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Alternative Starter Fertilization Strategies in Maize (*Zea mays* L.) Cultivation: Agronomic Potential of Microgranular Fertilizer and Plant Growth-Promoting Microorganisms and Their Impact on the Soil Native Microbial Community

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Abstract: Phosphorous (P) starter fertilization can increase maize (*Zea mays* L.) yield. Widespread application in soils with sufficient P availability leads to environmental risks. Subsequently, alternative strategies to support the maize plant's early development are needed to lower P surpluses. Here, we conducted field experiments comparing standard starter fertilizer diammonium phosphate (DAP) (20.1 kg P ha^{-1}) to microgranular fertilizer (MG) (2.4 kg P ha^{-1}) and combined in-furrow inoculation with *Bacillus atrophaeus* and mycorrhizal fungi (*Rhizoglomus irregulare*, *Funneliformis mosseae*, and *Funneliformis caledonium*), alone and in combination. The soil microbial community inside and between the maize rows was monitored by quantitative PCR (qPCR)-based quantification of eight fungal and bacterial groups. The yield did not vary between fertilization with DAP or MG and no fertilizer control. The combined microorganism inoculum (MO), however, enhanced the yield by 4.2%. The soil microbial community composition was not affected by the MO application. However, on one field site and inside the rows, it leads to a significant increase in overall microbial gene copy numbers by 9.3% and a significant decrease in the relative abundance of the bacterial phylum of *Bacillota* (*Firmicutes*) by 18%. The in-furrow MO application is thus a promising option for starter fertilizer replacement.

Keywords: phosphorous fertilization; starter fertilizer; plant growth-promoting microorganisms; arbuscular mycorrhizal fungi; maize; *Firmicutes*; *Acidobacteria*; *Bacillus atrophaeus*

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1. Introduction

Modern agriculture in Western Europe is characterized by high yields based on high inputs of energy and nutrients. The yearly phosphorous (P) surpluses applied to crops and grasslands in Germany in the past have led to elevated soil P concentrations, especially in areas with high livestock density [1,2]. At present, German agricultural soil surface P balance has been estimated to be roughly neutral on average, while the north-western regions with high livestock density still feature annual P surpluses of up to >10 kg P ha^{-1} [1,3]. High soil P levels and excessive P application fuel massive nutrient losses and environmental hazards like the eutrophication of surface water bodies [1,4]. Additionally, P is a limited resource whose reserves are unequally distributed worldwide, leading to risks for global food security [5,6]. It is thus of the utmost importance to reduce P fertilizer inputs, especially in regions already exhibiting high soil nutrient levels, and to close P cycles.

In maize (*Zea mays* L.) cultivation, starter fertilization is used to guarantee a sufficient supply of P in the early growth stages of the plant when its root system is still small. It is

usually applied at sowing in a band of 5 cm beneath and 5 cm beside and below the furrow (5 × 5 application). The amount of P applied with the starter fertilizer can vary depending on soil P concentrations. The effect of starter fertilization on yield depends on many factors, leading to inconsistent study results. While some studies find yield-increasing effects of starter fertilizer [7,8], others do not [9,10]. A recent meta-analysis has shown that starter fertilization increases maize yield by 5.2% on average, while this effect diminishes with rising soil test P levels [11]. The yield increase after starter fertilization or whole-field P fertilization reaches values below 1% at soil test P levels of over 15 mg 100 g^{-1} soil (soil test class E) [11,12]. In Germany, starter fertilization is a widespread practice, even in areas with high P-testing soils. It is thus important to investigate and promote the omission of starter fertilization or replacement with low-nutrient-input alternatives to lower P inputs in soils.

One strategy to reduce the nutrient input is the use of in-furrow starter fertilizers that are directly applied beneath the seed at less than half the N and P application rates of standard 5 × 5 band fertilization [11]. In-furrow starter fertilization has been shown to be equally effective in increasing maize grain yield compared to the 5 × 5 band fertilization when the principal nitrogen (N) fertilizer is given before or at sowing [8,11]. Organomineral microgranular fertilizers (MG) manufactured from waste materials like bone and whey protein have been proposed as in-furrow starter fertilizers. They have been shown to increase the yield and root density of young maize plants in pot experiments [13,14]. Two field studies have found maize yields after MG starter fertilization to be about equal to yields with standard diammonium phosphate (DAP) starter fertilization [15,16]. The widespread usage of MG as a starter fertilizer in maize could simultaneously lower nutrient inputs and be a step towards closing nutrient cycles.

Another strategy to reduce nutrient inputs while maintaining high yields is to make the nutrients in the soil more accessible for the crop. Two options to influence the nutrient availability of the plant are changing the soil pH and applying plant-beneficial microorganisms. Meta-analyses have shown that inoculation with arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) increases cereal yield in field experiments. They reported a maize grain yield increase (13%) after AMF inoculation [17] and a cereal yield increase (13%) after "biofertilizer" (AMF and PGPR) application, with both P and N use efficiency increasing by around 6 kg yield kg^{-1} N or P [18]. The reported yield increases could be due to increased nutrient availability mediated by the inoculated microorganisms or other plant growth-promoting mechanisms. Plant-associated microbes influence plant growth through the production of phytohormones and signaling molecules, fixation of N, mobilization of P and minerals, pathogen suppression, and stimulation of the plant immune system [19–21]. Subsequently, the application of PGPR has been shown to influence root biomass and architecture, influence plant development timepoints, enhance resistance to abiotic and biotic stressors, and even trigger induced systemic resistance against pathogens by activating the pattern recognition receptors [22–26]. AMF and PGPR can be applied in the furrow at sowing and are thus a promising option to act as a partial or complete replacement for starter fertilization.

