Article

Effects of Multi-Species Microbial Inoculants on Early Wheat Growth and Litterbag Microbial Activity

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Abstract: The use of microbial consortia (MC) with complementing features is considered to be a promising method of sustainable crop intensification, potentially trumping the limited performance of single-strain applications. We assessed the effect of two novel MC on early wheat growth and litterbag microbial activity in heated and unheated soil. Pot experiments were carried out in duplicate in a greenhouse over 63 days using a completely randomized design with six replications. A range of parameters of plant growth and nutrient uptake were regularly assessed and statistically analyzed by ANOVA. The litterbag-NIRS method was used to trace the microbial activity. Averaged over both trials, soil heating resulted in a significant increase in shoot biomass (+53%) and subsequent nitrogen uptake (+307 mg N pot^{-1}) but strongly reduced root development (−46%) compared with unheated soil. The application of MC had no effect on wheat growth in the heated soil. By contrast, in the unheated soil, shoot (+12%) and root (+15%) biomass and shoot nitrogen uptake (+11%) were significantly increased after double inoculation with MC compared with autoclaved MC. The litterbag-NIRS method confirmed clear effects of soil heating on microbial activity. Differences between MC application and the control were noted, indicating a buffering effect of MC.

Keywords: plant-growth-promoting microorganisms; microbial consortia inoculants; microbial fertilizer; plant-microbe interactions; pot experiments; greenhouse; litterbag-NIRS method

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1. Introduction

The targeted application of selected rhizosphere microorganisms with well characterized beneficial properties is a promising strategy for the sustainable intensification of cropping systems [1]. These beneficial microorganisms are commonly referred to as "plant-growth-promoting microorganisms" (PGPMs). This group of organisms predominantly colonizes the rhizosphere of crops and includes bacteria, fungi, actinomycetes, protozoa, and algae [2]. Among them, bacteria are the most abundant and have the greatest diversity in bulk soil and, especially, in the rhizosphere [3]. Plant-growth-promoting rhizobacteria (PGPR) are well known to stimulate plant growth by direct and indirect mechanisms [4,5]. Their reported functional mechanisms include: (i) the facilitated acquisition of water and nutrients (primarily N, P, and Fe); (ii) the modulation of concentrations of phytohormones, such as auxins, cytokinins, and gibberellins, leading, i.a., to an increased root growth; (iii) the release of volatile organic compounds and siderophores; (iv) the production of antipathogenic compounds, such as antibiotics, toxins, and lytic enzymes; and (v) the reinforcement of induced systemic resistance against pests and diseases [6–10].

Under experimental conditions, mainly pot trials, PGPR applications have shown yield increasing effects on various crops [8,11–15], while other studies indicated no effect of microbial applications on crop growth [16–18]. The reproducibility of beneficial microbial effects in real field applications still remains a considerable challenge for an ecologically and economically efficient use of PGPMs in agriculture [11,19]. Successful PGPM utilization is mainly defined by interactions between the specific microbial characteristics, the plant species and its susceptibility for mutualistic associations, the soil type, the inoculant density, and the specific environmental conditions [11,19].

To solve the problem of the lack of field efficacy of PGPR, it is increasingly suggested to use multifunctional microbial consortia (MC), which are better able to cause stable synergistic effects [11,20–22]. A high level of genetic diversity within consortia may improve the flexibility and resilience of microbial applications under different environmental conditions, resulting in a higher potential to promote plant growth [23]. In turn, this may improve the chances of success of the respective inoculants [24]. Microorganisms with similar functions may replace each other if a particular strain fails to establish in the rhizosphere [11,21,25]. According to Bargaz et al. [24], the successful establishment and persistence of applied MC is mainly affected by the functional, complementary, and synergistic properties of the selected strains. However, the targeted design of multifunctional MC when trying to quantify individual contributions to crop growth remains challenging. This holds true especially if further synergistic interactions occur under natural rhizosphere conditions, either among the introduced species themselves or with the native soil microbiome [19,26]. Recently, Tabacchioni et al. [22] designed new synthetic MC composed by strains able to interact synergistically with each other through the reciprocal provision of nutrients and the removal of inhibitory compounds. Based on positive interactions found in vitro, it is assumed that the beneficial microbial effects arising from the combined application of single strains exceed the sum of their individual contributions [22].

The objective of this study was to investigate the effect of two novel microbial consortia (MC_A and MC_B) under different soil conditions on early wheat growth in greenhouse pot experiments. Using the bottom-up approach [22], compatible single strains with different functional attributes and proven effects were identified and used to design two MC. To minimize the interspecific competition between the autochthonous soil microflora and the introduced microbial inoculants, and to evaluate the direct PGP mechanisms, heat steaming was included as an experimental factor. The effects of MC application on plant biomass production, leaf spectral indices, and shoot nutrient contents and uptake were evaluated on young wheat plants grown either in heated or unheated soil. In parallel, an indirect method for the evaluation of the soil microbial activity and dynamics was applied. The litterbag-NIRS method [27] is based exclusively on the allometry of the decay of a standard litter evaluated by NIR vibrational spectroscopy after sixty days of burial near living crops. From the NIR spectra, by means of chemometric models, it is possible to estimate a series of chemical-bromatological compounds and properties of the litter that reflect the microbial degradation. In particular, the action of microbial fertilizer has been highlighted, but it has also been repeatedly demonstrated that the method has a predictive capacity with regard to the fertility of the soil in relation to the yields obtained from different crops [27–30].

2. Materials and Methods

2.1. Selection, Cultivation, and Preparation of Microbial Consortia for Seed Inoculation

Two MC inoculants (MC_A and MC_B) were designed as part of the Horizon 2020 SIMBA project (Sustainable Innovation of Microbiome Applications in the Food System), as described in detail by Tabacchioni et al. [22]. The inclusion of a strain in a consortium depended on two criteria: first, a confirmed specific ability to promote plant growth with a known physiological mode of action and, second, a lack of antibiosis between the strains (see [22] and the references therein). The final consortia, MC_A and MC_B, consisted of six and five different microbial single strains, respectively, as shown in Table 1.

Table 1. Composition of MC used in the experiments, the properties of single strains, and the concentration of each active member measured after centrifugation and pellet resuspension (I) and stabilization in zeolite (II).

