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Unveiling the Impact of Soil Prebiotics on Rhizospheric Microbial Functionality in *Zea mays* L.

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Abstract: Prebiotics, a subset of biostimulants, have garnered attention for their potential to enhance soil conditions and promote plant growth, offering a promising alternative to conventional agricultural inputs. This study explores how two commercial prebiotics, K1® and NUTRIGEO L® (SPK and SPN), impact soil functions compared to a control (SP). The experiment involved agricultural soil amended with organic wheat straws and cultivated with *Zea mays* L. Previous research demonstrated substantial effects of these prebiotics on plant biomass, soil parameters, and microbial community ten weeks after application. The present study delves deeper, focusing on soil microbial abundance, enzyme activities, and metabolic diversity. Analysis revealed that SPN notably increased the fungi-to-bacteria ratio, and both prebiotics elevated the activity of several key enzymes. SPN enhanced α-glucosidase and β-galactosidase activities, while SPK increased arylsulfatase, phosphatase, alkaline phosphatase, and urease activities. Enzymatic indexes confirmed the positive impact on soil functional diversity and fertility. Additionally, prebiotic treatments showed distinct metabolic profiles, with SPK degrading eleven carbon sources more rapidly across five groups and SPN accelerating the decomposition rate of four carbon sources from three groups. These findings highlight the ability of prebiotics to shape microbial communities and enhance soil fertility by modulating their functional activity and diversity.

Keywords: prebiotics; biostimulants; soil fertility; microbial abundance; enzymatic activity; metabolic diversity

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1. Introduction

The agricultural sector is undergoing a crucial transition, necessitating increased crop yields while reducing reliance on conventional practices that negatively impact the environment, particularly soil health [1,2]. This agroecological transition involves exploring and implementing alternative approaches, among which biostimulants play a prominent role [3,4]. Biostimulants, comprised of one or several substances, microorganisms, or a combination thereof, are applied to seeds, plants, or the rhizosphere to stimulate natural processes [5]. Their objective is to enhance nutrient uptake and utilization efficiency, stress tolerance, and, ultimately, crop quality and yield [6].

Researchers are currently focused on studying biostimulants to comprehend their mode of action and enhance their efficacy [6–8]. Prebiotics, a subset of biostimulants composed of natural ingredients without living microorganisms, are primarily applied through soil drenching [9]. They exhibit the potential to improve plant growth, quality,

and yield by enhancing soil fertility and fostering a conducive environment for microbial proliferation and activity [10–12]. These enhancements encompass improvements in soil physicochemical characteristics, aggregate stability, nutrient availability, and the structure and diversity of microbial communities [13,14]. Prebiotics have been shown to modify indigenous soil microbial communities, increasing bacterial and fungal diversity and recruiting microorganisms with specific ecological functions [15,16]. This enriched microbial consortium enhances microbial communication and symbiotic interaction with roots, thereby improving water and nutrient uptake and significantly contributing to overall plant growth [17]. Furthermore, some of these microorganisms facilitate the degradation of recalcitrant organic sources, such as plant residues, thereby increasing soil carbon (C) content and supporting beneficial microorganisms over pathogenic ones [18]. The microbial taxa recruited and nourished by prebiotic application also play an essential role in soil nutrient cycling and bioavailability, potentially reducing dependency on chemical fertilizers and aiding in the recovery of degraded soils [13,19–23].

Despite the abundance of literature on biostimulants' direct effects on soil characteristics and plant growth, few studies have delved into their impact on soil functional activity and diversity [10,24]. Addressing this gap is crucial for monitoring the impact of these products on soil biochemical processes established by microbial communities [25]. Key biological indicators such as microbial abundance, enzymatic activity, and metabolic diversity play significant roles in understanding how subsurface soil ecosystems react to perturbations, making quantifying the soil microbial community an effective early indication of shifts in soil health and quality [26,27]. Soil enzymes have also been recognized as useful soil-state bioindicators, providing insights into the soil's ability to perform biogeochemical reactions, encompassing carbon C, nitrogen (N), phosphorus (P), and sulfur (S) cycles [28–30]. Community-level physiological profiling (CLPP) techniques, such as the Biolog EcoPlates™ (Hayward, CA, USA) assay, have emerged as efficient tools for assessing biostimulants' impacts on soil microbial activity and diversity [31–34]. These assays quantify soil microbial communities' metabolic capabilities by measuring their utilization of various C substrates, offering valuable insights into microbial community metabolic profiles and functional diversity in the rhizosphere [35,36]. Integrating multispectral analysis enhances understanding of post-biostimulant application results, shedding light on both structural and functional aspects of soil microbial communities [37,38].

In this context, our study extends prior research by investigating the effects of two commercial prebiotics, K1® and NUTRIGEO L® (SPK and SPN, respectively), under controlled conditions on soil amended with organic wheat straws and cultivated with *Zea mays* L., compared to a control group (SP), ten weeks post-application [39]. We aim to deepen our understanding of their mode of action and impact on soil microbial functionality by monitoring enzymatic and metabolic activities alongside microbial abundance. Specifically, we assessed twelve key enzymes and calculated enzymatic indexes reflective of soil quality and fertility. Additionally, using CLPP, we observed metabolic activity across treatments, revealing the effects on metabolic profiles and the degradation rates of thirty-one different C sources and their groups. Guided by the hypothesis that prebiotics enhance beneficial soil microorganisms, improve nutrient cycling enzymatic activities, and increase microbial metabolic diversity, our study aims to uncover their short-term positive effects on microbial functionality, soil fertility, and health. These insights pave the way for adopting innovative bioecological approaches to advance modern and sustainable agriculture [40].

2. Materials and Methods

2.1. Experimental Design and Harvesting

The experimental setup utilized in this study was consistent with our previous research [39]. Briefly, soil was collected from an agricultural field close to Ronchois in the Normandy region of France (49°43′56.2″ N, 1°36′41.6″ E), characterized by its silty texture

(silt 63.4%, sand 20.5%, clay 16.2%), with a neutral pH of around 7 and a water retention capacity (WRC) of 31.7%. After sampling, the soil underwent several preparatory steps. Initially, it was spread out on nylon sheets to facilitate aeration for three days. Subsequently, it was sieved at 5 mm to eliminate any debris or stones and then stored in opaque containers at a temperature of 4 °C. Approximately two weeks before launching the experiment (T0), the soil was sorted, hydrated to achieve 70% of the WRC, and mixed with organic wheat straws with 2.5% *w*/*w*. Then, 1.85 kg of this soil was filled into rectangular 2 L black plastic pots. Originally, the soil contained 13.26 g kg^{-1} and 0.98 g kg^{-1} of dry soil of total carbon (TC) and N, respectively, and it received an amount of 10.52 g kg^{-1} and 0.13 g kg^{-1} of dry soil of TC and N, respectively, from the wheat straws incorporation. The pots containing the soil–straw mixture were treated at T0 with the prebiotics K1® and NUTRIGEO L®, then sowed with organic *Zea mays* L. seeds of the variety DATABAZ (LOT: F0272 E9 09818 A) obtained from Soufflet seeds. These prebiotics were freely provided in liquid form by Gaïago and consisted of a blend of plant extracts and trace elements such as manganese (Mn), specific organic acids, and other trace elements like zinc (Zn) in K1® and boron (B) in NUTRIGEO L®. The experimental setup comprised a total of 90 pots, all filled with the same soil–straw mix and divided equally into three treatment groups: (i) planted soil without any treatment (SP), (ii) planted soil treated with K1® (SPK), and (iii) planted soil treated with NUTRIGEO L® (SPN). Each treatment group consisted of five blocks, each with three replicates. The harvesting date was scheduled for ten weeks post-prebiotics application. The prebiotics were applied at T0 to the soil through drenching at concentrations of 5 L per hectare (L ha^{-1}) for K1® and 25 L ha^{-1} for NUTRIGEO L® as recommended for field application. As for the control treatment (SP), it just received a water application. The pots were randomly arranged in the greenhouse and regularly irrigated to maintain the soil moisture at 70% of its WRC, thus preventing nutrient leaching. The greenhouse conditions were carefully controlled to meet the optimal growth requirements of maize plants, including temperature maintenance at 22 °C during the day and 18 °C at night, 16 h of light exposure, 8 h of darkness, and a humidity level of 70%. At the harvesting date, the system was disassembled by removing the plant from the pots, separating the shoots from the roots, and sieving the soil. The sieved soil samples were mixed well and divided according to downstream analysis. A total of 5 g of fresh soil was used for CLPP, and the rest were preserved in small plastic bags, sealed, and stored at −80 °C for microbial abundance and enzymatic activity analysis.

