

Article

Host–Pathogen–Biocontrol Interactions: Effects of *Bacillus velezensis* on the Suppression of Black Rot Disease in Cabbage cv. Futoški

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Abstract

Xanthomonas campestris pv. *campestris* (Xcc), the causal agent of black rot, has a significant impact on cabbage production worldwide. The goal of this research was to evaluate the effect of preventive foliar treatments with *Bacillus velezensis* strain RD-FC 88 on the primary and secondary metabolism of Xcc-infected cabbage cv. Futoški plants. Special attention was given to measuring metabolites' changes, aiming to determine the influence of the applied biocontrol treatment on the development of plant immune response and resistance to pathogen. This study reports the first comprehensive biochemical and physiological analysis of the interaction between host plant, biocontrol strain and pathogen, thus providing novel insight into black rot management. Pathogen inoculation caused a significant decrease in the majority of measured metabolites, including most free amino acids (Gln, Ala, BCAA), phenolics, and glucosinolates. Preventive application of *B. velezensis* strain in Xcc-infected plants restored the levels of aromatic amino acids, Asp, Glu, Leu, Val, and Ala to control values. A similar pattern was observed in aliphatic glucosinolates sinigrin and glucoiberin, as well as for the indolic glucosinolate 4-methoxy-glucobrassicin. Additionally, increased accumulation of hydroxybenzoic acids, hydroxycinnamic acids, and kaempferol derivatives was also observed in the plants treated with the biocontrol strain and subsequently infected with Xcc, compared to plants solely infected with Xcc. The obtained results imply that the RD-FC 88 strain holds potential as an efficient priming agent, capable of stimulating cabbage cv. Futoški defense responses and enhancing its resistance to Xcc.

Keywords: cabbage cv. Futoški; *Xanthomonas campestris* pv. *campestris*; biocontrol; free amino acids; phenolics; glucosinolates



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1. Introduction

Black rot disease, caused by the bacterium *Xanthomonas campestris* pv. *campestris* (Xcc), is one of the main destructive diseases affecting Brassicaceae crops worldwide. The most important hosts of Xcc belong to varieties of *Brassica oleracea*, including cabbage, cauliflower, kale, broccoli, and Brussels sprouts [1]. Their agricultural production and profitability of annual yields are frequently hampered by this plant pathogenic bacterium,

especially in the regions characterized by high humidity and temperature, resulting in substantial economic losses [2]. Conventional methods used to control the spread of black rot usually include different management strategies, such as agrotechnical measures (e.g., crop rotation, use of disease-free seed), physical treatments (e.g., seed treatment with hot water), and chemical treatments [3]. Chemical treatments mostly rely on the use of copper and copper-based compounds, which inevitably leads to environmental pollution and endangers food safety [4]. Therefore, in line with the growing concern for the devastating impact of chemical agents used in disease control on human health and the environment, the demand for promising biocontrol strains is increasing.

Efforts to combat *Xcc* have been mainly focused on identifying effective prevention and control measures due to the lack of resistant cabbage cultivars to black rot. Consequently, researchers were generally focused on studying pathobiology of black rot in cabbage, screening of resistance traits, resistance breeding, and the biochemical parameters associated with plant resistance [5]. Cabbage production is highly important in Serbia, particularly in the region of Futog (Vojvodina). This area holds the production of a unique autochthonous cabbage cultivar called Futoški, which has obtained protected geographical indication status [6]. In the recent study, the pronounced biocontrol potential of four indigenous *Bacillus velezensis* strains (P-FC 55, RD-FC 88, R-FC 102, and R-FC 114) against the black rot was demonstrated on the aforementioned cultivar [7]. Furthermore, the preventive foliar treatment, especially with the *B. velezensis* strain RD-FC 88, resulted in the most effective disease suppression under controlled greenhouse conditions. In the current study, we investigated the response in cabbage cv. Futoški infected with the indigenous *Xcc* strain and evaluated how the preventive application of the biocontrol strain of interest modulates plant defense mechanisms.

The possible mechanism of the applied preventive biological control's involvement in the stress response to black rot was explored through a comprehensive analysis of some of the main metabolites, known for their participation in the plant's response to biotic stress. This research shifts focus from disease prevention and management of black rot in cabbage toward the evaluation of host resistance to pathogen through an integrated analysis of key components of the plant's primary and secondary metabolism [4]. Plants respond to pathogen attack by activating a complex defense mechanism, where phytohormones play a key role. Their signalling is accompanied by the induction of secondary metabolites, plant defense molecules, which are synthesized via the phenylpropanoid pathway [8]. In the Brassicaceae family these metabolites mostly include phenolics and glucosinolates. Correlation between the induction of secondary metabolites and the infection caused by *Xcc* was previously reported in Brassicaceae plants [9–11]. However, more recent studies have demonstrated differential accumulation of metabolites in different varieties of analyzed plants, depending on the susceptibility/resistance to *Xcc* infection [8,12,13]. Namely, integrated transcriptome and metabolome analysis performed on *Brassica napus* upon infection with *Xcc* revealed the opposite mechanisms in susceptible and resistant rapeseed varieties [14].

Glucosinolates are the most important secondary metabolites with vast structural diversity that are involved in the defense against various insect pests and pathogen attacks in the Brassicaceae family. Based on their biosynthetic pathways, glucosinolates are classified into aliphatic, aromatic, and indolic groups, and upon hydrolysis, their degradation products, including thiocyanates and isothiocyanates, exhibit toxic effects against invading plant pathogens [15].

In the case of cruciferous plants, amino acids (AAs) metabolism is shifted to biosynthesis of glucosinolates which play an important role in plant defense against pathogens [16]. AAs are one of the pivotal players in plant immune response [17]. During plant–pathogen

interaction, the metabolism of AAs is reprogrammed toward the biosynthesis of various phytohormones, such as auxin and ethylene, as well as phenolic compounds [14,17]. During plant–pathogen interactions, amino acids are at the crossroads between plant growth and defense, and appear as essential contributors for supporting the immune signalling network and metabolic remodeling, aiming to develop plant disease resistance [18].

The objective of this study was to analyze how preventive foliar application of biocontrol *B. velezensis* strain RD-FC 88 affects immune response and the interaction between primary and secondary metabolism in cabbage cv. Futoški plants infected with *Xcc*. In this regard, the contents of free proteinogenic amino acids, glucosinolates, and phenolic compounds were analyzed to explore their role in cabbage responses to infection with *Xcc*, as well as in the presence of biocontrol strain. To the best of our knowledge, data on the content of these compounds in the autochthonous cabbage cv. Futoški are lacking. However, the key novelty of this study lies in the integrated multi-omics analysis of the plant/biocontrol/pathogen interactions, thereby underscoring the relevance of this research for understanding and improving resistance mechanisms to black rot.

2. Materials and Methods

2.1. Plant Material and Experimental Setup

Seeds of cabbage cv. Futoški were germinated in the commercial substrate (Potgrond H, Kasmann-Deilmann GmbH, Geeste, Germany) until they reached the 8–9 leaf stage (for approximately two months), after which they were subjected to experimental treatments. Prior to germination, seeds were disinfected by immersing in the 3% sodium hypochlorite solution for 5 min and washed with tap water. The research was conducted at the Institute for Multidisciplinary Research (University of Belgrade, National Institute of the Republic of Serbia), specifically within the Department of Life Sciences. The experimental setup involved controlled growth chamber conditions to evaluate the effects of the biocontrol strain on cabbage cv. Futoški, and the experiments were conducted during the 2024–2025 period. Plants were grown under controlled conditions of photoperiod (14/10 h day/night), temperature (27/20 °C day/night), photosynthetically active radiation (PAR) intensity of $250 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$ and relative humidity (50%). In our previous research, preventive foliar treatment with the whole (overnight) culture of *B. velezensis* strain RD-FC 88 was identified as the most effective in suppressing highly virulent *X. campestris* pv. *campestris* strain XcFC 231 on cabbage cv. Futoški plants under controlled greenhouse conditions [7]. Accordingly, these strains were further employed in the present study. The experiment included four plant groups depending on the applied treatment: (i) NB—plants sprayed only with Nutrient Broth (NB) used for growing bacteria (control); (ii) Bac—plants sprayed only with biocontrol *B. velezensis* strain RD-FC 88; (iii) Xcc—plants sprayed only with pathogenic strain XcFC 231 and (iv) Bac + Xcc—plants preventively sprayed with *B. velezensis* strain RD-FC 88 and subsequently with pathogenic strain XcFC 231. Preventive foliar treatment implied inoculation of cabbage cv. Futoški plants with whole culture of *B. velezensis* strain RD-FC 88 four days prior to inoculation with pathogenic strain XcFC 231. Suspensions of biocontrol and pathogenic strains (both 10^8 CFU mL^{-1}) were made in NB while shaking at 30 °C for 24 h and 27 °C for 48 h, respectively. All treatments were applied by foliar spraying until the leaves were uniformly covered with fine droplets. Two identical experiments were conducted, each with 7 biological replicates per treatment. The results reflect the total population of 14 plants per treatment. Two weeks following the inoculation of plants with the pathogenic strain XcFC 231, disease intensity was assessed using the Horsfall–Barrat (H–B) scale with scores 1–12 (1 = healthy plant; $2 \geq 0\text{--}3\%$; $3 \geq 3\text{--}6\%$; $4 \geq 6\text{--}12\%$; $5 \geq 12\text{--}25\%$; $6 \geq 25\text{--}50\%$; $7 \geq 50\text{--}75\%$; $8 \geq 75\text{--}87\%$; $9 \geq 87\text{--}94\%$; $10 \geq 94\text{--}97\%$; $11 \geq 97\text{--}100\%$; 12 = 100%) [19]. Scores were converted to percentages using

the midpoint of the percentage range corresponding to each score, following the Elanco method [20]. The efficacy of the applied preventive biocontrol treatment with the whole culture of *B. velezensis* strain RD-FC 88 was calculated using Abbott's formula: Efficacy (%) = (mean disease intensity in Xcc-inoculated plants—mean disease intensity in Bac + Xcc-treated plants)/mean disease intensity in Xcc-inoculated plants $\times$ 100 [21]. Following the evaluation of disease intensity, the 1–3 youngest fully developed leaves, with the removed midvein, were harvested from all plants/treatments. Samples were frozen in liquid nitrogen and stored at -80°C until further analysis.