Microbial Strain	MC [1]	Properties [22]	Pellet (I) CFU [2] mL^{-1} or Spores mL^{-1}	Zeolite (II) CFU g^{-1} or Spores g^{-1}
Azotobacter chroococcum LS132	A	N_2-fixation	3.00×10^{10}	2.80×10^{7}
Bacillus licheniformis PS141	A	IAA [3] production	3.20×10^{9}	1.80×10^{6}
Komagataella pastoris PP59	A	PGP [4]	1.50×10^{10}	1.80×10^{5}
Paraburkholderia tropica MDIIIAzo225	A	N_2-fixation	5.00×10^{9}	2.80×10^{3}
Pseudomonas granadensis A23/T3c	A	PGP	2.50×10^{11}	4.80×10^{6}
Trichoderma harzianum TH01 *	A	PGP	2.20×10^{10}	5.00×10^{7}
Azotobacter vinelandii DSM 2289	B	N_2-fixation, siderophore production	1.10×10^{11}	1.90×10^{7}
Bacillus amyloliquefaciens LMG 9814	B	Alpha-glucosidase, alpha-amylase, and isoamylase production	5.00×10^{9}	3.95×10^{6}
Bacillus sp. BV84	B	Biocontrol/PGP	1.00×10^{9}	4.00×10^{6}
Pseudomonas fluorescens DR54	B	Biocontrol	1.06×10^{11}	5.00×10^{6}
Rahnella aquatilis BB23/T4d	B	PGP	8.00×10^{11}	7.00×10^{5}

[1] MC: microbial consortium; [2] CFU: colony-forming unit; [3] IAA: indole acetic acid; [4] PGP: plant-growth promotion; * The concentration for *Trichoderma harzianum* TH01 is given as spores mL^{-1} and spores g^{-1}.

Both MC_A and MC_B were prepared in two main steps: (1) microbial cultivation and production of inoculum material for each active member under sterile conditions and (2) stabilization of microbial inoculum on sterile micronized zeolite (granulometry < 75 µm). Zeolites are natural hydrated aluminosilicates utilized in agriculture as carriers to support the survival of PGPR [31]. All microbial cultivation and production steps were performed in triplicate. In the first step, a loopful of each bacterial strain and the yeast *Komagataella pastoris* PP59 was picked up from a pure culture stored in cryotubes at −80 °C and then transferred into a sterile flask containing 3 mL of liquid nutrient broth (NB, Oxoid Limited, Hampshire, UK). The flasks were then incubated in an orbital shaker at 200 rpm and 28 °C for 24 h, except for the *Azotobacter vinelandii* strain, which was incubated for 72 h due to its significantly lower growth multiplication rate (data not shown, see [22]). The colony-forming units per mL (CFU mL^{-1}), measured by serial dilution and plating of microbial suspension [32], ranged from 1.10×10^{7} (*A. vinelandii* DSM2287) to 6.00×10^{9} (*P. granadensis* A23/T3c). To obtain the microbial inoculum, 1% of the pre-inoculum culture was transferred into sterile flasks containing 1.5 L of fresh NB and incubated at 200 rpm and 28 °C for 24–72 h, as described above. Each microbial culture was centrifuged at 8000× g for 10 min. The pellet was used for successive resuspension in sterile distilled water in order to achieve (1) the right volume of liquid for stabilization in zeolite with a 10% moisture content and (2) to reach a final minimum concentration (prior to stabilization) of each single strain of 1.00×10^{8} CFU mL^{-1}. To determine viable cell count for the strain pellet preparation, serial dilutions in distilled water of bacterial and yeast strains were performed. A total of one hundred microliters of each serial dilution was placed on nutrient agar (NA, Oxoid Limited, Hampshire, UK) that was incubated at 28 °C for 24–72 h. Cell density of each strain was determined by counting the number of viable cells on nutrient agar (Table 1) using the surface-viable count method [32]. Concerning the fungal strain *Trichoderma harzianum* TH01, a small agar plug of the mycelium (5 mm in diameter) cryopreserved at −80 °C in glycerol (30% v/v) was picked up and placed in the center of a Potato Dextrose Agar (PDA) (Potato Dextrose Agar, Oxoid Limited, Hampshire, UK) plate. After incubation at 28 °C in darkness for 9 days, the spore suspensions were prepared by adding sterile distilled water (10 mL) onto the plates and scraping the surfaces slightly with a sterile microbiological plastic loop. The resulting suspension was filtered through two layers of sterile gauze to remove residual hyphae. To achieve a sufficient density of fungal spores, centrifugation and resuspension in sterile distilled water was performed analogously to the procedure described for bacteria and yeast strains. Next, serial dilutions were performed and one hundred microliters of each dilution were plated on PDA to assess

the fungal concentration of the spore suspension by determining the germinating spores with emerging hyphae. The PDA dilution plates were incubated at 28 °C in darkness and checked daily for the colony count. The concentration was then adjusted to 1×10^8 spores per mL that was used for stabilization in zeolite.

In the second step, 100 mL of each microbial suspension was stabilized by mixing with 1 kg of sterile micronized zeolite, resulting in a specific humidity of ca. 10%. Twenty-four hours after stabilization, the cell density of each strain was determined by resuspension of the powder in sterile water and serial dilution and plating of suspension (Table 1). Finally, the stabilized strains were used to formulate MC_A and MC_B by adding each strain in equal proportions, in a 1:1 ratio, and storing them at room temperature in sterile plastic bags until application.

The MC were applied immediately before sowing. In order to obtain a sufficiently high density of viable cells in the area of the radicle, 1 g of inoculum material was placed directly below the seeds in each seeding hole. These comparably high amounts of inoculum material were used to ensure a minimum concentration of 10^6 CFUs for each microbial strain per seed. Target concentrations were achieved for all strains except P. *tropica* MDIIIAzo225 and *K. pastoris* PP59 in MC_A and R. *aquatilis* BB23/T4d in MC_B (Table 1). In addition, the inoculum was refreshed 21 days after sowing (DAS) in selected treatments (MC_A_2x and MC_B_2x, see Section 2.3. below) to counteract the potential decline in viable cells in the soil. For this purpose, both MC were dispersed in 100 mL tap water and again applied to the pots with the same concentration. All other pots were treated with tap water.

2.2. Substrate Characterization, Soil Properties, and Fertilization

Top soil (sandy alluvial loam) from a potato field on the organic experimental farm "Wiesengut" (50°47′12.8″ N, 7°16′30.0″ E) of the University of Bonn was collected from the *Ap* horizon to a depth of 20 cm. The soil was sieved with 2 millimeter mesh and homogenously mixed with fine quartz sand at a ratio of 2:1 (*w*:*w*) to avoid compaction in the pots. The chemical analysis indicated that the soil was comparatively well supplied with most macro and micronutrients [33] (Table 2). No additional fertilizers were applied.

Table 2. Chemical properties of the soil before mixing with sand.

	pH	P	K	Mg	Na	Fe	Cu	Mn	Zn	B	Ca	Mo	TN [3]
Value [1]	6.7	100	200	120	8	270	5.4	430	13	0.41	1731	<0.04	0.12
Level [2]	C	C	D	E	-	-	E	E	E	C	-	A	-

[1] Values are given in ppm, except for the pH and TN (%). [2] Regional supply levels for Germany according to the classification of the German Chamber of Agriculture: A = very low, B = low, C = normal, D = high, E = very high, "-" = not available. [3] Total nitrogen content (%).

