Green lettuce showed higher sugar concentrations across all main sugars, TSS, and TSI with the exception of polyol myo-inositol (Table 2). Sugar profile aligns with previous lettuce findings [71,72]. Fructose was the predominant sugar, alongside glucose and sucrose, matching the pattern and similar concentration ranges observed in the same cultivars during our autumn experiment [6]. Similarly, literature data report comparable TSS values [6,59,73] as well as similar TSI levels for autumn lettuce [6] or even higher TSI values compared to Fedeli et al. [74]. However, sucrose was absent in red lettuce in the winter trial, unlike our autumn experiment. This suggests cultivar-dependent differences in carbohydrate metabolism compared with Becker et al.'s [75] findings, where red and green lettuce accumulated sucrose alongside flavonoids under decreasing nitrogen conditions. Our results show cultivar-specific differences in carbohydrate and secondary metabolite accumulation under winter conditions, with green lettuce exhibiting higher sugar levels and red lettuce showing higher phenolic accumulation. Additionally, red lettuce showed higher myo-inositol levels, a compound associated

with stress responses and osmoprotection in plants. Myo-inositol acts as a precursor to inositol derivatives that play key roles in cell membrane biogenesis, programmed cell death, plant growth, hormone signaling, and stress responses [76]. Myo-inositol promotes cold stress tolerance in rapeseed seedlings via increased Ca^{2+} influx; however, the full mechanism remains to be fully elucidated [77]. From a plant breeding perspective, sugar composition is important as it modulates taste perception, particularly through glucose's strong correlation with sweetness [78], despite fructose being predominant sugar. The autumn experiment also showed higher myo-inositol levels in red cultivars [6], consistent with winter results (Table 2).

Biofertilizer application increased glucose by 42.9%, fructose by 39.1%, sucrose by 57.8%, and TSI by 40.5%, while decreasing myo-inositol by 23.3%, with no significant effect on TSS (Table 2). Conversely, our autumn greenhouse experiment showed no significant effects from EM Aktiv on main sugars and even increased myo-inositol levels [6], contrasting with winter results and suggesting that biofertilizer effects on sugar metabolism may be influenced by environmental conditions, particularly low-temperature. This aligns with literature showing that *Azospirillum brasilense* strains and *Pseudomonas* inoculation elevated total sugars in lettuce compared to non-inoculated plants [61], and with reports showing that foliar wood distillate (0.25%) increased glucose and fructose in pot-soil experiments [74]. Similarly to our results, application of different biostimulants based on fulvic acid, amino acid, and vermicompost showed no significant effect on TSS [79]. Additionally, application of *Ecklonia maxima* extracts in hydroponic leaf lettuce cultivation showed no significant effect on soluble solids [73]. However, regarding TSI, our biofertilizer-induced increase contrasts with findings of reduced or unchanged levels compared to the control [6], which may reflect differences in environmental conditions and possible stress conditions between experiments.

Significant cultivar $\times$ treatment interactions were observed for myo-inositol, sucrose, and TSS (Figure 3). For myo-inositol and TSS, the interactions exhibited crossover patterns, with EM Aktiv increasing concentrations in 'Aleppo' but decreasing them in 'Murai', indicating contrasting responses between cultivars. These opposite responses suggest genotype-dependent differences in metabolic adjustment under low-temperature conditions, with the two cultivars exhibiting contrasting responses to EM Aktiv application. For sucrose, EM Aktiv increased sucrose levels in 'Aleppo', whereas sucrose was not detected in 'Murai' under either treatment, indicating a strong genotype-dependent difference in sucrose accumulation. Overall, these interactions demonstrate that the effects of EM Aktiv on carbohydrate metabolism were metabolite- and genotype-dependent under winter growing conditions.

Correlation coefficients indicated a very strong positive relationship between the two dominant sugars (glucose and fructose), while no correlation was observed with sucrose (Supplementary Table 1, Supplementary Figure 1). Correlation analysis also revealed strong negative correlations between fructose and major phenolic acids and flavonoids. Similarly, positive correlation between glucose and fructose content was observed in lettuce cultivars [80]. In cabbage, glucose, fructose, and sucrose exhibited negative or non-significant correlations, highlighting cultivar-specific differences in sucrose hydrolysis to glucose and fructose [81].