The soil and rhizosphere microbiome is a complex network composed of a myriad of different fungi, bacteria, oomycetes, and archaea. The bulk soil microbiome is shaped by many different parameters, like soil texture, temperature, moisture, pH, organic matter content, and soil amendments like organic or inorganic fertilizers [27–30]. The rhizosphere microbiome depends on the bulk soil microbiome [31] and is additionally influenced by plant species, genotype, and development stage [19,32–37]. This becomes more complex as one contemplates the network character of the soil and rhizosphere microbiome. For example, AMF interacts with "mycorrhiza helper bacteria" and creates their own hyphosphere, which is mediated by hyphal exudates [38,39]. A recent review showed that fungal and bacterial inoculants have the capacity to either increase or decrease the diversity of native fungal and bacterial communities, while in almost half of the reviewed studies, the native communities' diversity was not changed following inoculation [40]. Only a few

studies have been published on the effect of inoculation with *Bacillus* strains or consortia containing *Bacilli* on the native soil microbial community [41].

Changes in the soil microbial community can regularly be detected at the phyla and class levels [42–44]. Often, next-generation sequencing is used to measure the relative abundance of bacterial phyla or classes. Quantitative real-time PCR is a cost-effective alternative and has been shown to detect changes in the soil microbial community due to, for example, different environments [45], cattle impacts [46], additions of biochar and readily available carbon [47], and C-mineralization rates [48].

There are few studies investigating the agronomic potential of MG as a starter fertilizer in the field. Also, there are very few studies using in-furrow inoculation with AMF and PGPR [15,16,49,50] as a replacement for starter fertilization. Little is known about the impact of AMF and PGPR inoculation on the native soil microbial community [41], although it plays a pivotal role in nutrient cycles and is an important factor in soil quality.

This study aimed to investigate the potential of starter fertilization with MG and a combined inoculum containing PGBP and AMF to substitute the standard DAP starter fertilization. A second objective was to shed some light on the impact of amendments to DAP, MG, and the microbial inoculum on the native soil microbial community and add to the general understanding of the soil microbiome in the field. Field experiments with a randomized plot design were conducted at two field sites in Wanna, Lower Saxony, Germany. The soil microbial community was monitored by quantitative polymerase chain reaction (qPCR)-based quantification of eight fungal and bacterial groups.

2. Materials and Methods

2.1. Site Description and Weather Data

The experiment was conducted at two field sites near Wanna (Lower Saxony, Germany), "Wanna Sand" (WS) (53.751389, 8.763083) and "Wanna Sand without biogas slurry" (WSo) (53.741333, 8.817417). The WS site was a deep podzol-gley with a 30 cm deep A-horizon, and the WSo site was a plaggic anthrosol above a podzol with a 70 cm deep A/E-horizon. The soil type is weakly silty sand (Su2 in the German Bodenartendiagramm (BK 1994)) at both sites (NIBIS Kartenserver, https://nibis.lbeg.de/cardomap3/, accessed on 25 October 2023). The WS site received a regular application of biogas slurry in the years before the experiment setup, while the WSo site did not. Soil parameters after the biogas application but before the experimental setup are summarized in Table 1.

Table 1. Soil parameters of the "Wanna Sand without biogas slurry" (WSo) and "Wanna Sand" (WS) sites before the experimental setup (after biogas digestate application to the WS site) (17 April 2021).

Site	**WS**			**WSo**		
Depth [cm]	**0–30**	**30–60**	**60–90**	**0–30**	**30–60**	**60–90**
TOC [%]	3.16 ± 0.30	2.19 ± 1.04	0.83 ± 0.49	2.57 ± 0.29	1.21 ± 0.12	0.44 ± 0.14
N_{tot} [%]	0.21 ± 0.02	0.12 ± 0.07	0.06 ± 0.01	0.23 ± 0.04	0.12 ± 0.03	0.06 ± 0.02
N_{min} [kg ha^{-1}]	102.3 ± 17.1	32.0 ± 9.3	12.7 ± 2.1	57.4 ± 29.9	47.7 ± 19.7	2.2 ± 0.84
P_{DL} [mg 100 g^{-1} soil]	19.1 ± 1.1	10.4 ± 4.8	4.7 ± 6.6	20.1 ± 1.9	20.0 ± 3.2	13.3 ± 4.2
K_{DL} [mg 100 g^{-1} soil]	22.8 ± 2.1	14.4 ± 3.5	8.9 ± 3.5	7.5 ± 2.5	5.0 ± 1.7	3.2 ± 0.6
Mg_{DL} [mg 100 g^{-1} soil]	9.4 ± 1.3	5.6 ± 0.6	3.5 ± 1.6	6.4 ± 2.0	5.0 ± 2.0	4.5 ± 2.1
pH	5.16 ± 0.08	5.37 ± 0.08	5.47 ± 0.08	4.61 ± 0.09	4.56 ± 0.15	4.66 ± 0.08

TOC—total organic carbon; N_{tot}—total nitrogen; N_{min}—mineral nitrogen; P_{DL}—plant-available phosphorous; K_{DL}—plant-available potassium; Mg_{DL}—plant-available magnesium.