To reduce the competition from the autochthonous soil microflora and to provoke stronger direct effects of MC, half of the soil substrate was steamed to a temperature of 100 °C (±1 °C) for at least 12 h using a soil pasteurizer (Harter Elektrotechnik, Schenkenzell, Germany). Soil steaming was expected to provide favorable conditions for the establishment and persistence of the applied MC in the soil, resulting in a prompt colonization of the rhizosphere. To provide a suitable habitat for the MC ("charosphere" [34]) wooden biochar was incorporated by default in all pots at a rate of 0.1 weight % in the upper soil layer (0–5 cm).

2.3. Experimental Setup and Culture Condition

Pot experiments were performed in an air-conditioned greenhouse at a constant daytime temperature of 19.3 °C (±0.9 °C) and night temperature of 14.7 °C (±0.5 °C) at Bonn University, Germany. The relative humidity was 54.4% (±3.1%) during the day and 65.4% (±2.4%) at night. Sodium vapor high-pressure lamps (400 W) with an active photon flow of 725 µmol s^{-1} and a luminous flux of 56,500 lm ran 12 h per day. The test crop was wheat (*Triticum aestivum* L.) cv. Butaro. Seeds had a germination rate of 98%.

Prior to sowing and microbial inoculation, the seed surfaces were carefully sterilized with ethanol (70%) and sodium hypochlorite (3.5%) by slightly shaking the seeds in each of the two chemicals for 90 s. Subsequently, the seeds were gently rinsed six times with sterile water for 30 s, air-dried under a sterile bench, and stored in sealed sterile petri dishes [35] until sowing.

Wheat plants were grown in pots (11 L, SOPARCO, Condé-sur-Huisne, France) filled with 14 kg of soil substrate. Prior to sowing, pots were surface-sterilized by submerging them into a 1.5% (v/v) solution of Menno Florades® (Menno Chemie-Vertrieb GmbH, Norderstedt, Germany) for a minimum of 24 h. After germination, plant density was adjusted to 20 plants per pot. Pots were automatically watered by drip irrigation. Soil moisture was continuously controlled by gravimetry and regularly adjusted to ~75% of the substrate's water-holding capacity. Pots were kept weed-free by manual weeding.

Wheat plants were harvested 63 DAS (duration trial 1: 18 November 2019–21 January 2020; trial 2: 2 December 2019–4 February 2020). The experimental setup included two factors: (1) soil treatment with two levels (I: unheated soil, II: heat-steamed soil) and (2) application of microbial consortia with six levels (I: MC_A_1x, II: MC_A_2x, III: MC_B_1x, IV: MC_B_2x, V: CON (uninoculated control), and VI: MC_A_AC (consortium A autoclaved)) with six replications. Autoclaved MC_A was included as a positive control treatment to assess the nutritional effect of the applied consortium A. The corresponding nutritional inputs arising from the application of each MC are indicated in Table S1.

2.4. Data Collection

To assess the soil microbial activity indirectly, the Litterbag-NIRS (LBN) method [27,36] was applied. At sowing, one hay litterbag was buried at the edge of each pot at a depth of approximately 10 cm. Each litterbag was filled with hay (Vitakraft pet care GmbH & Co. KG, Bremen, Germany) ground to 3 mm. An approximate total of 3.5 g hay was packed into half-empty square polypropylene nets (10 × 10 cm, 1.5 mm mesh size), which were re-sealed. At harvest, the litterbags were removed from the soil, dried at 40 °C for 48 h, gently cleaned, and finally stored at room temperature until further processing. For analysis, the litterbags were carefully opened to examine the spectral reflectance of the hay-litter sample, using the magnetic spacer capsule, measuring 9 × 40 mm of a smart miniaturized NIRS web-based wireless spectrophotometer (SCiO v. 1.2, Consumer Physics, Tel Aviv, Israel) in the range of 740–1070 nm. One spectrum was recorded on each side of the litterbag.

During the growing period, a range of morphological and physiological parameters was assessed to quantify the impact of the two MC on early wheat growth and development. Plant length was measured from the soil surface to the top of the plant stock using a folding rule. Furthermore, different vegetation indices, including the Normalized Difference Vegetation Index (NDVI) (1) [37],

$$\mathrm{NDVI} = \frac{\mathrm{R}_{NIR} - \mathrm{R}_{RED}}{\mathrm{R}_{NIR} + \mathrm{R}_{RED}}, \tag{1}$$

the Carotenoid Reflectance Index (CRI1) (2) [38],

$$\mathrm{CRI1} = \frac{1}{\mathrm{R}_{510}} - \frac{1}{\mathrm{R}_{550}}, \tag{2}$$

and the Photochemical Reflectance Index (PRI) (3) [39],

$$\mathrm{PRI} = \frac{\mathrm{R}_{531} - \mathrm{R}_{570}}{\mathrm{R}_{531} + \mathrm{R}_{570}}, \tag{3}$$

were measured using a handheld spectrophotometer (Polypen RP 410 UVIS, Photon Systems Instruments, Drásov, Czech Republic). The non-invasive measurements were carried out 28, 42, and 56 DAS with eight measurements per pot using different plants.

After a growing period of 63 days, plants were cut at the soil surface and weighed. Shoot dry matter yield was calculated after drying the samples in an oven at 105 °C until they reached a constant weight. Subsequently, biomass was ground in a vibratory disc mill for 40 s at 1400 rpm (RS200, Retsch® GmbH, Haan, Germany) for further analysis of the nitrogen (N), phosphorous (P), and potassium (K) content.

After microwave digestion with H_2O_2 and HNO_3 (220 °C for 30 min), wheat shoot P content was determined photometrically at 880 nm with the molybdenum blue method using a continuous flow autoanalyzer (QuAAtro39, SEAL Analytical, Southampton, UK). Shoot K content was quantified using an atomic absorption flame spectrometer (AAnalyst 200, Perkin Elmer, Waltham, MA, USA). Plant N contents were assessed using an elemental analyzer (EURO-EA3000, Eurovector, Pavia, Italy) subsequent to the chemical pulping of 4 mg shoot biomass with acetanilide. Plant roots were carefully washed out of the soil using a mesh size of 1 mm, oven-dried at 60 °C, and weighed.