2.2. Total Soil DNA Extraction and Quantification of Microbial Abundance

Genomic DNA from 0.5 g of soil samples was extracted, employing the FastDNA® spin kit for soil (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's instructions. The extracted DNA from the samples was subsequently quantified using the Hoechst 33258 staining dye kit from Biorad, marketed as the Florescent DNA Quantitation kit (Biorad, Hercules, CA, USA). The absorbance reading was carried out using an advanced spectral scanning multimode reader Varioskan® Flash (Thermo Fisher Scientific, Waltham, MA, USA) with a ratio of 360 nm excitation to 460 nm emission. Post quantification, the DNA samples were preserved at −20 °C for subsequent analysis. Following this step, bacterial and fungal abundances were measured utilizing a molecular method employing real-time quantitative polymerase chain reaction (qPCR) targeting 16S rDNA and 18S rDNA, respectively [41]. The concentrations of DNA obtained from various soil samples were normalized to a final concentration of 5 ng μL^{-1}. The qPCR mixture was prepared with 5 ng of soil DNA, 0.5 µmol of each primer, 0.25 mg ml^{-1} BSA (NEB), and 12.5 µL of Power SYBR™ Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA). The amplification protocol involved 40 PCR cycles, comprising 40 s at 95 °C, 45 s at 64 °C, and 30 s at 72 °C, conducted using the LightCycler 480 II (Roche). The primers utilized were as follows: (1) BU 16S2: 63f: 5′-CAGGCCTAACACATGCAAGTC-3′ according to [42] + BU16S4: 5′-CTGCTGCCTCCCGTAGG-3′ derived from 341F, [43] for 16S, and (2) FU18S1: 5′-GGAAACTCACCAGGTCCAGA-3′ derived from Nu-SSU-1196 and FU 18S2 = Nu-

SSU-1536: 5′-ATTGCAATGCYCTATCCCCA-3′ for 18S [44]. The overall efficiency of the qPCRs exceeded 93%. Standard curves were generated utilizing serial dilutions of linearized plasmids containing the rDNA, 16S, or 18S gene. The outcomes were expressed as the number of copies of the rDNA, 16S, or 18S gene per gram (g) of dry soil.

2.3. Enzymatic Activities Analysis

Enzyme activities were measured according to standards ISO 20130:2018 and ISO/TS 22939:2019 using soil samples that were sieved at 5 mm and stored at −80 °C [45,46]. A total of twelve key enzymes were tested from different nutrient cycles in the soil [47,48]. Five enzymes were associated with the C cycle: α-glucosidase (aGLU), β-glucosidase (bGLU), β-galactosidase (bGAL), cellulase (CEL), and laccase (LAC). Two enzymes were involved in the N cycle: N-acetylglucosaminidase (NAG) and urease (URE). Three enzymes were related to the P cycle: phosphatase (PHOS), acid phosphatase (ACP), and alkaline phosphatase (AKP). Arylsulfatase (ARYLS) was tested for the S cycle, and dehydrogenase (DEH) was tested for global enzymatic activity. A soil solution was prepared by adding 25 mL of the extraction buffer to three lots of 4 g of soil in sterile pots. Nearly all enzymes were extracted using deionized water adjusted to specific pH levels, except for ACP and AKP, which were extracted using 50 mM trizma HCl at pH 5, and pH 11, respectively (Table 1). Then, the soil solutions were homogenized using an orbital shaker for 10 min (min) at 250 rotations per minute (rpm). With stirring, 125 µL of the soil solution was withdrawn and placed on a 96-well microplate at the rate of four wells per sample. Next, 25 µL of substrate, specific to each enzyme, was added to three wells, leaving the fourth well without substrate as a control (Table 1)**.** The microplates were then incubated at 37 °C for a time varied according to each enzyme incubation conditions. To stop the reaction after incubation, 25 µL of $CaCl_2$ and 100 µL of 50 mM trizma pH 12 were added to all wells including the control, and then the substrate was added to the control wells. After centrifugation of the plate at 1500 rpm for 5 min, 200 µL of the supernatant was transferred to a new plate. After incubation, the enzyme reaction was stopped, and a color test was carried out. The microplates were then centrifuged for 5 min at 1500× *g*, and 200 µL of the supernatant was transferred to a new plate. The absorbance reading of all enzymes was measured at 405 nm using a spectrophotometer (Varioskan Flash-Thermo). Enzymatic activity was determined using a standard prepared with paranitrophenol (PNP).

Table 1. Substrates used for enzymatic activity assays.

Enzymes	Abbreviations	E.C. Numbers	Substrates (Buffer pH and Concentration)
C cycle enzymatic activities			
α-Glucosidase	aGLU	3.2.1.20	p-NP-β-D-glucopyranoside (pH 6; 25 mM)
β-Glucosidase	bGLU	3.2.1.21	p-NP-β-D-glucopyranoside (pH 6; 50 mM)
β-Galactosidase	bGAL	3.2.1.23	p-NP-β-D-galactopyranoside (pH 7; 0.02 M)
Cellulase	CEL	3.2.1.4	p-NP-β-D-cellobioside (pH 6; 10 mM)
Laccase	LAC	1.10.3.2	2,2′-azino-bis-(3 ethylbenzothiazoline-6-sulfonate) (pH 5; 0.5 mM)
N cycle enzymatic activities			
N-acetylglucosaminidase	NAG	3.2.1.30	p-NP-N-acetylglucosaminide (pH 6; 10 mM)
Urease	URE	3.5.1.5	Urea (pH 7; 0.05 mM)
P cycle enzymatic activities			
Phosphatase	PHOS	3.1.4.1	p-NP-phosphate (pH 7.5; 50 mM)
Acid phosphatase	ACP	3.1.3.2	p-NP-phosphate (pH 5; 50 mM)
Alkaline phosphatase	AKP	3.1.3.1	p-NP-phosphate (pH 11; 50 mM)
S cycle enzymatic activities			
Arylsulfatase	ARYLS	3.1.6.1	p-NP-sulfate (pH 7; 25 mM)
Global enzymatic activities			

Dehydrogenase	DEH	1.1.1.1	2,3,5 triphenyltetrazolium chloride (pH 7; 0.12 M)

E.C. Enzyme commission numbers, NP nitrophenyl.

The generated results were then used to measure the activity of each enzyme and to calculate the enzymatic indexes [49]. We have calculated two indexes:

1. Soil fertility index (*Mw*) reflects the soil fertility state where the higher the *Mw*, the more fertile the soil [50]. It was calculated according to this formula:

$$Mw = (URE \times 10^{-1} + DEH + ACP + AKP) \times \%SOC, \quad (1)$$

where *SOC* stands for the soil organic carbon measured in the soil samples determined in our previous work (Table S1) [39].

2. Geometric mean (*Gmea*) presents the soil functional diversity, with elevated *Gmea* corresponding to high functional diversity [51]. It was calculated according to this formula:

$$Gmea = \sqrt[12]{\text{aGLU} \times \text{bGLU} \times \text{bGAL} \times \text{CEL} \times \text{LAC} \times \text{NAG} \times \text{URE} \times \text{ARYLS} \times \text{PHOS} \times \text{ACP} \times \text{AKP} \times \text{DEH}} \quad (2)$$

2.4. Metabolic Profiling of Soil Microbial Communities

The CLPP or metabolic potential analysis was performed using Biolog EcoPlates™, specialized 96-well plates containing triplicate samples of 31 different C sources along with water control. To start the process, 5 g of fresh sieved soil was mixed with 45 mL NaCl 0.9% in 50 mL falcon tubes and shaken using the vortex TopMix FB 15024 (Thermo Fisher Scientific) at maximum (max) speed for 3 min at room temperature to suspend bacterial cells. Then, the falcon tubes containing the soil solution were centrifuged for 5 min at 1000 rpm using the Eppendorf® centrifuge 5810R (Eppendorf, Hamburg, Germany). After that, 7 mL were aliquoted from the supernatant and added to 33 mL NaCl 0.9% solution in another 50 mL Falcon tube which will be used to inoculate the Biolog EcoPlates™. Another 100 µL of the supernatant (10^{-1}) was transferred to 1.7 mL Eppendorf® tubes containing 900 µL of NaCl 0.9% solution (10^{-2}) to start a serial dilution reaching 10^{-5} concentration. Each of the Eppendorf® dilution tubes was vortexed to withdraw 100 µL of the diluted solutions to inoculate, in sterile conditions, the previously prepared plates containing 30 mL of (Reasoner's 2A agar) R2A culture medium. The diluted solution was well spread on the R2A culture medium, and the plates were well sealed and incubated in darkness at 20 °C. After 48 h of incubation, the inoculated plates were sorted out and the bacterial colonies were counted manually to quantify the number of culturable bacteria present in each diluted soil solution. The results were expressed in colony forming unit (CFU) values, considering the dilution, which results in a CFU ranging between 30 and 300. Returning to the 50 mL Falcon tubes, these tubes were then vortexed at max speed for 1 min using TopMix FB 15024 vortex (ThermoFisher Scientific) followed by transferring the whole mix into a reservoir to withdraw 150 µL using a multichannel pipette (~1500 microorganisms per well) and inoculate the Biolog EcoPlates™. These plates were then introduced to the Biolog Microstation® (Biotek Instruments, Winooski, VT, USA) to perform the first spectrophotometric read (590 nm) at T0 then returned to darkness at 20°C for incubation. Utilization of C sources was indicated by a color change resulting from the reduction of the redox dye tetrazolium. A second and a third read were then performed after 24 h (T24) and 48 h (T48) of incubation, respectively. The data from all reads was then treated using Spyder® software (Spyder, Anaconda 3, version 5.1.5). Briefly, to assess the overall metabolic activity, the average well color development (AWCD) was calculated for each plate using the formula:

$$AWCD = \sum ODi/31, \quad (3)$$

where *ODi* represents the corrected optical density (OD) obtained by subtracting the initial OD of each well (at T0) and the OD of the control well from the recorded optical OD

of each well. To standardize the data and mitigate inoculum density effects, the *ODi* for each well was divided by the *AWCD*. Any negative values were set to zero, and an *ODi* > 0.25 was considered a positive response. On the other hand, the richness of substrate utilization, or metabolic richness, was quantified as the number of oxidized substrates or the number of positive wells (pwells). Finally, the generated data from both CFU and Biolog analysis were then organized for further statistical analysis [52,53].

2.5. Statistical Analysis

Statistical analyses and the creation of figures were conducted using R version 4.2.0 (http://www.r-project.org/ (accessed on 20 January 2024) and MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/ (accessed on 7 February 2024). The results shown represent the average values with standard deviation (SD) derived from four determinations for each treatment across all soil parameters examined. Analysis of variance (ANOVA) for all data was performed using the "AOV" function from the "stats" package [54]. Subsequently, multiple comparisons among treatments were carried out using the least significant difference (LSD) test, and the grouping of treatments was achieved using the "LSD.test" function from the agricolae package [55]. A significance level of 5% ($p \leq 0.05$) was applied, and the Bonferroni method was utilized for probability value adjustment. To create the heatmaps for CLPP analysis, after treating the raw OD reads using the Spyder® software and preparing the results matrix, we created the metadata matrix and uploaded all data files to the MetaboAnalyst server [56]. Next, a data integrity check was performed using the "SanityCheckData" function, where names and formats of samples and features in addition to data values are checked [57]. Data normalization was implemented using the "Normalization" function, and the data were auto-scaled to be mean-centered and divided by the standard deviation of each variable [58]. The heatmaps were generated using the "PlotHeatMap" function under the following criteria: Euclidean distance measure, without clustering, and showing group averages [59]. All C substrates and C groups degradation levels were compared among treatments using the *t*-test/ANOVA statistical method at a *p*-value < 0.05. For comparison between metabolic diversity and enzymatic activity in correlation to treatments, the "PCA.Anal" function was used for principal component analysis (PCA) and to create the 2D Scores plot with 95% confidence regions display and the Biplot [60].

3. Results

3.1. Effect of Prebiotics on Soil Microbial Abundance

We investigated the impact of two prebiotic treatments on soil microbial abundance ten weeks after their application. We observed that the bacterial abundance, as indicated by the number of 16S rDNA copies per gram of soil, exhibited no significant change ($p < 0.05$) between the control (SP) and treatments (SPK and SPN) (Figure 1a). In contrast, the number of 18S rDNA copies showing the fungal abundance in soil increased with prebiotic treatments, significantly ($p < 0.05$) in SPN (1.48×10^8 18S copies g^{-1} soil) compared to SP (1.15×10^8 18S copies g^{-1} soil) (Figure 1b). Additionally, the ratio of fungi to bacteria demonstrated a significant difference ($p < 0.05$) between prebiotic treatments SPK (3.12) and SPN (3.77) (Figure 1c).

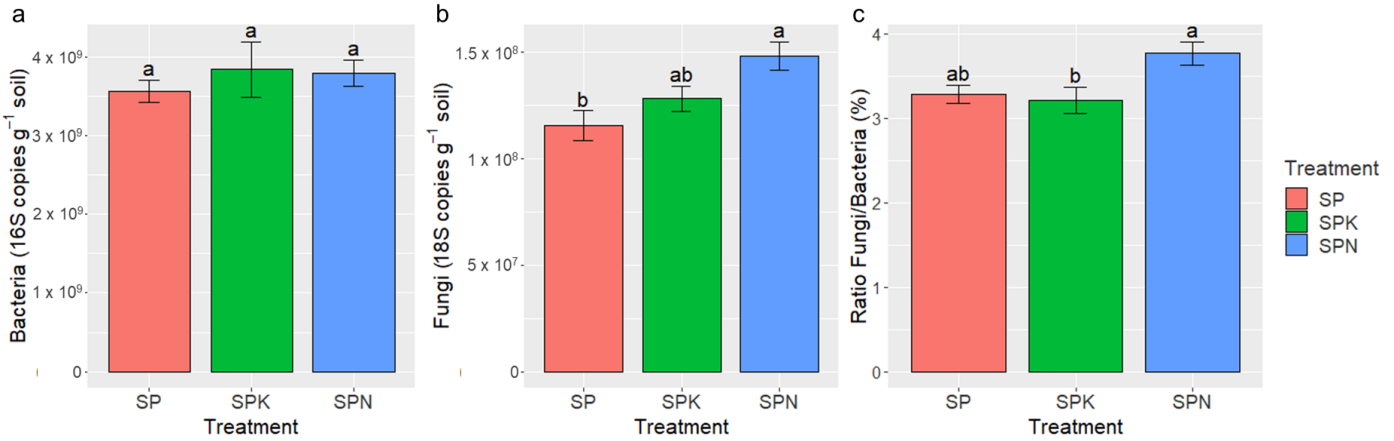


Figure 1. Histograms representing the effects of applying two soil prebiotics on soil microbial abundance compared to the control (**a**) bacterial abundance (16S), (**b**) fungal abundance (18S), and (**c**) fungi-to-bacteria ratio (%). SP plants without any treatment (control); SPK plants treated with K1®; SPN plants treated with NUTRIGEO L®. Each treatment is denoted by a color, where red is for SP, green is for SPK, and blue is for SPN. Data presented are means ± SD. Bars labeled with the same letters within each graph indicate no significant difference ($p < 0.05$) as determined using the ANOVA LSD test.

3.2. Effect of Prebiotics on Enzyme Activities in Soil

We studied the two prebiotic treatments' effect on soil functional diversity and fertility by monitoring various soil enzymes involved in C, N, P, and S biogeochemical cycles, as well as soil microbial activity.

Five enzymes associated with the C cycle were analyzed: aGLU, bGLU, bGAL, CEL, and LAC. Ten weeks after the application of prebiotics (SPK and SPN), these enzymes exhibited diverse responses compared to the control. SPN significantly increased aGLU activity ($p < 0.05$) by 5.9% compared to SPK, and bGAL activity by 7% compared to SP (Table 2). bGLU activity showed a significant increase ($p < 0.05$) in both SPK and SPN (9% and 7.3%, respectively) compared to the control (15.12 nmol PNP min^{-1} g^{-1} dry soil) (Table 2). Similarly, LAC activity significantly increased ($p < 0.05$) in both SPK and SPN by 24.4% and 26.2%, respectively, compared to SP (26.68 nmol ATBS ox min^{-1} g^{-1} dry soil) (Table 2). However, CEL activity did not significantly ($p < 0.05$) vary between treatments (SP, SPK, and SPN) (Table 2).

For the N cycle, we analyzed the activity of two enzymes: NAG and URE. The activity of the first one, NAG, did not show a significant ($p < 0.05$) difference between treatments. On the contrary, URE activity significantly ($p < 0.05$) increased with prebiotic treatments, particularly in SPK by 7.5% compared to SP (4.9 nmol NH_4Cl min^{-1} g^{-1} dry soil) (Table 2).

To assess the effects on the P cycle, we analyzed three enzymes: PHOS, ACP, and AKP. SPK treatment significantly increased ($p < 0.05$) the activity of PHOS, ACP, and AKP (7.5%, 3.7%, and 5%, respectively) compared to SP (50.41, 78.33, 104.59 nmol PNP min^{-1} g^{-1} dry soil, respectively). Meanwhile, SPN significantly increased ($p < 0.05$) ACP activity by 6.1% compared to SP (Table 2).