The region is classified as having a European Atlantic climate [51], characterized by mild winters and moderate summer temperatures. The average precipitation per year for Wanna is 735 mm, and the average annual temperature is 9.9 °C. Usually, more than 45% of

annual precipitation falls during the maize crop season from April to September. In April and May, the soil temperatures are often low due to cold night temperatures, which slows down early plant development. The weather data of the experimental year (2021) from the nearest weather station in Steinau (Lower Saxony, Germany) are displayed in Figure S1.

2.2. Preparation of Combined Plant-Beneficial Microorganism Inoculum

The combined microorganism inoculum (MO) was prepared with two different carrier materials: MG and bentonite. The commercially available AMF product INOQ Advantage Pellets (INOQ GmbH, Schnega, Germany) containing the three species *Rhizoglomus irregulare*, *Funneliformis mosseae*, and *Funneliformis caledonium* (144 propagules (ppg) g^{-1}) was ground to obtain a particle size similar to the particle sizes of MG and bentonite. The ground mycorrhiza product was then mixed together with MG or bentonite to reach a concentration of 0.12–0.128 Mio ppg per kg. The resulting mixtures were then sprayed with the commercially available product RhizoVital C5 (Abitep GmbH, Berlin, Germany) containing the strain *Bacillus atrophaeus* Abi05 (DSM 29418) (3.47×10^{10} cells ml^{-1}) at a rate of 0.08 L per kg. The product was then air-dried and mixed again.

2.3. Experiment Setup and Field Treatments

At the WS site, biogas slurry containing 4.3 kg m^{-3} total N, 0.56 kg m^{-3} of P, and 4.3 kg m^{-3} of potassium (K) was applied before sowing and experiment setup at a rate of 30 m^3 ha^{-1}, resulting in a nutrient input of 129.0 kg N ha^{-1}, 16.9 kg P ha^{-1} and 128.3 kg K ha^{-1}. The WSo site did not receive biogas slurry.

Startec microgranular fertilizer (De Ceuster Meststoffen NV (DCM), Bannerlaan 79, 2280 Grobbendonk, Belgium) was used as an MG. It can be classified as an organomineral fertilizer, of which 80% (of the original substance) is made up of the organic industrial by-products oil cake and deglued bone meal. The added mineral components comprise ammonium phosphate, ammonium sulfate, ethylenediaminetetraacetic acid chelated iron, manganese, zinc, zinc sulfate, and zinc oxide. The resulting product contains 7.5% nitrogen, 9.6% P, 3.3% K, 4% sulfur, 1.5% zinc, 0.5% iron and 0.5% manganese. DAP is a standard mineral fertilizer produced from rock phosphate and contains 18.0% total nitrogen and 20.1% P.

The plot experiment set up on each field site comprised six different treatments: control without starter fertilization or MO application, DAP, MG, MO, DAP + MO, and MG + MO. Each treatment was carried out with fivefold repetition in 8×20 m^2 sized plots following a randomized design. Application rates of starter fertilizers and MO, including the resulting nutrient inputs for each treatment, are summarized in Table 2.

Table 2. Rates of fertilizer and inoculum application and the resulting nutrient inputs.

Treatment	Fertilizer/Inoculum	Application Rate	N Input [kg ha^{-1}]	P Input [kg ha^{-1}]	K Input [kg ha^{-1}]
DAP	DAP	100 kg ha^{-1}	18.0	20.1	-
MG	MG	25 kg ha^{-1}	1.9	2.4	0.8
MO	MO inoculum in bentonite	25 kg ha^{-1}	-	-	-
MG + MO	MO inoculum in MG	25 kg ha^{-1}	1.9	2.4	0.8
DAP + MO	DAP	100 kg ha^{-1}	18.0	20.1	-
	MO inoculum in bentonite	25 kg ha^{-1}	-	-	-

MO—application of a combined inoculum containing three species of AMF and *B. atrophaeus*; DAP—starter fertilization with diammonium phosphate; MG—starter fertilization with organomineral microgranular fertilizer.

The experiment was conducted in the growing season of 2021. The AMAZONE single-grain seeder system (EDX 6000-2C precision air-seeder, Amazonen-Werke H. Dreyer SE Co. KG, Hasbergen, Germany) was used for simultaneous sowing, the application of starter fertilization and the application of microorganisms on 20 April 2021. The maize cultivar Amaroc S230 was sown with an 80 cm row distance and 85,000 seeds ha^{-1}. DAP was

applied in a band 5 cm below and 5 cm beside the grain. MG and MO were applied in the furrow a few centimeters beneath the grain. The application rate of MO resulted in an application rate of 6.94×10^{13} cells ha^{-1} *Bacillus atrophaeus Abi05* and 3–3.2 Mio ppg ha^{-1}.