2.5. Statistical Evaluation

Statistical data analysis was performed using the open-source program R (version 4.0.2) with RStudio (version 1.2.5033) [40]. A multiple linear model (*lm* function) was developed and applied to all agronomic parameters. Using a complete model, all possible interactions between all experimental factors (microbial inoculation x soil substrate x trial replicate) were considered. First, the datasets were analyzed for normal distribution of residuals by using the quantile-quantile method and the Shapiro–Wilk test. Homogeneity of variances was tested using the modified Levene test. Logarithmic ($\log_{10}$) or square-root transformation was conducted if needed to normalize data. Outliers were identified and removed by means of the quartile method [41]. Subsequently, a two-or three-factorial analysis of variance (ANOVA) was conducted and followed by a Tukey-HSD [42] test with adjusted p-value (α = 0.05) to assign significant differences. The results are presented as means and standard deviations (SD) are indicated where suitable. The visualization of results was performed according to the output of the statistical analysis for each response variable individually. Interactions are shown when significant. Otherwise, only the main effects are presented.

Twenty-two LBN variables derived from the reflectance spectra were analyzed for each treatment by EXCEL spreadsheets, expanded with the XLSTAT statistical software. The six individual biological replications were averaged for each treatment. Joint ANOVA over both trials and soils was performed where statistically permitted and suitable in terms of content. The ANOVA was followed by Tukey-HSD test to identify different treatments.

3. Results

3.1. Non-Invasive Evaluation of Microbial Performance—Plant Length and Vegetation Indices

Soil heating significantly affected the plant length at 42 and 56 DAS (Table 3). At 56 DAS, the plants grown in heated soil were 2.7 cm longer than those grown in unheated soil. Although, in all cases, the absolute values of plant length were lowest in the control, the application of both MC—in contrast to soil heating—only tended to increase the plant length.

In accordance with the results for the plant length, the vegetation indices NDVI, PRI, and CRI were significantly affected by soil heating, but not by MC applications. In five out of nine cases, soil heating compared with unheated soil resulted in a significantly higher index value (Table 3). Inoculations with either MC had no effect on any vegetation index at any measurement date.

Table 3. Plant length and vegetation indices (NDVI, PRI, and CRI) calculated from the leaf spectral data of wheat plants grown in heated or unheated soil. Plants were either inoculated once (1×) or twice (2×) with one of the two microbial consortia (MC_A or MC_B), not inoculated (CON), or inoculated with autoclaved MC (MC_A_AC). The table shows the main effects of duplicate trials. Different small letters after values indicate significant differences between plants from each treatment group at each measurement date; Tukey test ($p < 0.05$).

Analysis of Plant Length and Different Vegetation Indices of Wheat Plants											
Main Effects		**Microbial Inoculation**						**Soil Treatment**		**Trial Replication [5]**	
Parameter	**DAS [1]**	**MC_A_1x**	**MC_A_2x**	**MC_B_1x**	**MC_B_2x**	**CON**	**MC_A_AC**	**Unheated Soil**	**Heated Soil**	**Trial 1**	**Trial 2**
Plant length (cm)	28	43.7	44.0	43.7	44.0	43.4	42.9	43.8	43.4	43.1 **b**	44.1 **a**
	42	53.6	54.8	53.6	54.3	53.3	53.8	53.4 **b**	54.3 **a**	53.3 **b**	54.4 **a**
	56	60.1	60.5	61.1	61.0	58.7	59.2	58.8 **b**	61.4 **a**	58.8 **b**	61.3 **a**
NDVI [2]	28	0.574	0.575	0.571	0.561	0.568	0.574	0.555 **b**	0.586 **a**	0.566 **b**	0.575 **a**
	42	0.601	0.601	0.593	0.603	0.595	0.592	0.589 **b**	0.606 **a**	0.607 **a**	0.589 **b**
	56	0.611	0.609	0.605	0.606	0.609	0.608	0.601 **b**	0.615 **a**	0.611 **a**	0.604 **b**
PRI [3]	28	0.031	0.032	0.032	0.030	0.030	0.030	0.030 **b**	0.032 **a**	0.039 **a**	0.023 **b**
	42	0.031	0.032	0.031	0.032	0.032	0.031	0.032	0.031	0.020 **b**	0.043 **a**
	56	0.022	0.022	0.022	0.022	0.023	0.023	0.023	0.022	0.025 **a**	0.021 **b**
CRI [4]	28	2.701	2.718	2.701	2.628	2.687	2.711	2.649 **b**	2.734 **a**	2.436 **b**	2.946 **a**
	42	2.756	2.779	2.735	2.766	2.773	2.714	2.782 **a**	2.726 **b**	2.915 **a**	2.592 **b**
	56	3.093	3.029	3.033	3.041	3.083	3.057	3.134 **a**	2.978 **b**	3.103 **a**	3.009 **b**

[1] DAS: days after sowing; [2] NDVI: Normalized Difference Vegetation Index; [3] PRI: Photochemical Reflectance Index; [4] CRI: Carotenoid Reflectance Index; [5] Each trial had six replicates.

3.2. Plant Growth and Development—Shoot, Root, and Plant Biomass

The shoot, root, and total plant dry weights were significantly affected by soil heating and MC (Figures 1 and 2). In line with the results obtained for the non-invasive parameters, the relative effect of soil heating was stronger than the impact of both MC. Averaged over both trials, the shoot biomass was 53% higher in heated compared with unheated soil (Figure 1). The assumption of higher plant-growth-promoting effects upon MC application in heat-steamed and, thus partly disinfected soil was not confirmed in this experiment. By contrast, significant effects of MC on crop productivity were exclusively noted in the unheated soil. The dual application of novel MC to wheat grown in unheated soil significantly increased early crop growth (shoot (+12%), root (+15%), and total plant biomass (+15%)) compared to the effect of autoclaved MC_A. Furthermore, none of the experimental factors had an effect on plant phenology. At harvest, all plants were in the transition from tillering to stem elongation.

Similar to the results obtained for the shoot dry weight, the root growth was also significantly affected by soil heating and MC. However, in contrast to the shoot biomass, soil heating resulted in significantly lower root biomass (−46%) compared with the unheated soil. The root dry weight of plants inoculated twice with MC_A or MC_B and grown in unheated soil was significantly higher (+15%) compared with the non-inoculated and the autoclaved control (Figure 1). Significant differences in root growth between the single and double application of each MC were not observed. In analogy with the results obtained for the shoot dry weight, no root growth-promoting effects upon MC application were observed in heated soil (Figure 1).

With respect to the total biomass, no significant differences were detected in plants grown in heated soil (Figures 1 and 2). By contrast, in the unheated soil, the plants inoculated twice with MC_A or MC_B achieved significantly higher total plant dry weights compared with all the other treatments. The relative growth increase observed in the plants inoculated twice over the non-inoculated ones was about 15%. The dual inoculation of plants with either MC in unheated soil resulted in the same level of total biomass compared

with the plants cultivated in the heated soil. Hence, the MC were able to compensate for the growth advantage arising from soil heating by favoring root and shoot growth by the same order of magnitude (Figure 2). On average, plants grown in heated soil had an almost three times higher shoot—root ratio (3.2) compared to plants grown in unheated soil (1.1). This equal improvement in shoot and root growth was different from the single and simple nutritional effect of soil heating and, thus, might be an indicator of a long-lasting, resilient, and balanced growth increase. Furthermore, in unheated soil, a strong positive correlation was noted between wheat shoot and root biomass production with respect to the MC application frequency (Figure 2).