These correlations may reflect broader physiological patterns associated with low temperature, which is known to influence plant metabolism and growth in temperate climates. It is widely reported that under low-temperature conditions, plants enhance freezing tolerance through regulated photosynthesis, metabolism, and protein pathways, alongside accumulation of osmotic regulators and antioxidants like soluble sugars, proline, and polyamines that stabilize membranes and neutralize ROS [82]. Soluble sugars serve as osmoprotectants, increasing cellular osmotic concentration to counteract low-

temperature-induced shrinkage while protecting membranes from dehydration and freezing injury. Cold-tolerant *Pinus halepensis* seedlings accumulate approximately 25.0% more glucose and 33.0% more fructose under stress conditions, relative to cold-sensitive counterparts [83]. Sucrose serves as plants' primary transport sugar, translocating to different tissues for storage or metabolism. Invertases hydrolyze it into glucose and fructose, both requiring hexokinase phosphorylation for utilization. Alternatively, sucrose synthase transforms it into UDP-glucose (directly available for synthesis) and fructose (needing hexokinase activation). Sucrose functions as a readily mobilizable reserve carbohydrate for metabolic needs. Low temperatures impair photosynthetic assimilation less severely than metabolic processes, and diminished growth reduces photosynthate requirements, which may result in increased carbohydrate accumulation [84]. Moreover, metabolite enrichment analysis reveals cold-induced shifts in sucrose metabolism and flavonoid pathways between cold-tolerant and cold-sensitive lettuce cultivars [20], while fructose showed strong superoxide-scavenging ability and involvement in antioxidative defense in cold-stressed pea leaves [85]. Literature on lettuce under various stressors supports these sugar accumulation patterns: Low nitrogen conditions reduce the citrate cycle while increasing glucose and sucrose levels, providing excess carbon for phenolic compound biosynthesis [86].

Organic acids dominate the acidity in fruits and vegetables, profoundly influencing taste, flavor, and nutritional quality. In our study, cultivar effects significantly influenced the organic acid profile, with higher levels of the major organic acids in red lettuce than in green (Table 3). Literature data shows variations in organic acid content between green and red lettuce cultivars [87]. Similar organic acid profiles were observed for the same cultivars during the autumn experiment [6]. The major organic acids (malic, propionic, and citric) matched profiles from other lettuce studies [55,71].

Biofertilizer application with EM Aktiv increased malic acid by 23.2% and fumaric acid contents by 36.0%, while reducing propionic acid by 21.6% (Table 3). Literature on organic acid responses to biostimulants remains inconsistent. Plant-based biostimulants applied to lettuce containing legume-derived protein hydrolysates and tropical plant extracts similarly elevated malic acid levels without affecting tartaric acid [87], and arbuscular mycorrhizal fungi significantly increased citric acid while leaving malic acid unchanged [88]. In contrast, our autumn greenhouse experiment detected no significant changes in major organic acids [6]. *Spirulina* application significantly decreased fumaric and formic acids, while only slightly reducing malic acid, with other organic acids remaining stable [71]. Organic acids have been reported to contribute to stress responses through roles in nutrient chelation, ATP metabolism, redox balance, osmoregulation, and signaling processes [89]. Furthermore, exogenous malic acid application has been reported to enhance chilling tolerance through osmoprotective mechanisms [90]. Malic acid showed strong positive correlations with quercetin derivatives, fumaric acid, TPC, and lactucin, and a very strong correlation with citric acid (Supplementary Table 1, Supplementary Figure 1). These relationships may indicate coordinated changes in organic acid and secondary metabolite levels under these experimental conditions. Moreover, significant cultivar $\times$ biofertilizer interactions were observed for malic, citric, propionic, fumaric acids, and unidentified compound 2 (Figure 4), indicating cultivar-dependent metabolic responses to EM Aktiv application. Malic, citric, and fumaric acids exhibited crossover interaction patterns, with EM Aktiv increasing their concentrations in 'Aleppo' but decreasing them in 'Murai'. Propionic acid showed a non-crossover interaction, with EM Aktiv reducing its concentration in both cultivars, although the decrease was more pronounced in 'Murai'. Because malic, citric, and fumaric acids are key intermediates of the tricarboxylic acid cycle, these contrasting responses may reflect

cultivar-dependent regulation of respiratory metabolism and carbon distribution under winter growing conditions.