According to good agricultural practice, both sites were treated with the herbicides Spectrum Gold, Motivell Forte, Nagano, and Callisto at rates of 1.3 L ha^{-1}, 0.5 L ha^{-1}, 0.5 L $ha^{-1,}$ and 0.5 L ha^{-1}, respectively. The harvest took place with a Krone Big X 780 forage harvester in the version OptiMaize (Maschinenfabrik Bernard KRONE GmbH & Co. KG, Spelle, Germany) with integrated logging of fresh mass yield and several characteristics of the harvested crops via a near-infrared spectroscopy (NIR) sensor. The NIR-Scanner model was the NIR SmartSensor Krone (Dinamica Generale S.p.A., Poggio Rusco (MN), Italy).

2.4. Soil and Root Sampling

Soil samples were taken at three sampling dates in two sampling regimes. The sampling dates were t_2 (7 June 21), t_3 (20 July 21/21 July 21), and t_4 (16 August 21/17 August 21). The development stages of the maize plants according to the German Biologische Bundesanstalt für Land- und Forstwirtschaft, Bundessortenamt und Chemische Industrie (BBCH) scale [52] were 13–19, 33–35, and 69, respectively. In one sampling regime, four soil cores of 0–30 cm depth were taken in each plot near the maize rows and mixed thoroughly to form one sample per plot. This sampling regime was performed at sampling dates t_2 and $t_{3,}$ and the resulting samples were used to analyze the nutrient concentrations in the soil. In the second sampling regime, six soil cores of depth 0–30 cm were taken from a small area approximately in the middle of each plot. Three of them were taken in the maize row between four neighboring plants and thus inside the application area of the starter fertilizer and plant-beneficial microorganisms. The other three samples were taken in direct proximity to the first three samples, but in the middle between two rows and thus outside the application area. This sampling regime was performed on sampling dates t_3 and $t_{4,}$ and the resulting samples were used for the measurement of soil dry mass content and deoxyribonucleic acid (DNA) extraction. Along with the second sampling regime at sampling date t_3, the rootstock of five plants from each treatment was collected from the area of soil sampling.

The soil samples were cooled directly at the site and frozen at −20 °C upon arrival at the laboratory until further preparation. The rootstocks were transported without cooling and cooled at 5 °C upon arrival at the laboratory. Within one week of sample collection, a small heap of fine roots was collected from each rootstock, washed with tap water, and stored in 2 mL tubes filled with deionized water at −20 °C until further use.

2.5. Measurement of Soil Nutrient Concentrations

The plant available P concentration (P_{DL}) and mineral N content (N_{min}) in the soil were measured according to the standard protocol from the Association of German Agricultural Analytic and Research Institutes (VDLUFA, Speyer, Germany). To measure P_{DL}, 4 g of air-dried and sieved (2 mm) soil samples were extracted with 200 mL of double-lactate solution (calcium lactate (0.4 M $C_6H_{10}CaO_6$ * 5 H_2O)) and hydrochloric acid (0.5 M HCl) at pH 3.6. The suspension was shaken overhead for 90 min at 35 rpm. After filtration (cellulose round filter, from 3 to 5 µm), the P_{DL} concentration was determined via inductively coupled plasma–optical emission spectrometry (ICP-OES) (Optima 8300 D, PerkinElmer, Waltham, MA, USA). To determine N_{min}, 20 g of fresh soil was suspended in 200 mL of a 12.5 mM $CaCl_2$ solution and shaken overhead for 2 h. Soil extracts were analyzed for nitrate and ammonium via continuous flow analysis (AutoAnalyzer AA3, Seal Analytical, King's Lynn, UK).

2.6. Measurement of Mycorrhizal Colonisation Rate

The roots were stained using a modification of the method described by Vierheilig and Piché [53]. The roots were thawed at room temperature, then immersed in potassium hydroxide (10%) and shaken overnight in a New Brunswick Innova® 42 incubator shaker

(Eppendorf SE, Hamburg, Germany) at 50 °C. The roots were then rinsed with tap water several times and immersed in staining liquid (1:1:1 ratio of distilled water, glycerol, and DL-Lactic acid, 0.05% methyl blue). The flasks containing the roots and staining liquid were boiled in a water bath for 3–4 min. After staining, the roots were rinsed with tap water and stored in 100 µL lactoglycerol at 5 °C. The root mycorrhizal colonization rate was measured by the grid intersect method, as described by Giovannetti and Mosse [54], counting 100 intersects per sample at a SteREO Discovery.V20 microscope (ZEISS, Oberkochen, Germany) with 265× magnification.

2.7. Measurement of Dry Mass Content and DNA Extraction

Thawed soil samples were thoroughly mixed in the sample bag with a sterile spatula. The dry mass content of each sample was measured by drying approximately 5 g of soil at 70 °C in a drying cabinet. The samples were processed along with samples from organic soils not included in this study, leading to the choice of a 70 °C drying temperature [55].