2.2. Extraction and Analysis of Amino Acids

Amino acid content was analyzed according to Živanović et al. [22]. Leaf material was homogenized in liquid nitrogen and extracted in 50% methanol. Obtained homogenate was centrifuged for 10 min at 4°C at $14,000\times g$ and supernatants were saved at -80°C for future analyses. The aliquots of extracted amino acids were diluted 10 times and combined with the same volume of derivatization agents, which consisted of 0.5 M sodium borate buffer (pH 9.5), 0.4 M o-phthalaldehyde (OPA) and β -mercaptoethanol (9:1:0.2, *v:v:v*). Soluble amino acids were analyzed by HPLC-PDA (LC-20AB Prominence Liquid Chromatograph, Shimadzu, Kyoto, Japan) with fluorescence detection. The elution gradient was consisted of A solution: 20 mM sodium phosphate buffer pH 6.8, methanol and THF (90:9:1, *v:v:v*) and B solution: 20 mM sodium phosphate buffer pH 6.8, methanol and THF (40:59:1, *v:v:v*) and was applied in the following way: 0–5 min A = 100%, B = 0%; 5–20 min A = 100%, B = 0%; 20–35 min A = 60%, B = 40%; 35–48 min A = 50%, B = 50%; 48–60 min A = 40%, B = 60%; 60–70 min A = 30%, B = 70%; 70–80 min B = 100%; 80–90 min B = 100%. Injection volume was 20 μL , while the excitation and emission wavelength were set at 340 nm and 455 nm, respectively. AAs were quantified using a standard curve. Proline could not be detected by fluorescent HPLC assay applied for all other measured free AAs. Therefore, it was measured spectrophotometrically according to Živanović et al. [22]. Briefly, the extraction was performed in 3% sulfosalicylic acid (1:10, *w:v*) followed by centrifugation at $14,000\times g$ for 10 min at 4°C . Obtained supernatant was mixed with the same volumes of acidic ninhydrin and glacial acetic acid and incubated for 60 min at 100°C . The reaction mixture was stopped by placing samples in ice-bath, followed by the addition of toluene (1:1, *v:v*) and the upper organic phase was used for proline determination. The proline concentration was calculated based on absorbance at 520 nm using a standard curve.

2.3. Extraction of Glucosinolates and Phenolic Compounds

Leaf tissue samples were homogenized in liquid nitrogen using a mortar and a pestle. Phenolic compounds and glucosinolates were extracted from homogenized tissue using 80% methanol (1:10 *w/v*). Extraction was carried out for 60 min at 55°C , after which the homogenate was centrifuged for 10 min at 25°C at $16,000\times g$. The resulting supernatant was filtered through a 0.22 μm syringe filter before the analysis.

2.4. Ultra-High-Performance Liquid Chromatography Coupled to Quadrupole Time-of-Flight Tandem Mass Spectrometry (UHPLC Q-ToF MS) Analysis

Identification, characterization and quantification/semi-quantification of bioactive compounds derived from leaves of cabbage cv. Futoški was performed by high-performance liquid chromatography (Agilent 1290 Infinity liquid chromatography) coupled to a quadrupole time-of-flight mass spectrometer (6530C Q-ToF-MS, Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was carried out at 40°C , on a Zorbax C18 column (2.1 $\times$ 50 mm, 1.8 μm ; Agilent Technologies, CA, USA). Applied UHPLC method and used Q-ToF operating parameters were previously reported by Kilibarda et al. [23]. Briefly, mobile phase consisted of: (A) ultrapure water +0.1% formic acid (*v/v*);

and (B) 98% acetonitrile (MS grade) + 0.1% formic acid (*v/v*). According to the method, the following gradient of elution was applied: 2% B for the first 2.0 min, followed by a linear increase to 98% B (2–98% B), from 2.0 to 17.0 min. After that the gradient was returned to the initial conditions (2% B) for 5 min to re-equilibrate the column and prepare for the following injection. Flow rate was constant and set at 0.3 mL/min, while the injection volume was 5 µL.

The QToF-MS system was equipped with an Agilent Jet Stream electrospray ionization (ESI) source, operating in both negative and positive ionization modes. In this case, bioactive compounds of interest (glucosinolates and phenolic compounds) were recorded in negative ionization mode, using the Auto MS/MS acquisition mode (full-range acquisition of 100–1700 *m/z*; acquisition rate of 1 spectra/s; and acquisition time of 1000 ms/spectrum), and fixed collision energy at 30 eV. The operating parameters for ESI were set as follows: Nebulizer pressure of 45 psi; a drying gas temperature of 225 °C and a flow rate of 8 L/min; a sheath gas temperature of 300 °C and a sheath gas flow 10 L/min; a capillary voltage of 2500 V; a fragmentor energy of 175 V; a skimmer voltage of 65 V; and octopole RF peak of 750 V.

Agilent MassHunter software (AgilentMassHunter Qualitative Analysis 10.0) and MS DIAL (ver. 4.60) software (<http://prime.psc.riken.jp/>, accessed 20 November 2025) were used for screening of spectra, collection of data of interest (retention time, monoisotopic mass, area), evaluation, analysis, and presentation of MS data. Compounds were tentatively identified based on their monoisotopic masses and typical MS fragments, while some of them were confirmed by comparison with available standards (compounds labelled with *; Table 1). The CAS SciFinder-n (<https://scifindern.cas.org/>, accessed 20 November 2025) and PubChem (<https://pubchem.ncbi.nlm.nih.gov/>, accessed 20 November 2025) databases were applied to search formulas and structures of identified compounds. In order to obtain representative results of quantification/semi-quantification, previously prepared extracts for each plant were appropriately diluted to be in accordance with linear ranges of applied standards. Peak areas for each compound were compared among samples, normalized, processed under the same conditions and exported from MS DIAL software. Standards of glucosinolates and phenolic compounds (mix of standards by different concentrations) were prepared and analyzed under the same conditions as samples. Except 4-hydroxy-glucobrassicin (expressed as µg of glucobrassicin equivalents), other selected glucosinolates were quantified using available standards and their content was expressed in µg per g of fresh weight of each plant. Due to lack of specific standards, all tentatively identified phenolic compounds were semi-quantified, using structurally closest standards such as sinapic acid and kaempferol. Taking this into account, content of phenolic acids was expressed as µg of sinapic acid equivalents per g of raw mass of each plant, while content of selected flavonols was expressed as µg of kaempferol equivalents per g of fresh weight of each plant. Validation parameters of applied/used standards for quantification/semi-quantification of glucosinolates/phenolic compounds are shown in Table S1. In this table (Table S1) are presented following validation data: Regression equation ($Y = a \cdot X \pm b$) and its accompanying parameters including coefficient of determination (R^2); correlation coefficient (r); linearity range; LOD-limit of detection (3:1 Signal-to-noise ratio); LOQ-limit of quantification (10:1 signal-to-noise ratio); and repeatability (intra-day precision, $n = 6$) for each standard separately.

2.5. Fluorescence Measurements

Ice-cold methanol containing 0.1% HCl was used for extraction (1:10, *w:v*) from leaf tissue samples homogenized in liquid nitrogen. Extraction was carried out for 60 min on a shaker, on ice and in the dark, after which the homogenate was centrifuged for 10 min

at $14,000\times g$ at $4\text{ }^{\circ}\text{C}$. The obtained supernatant ($600\text{ }\mu\text{L}$) was mixed with deionized water ($400\text{ }\mu\text{L}$) and chloroform ($600\text{ }\mu\text{L}$) and incubated on a shaker in the dark and on ice for 1 h. The homogenate was then centrifuged for 10 min at $14,000\times g$ at $4\text{ }^{\circ}\text{C}$ and $80\text{ }\mu\text{L}$ of concentrated HCl was added to $400\text{ }\mu\text{L}$ of the obtained supernatant in order to carry out hydrolysis (60 min at $85\text{ }^{\circ}\text{C}$). The extracts were deoxygenated under a nitrogen stream [24].

Fluorescence spectra of cabbage samples (NB, Bac, Xcc, and Bac + Xcc) were measured using an FL3-221P spectrofluorometer (Jobin Yvon, Horiba, Longjumeau, France). Methanolic extracts were placed in 1 mL quartz cuvettes (10 mm path length) in a right-angle configuration. The instrument was equipped with a 450 W xenon (Xe) lamp as the excitation source and a photomultiplier tube for emission detection. To obtain excitation-emission matrices (EEMs), the following settings were used: excitation wavelengths from 280 to 400 nm and emission wavelengths from 335 to 580 nm. Slit widths for the entrance, exit, and intermediate slits were set to 3 nm bandpass.

Principal Component Analysis (PCA) was employed to classify treated cabbage samples based on differences in their characteristic fluorescence emission spectra. Before PCA, the fluorescence data were normalized and standardized, and the analysis was performed using XLSTAT 2018 software (Addinsoft Inc., New York, NY, USA). To validate the group separation observed in the PCA score plots, a permutation multivariate analysis of variance PERMANOVA was performed on the PCA scores. The analysis was conducted using the scores of the first two components, PC1 and PC2 ($F = 3.81$, $p = 0.007$). Additionally, PERMDISP was applied to confirm homogeneity of dispersion $F = 0.799$, $p = 0.492$) ensuring that dispersion differences between groups were no significant.

2.6. Statistical Analyses

Statistical analyses were performed using SPSS Statistics 26.0 (IBM, Armonk, NY, USA). Normality of data distribution within each treatment group was assessed using the Shapiro–Wilk test, and homogeneity of variances using Levene’s test. If at least one group deviated from normality ($p \leq 0.05$), the Kruskal–Wallis test was applied. When normality was met but variances were unequal, Welch’s ANOVA was used. Otherwise, one-way ANOVA was performed (Table S2). Appropriate post hoc tests (Tukey’s HSD or Games–Howell) were applied depending on variance homogeneity. Statistical significance was set at $p < 0.05$. The figures were created using Python 3.11.14 and the Seaborn library 0.13.2.).

3. Results

3.1. Disease Assessment

Two weeks after inoculation of cabbage cv. Futoški plants with the pathogenic strain XcFC 231, black rot symptoms were observed in both the Xcc-treated and Bac + Xcc-treated plant groups (Figure 1). Symptoms appeared as chlorotic to necrotic V-shaped lesions, typically starting at the leaf margins and progressing toward the leaf center, with blackened veins visible, indicating the pathogen presence. Control (NB) and Bac-treated plants remained healthy. The calculated disease intensity in Xcc-treated plants was $4.81 \pm 1.10\%$, whereas in Bac + Xcc-treated plants it was $1.78 \pm 0.44\%$, corresponding to a calculated biocontrol efficacy of 62.99%.