ARYLS, an enzyme involved in the S cycle, showed a significant increase ($p < 0.05$) in its activity in SPK by 3.8% compared to SP (5.62 nmol PNP min^{-1} g^{-1} dry soil). With respect to DEH, a general microbial activity enzyme, it did not significantly differ ($p < 0.05$) between treatments, ranging around 0.4 INTF nmol min^{-1} g^{-1} dry soil (Table 2).

Table 2. Effects of prebiotic treatments (SPK and SPN) on soil enzymatic activity ten weeks post-application in comparison to the control (SP).

		Treatment		
Enzymes	**Unit**	**SP**	**SPK**	**SPN**
aGLU	nmol PNP min^{-1} g^{-1} dry soil	4.09 ± 0.14 ab	4.06 ± 0.16 b	4.3 ± 0.12 a
bGLU	nmol PNP min^{-1} g^{-1} dry soil	15.12 ± 0.47 b	16.48 ± 0.4 a	16.23 ± 0.17 a
bGAL	nmol PNP min^{-1} g^{-1} dry soil	2.27 ± 0.17 b	2.43 ± 0.03 ab	2.52 ± 0.05 a
CEL	nmol PNP min^{-1} g^{-1} dry soil	6.55 ± 0.96 a	10.65 ± 5.51 a	9.68 ± 0.88 a
LAC	nmol ATBS ox min^{-1} g^{-1} dry soil	26.68 ± 4.42 b	33.19 ± 1.37 a	33.66 ± 4.99 a
PHOS	nmol PNP min^{-1} g^{-1} dry soil	50.41 ± 2.52 b	54.02 ± 2.07 a	53.56 ± 1.35 ab
ACP	nmol PNP min^{-1} g^{-1} dry soil	78.33 ± 1.79 b	81.2 ± 1.53 a	83.13 ± 1.36 a
AKP	nmol PNP min^{-1} g^{-1} dry soil	104.59 ± 3.42 b	109.83 ± 0.75 a	108.04 ± 3.9 ab
NAG	nmol PNP min^{-1} g^{-1} dry soil	4.12 ± 0.25 a	4.09 ± 0.13 a	4.35 ± 0.26 a
URE	nmol NH_4Cl min^{-1} g^{-1} dry soil	4.9 ± 0.14 b	5.27 ± 0.18 a	5.14 ± 0.24 ab
ARYLS	nmol PNP min^{-1} g^{-1} dry soil	5.62 ± 0.02 b	5.83 ± 0.04 a	5.7 ± 0.18 ab
DEH	INTF nmol min^{-1} g^{-1} dry soil	0.4 ± 0.02 a	0.4 ± 0.04 a	0.37 ± 0.02 a

aGLU α-glucosidase, bGLU β-glucosidase, bGAL β-galactosidase, CEL cellulase, LAC laccase, PHOS phosphatase, ACP acid phosphatase, AKP alkaline phosphatase, NAG N-acetylglucosaminidase, URE urease, ARYLS arylsulfatase, DEH dehydrogenase. SP untreated soil (control), SPK soil treated with K1®, SPN soil treated with NUTRIGEO L®. Means within columns followed by different lowercase letters are significantly different at $p < 0.05$ according to the two-way ANOVA LSD test.

The two calculated soil enzymatic indexes, namely, the geometric mean (*Gmea*) and soil fertility index (*Mw*), exhibited a significant variation ($p < 0.05$) between treatments (Figure 2). Gmea increased by 9.2% in both SPK and SPN compared to SP (Figure 2a). Mw also followed the same trend and increased by 11.64% in SPK and 15.19% in SPN compared to the control (Figure 2b).

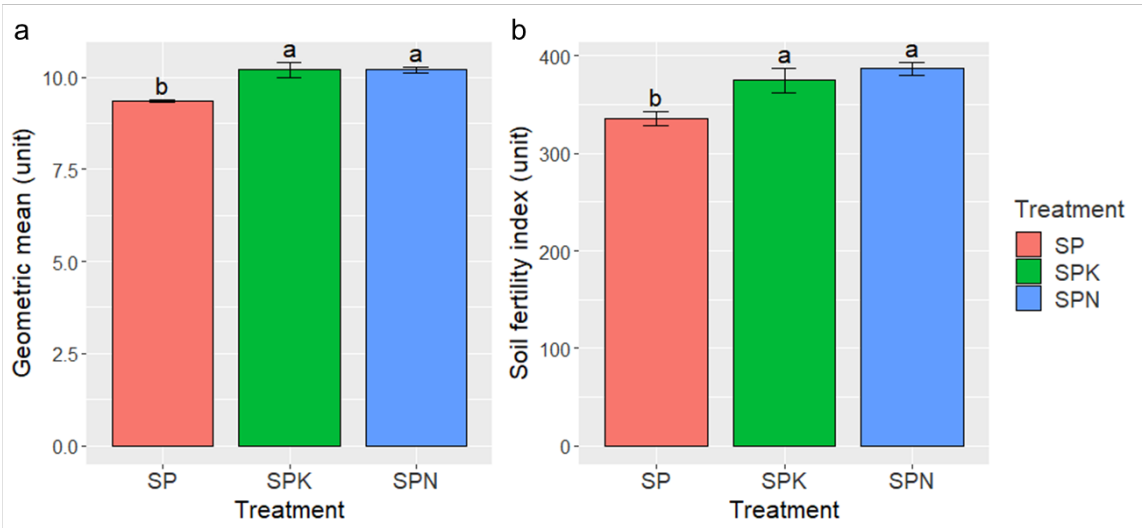


Figure 2. Histograms representing the effects of applying two soil prebiotics on soil enzymatic indexes compared to the control: (**a**) geometric mean or soil functional diversity index (Gmea) and (**b**) Soil fertility index (Mw). SP plants without any treatment (control); SPK plants treated with K1®; SPN plants treated with NUTRIGEO L®. Each treatment is denoted by a color where red is for SP, green for SPK, and blue for SPN. Data presented are means ± SD. Bars labeled with the same letters within each graph indicate no significant difference ($p < 0.05$) as determined using the ANOVA LSD test.

3.3. Effect of Prebiotics on the Metabolic Pattern in Soil

Community-level physiological profiling (CLPP), utilized for assessing the metabolic potential of soil bacterial communities through Biolog EcoPlates™, begins with the

enumeration of culturable bacteria extracted from soil. The number of colony-forming units (CFU) did not exhibit a significant ($p < 0.05$) difference between the treatments (SP, SPK, and SPN) (Figure 3a). Similarly, metabolic richness, calculated by the number of metabolized substrates or the number of positive wells with absorbance greater than 25 OmniLog units (OD > 0.25), mirrored this trend with no significant difference ($p < 0.05$) across all treatments, averaging 19 wells in SP and 20 wells in both SPK and SPN (Figure 3b). Regarding overall metabolic activity, soil treated with prebiotics demonstrated significantly higher ($p < 0.05$) average well color development (*AWCD*), with an increase of 27.61% and 18.3% for SPK and SPN, respectively, compared to SP (Figure 3c).