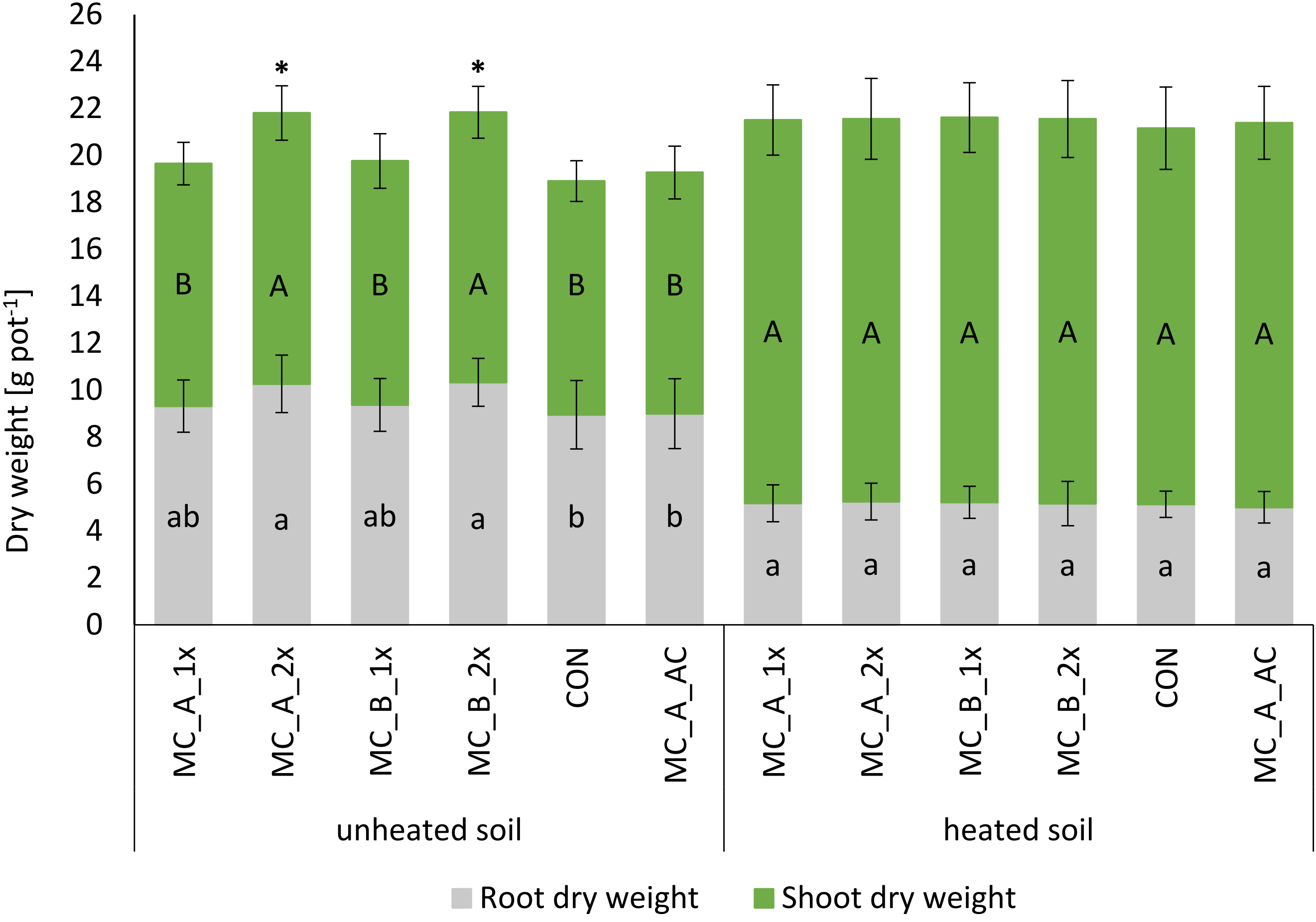

Figure 1. Contribution of shoots (green columns) and roots (gray columns) to total plant dry weight production per pot of wheat grown for 63 days in unheated or heated soil. Plants were either inoculated once (1×) or twice (2×) with one of the two microbial consortia (MC_A or MC_B), not inoculated (CON), or inoculated with autoclaved MC (MC_A_AC). Data represent the mean and standard deviation of duplicate trials with six replicates per trial. Different letters in the columns (small letters: root dry weight; capital letters: shoot dry weight) indicate significant differences between treatments within one soil substrate. Asterisks indicate significant differences in total plant dry weight within one soil treatment; Tukey test ($p < 0.05$).

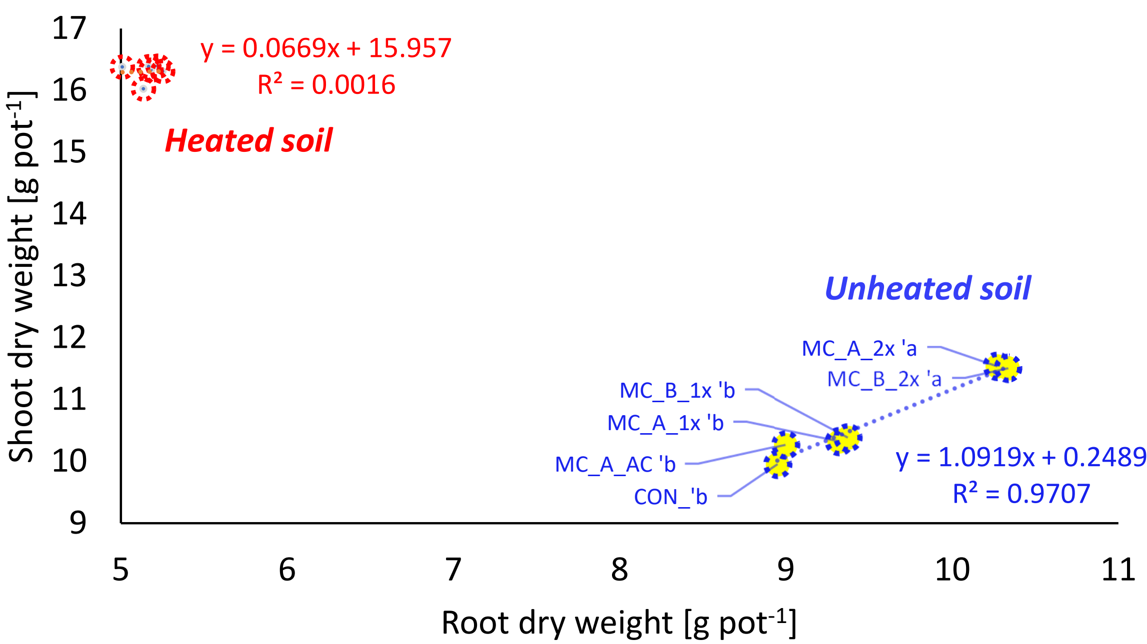

Figure 2. Regression of shoot and root dry weights of wheat plants measured at harvest and aggregated over both trials. Plants grown in heated or unheated soil were either inoculated once (1×) or twice (2×) with one of the two microbial consortia (MC_A or MC_B), not inoculated (CON), or inoculated with autoclaved MC (MC_A_AC). Different small letters (provided only in unheated soil) indicate significant differences in total biomass between treatments; Tukey test ($p < 0.05$).