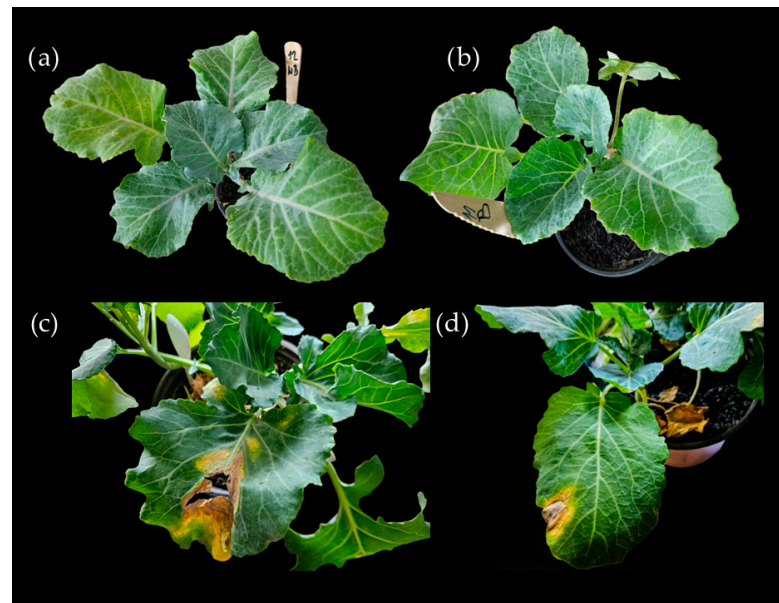


Figure 1. Cabbage cv. Futoški plants two weeks after inoculation with the pathogenic strain *X. campestris* pv. *campestris* XcFC231: (a) NB control treatment, (b) *B. velezensis* RD-FC 88 treatment (Bac), (c) preventive foliar treatment with *B. velezensis* RD-FC 88 followed by pathogen inoculation (Bac + Xcc), and (d) pathogen-only treatment (Xcc). Black rot symptoms absent in treatments (a,b) and present in treatments (c,d), appearing as typical V-shaped chlorotic to necrotic lesions starting from the leaf margins and spreading toward the leaf base.

3.2. Free Amino Acid Content

In this research, *Xcc*-inoculated cabbage plants exhibited a marked reduction in total free amino acid content relative to all other treatment groups (NB, Bac and Bac + *Xcc*). In all treatments, the most abundant amino acids were aspartic acid (Asp), glutamic acid (Glu), serine (Ser), glutamine (Gln) and leucine (Leu), accounting for about 80% of all measured amino acids (Figure 2).

While the accumulation of Asp, Glu and Leu was not significantly altered by *X. campestris* pv. *campestris* strain XcFC 231 infection, the levels of Ser and Gln were significantly diminished compared to the control (Figure 2a–e). A similar pattern in reduced Ser content was also noticed in Bac-treated plants (both cases) in comparison to control (Figure 2e). Regarding glutamine content, *Xcc*-infected plants exhibited a 40% reduction in comparison to plants treated only with the *B. velezensis* strain RD-FC 88. Moreover, the preventive application of biocontrol strain combined with subsequent application of pathogen led to restored Gln content close to values corresponding to control plants (NB).

Furthermore, the levels of branched-chain amino acids (BCAA)—specifically isoleucine (Ile), leucine (Leu), and valine (Val)—were significantly reduced in *X. campestris* pv. *campestris*-inoculated plants (*Xcc*) compared to all other treatment groups (NB, Bac, Bac + *Xcc*) (Figure 2d,f,g). Moreover, Leu was one of the most dominant amino acids in the total pull of measured AA, and interestingly, in the presence of *Bacillus* (Bac and Bac + *Xcc*), its content was almost twice as high compared to *X. campestris* pv. *campestris*-inoculated plants (*Xcc*) (Figure 2d). Upon *X. campestris* pv. *campestris* infection (*Xcc*), Val content decreased about 30% compared to control plants, and similar to Leu, Bac treatment restored its content to control conditions (Figure 2d,g). A similar trend was observed for alanine (Ala) content, with *Xcc* infection reducing its concentration by approximately 50% in comparison to control (Figure 2h).

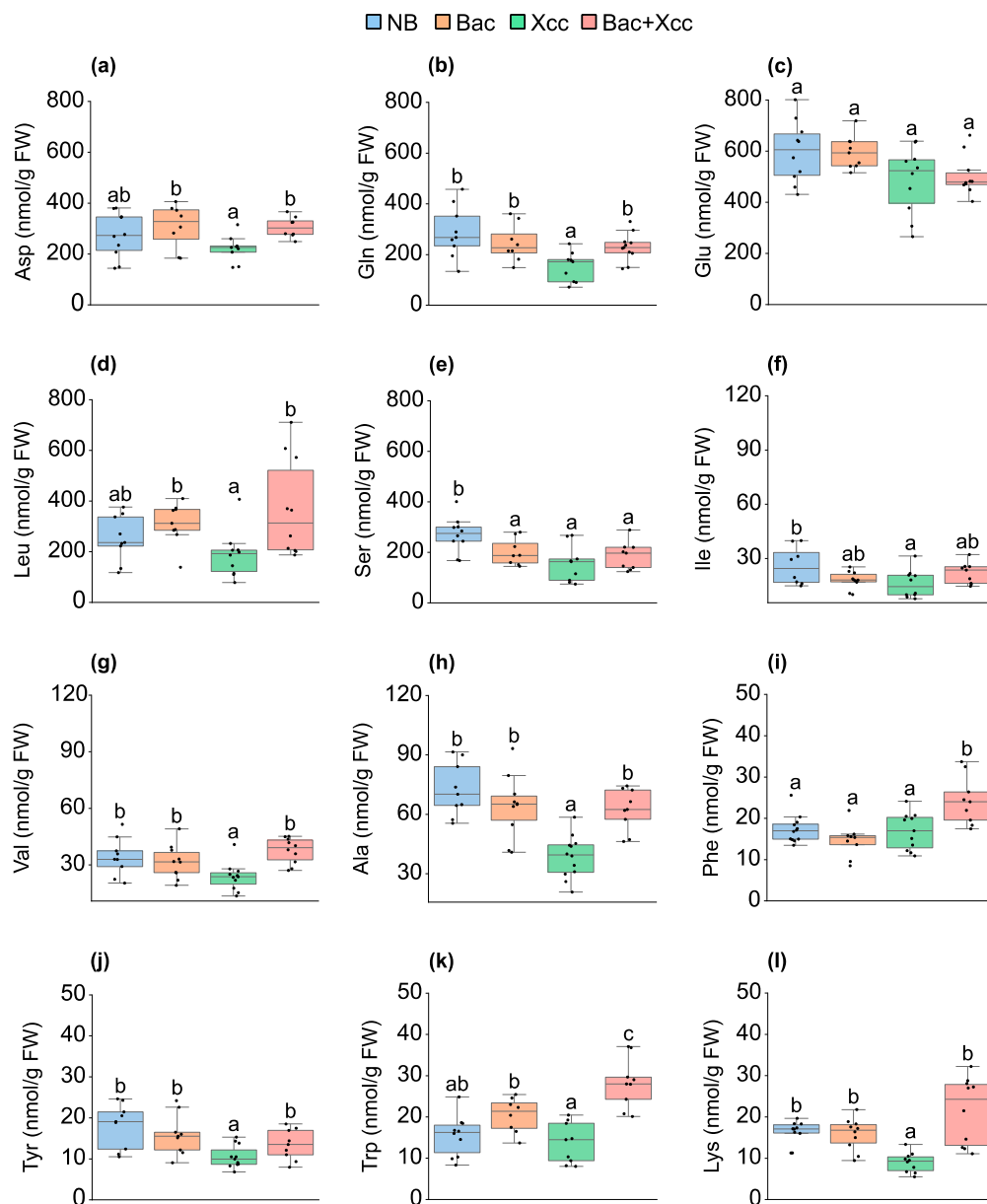


Figure 2. Free amino acid concentrations across four different treatments. The box plots illustrate the distribution of different amino acids (a–l) across four experimental treatments: NB (Nutrient Broth; blue), Bac (*B. velezensis* RD-FC 88; orange), Xcc (*X. campestris* pv. *campestris* XcFC 231; green), Bac + Xcc (*B. velezensis* + *X. campestris* pv. *campestris*; pink). Individual points represent measured values ($n \geq 7$). Different letters indicate statistically significant differences between the treatments ($p < 0.05$).

Infection with *X. campestris* pv. *campestris* (Xcc) led to a significant reduction in tyrosine (Tyr) and tryptophan (Trp) content compared to the control (NB) and biocontrol-treated groups (Bac + Xcc), respectively, while phenylalanine (Phe) content remained relatively stable in comparison to the control (Figure 2i–k). The most substantial accumulation of Phe was observed in biocontrol-treated Xcc-infected plants (Bac + Xcc), reaching levels significantly higher than those found in either the control or the Xcc-infected plants.

X. campestris pv. *campestris* infection (Xcc) reduced lysine (Lys) content by more than 50% in cabbage plants in comparison to control (NB), and to other treatments (Bac and Bac + Xcc) (Figure 2l). Interestingly, the biocontrol-treated Xcc-infected plants showed the highest Lys content which was almost threefold higher than in Xcc-infected plants.

3.3. Secondary Metabolites

Untargeted UHPLC Q-ToF MS analysis revealed 10 different glucosinolates and 22 phenolic compounds that were constitutively present in all samples (Tables 1 and 2, Figure 3).

3.3.1. Phenolic Content

To the best of our knowledge, the phenolic profile of cabbage cv. Futoški was characterized for the first time in the current study. Due to the absence of specific standards, all phenolic compounds were tentatively identified based on typical fragments (Table 1). Among those compounds three different classes emerged (i) hydroxybenzoic (HBA) group containing two compounds, (ii) hydroxycinnamic (HCA) group with 12 compounds representing the highest diversity and (iii) flavonol group containing 6 different glycosylated and/or acylated derivatives of the flavonol kaempferol. Phenolic acid derivatives were the most numerous and were found in the form of monohexosides (11, 12, 16, and 18), diglycosides (19 and 20), complex sinapoyl/feruloyl-hexosides (21–27) and esters with quinic acid (cinnamate esters) (13–15 and 17). The main fragment of phenolic acid monohexosides and diglycosides was deprotonated phenolic acid, as shown by the loss of hexosyl (and pentosyl) unit(s). On the other hand, complex sinapoyl/feruloyl-hexosides exhibited various MS fragments obtained by sequential losses of hexosyl units (-163 Da) and sinapoyl (-205 Da) and/or feruloyl (-175 Da) moieties. Cinnamate esters (coumaroyl-; caffeoyl-; and feruloyl-quinic acid) showed typical fragments originating from deprotonated quinic (191 m/z ; 173 m/z [$-H_2O$]; 155 m/z [$-2 \times H_2O$]; 127 m/z [$-2 \times H_2O$; $-CO$]; 111 m/z [$-2 \times H_2O$; $-CO_2$]) and/or specific phenolic acid. Except of phenolic acid derivatives, several kaempferol glycosides (28 and 29) and kaempferol monoacylated trihexosides (30–33) were also found. Compound 29 was recognized as kaempferol 3-O-sophoroside-7-O-hexoside, with main fragment at 609 m/z obtained by loss of hexosyl unit in C-7 position. Fragment at 429 m/z indicating on $1 \rightarrow 2$ interglycosidic linkage between two hexoses (sophoroside). All kaempferol monoacylated glycosides had unique monoisotopic mass and MS fragmentation, depending on hydroxycinnamoyl moiety related to sophoroside. Main fragment for each of these derivatives was obtained by loss of hexosyl unit from C7-position, specifically 771 m/z (30), 785 m/z (31), 801 m/z (32) and 815 m/z (33). Common fragment at 609 m/z was obtained by loss of specific hydroxycinnamoyl moiety (caffeoyl-; feruloyl-; hydroxyferuloyl-; or sinapoyl-moiety), indicating its position and linkage with sophoroside.