A total of 500 g of fresh soil was used for DNA extraction with the NucleoSpin™ Soil Kit (Macherey Nagel™, Düren, Germany) using the lysis buffer SL1 and no enhancer. The bead tubes were shaken at 5G for 2 × 30 s in a FastPrep-24™ 5G homogenizer (MP Biomedicals, Santa Ana, CA, USA). Before the elution step, the membranes were left to dry at room temperature for 10 min. The DNA was eluted in two steps with 30 µL and 15 µL of the elution buffer. The DNA concentration and A260/A280 ratio of each sample were measured in duplicate with the NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The A260/A280 ratio was above 1.8 for all samples. The DNA yield was between approximately 75 ng μL^{-1} and 200 ng μL^{-1}.

2.8. qPCR Analysis

The extracted DNA was used in nine different qPCR assays to quantify the abundance of six bacterial taxa (*Acidobacteria*, *Actinobacteria*, *α-Proteobacteria*, *β-Proteobacteria*, *Firmicutes*, and *Cyanobacteria*), the total fungi (ITS), the fungal genus *Fusarium*, and the inoculated bacterial strain *Bacillus atrophaeus* Abi05 in each soil sample. The qPCR conditions of all assays are summarized in Table A1 in Appendix A. The inoculated strain was quantified by qPCR with primers designed by ABiTEP GmbH. The primers are specific to the strain Abi05 and are able to discriminate it from other *B. atrophaeus* strains. The qPCR assays were conducted in polypropylene 96-well plates using the QuantStudio™ 12K Flex Real-Time PCR System (Life Technologies, Grand Island, NY, USA). Two technical replicate measurements were performed for each assay and DNA sample. If the measured abundance differed by more than 20% between the replicates, two more replications were performed.

The calibration curves were calculated from a series of dilutions of standard-strain DNA on each plate. Each dilution series consisted of at least five steps and spanned the complete range of template concentrations. The standard strains were bacterial or fungal strains sourced from culture collections of microorganisms of colleagues and the DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Inhoffenstraße 7 B, 38,124 Braunschweig). To obtain standard DNA, the standard strains were grown on agar plates, the cultures were scraped off with a sterile spatula, and their DNA was isolated and quantified in the same manner as the soil samples. The standard DNA was kept at 5 °C; dilutions were freshly made every day in ultra-pure water (ELGA PURELAB® flex 3, Veolia Water Technologies Deutschland GmbH, Celle, Germany).

Each assay's annealing temperature and time were optimized using melting curves. The specificity of each assay was checked using all the standard strains described in Table A1 and additional strains of *Streptomyces avermitilis* (*Actinobacteria*), *Rhizobium azooxidifex* (*α-Proteobacteria*), *Burkholderia phymatum* (*β-Proteobacteria*), and *Paenibacillus uliginis* (*Firmicutes*) as negative/positive controls. Additionally, each assay product was checked for undesired fragments with gel electrophoresis. All assays except the *β-Proteobacteria* and *Acidobacteria* produced one band of the expected size. The *β-Proteobacteria* exhibited two bands that can both be attributed to the targeted group according to the basic local alignment search tool

(BLAST). The *Acidobacteria*-specific assay exhibited several bands. As a 100% specificity was published for the primer pair used in soil samples [45], a reasonable specificity was nonetheless assumed.

qPCR inhibition was detected by spiking with standard DNA when undiluted template DNA was used. No inhibition was detected when template DNA was used in a 1:10 or 1:25 dilution. To correct for the inhibition in assays that use undiluted template DNA, an additional inhibition assay was performed using an artificial DNA fragment and matching primers [56]. The abundance of the artificial DNA fragment was measured in the presence and absence of template DNA, and an inhibition factor (f_{inhib})was calculated for each sample using Formula (1):

$$f_{inhib} = \frac{log10(Abu_{inhib-base})}{log10(Abu_{inhib-sample})}, \quad (1)$$

with $Abu_{inhib\text{-}base}$ being the abundance of the DNA fragment measured in the absence of the template DNA and $Abu_{inhib\text{-}sample}$ being the abundance of the DNA fragment measured in the presence of the template DNA. Then, the abundances measured with the group-specific qPCR assays in question were corrected according to Formula (2):

$$Abu_{korr} = 10^{(Abu_{meas} \times f_{inhib})} \times \frac{Eff_{assay}}{Eff_{inhib}}, \quad (2)$$

with Abu_{meas} being the abundance measured with a group-specific assay, Abu_{korr} being the group-specific abundance corrected for inhibition, Eff_{assay} being the average efficiency of the corrected assay, and Eff_{inhib} being the average efficiency of the inhibition assay. An average efficiency, calculated from 10 plates of each assay, was used.

2.9. Statistical Analysis

Data organization and visualization were performed using R Statistical Software (v4.2.0; R Core Team 2022). The effects of the treatments on dry mass yield and mycorrhizal colonization rate were analyzed with a three-factorial analysis of variance (ANOVA) with the factors site, MO application, and starter fertilization. The effects of the treatments on soil N_{min} and P_{DL} concentrations were analyzed with a four-factorial ANOVA, with sampling date as an additional factor. The ANOVA was followed up by Tukey's honestly significant difference (HSD) post hoc tests where necessary. The normality of residues and variance homogeneity were checked for every model. HSD was carried out with the rstatix R package [57], and ANOVA and model diagnosis were performed with base R functions.