Phenolic compounds serve as key antioxidants during low-temperature stress through ROS scavenging and membrane stabilization. Their antioxidant activity depends on chemical structure, encompassing key classes such as phenolic acids, flavonoids, tannins, chalcones, and coumarins. Major phenolic acids in our lettuce samples included chicoric, chlorogenic, caffeoylquinic acid glucoside, protocatechuic acid, and caffeic acid derivatives (Table 4). Major flavonoids consisted of quercetin derivatives (quercetin-3-glucoside, quercetin-3-O-(6''-O-malonyl)-glucoside, and quercetin 3-O-acetyl-glucoside), alongside kaempferol and its derivative (Table 5).

Compounds found in our samples correspond with literature lettuce phenolic profiles [8,91,92]. Red lettuce exhibited higher concentrations of chicoric acid, chlorogenic acid, caffeoylquinic acid glucoside than the green cultivar, demonstrating a significant cultivar effect on these key compounds (Table 4). Beyond genotype, polyphenol content in lettuce responds to growing treatments and nutrient availability, with quarter-strength reduced-nutrient solutions increasing polyphenol accumulation relative to full-strength conditions [93].

Phenolic acids are usually present in lettuce in bound forms, primarily as glycosylated derivatives or esters. Reported comprehensive metabolomic analysis detected 1905 compounds in lettuce, predominantly flavonoids (18%), phenolic acids (15%), and lipids (13%), with flavonols and flavones being the most abundant flavonoid subclasses [94]. Phenolic acids and flavonoids derive from the phenylpropanoid pathway, where phenylalanine ammonia-lyase (PAL) catalyzes the conversion of L-phenylalanine to cinnamic acid, leading to the formation of stress-related metabolites, which have been reported to increase under cold stress conditions [95,96]. Untargeted metabolomic analysis identified potentially active different flavonoid derivatives (such as quercetin and kaempferol) in cold stress resistance, with higher accumulation observed in cold resistant cultivars [20].

Chicoric acid, the predominant phenolic acid, showed very strong positive correlations with other major phenolic acids, all detected flavonoids, and TPC, and strong positive correlations with citric acid, propionic acid, and lactucin (Supplementary Table 1, Supplementary Figure 1). Conversely, it exhibited strong negative correlations with glucose and fructose content, indicating opposite accumulation trends. A similar pattern emerged for quercetin 3-O-glucoside, the predominant flavonoid, which showed strong negative correlations with sucrose but very strong positive correlations with the aforementioned compounds.

Application of EM Aktiv substantially decreased phenolic acid content, including chicoric acid by 66.9%, chlorogenic acid by 81.2%, caffeoylquinic acid glucoside by 32.6%, and caffeic acid glucuronide A by 21.8%, and reduced all flavonoids by 30.1–70.8%, except kaempferol, which was unaffected (Tables 4 and 5). In our autumn study, EM Aktiv showed no significant effects on phenolic acids or flavonoids except for an increase in kaempferol [6]. Significant cultivar $\times$ biofertilizer interactions for phenolic acids and flavonoids indicate a strong genotype-dependent regulation of secondary metabolism under EM Aktiv application. In general, the red cultivar 'Murai' showed a stronger sensitivity to the treatment, with most phenolic acids and flavonoids decreasing under EM Aktiv, whereas the green cultivar 'Aleppo' exhibited more stable or slightly increased levels depending on the compound. This contrasting behavior could be associated with differences in the capacity of the two cultivars to maintain phenylpropanoid pathway activity under biofertilizer treatment during winter growth conditions. The observed crossover responses in several phenolic acids and quercetin-based flavonoids may indicate cultivar-dependent differences in metabolic regulation, where EM Aktiv