Considering that phenolic compounds were identified in all analyzed cabbage samples, differences in their concentration/accumulation can contribute to the understanding of plant immune response caused by applied biocontrol treatments. However, the absence of typical standards limits the quantification of the identified phenolic compounds. In order to overcome this, phenolic compounds were semi-quantified using structurally closest and available standards, while their content was expressed in equivalents of selected standards. Taking this into account, phenolic acid derivatives were expressed in sinapic acid equivalents due to the fact that the most identified and abundant compounds from this group belong to sinapic acid glycosides and complex sinapoyl derivatives. On the other hand, all identified flavonoids (flavonols) correspond to kaempferol di(tri)glycosides and kaempferol monoacylated triglycosides, and consequently they were semi-quantified and expressed in kaempferol aglycone equivalents.

Table 1. Untargeted UHPLC Q-ToF MS profile of bioactive compounds derived from leaves of cabbage vs. Futoški. Compound name, retention time (RT), molecular formula, calculated and m/z exact mass, mean mass accuracy (mDa), and MS fragments are presented in Table 1.

No.	RT	Compounds	Formulas	Calculated Mass	m/z Exact Mass	mDa	MS Fragments (% Main Fragment)	Ref. **
Glucosinolates (GLSs)								
1	0.78	Sinigrin *	$C_{10}H_{16}NO_9S_2^-$	358.0266	358.0263	−0.35	116.017(100), 101.0243, 138.9702, 161.9855, 164.9659, 195.0313, 259.0114, 274.9880, 240.9985	Std.
2	1.35	Gluconapin	$C_{11}H_{18}NO_9S_2^-$	372.0423	372.0415	−0.8	101.024(100), 130.032, 119.0339, 138.9712, 158.9373, 178.9823, 259.0079, 274.9858	[25]
3	3.61	Glucotropaeolin *	$C_{14}H_{18}NO_9S_2^-$	408.0423	408.0398	−2.50	166.0315(100), 138.9667, 206.8495, 132.0458, 174.9496, 194.6512, 206.8495, 259.0067, 274.9838	Std.
4	4.17	Glucoerucin *	$C_{12}H_{22}NO_9S_3^-$	420.0457	420.0431	−2.57	274.9923(100), 259.0102, 195.0336, 178.0325, 127.924	Std.
5	0.66	Glucoiberin *	$C_{11}H_{20}NO_{10}S_3^-$	422.0249	422.0267	1.77	195.9735(100), 358.0258, 259.0119, 406.9998, 274.9895, 228.967, 180.015, 164.0015, 135.9714, 116.0174	Std.
6	6.19	Gluconasturtiin	$C_{15}H_{20}NO_9S_2^-$	422.0579	422.0567	−1.25	259.0109(100), 180.0482, 138.9696, 101.0245, 228.9968, 274.9862, 339.0327	[25]
7	5.26	Glucobrassicin *	$C_{16}H_{19}N_2O_9S_2^-$	447.0532	447.0519	−1.30	259.0110(100), 205.0419, 138.9703, 274.9876, 241.0031, 195.0329, 172.0219, 127.9259	Std.
8	1.76	4-hydroxyglucobrassicin	$C_{16}H_{19}N_2O_{10}S_2^-$	463.0481	463.0466	−1.51	169.0403(100), 221.0368, 160.0399, 144.0437, 187.0515, 221.0368, 274.9904, 259.0130, 285.0145, 267.0092	[25]
9	6.67	4-methoxyglucobrassicin *	$C_{17}H_{21}N_2O_{10}S_2^-$	477.0638	477.0619	−1.86	477.0600(100), 259.0105, 274.9875, 235.0532, 195.0316, 163.0573, 138.9685, 119.0354, 406.1440	Std.
10	7.36	Neoglucobrassicin *	$C_{17}H_{21}N_2O_{10}S_2^-$	477.0638	477.0621	−1.66	154.0527(100), 259.0139, 195.0292, 170.0470, 141.0473, 205.0414, 223.9998, 274.9860, 446.0442	Std.

Table 1. Cont.

No.	RT	Compounds	Formulas	Calculated Mass	<i>m/z</i> Exact Mass	mDa	MS Fragments (% , Main Fragment)	Ref. **
Phenolic compounds								
Phenolic acid and derivatives								
11	5.90	Hydroxybenzoic acid hexoside	C ₁₃ H ₁₅ O ₈ [−]	299.0767	299.0760	−0.69	137.0228(100)	[23]
12	2.50	Dihydroxybenzoic acid hexoside	C ₁₃ H ₁₅ O ₉ [−]	315.0716	315.0715	−0.11	152.0104(100), 108.0206, 153.0156, 109.0282	[23]
13	5.99	Coumaroylquinic acid	C ₁₆ H ₁₇ O ₈ [−]	337.0923	337.0914	−0.94	119.0497(100), 163.0394, 191.0551, 173.0454, 111.0440, 155.0334, 145.0279	[26]
14	4.88	Caffeoylquinic acid is. I	C ₁₆ H ₁₇ O ₉ [−]	353.0873	353.0866	−0.66	191.0555(100), 135.0444, 179.0343, 161.0238, 111.0443, 173.0451, 155.0332	[27]
15	6.45	Caffeoylquinic acid is. II (Chlorogenic acid) *	C ₁₆ H ₁₇ O ₉ [−]	353.0873	353.0870	−0.26	135.0445(100), 191.0555, 173.0446, 179.0338, 161.0238, 111.0450, 155.0351, 127.0400	[27]
16	6.74	Ferulic acid hexoside	C ₁₆ H ₁₉ O ₉ [−]	355.1029	355.1015	−1.41	160.0159(100), 134.0368, 175.0391, 193.0481, 149.0575	[28]
17	6.47	Feruloylquinic acid	C ₁₇ H ₁₉ O ₉ [−]	367.1029	367.1025	−0.41	134.0362(100), 193.0500, 149.0594, 191.0546, 173.0484, 155.0326	[28]
18	6.87	Sinapic acid hexoside	C ₁₇ H ₂₁ O ₁₀ [−]	385.1135	385.1121	−1.37	190.0266(100), 205.0497, 164.0468, 149.0234, 223.0611, 175.0031	[23,29]
19	6.43	Hidroxybenzoic acid pentosyl hexoside	C ₁₈ H ₂₃ O ₁₂ [−]	431.1190	431.1173	−1.65	137.0228(100)	-
20	6.55	Sinapic acid dihexoside	C ₂₃ H ₃₁ O ₁₅ [−]	547.1663	547.1660	−0.30	205.0493(100), 223.0600, 164.0459, 149.0246, 190.0259, 179.0655, 385.0848	[29]
21	8.71	Diferuloyl-dihexoside	C ₃₂ H ₃₇ O ₁₇ [−]	693.2031	693.2000	−3.07	175.0383(100), 193.0490, 160.0153, 217.0492, 235.0580, 134.0361, 499.1420	[26]
22	8.62	Sinapoyl feruloyl dihexoside	C ₃₃ H ₃₉ O ₁₈ [−]	723.2136	723.2114	−2.24	175.0391(100), 499.1424, 193.0493, 205.0495, 223.0600, 235.0605, 160.0171, 217.0495	[27]
23	7.41	Disinapoyl-dihexoside is. I	C ₃₄ H ₄₁ O ₁₉ [−]	753.2242	753.2209	−3.30	205.0492(100), 223.0596, 529.1557, 190.0254, 247.0605, 265.0688, 179.0703, 149.0213, 427.1262, 487.1495	[27]

Table 1. Cont.

No.	RT	Compounds	Formulas	Calculated Mass	<i>m/z</i> Exact Mass	mDa	MS Fragments (% , Main Fragment)	Ref. **
24	8.54	Disinapoyl-dihexoside is. II	C ₃₄ H ₄₁ O ₁₉ [−]	753.2242	753.2238	−0.40	205.0485(100), 223.0590, 190.0248, 247.0581, 190.0248, 265.0690, 529.1517	[27]
25	7.42	Sinapoyl feruloyl trihexoside	C ₃₉ H ₄₉ O ₂₃ [−]	885.2665	885.2662	−0.26	499.1436(100), 723.2108, 205.0483, 885.2700, 529.1599, 223.0596, 193.0488, 175.0381, 149.0556	-
26	9.16	Disinapoyl feruloyl dihexoside	C ₄₄ H ₄₉ O ₂₂ [−]	929.2715	929.2692	−2.35	705.2002(100), 175.0392, 529.1550, 205.0490, 223.0602, 193.0495, 247.0596, 499.1437, 481.1309, 735.2098	[27]
27	9.09	Trisinapoyl-dihexoside	C ₄₅ H ₅₁ O ₂₃ [−]	959.2821	959.2794	−2.71	735.2076(100), 205.0483, 529.1530, 223.0592, 247.0578, 265.0699, 190.0253, 511.1435, 487.1394, 753.2252	[27]
Flavonol glycosides and acyl derivatives								
28	7.44	Kaempferol 3,7-di-O-hexoside	C ₂₇ H ₂₉ O ₁₆ [−]	609.1456	609.1448	−0.76	609.1424(100), 283.0235, 446.0836, 447.0899, 255.0284, 285.0399	[27]
29	6.34	Kaempferol 3-O-sophoroside-7-O-hexoside	C ₃₃ H ₃₉ O ₂₁ [−]	771.1984	771.1977	−0.68	609.1414(100), 446.0829, 284.0291, 429.083, 327.0430, 255.0258, 178.9987, 285.0378, 447.0866	[27]
30	6.61	Kaempferol 3-O-caffeoyl-sophoroside-7-O-glucoside	C ₄₂ H ₄₅ O ₂₄ [−]	933.2301	933.2288	−1.28	771.1707(100), 609.1415, 933.2227, 446.0880, 285.0368, 429.0906	[27,30]
31	6.94	Kaempferol 3-O-feruloyl-sophoroside-7-O-glucoside	C ₄₃ H ₄₇ O ₂₄ [−]	947.2457	947.2441	−1.63	785.1905(100), 609.1441, 947.2438, 284.0315, 446.0847, 591.1323, 429.0784, 255.0283, 227.0300, 193.0516, 175.0407	[30]
32	6.55	Kaempferol 3-O-hydroxyferuloyl-sophoroside-7-O-glucoside	C ₄₃ H ₄₇ O ₂₅ [−]	963.2406	963.2391	−1.54	801.1817(100), 609.1410, 963.2328, 284.0312, 429.077, 591.1274, 446.0789	[27,30]
33	6.87	Kaempferol 3-O-sinapoyl-sophoroside-7-O-glucoside	C ₄₄ H ₄₉ O ₂₅ [−]	977.2563	977.2555	−0.79	815.2012(100), 977.2531, 609.1436, 591.1310, 529.1569, 446.0835, 429.0844, 284.0315, 255.0294, 227.0372, 205.0490, 178.9999	[30]