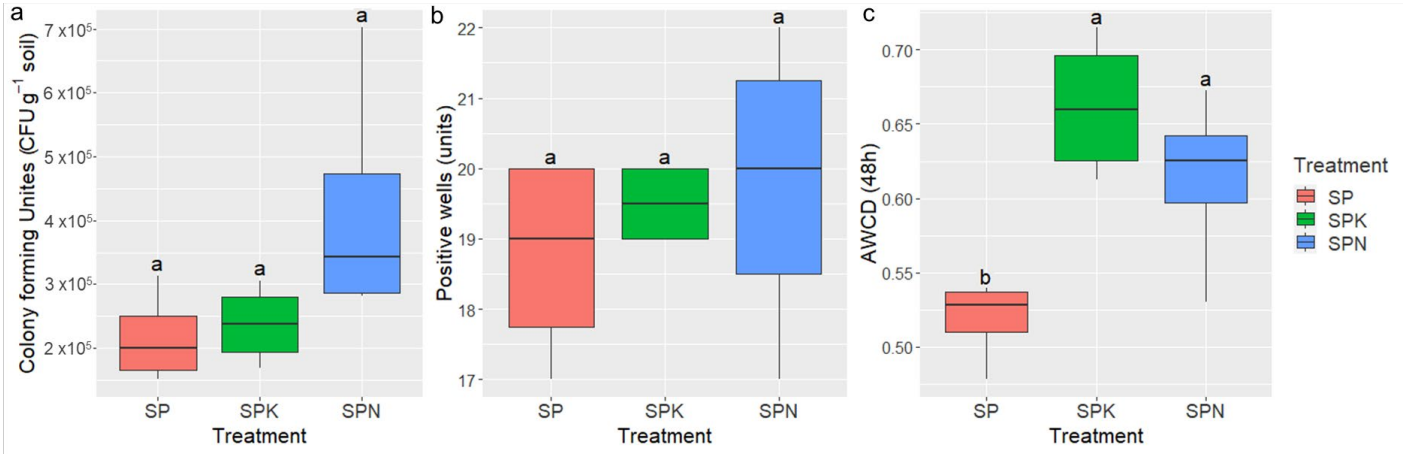
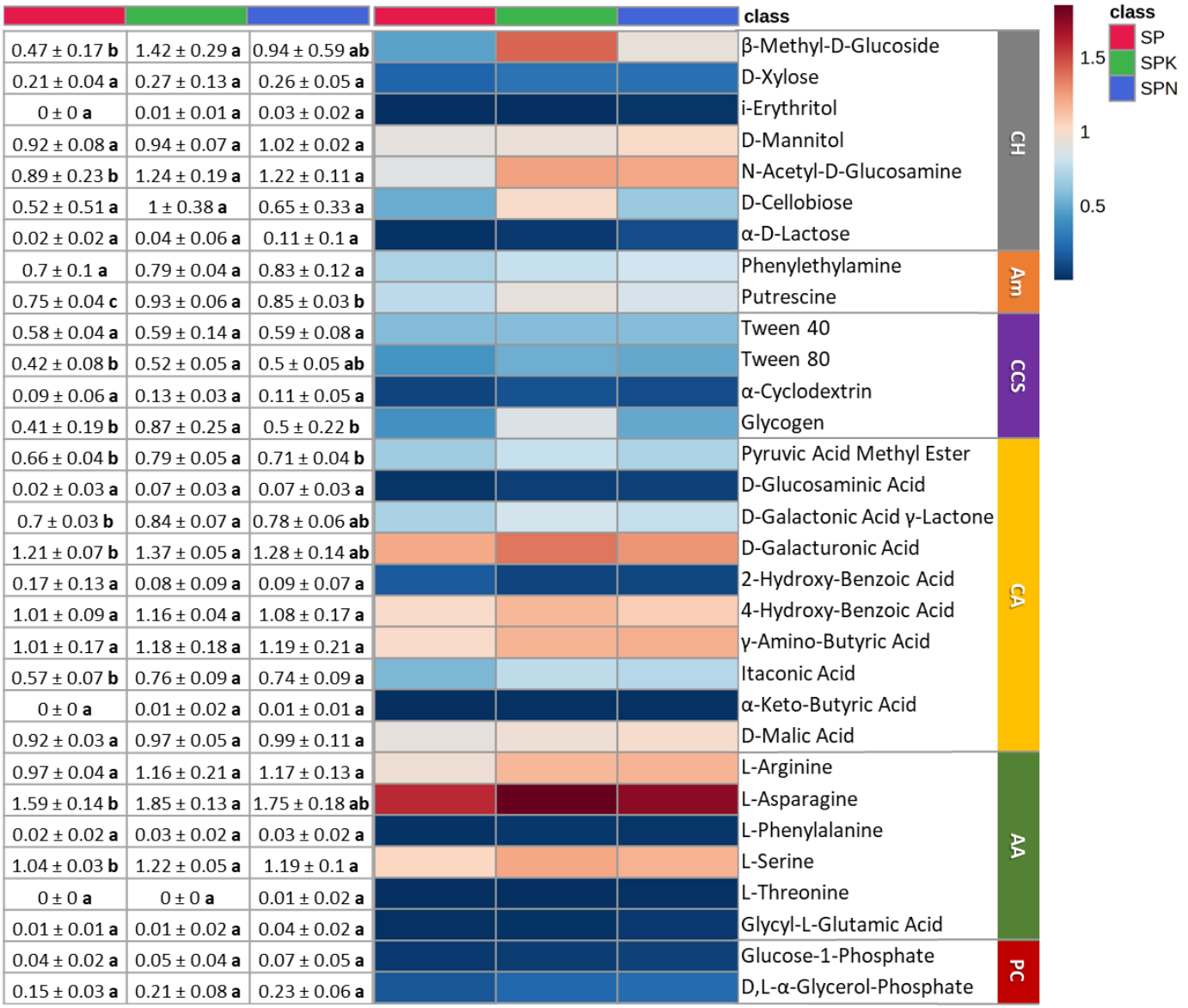


Figure 3. Boxplots representing the effects of applying two soil prebiotics on soil community-level physiological profiling (CLPP) measures compared to the control: (**a**) colony forming units (CFUs), (**b**) metabolic richness or number of positive wells (Units) with optical density (OD) > 0.25, and (**c**) metabolic activity or average well color development (AWCD), which is measured at 48 h (h) post plates' inoculation. SP plants without any treatment (control); SPK plants treated with K1®; SPN plants treated with NUTRIGEO L®. Each treatment is denoted by a color, where red is for SP, green is for SPK, and blue is for SPN. Data presented are means ± SD. Bars labeled with the same letters within each graph indicate no significant difference ($p < 0.05$) as determined using the ANOVA LSD test.

Examining the metabolic fingerprint reaction patterns of the soil bacterial community, we calculated the rate of color change in each well, reflecting the degradation rate of individual C sources. Among the twenty positive wells (OD > 0.25), both prebiotic treatments (SPK and SPN) significantly increased ($p < 0.05$) the degradation of four C sources: N-Acetyl-D-Glucosamine (39.13% and 36.82%, respectively), Putrescine (22.96% and 12.39%, respectively), Itaconic acid (33.37% and 28.95%, respectively), and L-serine (16.82 and 13.94, respectively) compared to the control (Figure 4). On the other hand, seven C sources: β-Methyl-D-Glucoside, Tween 80, Glycogen, Pyruvic Acid Methyl Ester, D-Galactonic acid γ-lactone, D-Galacturonic acid, and L-asparagine were significantly ($p < 0.05$) more degraded in SPK by 199.49%, 24.44%, 112.72%, 18.83%, 19.58%, 12.77%, 16.12%, respectively, compared to SP (Figure 4). The degradation of the remaining nine substrates with OD > 0.25: D-Xylose, D-Mannitol, D-Cellobiose, Phenylethylamine, Tween 40, 4-Hydroxy-Benzoic Acid, γ-Amino-Butyric Acid, D-Malic Acid, and L-Arginine did not vary significantly ($p < 0.05$) between treatments (Figure 4).