shifted secondary metabolism toward reduced accumulation in 'Murai' but maintained or partially enhanced levels in 'Aleppo'. Such patterns may reflect cultivar-dependent differences in carbon allocation between primary metabolism and phenolic biosynthesis, potentially linked to stress responsiveness under low-temperature conditions. In contrast, kaempferol(6-O-malonyl) glucoside showed a non-crossover response, with EM Aktiv reducing its concentration in both cultivars, although the magnitude of reduction varied between genotypes. Overall, these results indicated that EM Aktiv does not exert a uniform effect on phenolic metabolism in the two cultivars but is associated with genotype-dependent differences in flavonoid accumulation, which may reflect differences in metabolic flexibility and responsiveness between green and red cultivars under winter conditions. In this sense, the effect of EM Aktiv appears to be highly cultivar-dependent rather than universally applicable across genotypes.

Different lettuce responses to biofertilizers are observed in the literature: AMF significantly enhances caffeic and chlorogenic acids, while protocatechuic acid level stay unchanged [88]; *Rhodotorula mucilaginosa* and *Candida guilliermondii* with nanoparticles increase caffeic, chlorogenic, and chicoric acids compared to the non-inoculated control [12]; *Rhizobium laguerreae* HUTR05 enhances levels of chicoric acid, quercetin-3-glucoside, and quercetin-3-O-(6"-O-malonyl)-glucoside under non-stressed conditions but not in the case of saline stress [97]; sulfur nutrition did not impact levels of chicoric, caffeic, and quercetin derivatives [98].

Sesquiterpene lactone concentration and composition vary by lettuce type and genetic background [78], as demonstrated in different Little Gem cultivars exhibiting distinct lactone levels [99]. The lactone profiles from our samples confirmed lactucin as the main compound, followed by lactucopicrin (Table 6). A similar profile has been observed in various lettuce cultivars, with lactucopicrin recognized as the major contributor to bitter taste [100,101]. Green lettuce showed higher levels of lactucopicrin and lactucopicrin-15-oxalate, reflecting cultivar effects. These results contradict experiments that revealed higher concentrations in red lettuce compared to green [6,101]. Correlation analysis revealed strong positive correlations between lactucin and quercetin and kaempferol derivatives, chlorogenic, chicoric, protocatechuic acids, malic and citric acids, and TPC (Supplementary Table 1 and Supplementary Figure 1). Given the exploratory nature of the correlation analysis and the large number of metabolite comparisons, some associations may reflect potential false positives and should therefore be interpreted with caution.

Literature data are scarce regarding biofertilizer effects on lactone profiles and content in lettuce. In this study, application of EM Aktiv did not significantly affect lactone content during winter, but significant cultivar $\times$ treatment interactions were observed for all tested lactones (Table 6). The interaction pattern revealed that EM Aktiv tended to increase lactone accumulation in 'Aleppo' while reducing concentrations in 'Murai' (Figure 7). Similar to the responses observed for phenolic acids and flavonoids, this suggests genotype-dependent differences in lactone accumulation patterns under winter conditions. The lack of a significant effect of EM Aktiv on lactone content may reflect genotype-dependent metabolic adjustments under cold conditions, consistent with broader shifts in primary and secondary metabolism observed in EM-treated plants. Acclimation with organic acids—fumaric acid accumulation is essential for cold acclimation in *Arabidopsis thaliana* [102], while malic acid serves as an osmotic adjuster and carbon source protecting photosynthesis under low temperature stress in *Secale cereale* [103]. A study with EM Aktiv and *Trichoderma*-based fertilizer, as well as their combination, showed positive effects on increasing contents of lactucin, lactucopicrin, and lactucopicrin-15-oxalate during an autumn greenhouse experiment [6]. Sesquiterpene lactone

production varies with mineral nutrition, nitrogen form, and cultivation system [104,105]. Future field studies and simulations mimicking natural conditions are needed to better understand the regulation of sesquiterpene lactone biosynthesis under different environmental variables [106].