Abbreviations: “is.”—isomers; Std.—Standard; * Compounds additionally confirmed by available standards. ** Previously reported in various *B. oleracea* varieties or other plant from Brassicaceae family.

In response to *X. campestris* pv. *campestris* infection, the group of benzoic acids exhibited a significant downward trend, with the concentrations of both detected hydroxybenzoic acid derivatives being significantly reduced compared to those in the control plants (NB) (Table 2). The group of phenolic acids belonging to the derivatives of various hydroxycinnamic acids (HCA) showed similar behavioural trends as the hydroxybenzoic group of compounds. Specifically, compounds labelled 14, 15, 17, 18, 22–24, 26–27 (Table 1) all showed significantly reduced content upon infection with *X. campestris* pv. *campestris* strain XcFC 231(Xcc).

The results of the flavonoid contents observed in the four plant groups investigated in this study are shown in Table 2. The majority of kaempferol derivatives identified in this study did not differ significantly across the various treatment groups (Table 2). However, a significant reduction was specifically observed in the plants preventively treated with *Bacillus* and subsequently infected with Xcc (Bac + Xcc). This notable decline was localized to specific compounds, namely kaempferol 3-O-sophoroside-7-O-hexoside (29), kaempferol 3-O-caffeoyl-sophoroside-7-O-glucoside (30), and kaempferol 3-O-sinapoyl-sophoroside-7-O-glucoside (33), which exhibited significantly lower concentrations compared to the control group (Tables 1 and 2).

Table 2. Concentration of measured phenolic compounds across four different treatments. Results are shown as mean $\pm$ SE across four experimental treatments: NB (Nutrient Broth), Bac (*B. velezensis* RD-FC 88), Xcc (*X. campestris* pv. *campestris* XcFC 231), Bac + Xcc (*B. velezensis* + *X. campestris* pv. *campestris*), $n \geq 7$ per group. Different letters indicate statistically significant differences ($p < 0.05$).

Compound ($\mu\text{g/g}$ FW)		NB	Bac	Xcc	Bac + Xcc
Phenolic acids and their derivatives	Hydroxybenzoic acid hexoside	10.99 $\pm$ 2.63 b	33.83 $\pm$ 7.01 c	3.36 $\pm$ 0.56 a	17.49 $\pm$ 2.35 c
	Dihydroxybenzoic acid hexoside	22.54 $\pm$ 4.63 b	38.20 $\pm$ 6.03 c	13.25 $\pm$ 2.78 a	33.14 $\pm$ 3.83 c
	Coumaroylquinic acid	121.03 $\pm$ 42.50 a	171.60 $\pm$ 44.93 a	179.59 $\pm$ 18.56 a	129.68 $\pm$ 24.34 a
	Caffeoylquinic acid is. I	129.26 $\pm$ 24.37 c	149.33 $\pm$ 32.56 c	17.70 $\pm$ 4.94 a	39.70 $\pm$ 7.95 b
	Caffeoylquinic acid is. II (Chlorogenic acid)	69.24 $\pm$ 8.18 b	70.76 $\pm$ 12.06 b	24.48 $\pm$ 4.42 a	50.19 $\pm$ 7.85 b
	Ferulic acid hexoside	26.16 $\pm$ 4.83 a	78.45 $\pm$ 19.39 b	30.44 $\pm$ 7.81 ab	41.71 $\pm$ 6.98 ab
	Feruloylquinic acid	21.09 $\pm$ 3.51 b	28.96 $\pm$ 5.36 b	10.61 $\pm$ 2.17 a	20.71 $\pm$ 2.63 b
	Sinapic acid hexoside	117.23 $\pm$ 12.28 b	147.95 $\pm$ 23.16 b	69.10 $\pm$ 11.55 a	110.24 $\pm$ 13.85 b
	Sinapic acid dihexoside	36.59 $\pm$ 2.62 ab	47.91 $\pm$ 7.89 b	26.18 $\pm$ 4.62 a	29.53 $\pm$ 3.67 a
	Diferuloyl-dihexoside	7.57 $\pm$ 0.67 a	19.99 $\pm$ 3.82 b	11.01 $\pm$ 2.21 ab	10.26 $\pm$ 1.66 ab
	Sinapoyl feruloyl dihexoside	140.96 $\pm$ 19.94 b	181.60 $\pm$ 29.52 b	62.62 $\pm$ 8.90 a	95.68 $\pm$ 13.91 ab
	Disinapoyl-dihexoside is. I	8.69 $\pm$ 0.97 c	12.44 $\pm$ 1.25 d	2.79 $\pm$ 0.38 a	5.63 $\pm$ 0.79 b
	Disinapoyl-dihexoside is. II	113.72 $\pm$ 12.45 b	149.93 $\pm$ 20.98 b	41.32 $\pm$ 6.40 a	69.36 $\pm$ 11.03 a
	Sinapoyl feruloyl trihexoside	10.26 $\pm$ 2.67 a	15.72 $\pm$ 3.92 a	5.77 $\pm$ 1.47 a	11.30 $\pm$ 1.94 a
	Disinapoyl feruloyl dihexoside	92.25 $\pm$ 11.53 c	129.58 $\pm$ 17.85 c	28.56 $\pm$ 4.24 a	39.53 $\pm$ 5.87 b
	Trisinapoyl-dihexoside	72.32 $\pm$ 9.86 b	98.16 $\pm$ 11.51 b	21.98 $\pm$ 3.78 a	31.29 $\pm$ 5.39 a
Flavonols	Kaempferol 3,7-di-hexoside	2.15 $\pm$ 0.30 a	1.89 $\pm$ 0.23 a	1.51 $\pm$ 0.14 a	1.57 $\pm$ 0.20 a
	Kaempferol	3.08 $\pm$ 0.57 b	2.57 $\pm$ 0.58 ab	1.61 $\pm$ 0.21 ab	1.37 $\pm$ 0.18 a
	3-O-sophoroside-7-O-hexoside				
	Kaempferol 3-O-caffeoyl-sophoroside-7-O-glucoside	3.70 $\pm$ 0.62 b	3.32 $\pm$ 0.93 ab	2.06 $\pm$ 0.28 a	2.00 $\pm$ 0.25 a
	Kaempferol 3-O-feruloyl-sophoroside-7-O-glucoside	3.70 $\pm$ 0.58 a	3.25 $\pm$ 0.85 a	2.52 $\pm$ 0.39 a	2.27 $\pm$ 0.32 a
	Kaempferol 3-O-hydroxyferuloyl-sophoroside-7-O-glucoside	3.17 $\pm$ 0.64 a	2.71 $\pm$ 0.66 a	1.84 $\pm$ 0.30 a	1.70 $\pm$ 0.22 a
	Kaempferol 3-O-sinapoyl-sophoroside-7-O-glucoside	4.97 $\pm$ 0.68 c	3.90 $\pm$ 0.95 bc	2.27 $\pm$ 0.39 ab	2.02 $\pm$ 0.36 a

3.3.2. Glucosinolate Content

As previously mentioned, glucosinolates (GLSs) represent a major class of secondary metabolites in *Brassicaceae* family responsible for plants' resistance to various pathogens,

such as *Xanthomonas campestris* pv. *campestris* [1]. Untargeted UHPLC Q-ToF MS analysis conducted in the present study identified 10 glucosinolate compounds in leaves of cabbage cv. Futoški (Table 1). In this study, ten well-known glucosinolates were detected and characterized, and some of them were additionally monitored and quantified. Mentioned GLSs (1–10) were detected based on unique monoisotopic masses, and typical/common fragments formed by cleavage of thioglucose and transfer of the sulphate group (275 *m/z*; 259 *m/z*; 195 *m/z*; and/or 163 *m/z*). Except gluconapin (2), gluconasturtiin (6) and 4-Hydroxyglucobrassicin (8), other detected GLSs (1, 3–5, 7, 9, 10) were additionally confirmed by comparison with available standards. Sinigrin, glucoiberin, glucoerucin, and gluconapin were detected from aliphatic glucosinolates, while glucobrassicin, 4-hydroxyglucobrassicin, 4-methoxy-glucobrassicin, and neoglucobrassicin were detected from indolic.

From the group of aromatic glucosinolates whose metabolic pathway begins with the aromatic amino acids Phe and Tyr, only gluconasturtiin and glucotropaeolin were detected and that in a negligible concentration. Also, the above-mentioned gluconapin, glucoerucin and neoglucobrassicin were detected in traces, and not even in all treatments. Constitutively, the most abundant aliphatic glucosinolate was glucoiberin reaching around 300 nmol g^{−1} FW, almost 5 times more in comparison to sinigrin (Figure 3a,b). *Xcc* infection resulted in a significant drop in the concentrations of both major aliphatic glucosinolates (glucoiberin and sinigrin), although the inhibitory effect was markedly more pronounced for glucoiberin than for sinigrin (Figure 3a,b). Infection with *Xcc* caused/induced a significant reduction in the levels of both aliphatic glucosinolates, though the inhibitory effect was markedly more pronounced/evidently stronger for glucoiberin than for sinigrin (Figure 3a,b). Interestingly, in biocontrol-treated *Xcc*-infected plants, the content of both glucosinolates was elevated in comparison to *Xcc*-inoculated plants, although with a slightly different pattern. In the case of sinigrin, biocontrol restored its accumulation to control values, whereas glucoiberin content was higher than in *Xcc*-infected cabbage plants, but remained at basal levels.