0.47 ± 0.17 **b**	1.42 ± 0.29 **a**	0.94 ± 0.59 **ab**				β-Methyl-D-Glucoside	CH
0.21 ± 0.04 **a**	0.27 ± 0.13 **a**	0.26 ± 0.05 **a**				D-Xylose	CH
0 ± 0 **a**	0.01 ± 0.01 **a**	0.03 ± 0.02 **a**				i-Erythritol	CH
0.92 ± 0.08 **a**	0.94 ± 0.07 **a**	1.02 ± 0.02 **a**				D-Mannitol	CH
0.89 ± 0.23 **b**	1.24 ± 0.19 **a**	1.22 ± 0.11 **a**				N-Acetyl-D-Glucosamine	CH
0.52 ± 0.51 **a**	1 ± 0.38 **a**	0.65 ± 0.33 **a**				D-Cellobiose	CH
0.02 ± 0.02 **a**	0.04 ± 0.06 **a**	0.11 ± 0.1 **a**				α-D-Lactose	CH
0.7 ± 0.1 **a**	0.79 ± 0.04 **a**	0.83 ± 0.12 **a**				Phenylethylamine	Am
0.75 ± 0.04 **c**	0.93 ± 0.06 **a**	0.85 ± 0.03 **b**				Putrescine	Am
0.58 ± 0.04 **a**	0.59 ± 0.14 **a**	0.59 ± 0.08 **a**				Tween 40	CCS
0.42 ± 0.08 **b**	0.52 ± 0.05 **a**	0.5 ± 0.05 **ab**				Tween 80	CCS
0.09 ± 0.06 **a**	0.13 ± 0.03 **a**	0.11 ± 0.05 **a**				α-Cyclodextrin	CCS
0.41 ± 0.19 **b**	0.87 ± 0.25 **a**	0.5 ± 0.22 **b**				Glycogen	CCS
0.66 ± 0.04 **b**	0.79 ± 0.05 **a**	0.71 ± 0.04 **b**				Pyruvic Acid Methyl Ester	CA
0.02 ± 0.03 **a**	0.07 ± 0.03 **a**	0.07 ± 0.03 **a**				D-Glucosaminic Acid	CA
0.7 ± 0.03 **b**	0.84 ± 0.07 **a**	0.78 ± 0.06 **ab**				D-Galactonic Acid γ-Lactone	CA
1.21 ± 0.07 **b**	1.37 ± 0.05 **a**	1.28 ± 0.14 **ab**				D-Galacturonic Acid	CA
0.17 ± 0.13 **a**	0.08 ± 0.09 **a**	0.09 ± 0.07 **a**				2-Hydroxy-Benzoic Acid	CA
1.01 ± 0.09 **a**	1.16 ± 0.04 **a**	1.08 ± 0.17 **a**				4-Hydroxy-Benzoic Acid	CA
1.01 ± 0.17 **a**	1.18 ± 0.18 **a**	1.19 ± 0.21 **a**				γ-Amino-Butyric Acid	CA
0.57 ± 0.07 **b**	0.76 ± 0.09 **a**	0.74 ± 0.09 **a**				Itaconic Acid	CA
0 ± 0 **a**	0.01 ± 0.02 **a**	0.01 ± 0.01 **a**				α-Keto-Butyric Acid	CA
0.92 ± 0.03 **a**	0.97 ± 0.05 **a**	0.99 ± 0.11 **a**				D-Malic Acid	CA
0.97 ± 0.04 **a**	1.16 ± 0.21 **a**	1.17 ± 0.13 **a**				L-Arginine	AA
1.59 ± 0.14 **b**	1.85 ± 0.13 **a**	1.75 ± 0.18 **ab**				L-Asparagine	AA
0.02 ± 0.02 **a**	0.03 ± 0.02 **a**	0.03 ± 0.02 **a**				L-Phenylalanine	AA
1.04 ± 0.03 **b**	1.22 ± 0.05 **a**	1.19 ± 0.1 **a**				L-Serine	AA
0 ± 0 **a**	0 ± 0 **a**	0.01 ± 0.02 **a**				L-Threonine	AA
0.01 ± 0.01 **a**	0.01 ± 0.02 **a**	0.04 ± 0.02 **a**				Glycyl-L-Glutamic Acid	AA
0.04 ± 0.02 **a**	0.05 ± 0.04 **a**	0.07 ± 0.05 **a**				Glucose-1-Phosphate	PC
0.15 ± 0.03 **a**	0.21 ± 0.08 **a**	0.23 ± 0.06 **a**				D,L-α-Glycerol-Phosphate	PC

Figure 4. Heatmap representing the effects of applying two prebiotics on soil metabolic activity, measured by the degradation level of 31 carbon (C) sources present in the Biolog EcoPlates™, compared to the control. Metabolic activity or C sources degradation is calculated from the difference between the optical density (OD) measured at the time of plates' inoculation (T0) and at 48 h post-inoculation. The degradation level is presented in the colored bar, where dark red indicates high degradation, white for medium degradation, and dark blue for low degradation. Wells or C sources with an OD < 0.25 were considered as not degraded. SP plants without any treatment (control); SPK plants treated with K1®; SPN plants treated with NUTRIGEO L®. The 31 C sources belong to 6 C groups: CH Carbohydrates, Am Amines, CCS Complex C sources, CA Carboxylic acids, AA Amino acids, and PC Phosphate-C. Each treatment is denoted by a color, where red is for SP, green is for SPK, and blue is for SPN. Data presented are means ± SD. Values sharing the same letters in each C source are not significantly different ($p < 0.05$) according to the ANOVA LSD test.

The 31 different C sources or substrates present in the EcoPlates™ could be categorized into six C families or groups: carbohydrates (CH), amines/amides (Am), complex carbon sources (CCS), carboxylic acids (CA), amino acids (AA), and phosphate-carbons (PC). The metabolic fingerprint of CH and CCS in SPK was significantly higher ($p < 0.05$) by 62.88% and 41.26%, respectively, compared to SP (Figure 5). The Am, CA, and AA groups showed a significantly ($p < 0.05$) higher metabolic activity in both SPK (18.25%, 14.93%, and 17.52%, respectively) and SPN (15.22%, 10.42%, and 14.97%, respectively)

compared to SP (Figure 5). However, both treatments (SPK and SPN) did not significantly ($p < 0.05$) impact the PC degradation rate compared to SP (Figure 5). When combining the enzymatic and metabolic activities data using the principal components analysis (PCA), we considered the first and second principal components (PC 1 and 2) displayed on the x-axis and y-axis, respectively, to explain the maximal amount of variance between treatments (Figure 6a). In the Scores plot, untreated soil is clearly separated from prebiotics' treated soil, explained by 43.1% on the x-axis (PC 1) (Figure 6a). A small separation is also noted between the two prebiotic treatments (SPN and SPK), explained by 15.4% on the y-axis (PC 2) (Figure 6a). This divergence between untreated soil and the two prebiotics was explained in the Biplot by almost all measured enzymatic and metabolic activities except for DEH (Figure 6b). Among the measured enzymes, the vectors of aGLU, bGAL, ACP, and bGLU were the strongest or most featured (Figure 6b). On the other hand, the vectors of the AA, CA, and CH carbon groups revealed the highest contribution to the divergence of treatments with respect to metabolic activity (Figure 6b).

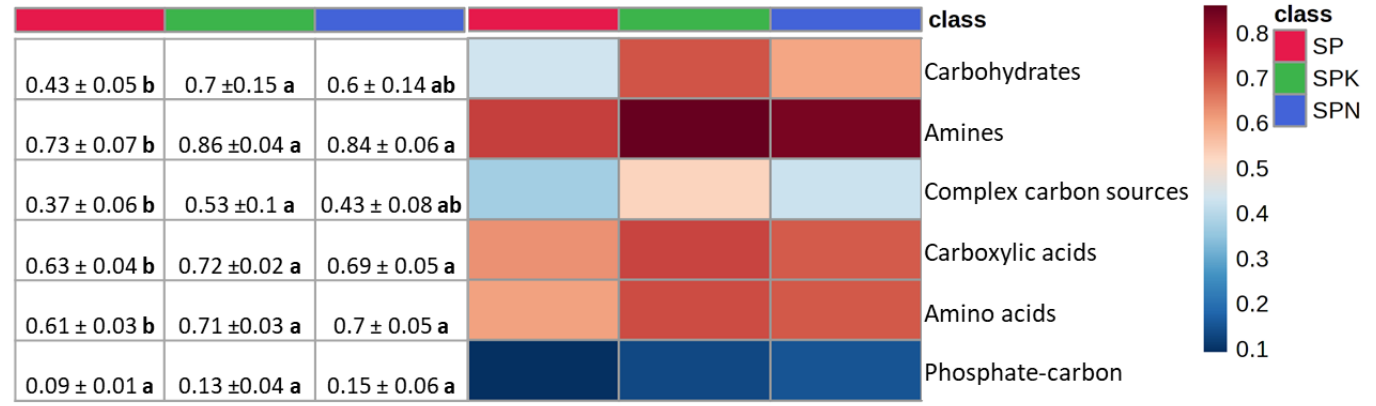


Figure 5. Heatmap representing the effects of applying two prebiotics on soil metabolic activity, measured by the degradation level of 6 carbon (C) groups, compared to the control. The C 'group's degradation level was calculated from the sum of the optical densities measured at the time of plates' inoculation (T0) and at 48 h post-inoculation of the C sources belonging to each group. The degradation level is presented in the colored bar, where dark red indicates a high degradation, white for medium degradation, and dark blue for low degradation. C groups with an OD < 0.25 were considered as not degraded. SP plants without any treatment (control); SPK plants treated with K1®; SPN plants treated with NUTRIGEO L®. The 31 C sources belong to 6 C groups: CH carbohydrates, Am amines, CCS complex carbon sources, CA carboxylic acids, AA amino acids, and PC phosphate-carbon. Each treatment is denoted by a color, where red is for SP, green is for SPK, and blue is for SPN. Data presented are means ± SD. Values sharing the same letters in each C source are not significantly different ($p < 0.05$) according to the ANOVA LSD test.