Our results partially support the hypothesis that biofertilizer treatment leads to an increase in primary and secondary metabolite levels. The observed metabolite changes are consistent with a shift in metabolic allocation patterns rather than direct evidence of stress alleviation or induction. Although increases in selected sugars and organic acids may be consistent with osmotic adjustment, and reductions in phenolic compounds may suggest altered investment in secondary metabolism, these interpretations remain indirect because enzymatic and gene expression data were not assessed. Therefore, specific pathway-level regulation, including the phenylpropanoid pathway or antioxidant enzyme systems, cannot be confirmed from our dataset. Instead, the results indicated that biofertilizer application is associated with cultivar-dependent changes in primary and secondary metabolite profiles under winter conditions. Within this framework, reductions in phenolic compounds likely reflect lower accumulation of secondary metabolites, while increases in soluble sugars and organic acids suggest shifts in carbon distribution and metabolic balance. Importantly, the metabolic responses were not uniform but strongly dependent on genotype. The two lettuce cultivars exhibited distinct baseline metabolic profiles, which shaped their responsiveness to biofertilizer application. 'Aleppo' was generally characterized by higher soluble carbohydrate levels and comparatively lower phenolic content, whereas 'Murai' showed higher levels of phenolic acids, flavonoids, and antioxidant-related metabolites. In addition, cultivar-specific differences in metabolic responsiveness were evident under EM Aktiv application, where 'Murai' generally exhibited stronger reductions in phenolic and flavonoid contents, while 'Aleppo' showed more compound-dependent responses, including maintenance or increases in selected secondary metabolites. Overall, these findings suggest that biofertilizer effects are context-dependent and shaped by genotype $\times$ biofertilizer interactions rather than acting as uniform modulators of plant metabolism. EM Aktiv was associated with changes in primary and secondary metabolite profiles, with responses strongly dependent on cultivar. This study revealed several limitations that warrant consideration. The experiment was conducted during a single winter growing season under one set of environmental conditions; therefore, the consistency of the observed responses across years and production environments remains to be determined. Although significant treatment and interaction effects were detected for several metabolites, the molecular mechanisms underlying these responses require further investigation. In addition, enzymatic antioxidant assays, including PAL, peroxidase (POD), and superoxide dismutase (SOD), were not performed, limiting direct mechanistic interpretation of the observed metabolic changes.

5. Conclusions

Cultivar selection creates distinct metabolic profiles: Red 'Murai' accumulates carotenoids, phenolic acids, and flavonoids, while green 'Aleppo' prioritizes sugars and sesquiterpene lactones. Biofertilizer application (EM Aktiv), as a main effect, was associated with increased levels of primary metabolites, particularly sugars and selected organic acids, whereas control plants generally accumulated higher levels of phenolic compounds across cultivars. Significant cultivar $\times$ biofertilizer interactions indicated genotype-dependent responses to EM Aktiv. In 'Murai', EM application was associated with lower levels of secondary metabolites compared with control plants, whereas in 'Aleppo', secondary metabolites were generally maintained or showed compound-dependent increases, alongside relatively higher levels of primary metabolites, particularly sugars and selected organic acids.

These results indicate that EM effects on metabolism are strongly dependent on genetic background under winter conditions. From a production perspective, these results suggest that the effectiveness of microbial biofertilizers under winter greenhouse conditions is not universal but depends strongly on cultivar selection. Therefore, matching cultivar $\times$ biofertilizer combinations may be important for optimizing quality under low-temperature cultivation. Although these patterns are consistent with physiological responses reported during winter production, they should be interpreted as associations rather than evidence of direct regulatory mechanisms. Future studies incorporating validation across growing seasons and greenhouse production environments, together with transcriptomics, proteomics, metabolomics, NMR, and advanced LC-MS techniques, would provide a more comprehensive understanding of mechanisms associated with response to biofertilizer application under low-temperature conditions. Additionally, increasing the number of biological replicates would enhance the robustness of the dataset, enable more reliable multivariate analyses, and strengthen statistical confidence in metabolite-level interpretations.

Author Contributions

Conceptualization: MS. Methodology: JDM, VM. Formal analysis: MS, DM, VM. Investigation: MS, DM, VM. Writing-original draft: MS. Writing-review & editing: MS, JDM, VM.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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