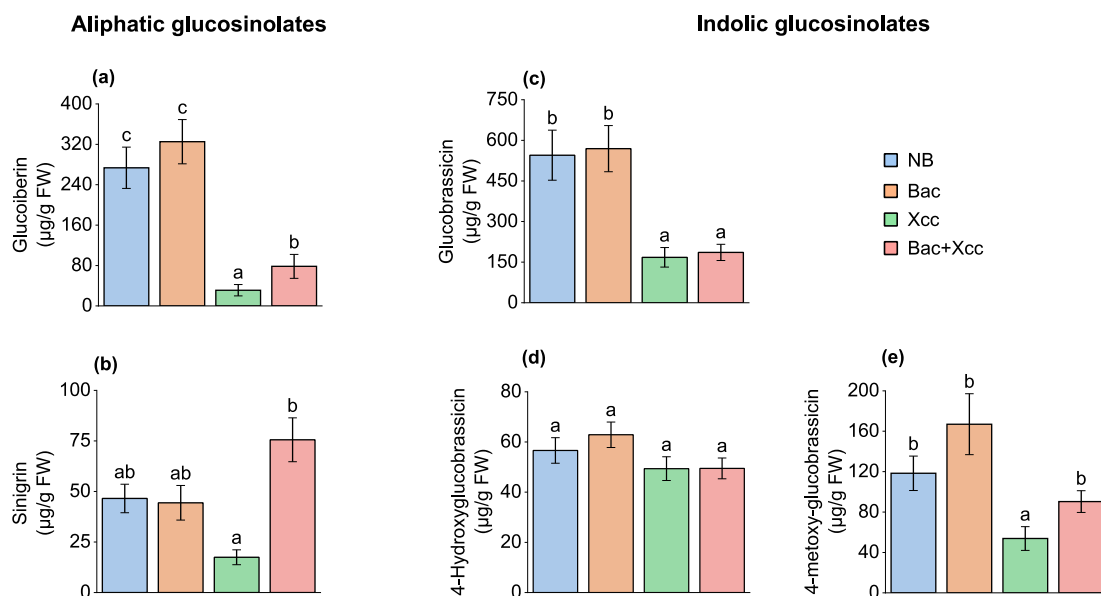


Figure 3. Glucosinolate concentrations in four different treatments. The bar graphs show the concentrations of aliphatic (a,b) and indolic glucosinolates (c–e) across four experimental treatments: NB (Nutrient Broth; blue), Bac (*B. velezensis* RD-FC 88; orange), Xcc (*X. campestris* pv. *campestris* XcFC231; green), Bac + Xcc (*B. velezensis* + *X. campestris* pv. *campestris*; pink), $n \geq 7$ per group. Different letters indicate statistically significant differences ($p < 0.05$).

Regarding indolic glucosinolates, glucobrassicin was the most dominant compound detected in all four treatments: NB, Bac, Xcc, Bac + Xcc (Figure 3c–e). Together with glucoiberin, glucobrassicin accounted for more than 75% of all detected glucosinolates in control cabbage samples. Moreover, basal values of glucobrassicin were 5–10 times higher than those of 4-hydroxyglucobrassicin and 4-methoxy-glucobrassicin. All three measured indole glucosinolates showed a distinct behaviour during infection with *X. campestris* pv. *campestris* (Xcc), as well as in the case of biocontrol-treated *X. campestris* pv. *campestris*-infected plants (Bac + Xcc). The content of 4-hydroxyglucobrassicin remained unaffected by either the pathogen or the biocontrol strain across all treatment groups (Xcc, Bac, Bac + Xcc) (Figure 3d). In contrast, glucobrassicin level significantly declined following *X. campestris* pv. *campestris* infection (Xcc), and this reduction was not reversed in the plants that received the preventive biocontrol treatment (Bac + Xcc) (Figure 3c). Conversely, while 4-methoxy-glucobrassicin concentrations also decreased significantly under pathogen stress (Xcc), the preventive application of the *Bacillus* strain (Bac + Xcc) effectively restored its content to levels comparable to the control group (Figure 3e).

3.4. Fluorescence Analysis

Principal Component Analysis (PCA) based on fluorescence spectra of cabbage samples showed evident separation between tested groups (Figure 4). The first two principal components accounted for 93.46% of the total variations (PC1 = 52.03% and PC2 = 41.43%). The score plot (Figure 4) demonstrated clear group discrimination. Control samples (blue circles) were mainly distributed along the positive axis of PC1, reflecting shared characteristics captured by this component, while their greater intracluster dispersion indicates variability within the control group. Bac + Xcc samples (red circles) also occupy the positive side of PC1, suggesting partial similarity in the variance captured by this component, but their separation along PC2 highlights component-specific differences distinguishing them from the controls. It is observed that samples from the Bac + Xcc treatment were mainly positioned in the positive PC2 region of PC2, while control samples were predominantly located in the negative PC2 region (Figure 4). NB and Bac treatments are projected near the origin of PCA plot, proposing minimal variation in emission profiles. PCA loadings indicate which spectral regions contribute most to the separation of treatment groups, reflecting relative differences in metabolic responses among samples. The loading plot (Figure S1) revealed that wavelengths in the 560–590 nm region were strongly associated with PC1, while wavelengths in the 400–430 nm region contributed predominantly to PC2. Wavelengths between 440 and 460 nm were positively associated with PC1 and negatively with PC2. The long vectors positioned near the unit circle indicate strong contributions of these wavelengths to the explained variance. High squared cosine ($\cos^2$) values for the key wavelengths and well-separated sample groups confirmed that the first two principal components provided a reliable representation of the data structure (Inset in Figure 4).

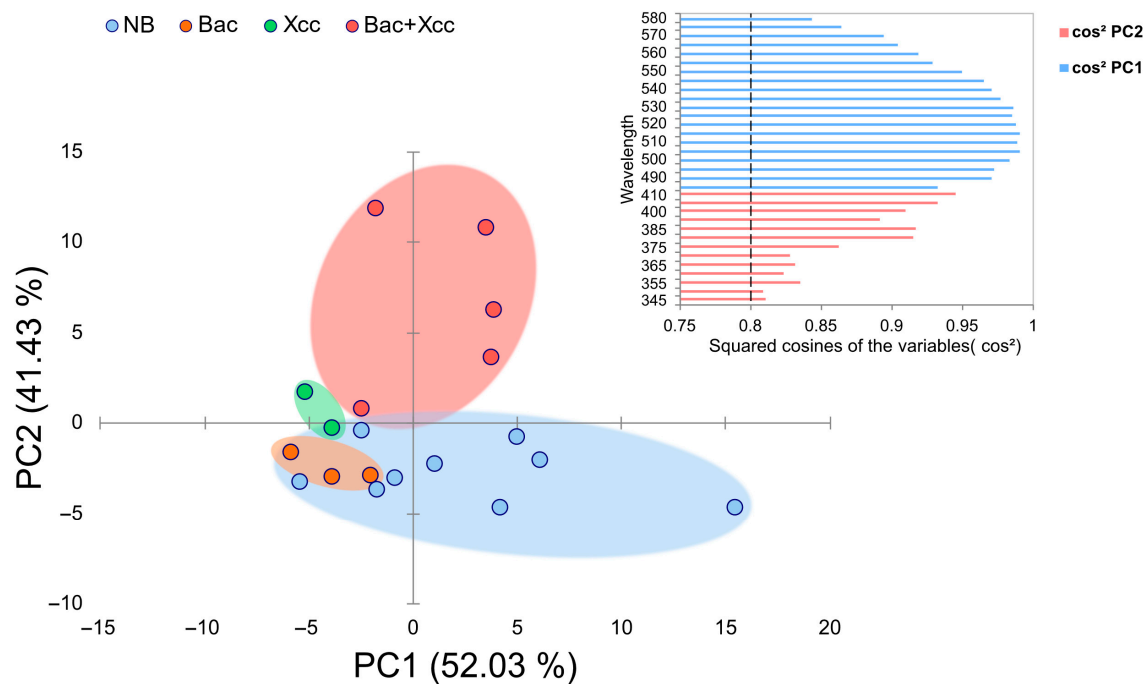


Figure 4. PCA score plot of the fluorescence emission spectra of cabbage samples subjected to different treatments: NB (Nutrient Broth; blue), Bac (*B. velezensis* RD-FC 88; orange), Xcc (*X. campestris* pv. *campestris* XcFC 231; green), Bac + Xcc (*B. velezensis* + *X. campestris* pv. *campestris*; red). The inset shows a bar chart of squared cosine ($\cos^2$) values for emission wavelengths with high contribution to PC1 (blue) and PC2 (red). Only wavelengths with $\cos^2 > 0.80$ are shown.

4. Discussion

4.1. Free Amino Acid Content

Numerous studies have shown that the accumulation of amino acids or upregulation of their differentially expressed genes (DEGs) in cruciferous plants after Xcc infection is time-dependent and also depends on whether the plant line is resistant or susceptible [14,31]. Also, numerous studies have reported that treatment of plants with selected *Bacillus* strains has a beneficial effect on the accumulation of amino acids and their derivatives in leaves or root exudates [32,33].

Although numerous studies have shown that pathogen attack induced glutamine accumulation and elevated glutamine synthetase (GS) activity [34,35], it has also been demonstrated that, during plant senescence, pathogen invasion disrupts primary metabolism in chloroplasts and leads to downregulation of GS. This process likely results in glutamine decrease and potential alternation in nitrogen metabolism, rather than implying a disturbance in nitrogen assimilation [36]. Indeed, restored glutamine content in biocontrol-treated Xcc-infected cabbage plants observed in this study indicates that the selected *Bacillus* strain holds potential to modulate nitrogen metabolism, thus contributing to the resistance development to Xcc (Figure 1b). Furthermore, the infection with Xcc resulted in a significant decline in the Gln/Glu ratio (Table S3), primarily caused by reduced Gln content following Xcc infection, indicating limitations in nitrogen assimilation [26]. In contrast, the restoration of this ratio to control levels in plants receiving the preventive biocontrol treatment (Bac + Xcc) suggests an improved plant defense response to pathogen attack.

Bacillus-treated plants (Bac and Bac + Xcc) showed over 30% higher aspartate accumulation than those treated only with Xcc (Figure 2a). Unchanged aspartate content in Xcc-infected cabbage plants was noted in both tolerant and resistant cabbage cultivars [31], while Sun et al. observed that Xcc affected the reduction in aspartate content only in a susceptible cultivar [13]. Apart from glutamate, aspartate is the primary acceptor of

inorganic nitrogen needed for glutamine biosynthesis and also plays a key role in auxin biosynthesis in plants [27].