For analysis of the qPCR data, the genome copy numbers (gcns) measured with the group-specific qPCR assays were summed up to obtain the total genome copy number (tot-gcn). This tot-gcn was used as an indicator of community size, as the bacterial groups monitored comprised many of the typically most abundant phyla in soil bacterial communities. The relative abundances of each group were then calculated by dividing the gcn measured with the group-specific qPCR assays by the tot-gcn. The relative abundances calculated this way are not the relative abundances in the overall bacterial community. Relative abundances were analyzed instead of absolute abundances to correct for size variation in the overall microbial community. This has been shown to help in analyzing qPCR-based microbial compositions [46]. All further analyses were carried out with the software PRIMER (PRIMER-e, Auckland, New Zealand) with the permANOVA add-on. First, the relative abundances were transformed by the natural logarithm (ln). Then, the data were checked for univariate outliers with histograms and for multivariate outliers with non-metric multidimensional scaling (NMDS). Out of 6480 measured abundances, eight very extreme univariate outliers were replaced by the average of the remaining two replicate samples. Out of 720 samples, two that were extreme multivariate outliers were removed from the dataset. Then, averages of the three replicate samples from each plot, sampling date, and sampling area (inside or outside the application area) were calculated for each microbial group. To detect changes in the soil microbial community structure,

permutational ANOVA (permANOVA) analysis was performed on the averaged data. The samples inside and outside of the application area were analyzed separately. The permANOVA was carried out with a multifactorial nested design with a fixed-factors site, sampling date, MO application, and type of starter fertilization. An additional random factor plot was needed because of the repeated sampling of every plot and was nested in the factors site, MO application and fertilizer type. Multivariate permANOVA and principal coordinate analysis (PCO) were performed with Bray–Curtis dissimilarity as a distance metric. Univariate permANOVAs of single microbial groups and the tot-gcn were performed using Euclidean distance as a distance metric. A significance level of 5% was chosen. Significant permANOVAs were followed up by post-hoc tests, also using the permANOVA add-on of PRIMER. Significant interactions without significant post-hoc tests were not further considered.

To identify drivers of the maize dry mass yield, the Kendall–Tau correlation coefficients were calculated between the maize yield and the following parameters: soil DM, P_{DL}, N_{min}, tot-gcn, and the relative abundances of the individual microbial groups, including the inoculated bacterial strain *B. atrophaeus* Abi05. The coefficients were separately calculated for each site and sampling date with R.

3. Results

3.1. The Effect of Starter Fertilization and Inoculation with Plant-Beneficial Microorganisms on Concentrations of Soil-Available Nutrients and Maize Yield

After biogas slurry application to the WS site (WSo did not receive biogas slurry), but before sowing, the P_{DL} level in the top 30 cm of the soil was about 20 mg P 100 g^{-1} soil at both sites (Table 1). Other parameters differed, however (Table 1). For the WS site, the concentrations of N_{min} in the top 30 cm of the soil were about double, and the concentration of plant-available Kalium (K_{DL}) was about triple the concentrations found at the WSo site. It is further noticeable that N_{min} and P_{DL} concentrations in the 30–60 cm layer are similar to those in the top layer at the WSo site, while the concentrations considerably decrease with depth at the WS site. The soil dry mass content in the top 30 cm was significantly higher at the WSo site compared to the WS site at all sampling dates ($p < 0.0001$, WSo: 87.1 ± 3.0%, WS: 84.5 ± 2.5%).

The maize dry mass yield was 13.6% higher ($p < 0.001$) at the WS site (18.4 ± 1.2 t ha^{-1}) compared to the WSo site (16.2 ± 1.4 t ha^{-1}). The starter fertilization did not influence the yield at either site. The MO application enhanced the yield by, on average, 4.2% ($p = 0.041$) (Figure 1a). The yield effect of the MO application did not significantly vary with the factors of starter fertilization and site, but some trends were observed. While the MO application led to a 6.1% dry mass yield increase at the WSo site, the yield increased only by 2.6% at the WS site. In addition, the MO effect on yield was highest for the treatment without additional starter fertilization at both sites (WSo: 11%, WS: 5.7%).

At sampling dates t_2 and t_3, the N_{min} concentration in the top 30 cm of the soil did not differ between sites (Table 1), while before sowing, it was higher for the WS site, which received an application of biogas slurry. The P_{DL} concentration in the top 30 cm of the soil was higher at the WS site compared to the WSo site at the sampling dates t_2 and t_3 ($p = 0.004$) (Figure 1b), while no difference was found before sowing. Between the sampling dates t_2 and t_3, the concentration of N_{min} decreased at both sites ($p < 0.001$), while the concentration of P_{DL} increased ($p < 0.001$) (Figure 1b). However, the differences were relatively small, considering the absolute P_{DL} concentrations at both sites. Both N_{min} and P_{DL} did not significantly change due to the starter fertilization or the MO application, except for the higher N_{min} concentration found at sampling date t_2 at the WS site following starter fertilization with DAP (DAP vs. control: $p = 0.010$; DAP vs. MG: $p = 0.003$). In summary, the starter fertilization and MO application seemed to have a very limited effect on the concentrations of plant-available nutrients in the soil.