3.3. Shoot Nutritional Status—Shoot Nutrient Contents and Uptake

The impact of soil heating on N, P, and K shoot nutrient contents (%) and uptake (mg pot^{-1}) was considerably higher than the effect of both MC. Compared to those grown in unheated soil, plants grown in heated soil were characterized by significantly higher shoot nutrient contents for all analyzed elements (Table S2). Accordingly, at harvest, the shoot nutrient contents of plants cultivated in heated soil had—in contrast to those grown in unheated soil—partly increased up to levels laying above the critical thresholds for adequate plant nutrition [43] (Table S2). The impact of both MC on shoot nutrient contents was, however, mostly insignificant, and differed partly between both trial replicates.

The shoot nutrient uptake, calculated by multiplying the shoot nutrient content and biomass, showed largely similar results. The shoot N, P, and K uptake was significantly higher in plants grown in heated compared with unheated soil (Table 4). The shoot N uptake in heated soil was, on average, more than twice as high as in the unheated soil. Likewise, soil heating resulted in a significant increase in shoot P and K uptake, by about 70%, compared to the unheated soil. Even though a double application of MC_A also resulted in a significantly higher N uptake of wheat in unheated soil compared with the non-inoculated control, the additional N uptake upon MC_A application was much lower than the effect of soil heating (Table 4). In contrast to the N uptake, the P uptake was not significantly affected by either MC. The K uptake of the non-inoculated control plants was significantly lower in the second trial compared to all living (i.e., non-autoclaved) MC treatments.

Table 4. Shoot nutrient uptake (mg pot^{-1}) of wheat grown in heated or unheated soil being inoculated once (1×) or twice (2×) with one of the two microbial consortia (MC_A or MC_B), not inoculated (CON), or inoculated with autoclaved MC (MC_A_AC). The data show the mean of the main effects for each trial. Different small letters after values indicate significant differences between treatments; Tukey test ($p < 0.05$).

Wheat Shoot Nutrient Uptake (mg pot^{-1})						
	N Uptake		P Uptake		K Uptake	
Parameters	**Trial 1**	**Trial 2**	**Trial 1**	**Trial 2**	**Trial 1**	**Trial 2**
MC_A_1x	354.9 **bc**	376.5 **abc**	44.6	35.4	746.0	722.8 **a**
MC_A_2x	385.1 **a**	400.0 **a**	47.9	39.4	782.3	742.5 **a**
MC_B_1x	376.5 **ab**	388.1 **ab**	46.4	38.4	788.7	722.0 **a**
MC_B_2x	366.9 **abc**	398.9 **ab**	45.0	40.8	810.8	755.6 **a**
CON	344.5 **c**	340.1 **c**	47.1	38.4	774.7	639.5 **b**
MC_A_AC	346.1 **bc**	352.3 **bc**	48.6	42.6	806.4	694.0 **ab**
Unheated soil	223.1 **b**	207.9 **b**	34.8 **b**	28.6 **b**	557.4 **b**	528.4 **b**
Heated soil	501.6 **a**	544.1 **a**	58.3 **a**	49.7 **a**	1012.2 **a**	897.1 **a**

However, a general and simple chemical (i.e., fertilization) effect based on dead or inactive MC, triggered by their application and carrier material, without any further biological effect, is highly unlikely. The additional nutrient input due to the double application of MC_A and B was, in terms of N, for example, only 1.05 mg and 0.35 mg. Hence, it was low compared with the increase in N uptake by the wheat shoots of up to 48 mg N pot^{-1} (Table 4 and Table S1). Furthermore, we did not observe any significant differences between the autoclaved MC_A and the non-inoculated control in terms of root and shoot biomass (Figure 1) or shoot N uptake (Table 4).

3.4. Litterbag NIRS Analysis to Estimate Micorbial Acitivity

The responses to the microbial inoculation in terms of the LBN variables were strongly affected by the management factors. In Table 5, the LBN variables are ordered by increasing values of their percentage decay from the original hay, starting from the most "r-rapid", ADL (−41%), to the most "k-constant", ash (+201%), for all the observations. The trial effect on the LBN variables was more pronounced than the soil heating effect. Six variables increased and seven decreased in trial 2, while only three and two in heated soil, respectively (Table 5). The average values of the r- and k-variables, as well as the contrasts (inoculation vs. control), indicate that the heated substrate in the control determined the strong transformation activity of the litter, while the microbial treatments instead buffered this activity (data not shown).

The MC application in inoculated plants, compared to the non-inoculated control plants, delayed the decrease in the rapid r-variables in four cases and increased the decay of the constant ones in two cases. In short, analyzing the r- and k-variables, the inoculation reduced both the r (−0.9), k (−0.5), and their difference (−0.4), thus showing a clear sign of buffering activity in the decay of the litterbags. The autoclaved inoculum acted in a similar way to the non-inoculated control. However, the k-fingerprint variables were somewhat less affected (Table 5).

Table 5. Composition of the litter-hay and of the degraded litter predicted from the litterbag-NIRS method, contrasts between the average microbial consortia (MC) and the control (CON) in unheated and heated substrate, including the ANOVA results (n = 240). The variables are ordered by % of decay from hay to litter.