Studies on Arabidopsis showed that during *Pseudomonas syringae* pv. *tomato* infection aspartate content was reduced at 18 h post infection (hpi) [37]. Moreover, elevated activation of aspartate oxidase involved in reactive oxygen species (ROS) production and hypersensitive response was noticed upon infection of wheat with *Xanthomonas translucens* [38]. Interestingly, cucumber root treatment with biocontrol, plant-growth promoting (PGPB) *Bacillus subtilis* strain Mbi600 led to aspartate accumulation [39], which is in accordance with our results. A similar observation was noticed in the research on wheat, where priming with *Bacillus velezensis* strain 5113 induced enhanced aspartate metabolism during different abiotic stresses [40].

X. campestris pv. *campestris*-infection (Xcc) significantly reduced branched-chain amino acids (BCAA: Ile, Leu, Val) and alanine (Ala) (Figure 2). Leu, a dominant amino acid, was twice as high in *Bacillus*-treated plants (Bac and Bac + Xcc) compared to *X. campestris* pv. *campestris*-inoculated plants (Xcc) (Figure 2). While *X. campestris* pv. *campestris* infection decreased Val by 30% and Ala by 50%, Bac treatment restored these levels, indicating their potential role in mediating the plant's stress response to the pathogen (Figure 2h). However, the opposite results were reported in a study on Arabidopsis, where treatment with *P. syringae* pv. *tomato* induced a significant accumulation of BCAA [37]. Also, Vega-Álvarez et al. [22] demonstrated that *X. campestris* pv. *campestris* infection affected Val and Ala accumulation differently, depending on the cultivar's pathogen tolerance and the sampling time. Accumulation of these amino acids was observed in the susceptible cabbage line during prolonged infection, whereas in the resistant line, Xcc did not affect Ala content, and Val content was reduced during the first sampling time. This discrepancy could be explained as a defense strategy of the sensitive cultivar to provide primary metabolites for a delayed response to the pathogen, or, in the case of the resistant cultivar, to allocate metabolic resources towards the upregulation of secondary metabolism. While the resistant cabbage line responded to Xcc infection with elevated Leu content during the early infection phase, in the susceptible line, Leu content increased during the prolonged infection phase. Restoration of the content of Ala, Val, and Leu to levels present in biocontrol-treated *X. campestris* pv. *campestris*-infected plants (Bac + Xcc) might suggest a potential role for these AAs in mediating plants' stress response to pathogen attack.

Phenylalanine (Phe) as a starting compound of the phenylpropanoid pathway is involved in the synthesis of various compounds such as flavonoids, hydroxycinnamic acids, lignin and their conjugates. Moreover, chorismate, the precursor for Phe biosynthesis, is also a precursor for the salicylic acid's biosynthesis, which is one of the key components involved in mediating plants' response to pathogens [41,42]. Along with the other aromatic amino acids (AAAs) from the shikimate pathway, Phe participates in the biosynthesis of various metabolites with defensive functions against pathogens [17]. Killiny and Hijaz showed that the content of AAAs depended on the resistance to *Candidatus Liberibacter asiaticus* in xylem sap of different lemon cultivars; i.e., tolerant cultivars exhibited higher AAAs levels in comparison to sensitive ones [43].

The unchanged Phe content in Xcc-infected plants compared to all other controls was contrary to the results reported by Vega-Álvarez et al. [31], who detected elevated Phe levels in the susceptible *B. oleracea* line infected with Xcc. Increased phenylalanine accumulation was also recorded in the research of Ward et al. [37], where the treatment of Arabidopsis plants with strains of *P. syringae* pv. *tomato* DC3000 and the *hrpA* induced the accumulation of certain amino acids, including phenylalanine. The distinct pattern of Phe accumulation during Xcc infection in the cabbage cultivar Futoški may result from its moderate activation of phenylpropanoid pathway, which is a consequence of its different

basal Phe content. This suggests that priming with the selected *Bacillus* strain has a greater effect than *Xcc* infection itself. The upregulation of AAAs metabolism under these conditions can be considered part of the plant's immune response to overcome the consequences of pathogen invasion, serving as a source for the biosynthesis of various defense-related metabolites [44]. Reduced accumulation of AAAs during pathogen treatment could potentially be a consequence of their higher demand, and/or limited production for the synthesis of indole glucosinolates [12,14,45]. In contrast, restoration of Phe and Trp content to control values (or higher) in biocontrol-treated *Xcc*-infected plants (for more than 30%) can also be considered as a plant's immune strategy to overcome pathogen invasion. Research on the tomato plants infected with *Phytophthora capsici* and primed with *Pseudomonas fluorescens* N04 and *Paenibacillus alvei* T22 PGPR strains showed an increased synthesis of Phe and Trp in the roots [46]. Moreover, the treatment of cucumbers with the biocontrol strain *Bacillus* 1JN2 significantly reduced the disease intensity caused by *Fusarium*, while the content of certain amino acids increased significantly in root exudates of plants, including tryptophan [47].

As our results showed, infection reduced lysine content by over 50% compared to controls (NB), while biocontrol-treated *Xcc*-infected plants exhibited the highest Lys levels—nearly threefold more than those with *Xcc* alone (Figure 21). During pathogen infection, lysine catabolism occurs leading to its conversion to pipecolic acid, which has been shown to participate in systemic acquired resistance (SAR) [48]. Study on a sensitive kohlrabi cultivar infected with *Xanthomonas campestris* pv. *campestris* suggests that, due to triggered lysine catabolism after 15 days of pathogen attack, pipecolic acid accumulates, while there is no clear evidence of developing SAR response [49]. Additionally, studies on pea roots showed that treatment of plants with *Aphanomyces euteiches* induced lysine catabolism and upregulation of pipecolic metabolism which leads to activation of immunological response, while the content of pipecolic acid in biocontrol-treated plants was restored to control values [50].

4.2. Secondary Metabolites

Phenolics and glucosinolates (GLSs) represent the main classes of secondary metabolites in the *Brassica* plants [51]. These metabolites are known for their involvement in the plant's defense response to pathogens and other stressors during abiotic and biotic stresses [9,52].

The phenolic compounds most prevalently found within other cabbage varieties included a variety of phenolic acids such as ferulic, *p*-coumaric (*p*CA), synapic, chlorogenic, vanillic, *trans*-cinnamic, syringic, caffeic, and protocatechuic acid, but also different compounds of the flavonoid moiety such as epigallocatechin gallate, epicatechin, quercetin, kaempferol, rutin, and apigenin [53].

Numerous studies report glucosinolate and phenolic content in different *Brassica* species *B. rapa* [51], *B. oleracea* var. *acephala*, *B. oleracea* var. *capitata*, *B. napus* ssp. *pabularia* [54], *Brassica campestris* ssp. *pekinensis*, *Raphanus sativus*, *Brassicoraphanus* [55], *Brassica napus* ssp. *rapifera*, *Brassica napus* ssp. *oleifera* [56]. All of the identified glucosinolates were previously reported in various *B. oleracea* varieties or other plants from the *Brassicaceae* family [23,25–27,30,57,58].

4.2.1. Phenolic Content

Hydroxybenzoic acids (HBAs), particularly *p*-HBA, belong to a group of wall-bound phenolic acids that play a major role in the plant's response to pathogen infection [59,60]. HBAs have been recognized as one of the main antimicrobial components that modulate virulence in important phytopathogens [61]. The preventive treatment with *B. velezensis*

resulted in the accumulation of these two compounds with and without *Xcc* (Bac and Bac + *Xcc*), but with a much higher intensity in Bac group, indicating that the plant was successfully adapted to the infection, thus restoring the levels of these HBA derivatives in response to *Xcc* with applied biocontrol agent. Biosynthesis of 4-HBA was shown to be promoted in cell cultures of carrot and potato by applying pathogenic fungal elicitors [59]. *Xcc* infection triggered the accumulation of 4-HBA in cabbage and Chinese radish, followed by the subsequent degradation of 4-HBA occurring via the *pobA/pobR* locus in *Xcc* that promotes higher virulence [60].

HCAs showed similar behavioural trends to the HBAs group of compounds. These compounds are known for their involvement in cell wall rigidification and/or lignification in response to pathogen attack [8]. In a well-orchestrated attack, *Xcc* strikes, among other strategies, with the production of enzymes that degrade the cell wall, generating a higher concentration of soluble sugars, but also other constituents of the plant cell wall, such as ferulic, sinapic, and *p*-coumaric acids [60]. Research has shown that these compounds accumulate locally, forming a physical barrier to prevent further penetration of the pathogen and also to stimulate lignification and cell wall strengthening [62]. The role of phenolics and glucosinolates was assessed during infection and colonization by *Verticillium dahliae* in two cruciferous species, broccoli and cauliflower. The speed of lignification and the higher concentration of lignin and phenolic compounds in broccoli were found to be in correlation with enhanced resistance to *V. dahliae* [62]. Studies showing differing accumulation of secondary metabolites and/or differentially expressed genes in transcriptome analysis in relation to disease resistance to *Xanthomonas campestris* pv. *campestris* in cruciferous plants were recently published [11–14]. An integrated metabolomic and transcriptomic approach on susceptible (S) and resistant (R) cabbage lines was employed to elucidate host-*Xcc* interaction mechanisms and plant defense strategies following infection with *Xcc* [13]. Comparative analysis of transcripts and metabolites in R and S lines demonstrated differential regulation of the phenylpropanoid and flavonoid pathways. Enhanced phenylpropanoid biosynthesis related gene expression encoding the enzymes chalcone synthase-CHS, chalcone isomerase-like-CHIL, flavonoid 3'-monooxygenase-F3'M, and flavonol synthase-FLS in the phenylpropanoid pathway was observed in the R line *Xcc*-infected samples compared with the S line. The metabolic analysis also showed preferential accumulation of the detected HCAs and flavonoids in cabbage line R compared to the S line [13].