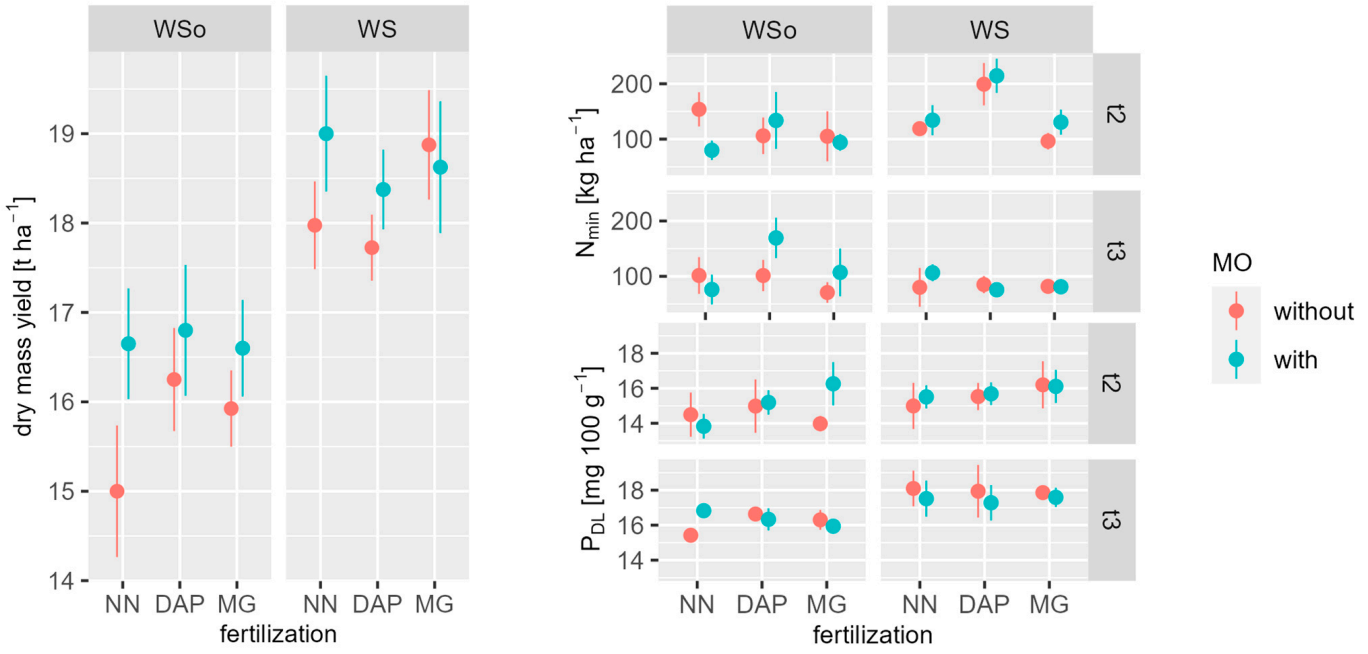


Figure 1. (**a**) Maize dry mass yield (t ha^{-1}) on the "Wanna Sand without biogas slurry" (WSo) and "Wanna Sand" (WS) sites in 2021. (**b**) Concentrations of mineral nitrogen (N_{min}) and plant-available phosphorous (P_{DL}) in the soil at the sampling dates t_2 (June) and t_3 (July). MO—application of a combined inoculum containing three species of AMF and *B. atrophaeus*; NN—no starter fertilizer; DAP—starter fertilization with diammonium phosphate; MG—starter fertilization with organomineral microgranular fertilizer. The values are depicted as the mean and 95% confidence interval.

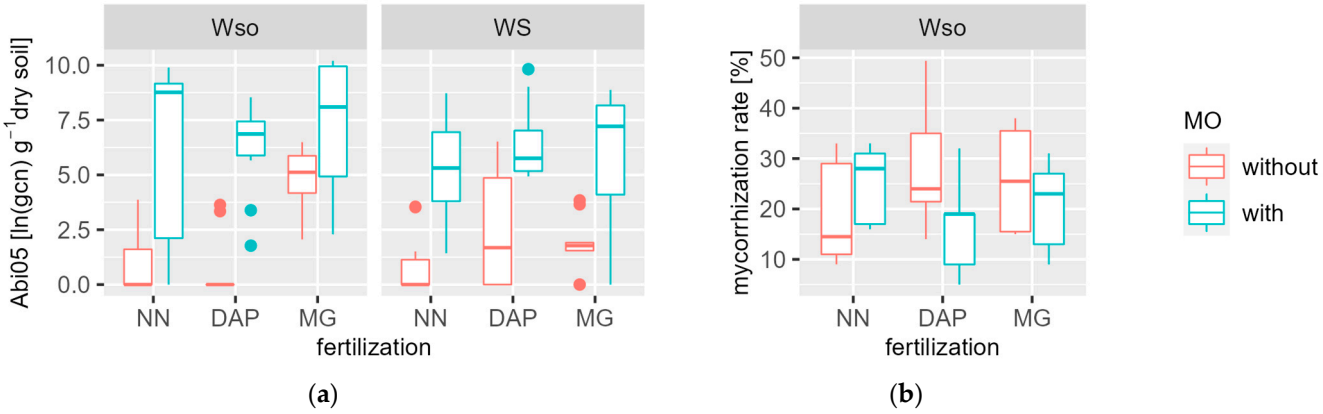
3.2. Root Mycorrhizal Colonisation Rate and Recovery of Inocluated Bacteria