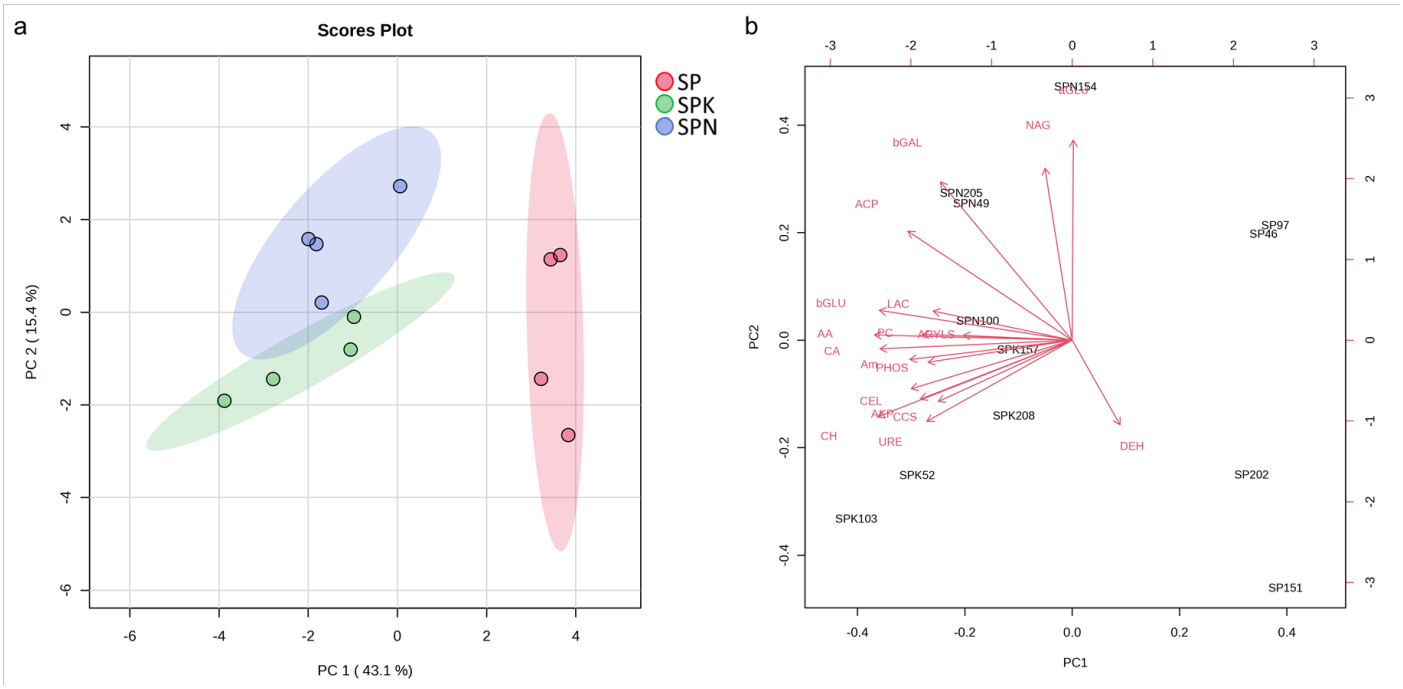


Figure 6. Principal component analysis (PCA) plots representing the effect of prebiotics' application on soil enzymatic activity and metabolic diversity compared to the control (**a**) Scores plot and (**b**) Biplot. SP plants without any treatment (control); SPK plants treated with K1®; SPN plants treated with NUTRIGEO L®. The data used are mean values of all enzymatic activity and carbon groups' degradation measured. aGLU α-glucosidase, bGLU β-glucosidase, bGAL β-galactosidase, CEL cellulase, LAC laccase, NAG N-acetylglucosaminidase, URE urease, PHOS phosphatase, ACP acid phosphatase, AKP alkaline phosphatase, ARYLS arylsulfatase, DEH dehydrogenase, CH carbohydrates, Am amines, CCS complex carbon sources, CA carboxylic acids, AA amino acids, PC phosphate-carbon. In the Scores plot, each treatment is denoted by a color, where red is for SP, green is for SPK, and blue is for SPN. The percentage of difference is indicated on the x-axis (PC 1) by 43.1% and the y-axis (PC 2) by 15.4%. Ellipses with a 95% confidence level are presented for each treatment. In the Biplot, the red arrows or vectors indicate the effect and direction of each factor measured and their contribution with respect to treatment dispersion and explanation.

4. Discussion

The use of soil biostimulants in agriculture has gained significant attention due to their potential to enhance soil fertility and crop productivity. In our study, we investigated the impact of two commercial soil biostimulants, K1® (SPK) and NUTRIGEO L® (SPN), on soil health, specifically focusing on microbial abundance, enzymatic activity, and metabolic diversity. K1® is formulated with specific organic acids and trace elements, while NUTRIGEO L® contains plant extracts and trace elements. These prebiotics were compared to untreated soil (control, SP) cultivated with *Zea mays* L. Ten weeks post-application, we assessed the effects of these prebiotics to gain a deeper understanding of how they influence soil functional diversity.

4.1. Prebiotics' Application Increase Soil Enzymatic Activity

Numerous studies have highlighted the importance of microbial activity, particularly enzymatic activity, for monitoring soil conditions, quality, and health, as they reflect internal changes [61,62]. This knowledge is crucial for developing soil restoration, preservation, and fertility management plans aligned with agricultural transition goals [37,63,64]. Our study demonstrated that biostimulants significantly influenced enzymatic activities crucial for nutrient cycling and soil health. We assessed twelve key oxidative and hydrolytic enzymes involved in C, P, N, and S nutrient cycling [63,65,66]. The results showed increased enzymatic activity ten weeks post-application, with SPK significantly increasing

aGLU, PHOS, and URE activities, and SPN increasing bGAL activity. Both prebiotics also increased LAC, bGLU, ARYLS, and ACP activities. These findings support existing literature on biostimulants' positive effects on soil physical properties and biochemical processes across various soil types and crops [5,67].

However, it is crucial to recognize that while biostimulants did enhance enzymatic activity, the effects were not overwhelming. For instance, studies on creeping bentgrass found that biostimulants improved visual quality by enhancing plant tolerance to heat and moisture, rather than significantly altering the microbial community [68]. Similarly, research on seaweed-based biostimulants demonstrated varied effects on soil enzymatic activities, enhancing DEH activity in some cases but showing no significant impact in others [69]. These mixed results underscore the complexity of biostimulant interactions with soil systems and the need for more precise application strategies.

Our previous findings showed that SPK and SPN prebiotics increased soil microbial diversity and altered bacterial and fungal community structure [39], which is consistent with the notion that a diverse microbial community is more resilient and efficient in nutrient cycling [70,71]. The synergistic effects of biostimulants on enzymatic activities and microbial diversity suggest enhanced soil functionality, fostering a balanced and dynamic soil ecosystem. However, the practical implications for soil health and fertility management require further investigation [72].

Studies have also highlighted the varying efficacy of different biostimulants. For example, Kelpak® applied to soil did not consistently affect DHE, peroxidase, catalase, and N reductase activities in winter oilseed rape soil [73]. In contrast, biostimulants derived from sewage sludge and chicken feathers were effective in mitigating the negative impacts of insecticides on soil enzymatic activities and biodiversity, demonstrating their potential for soil remediation [74,75]. Similarly, biostimulants made from wheat condensed distiller soluble (WCDS) enhanced adenosine triphosphate (ATP) formation and enzymatic activities but did not significantly alter the bacterial community [67]. These discrepancies highlight the importance of biostimulant composition and application context and may explain the difference observed in the impact of prebiotics (SPK and SPN) on soil enzymatic activity. The variability in biostimulant effects can be attributed to factors like their types, ingredients, soil and plant characteristics, application methods, and environmental conditions [76–79].

Research has shown that rhizogenic biostimulants increased soil organic matter, nutrient content such as calcium (Ca), potassium (K), magnesium (Mg), P, iron (Fe), and copper (Cu), microbial activity, and enzymatic activity in sandy and sandy loamy soils [80]. These findings coincide with our results, where SPK and SPN treatments enhanced soil minerals, leading to increases in electrical conductivity (EC), cation exchange capacity (CEC), and carbon storage [39]. The enhancement of soil nutrient bioavailability in the rhizosphere promotes plant root vitality, P efficiency, and soil microbial activity and functional diversity through enhanced N mineralization [81,82]. Prebiotics also increased mycorrhization in maize roots, even under low or no fertilization, highlighting the plant's reliance on arbuscular mycorrhizal fungi (AMF) for water and nutrient uptake [39,83]. Biostimulants derived from plants can also mimic hormonal functions, such as auxin, enhancing the plant rooting process [84]. The composition, stability, and physicochemical properties of biostimulants, such as humic substances, affect soil microbial community structure and functions, thereby influencing plant growth directly and indirectly [25,85]. However, their effects vary; for instance, some biostimulants like Tytanit® and Rooter® may not consistently improve crop and soil fertility indexes [86]. Additionally, while they can stimulate certain enzyme activities, this might lead to enzyme deterioration over time [86]. Long-term studies are essential to assess the sustainability and resilience of enzymatic enhancements induced by biostimulants, helping in the development of effective soil management strategies [87–90].