The decrease of almost all phenolics in cabbage cv. Futoški upon infection with *Xcc* suggests a moderate activation of phenolic-based defense responses which could be associated with increased vulnerability of this particular cultivar to pathogen infection, without unambiguous evidence of tolerance behaviour. This is in accordance with obtained values for disease intensity of *Xcc*-treated plants. Furthermore, in the case of *B. napus*, pretreatment with *p*-coumaric acid (*pCA*) induced jasmonic acid-mediated phenolic accumulation and enhanced resistance to infection, confirming the importance of phenolic compounds in the plant's defense response to stress [8]. Namely, the content of the soluble forms of *pCA*, sinapic acid, and ferulic acid all markedly decreased in response to *Xcc* inoculation, whereas pretreatment with *pCA* led to restored or even increased values compared to controls. Saleem and coauthors [63] revealed that seed treatment with *Serendipita indica*, a plant growth promoter and a biocontrol agent, also restored values for total phenolics compared to seeds inoculated with just *Xcc*. Our results show that the preventive application of *Bacillus* combined with the subsequent *Xcc* infection (Bac + *Xcc*) resulted in increased accumulation of the majority of the identified HCAs compared to control, and especially to *Xcc*-infected plants (Table 2). This metabolic response is in line with efficacy of almost 50%

of applied RD-FC 88 strain. Likewise, to the HBAs, this implies that the plant pre-treated with biocontrol strain RD-FC 88 was better adapted to combat the pathogen attack.

Kaempferol derivatives generally remained unchanged across treatments, except for a decrease in compounds 29, 30, and 33 in the Bac + Xcc group (Table 1). This decline aligns with HBAs and HCAs trends during Xcc infection of cabbage cv. Futoški and may reflect infection-associated metabolic adjustments which are consistent with phenolic responses reported in the literature [11,13,14]. The study published by Zhou and coauthors [14] explored the regulation of transcriptomic and metabolic changes in the leaves of susceptible and resistant rapeseed varieties upon Xcc infection. Contrasting responses were detected after Xcc infection, with upregulation of the flavonoid content in resistant rapeseed and downregulation in susceptible rapeseed. The enhanced accumulation of phenolic acids and kaempferol derivatives in cabbage plants pretreated with RD-FC 88 biocontrol strain followed by Xcc infection is in line with the proposed activation of phenylpropanoid-mediated defense responses. As previously stated, these compounds are established as components of the pathogen resistance mechanisms through both structural and antimicrobial mechanisms, implying that *B. velezensis* primes specific defense pathways rather than inducing a non-targeted stress response.

4.2.2. Glucosinolate Content

Although many studies have reported an increase in glucosinolate levels in response to pathogen infection [15,64,65], the opposite trend was observed in this experiment. This discrepancy may result from cultivar-specific responses, since it has been shown that both the constitutive level of glucosinolates and their pathogen-inducible accumulation are highly dependent on cultivar susceptibility. Concerning this, sensitive cultivars usually possess constitutively higher amounts of indolic glucosinolates, which have the potential to influence the response of plants to infection. Moreover, resistant cultivars have a higher capacity for induction of indolic glucosinolates [12]. Glucosinolate accumulation also depends on Xcc suspension concentration and sampling time. Applying a fivefold higher Xcc suspension resulted in a different glucosinolate accumulation pattern compared to our results, highlighting the importance of bacterial inoculum concentration and the dynamics of the plant defense response [64]. Moreover, the timing of sampling is one of the key factors in the plant response development and glucosinolate accumulation. The highest increase in glucosinolate concentration was observed during the earlier infection phase (15 days after infection), while at 30 days after infection, a decrease in glucosinolate accumulation was noted, tending to return to control values [12].

In line with our research, Rubel and coworkers [45] demonstrated that certain aliphatic glucosinolates content, such as sinigrin, glucoiberberin, and glucoerucin, was reduced in susceptible cabbage cultivar. Absence in indolic glucosinolate accumulation, or even a reduction in their content (glucobrassicin, neoglucobrassicin and 4-methoxy-glucobrassicin) was also noticed. Additionally, 4-methoxy-glucobrassicin originates from Trp; the obtained results are in accordance with the elevated content of Trp in biocontrol-treated *X. campestris* pv. *campestris*-infected plants (Bac + Xcc).

Glucosinolate accumulation was also not observed in two susceptible cabbage cultivars, white and tronchuda cabbage, in which Xcc inoculation did not affect glucosinolate content [9].

Moreover, in the paper of Tortosa et al. [66] it was demonstrated that following the infection with Xcc in “Early Big” broccoli line, the induction of specific GDSL-like lipase/acylhydrolase protein superfamily members occurred. These proteins are indirectly involved in the activation of myrosinase degradation system and in glucosinolate degradation, resulting in activation of plants immune response. In addition, upregulation of various

transcriptional factors and genes involved in the biosynthesis of glucosinolates does not necessarily indicate increased accumulation of these compounds, but rather susceptibility of the cultivar to the disease, and not the development of resistance to it [45]. Studies on *Arabidopsis* demonstrated that seed and root pretreatment with beneficial bacteria *Pseudomonas fluorescens* SS101 leads to upregulation of genes involved in glucosinolate metabolism, especially genes associated with methionine pathway, implying the biosynthesis of aliphatic glucosinolate is initiated. In this way, contribution of glucosinolate compounds in systemic resistance induction to *P. syringae* pv. *tomato* DC3000 was indicated [67]. Similar results were also obtained in the study of Jeon and coworkers [68], where pretreatment of *Arabidopsis* and broccoli seedlings with PGPB *P. fluorescens* SS101 strain induced higher content of long-chain aliphatic glucosinolates and indolic glucosinolates, respectively, contributing to an improved immunological response to pathogen invasion. On the other hand, pretreatment of oilseed rape with beneficial fungus *Trichoderma atroviride* LU132 did not affect glucosinolate content [69]. Taken together, the decrease in glucosinolate content after *Xcc* infection might be explained by plants developing a defense–growth trade-off strategy, in which downregulation of GLS biosynthetic genes is suppressed to conserve resources for primary metabolism and survival rather than for defense. Moreover, the partial recovery of GLS content after biocontrol treatment may suggest that priming with the selected *Bacillus* strain helps the plant to alleviate *Xcc*-induced metabolic disturbance and better balance its resources between growth (primary metabolism) and defense (secondary metabolism, GLS). It should be noted that, in the absence of phytohormone analyses as well as transcriptomic and proteomic data, the mechanism underlying the effects of *Bacillus velezensis* cannot be conclusively attributed to a classical priming effect and may reflect an overall improvement in plant condition under pathogen attack.

4.3. Fluorescence Analysis

The loading plot shows that wavelengths in the 560–590 nm range are associated with PC1, while 400–430 nm wavelengths mainly contribute to PC2. In this study, Bac + *Xcc*-treated samples were distinctly separated from control samples along PC2, suggesting that PC2 reflects treatment-specific spectral changes. PC2 was mainly influenced by emission wavelengths in the 400–430 nm region, which are commonly associated with coumarin-like compounds. It suggests the induction of defense-related metabolic pathways, which is in line with the priming effect induced by *Bacillus* that improves plant resistance to pathogen attack. Emission in the 400–430 nm region is typically attributed to UV-induced fluorescence of phenolic compounds, mainly hydroxycinnamic acids bound to cell wall [70,71]. Increased blue fluorescence has been associated with activation of phenylpropanoid metabolism during stress responses, including pathogen attack [72]. Therefore, the positive PC2 scores observed in Bac + *Xcc* samples likely reflect stress-induced accumulation or structural modification of phenolic fluorophores.

In this study only one cultivar of cabbage, although very important due to its cultural and nutritive traits, was analyzed. Other cabbage cultivars show considerable variability in their metabolite profiles and levels of resistance to *Xanthomonas campestris* pv. *campestris*. Given these facts, the metabolic patterns of response to *Xcc* and *Bacillus velezensis* RD-FC 88 measured in this research should be considered as cultivar-specific. Further studies with additional cultivars with contrasting susceptibility to black rot disease are needed to confirm the wide-ranging applicability of these conclusions and to assess the host/*Xcc*/biocontrol interactions.

5. Conclusions

Studying the specific metabolic response of host plants to *X. campestris* pv. *campestris* strain XcFC 231 and *B. velezensis* strain RD-FC 88 contributes to revealing the biocontrol defense mechanisms that confer plant resilience and help increase disease resistance. The profiles of all measured primary and secondary metabolites in this study were reported for the first time in cabbage cv. Futoški. The observed trends upon infection with *X. campestris* pv. *campestris* and preventively applied biocontrol strain in measured key metabolites from primary and secondary metabolism implies that the cabbage cv. Futoški used in this study most likely belongs to a group of sensitive cabbage cultivars. Several amino acids showed a significant decrease in their content in Xcc-infected plants (Xcc), while the presence of the *B. velezensis* strain RD-FC 88 led to restoration to control levels (Bac + Xcc). The aromatic amino acids Phe and Trp showed a distinct response in the plants pretreated with *B. velezensis* strain RD-FC 88 and subsequently treated with *X. campestris* pv. *campestris* strain XcFC 231 that differed compared to other free amino acids. The aromatic amino acids Phe and Trp showed a distinct response in the plants pretreated with *B. velezensis* strain RD-FC 88 and subsequently treated with *X. campestris* pv. *campestris* strain XcFC 231 that differed compared to other free amino acids. The induced accumulation of Phe in Bac + Xcc plants is in line with an elevated availability of precursors for phenylpropanoid metabolism, which is commonly associated with plant defense responses. While *X. campestris* pv. *campestris* infection did not affect the Glu content; the content of Gln was reduced, suggesting a potential alternation in nitrogen metabolism. Moreover, reduced Lys content in *X. campestris* pv. *campestris*-infected plants indicates its increased catabolism, at the expense of biosynthesis of pipecolic acid which is involved in resistance acquisition. The content of key cabbage secondary metabolites, i.e., glucosinolates and phenolics, also displayed a significant reduction upon infection, which was restored upon biocontrol application, which is in accordance with reduced disease intensity after Bac treatment. Taken together, all results revealed the potential of this particular *B. velezensis* strain to serve as a priming agent to enhance the cabbage constitutive resistance.

The application of the proposed biocontrol strain could lead to a more sustainable agricultural production with less negative impact on the environment. Taken together, obtained findings suggest that the preventive application of *B. velezensis* strain RD-FC 88 enhanced the capacity of cabbage cv. Futoški to respond to pathogen attack and alleviate the stress imposed by black rot infection. Further studies with field trials with single or multiple applications of the beneficial bacterium are necessary to unveil the potential of this sustainable approach in a broader context.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture16050544/s1>, Table S1: Regression equation and other accompanying parameters for standards used for quantification or semi-quantification of glucosinolates and phenolic compounds.; Table S2: Results of normality (SW: Shapiro–Wilk) and homogeneity of variances (Levene) tests and choice of statistical test by treatments.; Table S3. Concentration of measured free amino acids, Gly/Ser and Gln/Glu ratio and total amino acid content across four different treatments. Figure S1. PCA loading plot showing the contribution of specific wavelengths to the first two principal components.

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