					LS Means for Microbial Consortia (MC) Treatments						Contrasts		Experimental Effects	
AK [1]	**T** [2]	**Variables** [3]	**Hay**	**Decay**	**MC_ A_1x**	**MC_ A_2x**	**MC_ B_1x**	**MC_ B_2x**	**CON**	**MC_ A_AC**	**MC** [5] **vs. CON**	**MC_A_AC vs. CON**	**Trial 2 vs. GM** [6] **in %**	**Heated vs. GM** [6] **in %**
ADL	r	Acid Detergent Lignin	13.03	−41%	7.80	7.69	8.13	7.56	7.11	7.74	0.68 *	0.63 *	−5.0%	−1.7%
CF	r	Crude Fiber	21.54	−38%	13.48	13.43	14.26	12.87	12.02	13.76	1.49	1.74	−10%*	−3.3%
ADF	r	Acid Detergent Fiber	37.96	−22%	31.00	29.13	31.51	28.90	27.80	29.75	2.33 *	1.95	−3.2%	−1.2%
NDFD	r	NDF Digestibility	67.52	−17%	55.67	55.46	56.90	55.38	54.83	56.55	1.02	1.72 *	1.4%*	−0.9%
DNDF	r	Digestible NDF	30.40	−17%	25.31	25.23	25.90	25.22	24.89	25.71	0.52	0.82 *	1.3%*	−0.9%
Cell	r	Cellulose	24.44	−10%	23.19	21.44	23.38	21.34	20.69	22.01	1.65 *	1.32	−2.6%	−1.1%
E	r	Gross Energy [4]	16.95	−6%	16.00	15.97	16.06	15.98	15.88	15.98	0.12 *	0.09	−0.3%	−0.1%
EE	r	Ether Extract	2.98	−5%	2.83	2.82	2.82	2.81	2.84	2.83	−0.02	−0.01	−0.5%	−0.1%
NDF	k	Neutral Detergent Fiber	45.02	1%	45.46	45.48	45.52	45.54	45.40	45.47	0.10 *	0.07	0.0%	0.0%
NFE	k	N-Free Extract	45.92	6%	48.53	48.65	48.28	48.60	48.70	48.41	−0.19	−0.30	0.7%*	0.2% *
IVTD	k	Total Digestibility	67.58	20%	80.77	81.66	80.92	81.76	81.50	81.28	−0.22	−0.22	0.2%	0.1% *
INDF	k	Indigestible NDF	14.62	37%	20.15	20.25	19.62	20.32	20.51	19.75	−0.42	−0.75 *	−1.8%*	1.2% *
CP	k	Crude Protein	7.88	65%	12.64	13.27	12.60	13.20	13.34	12.99	−0.41	−0.34	1.0%	0.5%
CMI	k	Crop Mat. Index	0.45	120%	0.98	0.98	1.01	0.96	1.00	0.96	−0.01	−0.04	−1.7%	1.5%*
Hemi	k	Hemicellulose	7.06	124%	14.46	16.35	14.01	16.64	17.60	15.72	−2.2 *	−1.88	6.1%	2.5%
Ash	k	Ash	6.05	201%	17.80	18.33	17.44	18.62	19.10	18.15	−1.1 *	−0.95	3.6%	1.3% *
r	m	r_Fingerprinting			4.94	5.46	4.48	5.59	6.09	5.06	−0.97 *	−1.03 *	5.4%*	4.8% *
k	m	k_Fingerprinting			5.78	6.30	5.60	6.38	6.57	6.02	−0.55 *	−0.55	3.5%	2.2% *
r-k	m	r-to-k Difference			−0.83	−0.84	−1.12	−0.79	−0.48	−0.96	−0.42 *	−0.48 *	−106%*	−145%*

[1] Acronyms; [2] r = rapid variable, k = constant variable, m = microbial variable; [3] %DM or %; [4] values in MJ kg/DM; [5] MC = average effect of four microbial consortia; [6] GM = general mean; * $p < 0.05$.

4. Discussion

4.1. Effects of Microbial Consortia on Crop Growth

The growth-promoting effects of both MC were most evident in the increased root and shoot biomass. Beneficial microbial effects on crop growth have been reported for numerous plant species grown under various environmental conditions [11–13,15,44–47]. Among the most frequently reported mechanisms by which bacterial strains similar to those used in our MC can enhance plant growth is an improved plant nutrition due to N_2 fixation and the solubilization and mobilization of P. Furthermore, increases in enzyme activity in the rhizoplane and the production and secretion of plant hormones such as, for example, auxin derivates, into the rhizosphere upon MC application are repeatedly reported in the literature [12–15,22,44]. Based on a pre-screening of the applied strains, as described in detail by Tabacchioni et al. [22], every strain used for the formulation of our MC had a proven mode of action to promote plant growth (see also Table 1). Apart from the evident effect of nitrogen on crop growth, it can be assumed that other mechanisms, such as those mentioned above, influenced our results as well [12,15,47].

Microbially induced root growth increases similar to those observed in this study were frequently reported for numerous crop species subsequent to inoculation with different microbial strains [15,44,45,47–49]. The production of higher root biomass is also usually closely connected to a plethora of beneficial morphological changes in root architecture, leading to an increased root surface area [50,51]. Since shoot and root growth were equally affected by the MC, the shoot–root ratio of inoculated wheat plants remained unchanged (Figure 2). This result is in accordance with the observations of other authors using the same microbial genera and wheat as a host plant [45,52,53].

Microbially induced improvements in plant nutrition (content and uptake) can differ in origin. Some microbial genera are known to promote plant nutrition by decreasing plants´ dependency on soil nutrients (e.g., biological N_2 fixation by *A. chroococcum* [54,55] and *A. vinelandii* [56,57] strains or *P. tropica* [22] strains), while others increase their availability in the soil (e.g., the solubilization of phosphates by *A. chroococcum* strains [46,58]

or *T. harzianum* [59,60] strains) or facilitate plants' access to nutrients (e.g., by increasing the root surface area through microbially mediated changes in the plant's hormonal balance [50]) [4,9]. Compared with the non-inoculated controls, the plants that received a double application of MC_A achieved a significantly higher shoot N uptake in both trials (Table 4). As the shoot N content of the inoculated plants tended to be higher as well, the higher shoot N uptake induced by MC application is assumed to have been a combined effect of both changes. For *A. chroococcum*, *A. vinelandii*, and *P. tropica*, increasing the bioavailability of N is known to be a major mechanism inducing plant growth [54,55,57,61]. After a growing period of two months, the plants treated twice with either MC averaged an approximately 13% higher N uptake compared to the non-inoculated plants over both trials (Table 4). This improved N supply is probably the key factor in explaining the increased crop growth. However, it is still unclear as to how far this effect is relevant over a whole growing season. Under field conditions, the quantity of direct nitrogen supply via mutualistic rhizobacteria (e.g., *A. chroococcum* or *A. vinelandii* strains) tends to be comparatively low [7], usually providing less than 20 kg N ha^{-1} $year^{-1}$ [62].

In contrast to that of nitrogen, the wheat shoot P content and uptake were not affected by MC application, contradicting various other studies using the same species [4,12,15,44]. The ability of different microbial strains belonging to the same species to improve plant P nutrition can vary markedly [13,45]. The significant impact of MC on the K content and uptake of plants is most likely to have been due to the nutritional load stemming from the zeolite, the carrier material for the microbial strains (see Table S1). Since the K uptake did not differ between plants that received the viable MC and those that received the autoclaved consortia, the necessity of considering both the autoclaved microbial material and the carrier as potential and readily available nutrient sources became evident in this study.