Our assessment of soil quality indexes, namely, geometric mean (*Gmea*) and soil fertility index (*Mw*), based on enzymatic activity, showed a significant increase in prebiotic-

treated soil compared to the control, indicating enhanced soil quality and fertility [91–93]. The stimulation of diverse enzymatic activities by prebiotics, encompassing carbohydrate, lignin, S, P, and N cycling, contributes to improved nutrient availability and overall soil health, underscoring the comprehensive impact of biostimulants on the soil microbial community [33,94]. The correlation between enhanced soil enzymatic activity and microbial diversity supports the concept of a functionally resilient and efficient microbial community, highlighting the potential of biostimulants to improve soil functionality holistically [14,19,95].

4.2. Prebiotics' Application Increases the Abundance of Soil Fungi and the Metabolic Activity of Heterotrophic Culturable Soil Bacteria

The application of biostimulants revealed shifts in soil microbial community composition, indicating changes in metabolic diversity [96]. The increase in the abundance of beneficial microorganisms, crucial for nutrient cycling, corroborates prior research on biostimulants' efficacy in stimulating their growth [97,98]. These microorganisms play a pivotal role in enhancing nutrient availability, suppressing diseases, and promoting overall soil and plant health [16,99]. In our prior research [39], we found that SPK had a more generalized effect, favoring specific bacteria and fungi, while SPN predominantly affected the soil fungal community, promoting several species. These selected microorganisms were identified as saprotrophs, plant growth-promoting rhizobacteria and fungi (PGPR and PGPF), endophytes, endohyphal bacteria (EHB), and symbiotic microbiota. The shift in microbial communities elucidates the mode of action of each prebiotic and their effects on soil structure and plant growth [39].

Our current study noted a rise in the fungi-to-bacteria ratio post-prebiotic application, particularly with SPN, due to increased fungal abundance. However, no significant increase was noted in the soil bacteria [100]. This increase in microbial abundance may stem from improvements in soil physicochemical properties (EC, CEC, and nutrient solubility), creating a favorable environment for microbial growth and activity [2,65]. Biostimulants also influence soil structure, including aggregate stability, aeration, and humidity, by promoting root growth and facilitating rhizodeposition, microbial glomalin and exopolysaccharide production, and necromass turnover [101–104].

To address microbial functional diversity post-prebiotic treatments, Biolog EcoPlates™ were used to generate CLPPs for each treatment group [33]. This method assesses microbial utilization of various C substrates (31 substrates) [31,52]. It is important to note that microbial behavior in vitro may differ from their natural habitat and observed metabolic potential in CLPP assays might not reflect environmental conditions [36]. Furthermore, only a fraction of the total community may respond during the assay, although some responses from non-culturable species can be detected [105]. Significant variations in CLPP among different soil types, crop varieties, and plant developmental stages have been demonstrated in prior studies [106–108]. In our study, we used consistent soil, maize variety, and conditions across all treatments. Data from Biolog EcoPlates™ revealed significantly higher metabolic activity (AWCD) in the rhizosphere microbial community of plants treated with prebiotics (SPK and SPN) compared to untreated ones [109]. All treatments exhibited similar metabolic profiles (CLPP), indicating comparable degradation of C sources with minor variations. Differences resulted from the unique effects induced by each prebiotic (SPK and SPN) on the native microbial community structure, diversity, and functionality [16,39].

The increased degradation rate of C sources (*AWCD*) significantly rose in SPK treatment for eleven C sources (β-Methyl-D-Glucoside, N-Acetyl-D-Glucosamine, Putrescine, Tween 80, Glycogen, Pyruvic Acid Methyl Ester, D-Galactonic acid γ-lactone, D-Glalacturonic acid, Itaconic acid, L-asparagine, and L-serine) and in SPN for four C sources (N-Acetyl-D-Glucosamine, Putrescine, Itaconic acid, and L-serine) compared to the control [72]. Thus, prebiotic application did not alter metabolic richness but increased the degradation rate of oxidized C sources, leading to higher metabolic activity [110]. The CLPP

concept quantifies the total amount of C consumed, revealing the microbial biomass involved in utilizing specific C sources [105]. The increased metabolic activity suggests a more functionally active microbial community, contributing to improved nutrient cycling and bioavailability [111–113]. This aligns with the significant increase in soil enzymatic activities and indexes monitored after ten weeks post-prebiotic application, especially SPK. The broader impact of SPK on five out of six C groups (carbohydrates, complex C sources, carboxylic, amines, acids, and amino acids), while SPN increased the degradation rates of carboxylic acids, amines, and amino acids, underscores the prebiotics' significant and varied impact on soil microbial communities [39]. Enhanced degradation of complex C sources and carbohydrates, resembling lignocellulosic substrates, suggests an improvement in organic matter turnover [114]. This has implications for nutrient cycling and the release of essential nutrients like N, P, and S [25,80,115].

Biostimulants promote a nutrient-efficient soil environment by increasing microbial abundance and activity, enhancing soil physicochemical characteristics, and stimulating enzyme production for degrading various C sources [14,19,116]. The complementarity between increased enzymatic activities (glucosidases, LAC, phosphatases, ARYLS, and URE) and metabolic potential, as shown by CLPPs, underscores the multifaceted impact of biostimulants on the soil microbial community and biochemical processes, and, thus, on soil health and fertility [117,118]. To gain deeper insights into the mechanisms through which biostimulants are influenced by various factors, it is essential to leverage advanced analytical methods such as metabolomics and metatranscriptomics [119–121]. These methodologies offer a more comprehensive understanding of how biostimulants interact with soil microbiota and biochemical pathways, optimizing their application for sustainable agricultural practices.

5. Conclusions

The potential of biostimulants, particularly prebiotics, to reduce reliance on chemical fertilizers, mitigate environmental impacts, and enhance overall soil health positions them as promising tools for achieving agricultural sustainability. Our study contributes to this understanding by examining the short-term effects of prebiotics K1® (SPK) and NUTRIGEO L® (SPN) on soil functional diversity, building upon previous research. We observed a significant increase in key enzyme activity related to nutrient cycles in prebiotic-treated soils, particularly with SPK, indicating a positive influence on soil enzymatic activities. Additionally, community-level physiological profiling analysis highlighted the substantial impact of prebiotics, notably SPK, on soil metabolic activity and carbon source degradation rates. These results underscored the pivotal role of prebiotics in shaping the biochemical and metabolic landscape of soil ecosystems. Our study elucidated the distinct effects of each prebiotic, with SPK demonstrating a broader influence on soil microbial community structure and functionality, enhancing mineralization and decomposition processes, while SPN exerted a more targeted impact on soil fungal communities. These insights underscored the complexity of soil–plant–microbe interactions and emphasized the need for tailored approaches in soil management practices. Furthermore, our study underscores the importance of soil functional diversity, complementing our holistic approach to understanding prebiotics' mode of action and providing robust evidence of their efficacy in agricultural settings. Future research should investigate the long-term effects of prebiotics across different soil types, plant species, and growth conditions to validate sustainability and optimize soil health and productivity. As agriculture evolves towards sustainable practices, understanding the nuanced effects of biostimulants on both soil structure and function becomes crucial for designing targeted strategies that support resilient and nutrient-efficient soil ecosystems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14071115/s1, Table S1: Effects of prebiotic treatments (SPK and SPN) on soil organic carbon (SOC) ten weeks post-application in comparison to the control (SP).

Author Contributions: Conceptualization, A.A., B.T., A.B.-M., K.L. and I.T.-G.; methodology, A.A., L.E., M.B., L.C., W.R.-A. and N.B.-L. software, A.A.; validation, A.A., W.R.-A. and N.B.-L.; formal analysis, A.A., L.E. and L.C.; investigation, A.A., L.E. and M.B.; resources, W.R.-A., N.B.-L., A.B.-M., K.L. and I.T.-G.; data curation, A.A.; writing—original draft preparation, A.A.; writing—review and editing, A.A., L.E., M.B., L.C., W.R.-A., N.B.-L., B.T., A.B.-M., K.L. and I.T.-G.; visualization, A.A., B.T., A.B.-M., K.L. and I.T.-G.; supervision, A.B.-M., K.L. and I.T.-G.; project administration, A.A., B.T., A.B.-M., K.L. and I.T.-G.; funding acquisition, A.A., B.T., A.B.-M., K.L. and I.T.-G. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author.

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Conflicts of Interest: The author, Aude Bernardon-Mery, is an employee of the company Gaïago SAS. The funder had a role in the design of the study, the revision of the manuscript, and the decision to publish the results. The funder had no other roles in the research than the ones declared above. UniLaSalle reserves the right to terminate the contract immediately if the company's requirements do not align with its ethical standards, research rigor, and scientific principles. Apart from what has been stated above, the authors have no relevant financial or non-financial interests to disclose.

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