The data collected for all non-invasive parameters showed no response to inoculation with either MC. Although they were statistically insignificant, the longest plant lengths were found in plants treated twice with either consortium. These results are in accordance with the findings of Javaid et al. [16], who observed only slight effects of a mixture of different "effective microorganisms" on wheat plant length at 60 and 150 DAS. By contrast, other studies using different single strains—among them the genera of *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Azotobacter*, and *Stenotrophomonas*—partly observed significantly higher wheat plant heights compared with non-inoculated plants [13,15,46]. Plant vitality and robustness, as indicated by the NDVI, seemed not to be affected by MC application [63].

The lack of effects of the tested MC in heat-steamed soil was probably due to the overcompensation of MC effects by the release of nutrients induced by soil heating (see Section 4.3). It is well documented that with increasing fertilization rates, the beneficial effects of PGPMs tend to be masked [48,64–67]. With the increased application of a mineral NPK fertilizer up to its recommended dose for wheat, Shaharoona et al. [67] observed a linear decrease in the growth-promoting effect of *P. fluorescens*. In accordance with our results, the PGP effects of different PGPMs tend to be more pronounced under poor soil-fertility conditions [48] or more specifically, they tend to be higher in challenging or harsh environments with a strong differentiation in growth conditions [11]. In addition, achieving successful root–microbe interactions is highly dependent on the conditions created by hospitable microbes in the soil. A recurring obstacle and current challenge is the basic obstinacy of the soil ecosystem that often functions as a buffer against any introduced or incoming (micro)organism [68].

4.2. Effects of Microbial Consortia on Litterbag Microbial Activity and Traceability

Despite considerable advances in the exploration of the soil microbiome, rapid and cost-effective methods for the evaluation of soil microbiota and their activity are still lacking [28]. The litterbag-NIRS method is a relatively new approach for the indirect characterization and tracing of MC as biofertilizer in terms of soil and litter (degradation) microbial activity [27]. However, it must be assumed that the bio-variability of litterbags is not equal to that of soil [36]. Nonetheless, and regardless of potential differences in

bio-variability, the results obtained by using the LBN method can help to analyze the general impact of artificially applied MC on the microbial activity and dynamics within soil. The accelerated degradation of soil organic matter and, thus, improvements in soil fertility and plant nutrition are, along with direct PGP mechanisms, potential benefits of MC applications [28,36].

As indicated by the qualitative decay of hay to litter, the two MC significantly buffered the native microbes' activities when compared to the semi-sterility conditions, in which the autochthonous microflora of the litterbag (non-sterilized) enhanced their degradation activity. The results observed in our study contradict, in part, the observations of Baldi et al. [36], who found that the application of *Trichoderma* ssp. based microbial inoculants induced a faster and higher degradation of litter in apricot orchards compared to mycorrhizal inoculants and controls. The authors assumed that PGPMs such as *T. harzianum* [36], which accelerate the degradation of the fiber components of organic materials (i.e., PGPMs that have high-digestibility properties), also tend to favor the growth of plants the most [28]. The significantly higher degradation in the orchards with a test duration of one year was also accompanied by changes in the microbial community of the soil and litter monitored by NGS metabarcoding. In this context, it was also reported that, in addition to the time of the litterbag's burial in the soil, the quality (mainly the C/N ratio) of the 'starting material' and the composition of the microbial inoculum can have a strong impact on the results, potentially explaining the contrasting observations between the different experiments [36].

4.3. Effects of Soil Heating on Crop Growth

For all assessed growth parameters, the impact of soil heating was significantly higher than the microbially induced growth increase. It is well documented that soil heating using humid heat affects the chemical and physical properties of soil and causes considerable shifts in the abundance and composition of the autochthonous soil microbiome [69–71]. Increased crop growth and nutrient supply upon soil heating have been reported in several pot experiments for different crop species, including, for example, *Zea mays* L. [72] or *Plantago lanceolata* [73]. Moreover, soil heating with subsequent *Triticum aestivum* cultivation caused an early established growth advantage that persisted over the whole life cycle, resulting in a significant increase in grain yield [16].

Interestingly, in most of the aforementioned studies, root growth at early growth stages was also significantly increased after soil heating [16,73], while in this study, we noted a decreased root–shoot ratio. The decreased root growth in heated soil could be explained, at least in part, by an improved availability of soil nutrients [71,73]. Plants that are well supplied with water and nutrients, particularly nitrogen, tend to invest in shoot rather than root growth, which favors the generative growth [74–76]. This observation is in line with our finding of a threefold higher shoot–root ratio in plants grown in heated, compared with unheated, soil.

The enhancement of soil nutrient contents and, thus, of plant nutrient uptake induced by soil heating to about 100 °C for ca. 12 h is well known and especially pronounced in terms of nitrogen (ammonium and nitrate) and manganese [71–73,77,78]. A positive impact of soil heating on phosphorous [73] and potassium uptake [71,78] is also frequently reported in the literature. The increased shoot nutrient contents of plants grown in heated soil, in particular of N, can also explain higher plant length and vegetation indices [63]. The results obtained for the plant length are in accordance with the 25% increase in height observed by Javaid et al. [16] in wheat plants 60 DAS.

5. Conclusions

Our study showed that the application of selected microbial consortia with different functional properties can promote the early growth of both the shoots and roots of wheat. The higher shoot growth was probably mainly due to the improved nitrogen supply induced by the application of MC, while the root growth promotion remains unexplained. The tested MC thus satisfy a necessary, but not sufficient, condition for practical use in

farming. To achieve this, field trials over the whole crop cycle are necessary. Furthermore, our research underlines the importance of including autoclaved positive controls of MC applications, which is not currently a typical practice in this type of experiment. The LBN method helped to trace the functional changes in the litterbag microbial activity that occurred in response to inoculation, allowing a clear distinction between MC_A and the controls. Tracing the MC applications with the LBN method is a first step towards the monitoring of both functional changes in soil microbial activity and the fate of inoculants. However, a more precise monitoring of MC applications still requires future support from a detailed metagenomic analysis of the rhizoplane and rhizosphere. Identifying the environmental constraints of PGPM applications and developing targeted solutions could provide a major step towards the successful integration of MC in agriculture.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy12040899/s1, Table S1: Nutritional loads per pot arising from the application of novel microbial consortia and biochar, Table S2: Shoot nutrient contents (%) of wheat plants grown in heated or unheated soil being inoculated once (1×) or twice (2×) with one of the two microbial consortia (MC_A or MC_B), not inoculated (CON), or inoculated with autoclaved MC (MC_C_AC). The data show the mean of the main effects for each trial. Different small letters after values indicate significant differences between treatments; Tukey test ($p < 0.05$). Reference [43] is cited in the supplementary materials.

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