Inside the application area (in the maize rows), the MO application significantly ($p < 0.001$) increased the genome copy numbers (gcn) of the inoculated bacterial strain *B. atrophaeus* Abi05 (ABiTEP GmbH, Berlin, Germany). It was 30 times higher in inoculated plots (mean 8116 gcn g^{-1} dry soil) compared to non-inoculated plots (mean 285 gcn g^{-1} dry soil). This effect occurred regardless of the site and sampling date. Thus, at sampling date t_3, 10 weeks after planting, the bacteria had established themselves, and their number did not significantly change within the next month.

Additionally, at the WSo site, the Abi05 abundance was influenced by the starter fertilizer ($p = 0.046$). Plots fertilized with MG harbored significantly more Abi05 bacteria than the plots fertilized with DAP ($p = 0.004$) and without fertilization ($p = 0.044$). This was observed regardless of inoculation with Abi05, meaning that fertilization with MG increased Abi05 abundance in non-inoculated plots (Figure 2a).

Outside the application area (in the middle between the maize rows), no significant change in Abi05 bacterial gcn was detected after MO application at any sampling date. Thus, the bacterial strain did not spread into the space between the rows (40 cm distance from both rows), and the inoculation effect likely remains spatially restricted to the application area of the bacteria.

The mycorrhizal colonization rate was analyzed solely at the WSo site at sampling date t_3. It ranged between about 10 and 40% (Figure 2b) and did not significantly change due to the treatments.



(**a**) (**b**)

Figure 2. (**a**) Abundance of the inoculated strain *B. atrophaeus* Abi05 found inside the application area on the "Wanna Sand without biogas slurry" (WSo) and "Wanna Sand" (WS) sites at the sampling dates t_3 (July) and t_4 (August) (natural logarithm of genome copy numbers). (**b**) Root mycorrhizal colonization rate at sampling date t_3 (July) at the WSo site. NN—no starter fertilizer; DAP—starter fertilization with diammonium phosphate; MG—starter fertilization with organomineral microgranular fertilizer; MO—application of combined inoculum containing AMF and *B. atrophaeus*.

3.3. Treatment Effects on the Soil Microbial Community

Inside the application area, the community size (described by the tot-gcn) was affected by an interaction between the site and the MO application (Table 3). At the WSo site, tot-gcn was increased by about 9.3% following MO application (p = 0.039), regardless of the sampling date (Figure S2). At the WS site, however, this effect was not observed.

According to the multivariate analysis, the community composition inside the application area was not affected by the factors of fertilization and MO application at any site. At the level of the individual microbial groups, significant reactions of *Fusaria* and *Firmicutes* were observed (Table 3). The relative abundance of *Fusaria* was influenced by fertilization, depending on the site. The share of *Fusaria* in tot-gcn was doubled in WSo plots fertilized with DAP compared to WSo plots fertilized with MG (p = 0.021, perms = 9839). In contrast, for the WS site, the share of *Fusaria* in plots fertilized with DAP was only half as high as in the plots without fertilization (p = 0.020, perms = 9863). The site effect was also obvious regarding the influence of MO application on the relative abundance of *Firmicutes*. In the WSo site, the share of *Firmicutes* was 18% lower in plots with MO application compared to the non-inoculated plots (p = 0.002, perms = 9847) (Figure S2), while no effect was detected for the WS site.

Outside the application area, neither the soil microbial community's size nor structure was affected by starter fertilization or MO application (Table S1).

Table 3. Five-factorial permANOVA analysis of the soil microbial community composition inside the application area of starter fertilizers and MO. The analyzed parameters were total gene copy number (tot-gcn, the sum of the gene copy numbers of all investigated microbial groups), multivariate community composition (Bray–Curtis dissimilarity), and the relative abundance of the investigated microbial groups.

Source of Variation	tot-gcn	Multivariate	Acido	Actino	Firmi	Alpha	Beta	Cya	ITS	Fusa
site	<0.001	<0.001	0.915	0.632	<0.001	0.195	0.047	0.123	0.004	0.003
time	0.005	<0.001	0.030	0.686	<0.001	<0.001	0.002	<0.001	<0.001	0.095
fert	0.394	0.619	0.757	0.895	0.514	0.516	0.672	0.565	0.718	0.167
MO	0.130	0.315	0.392	0.518	0.022	0.304	0.411	0.147	0.860	0.434
site:time	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.300	0.178	0.173	0.451
site:fert	0.996	0.899	0.510	0.387	0.430	0.671	0.709	0.781	0.725	0.003

Table 3. *Cont.*

Source of Variation	tot-gcn	Multivariate	Acido	Actino	Firmi	Alpha	Beta	Cya	ITS	Fusa
site:MO	0.027	0.085	0.107	0.299	0.027	0.294	0.056	0.404	0.426	0.823
time:fert	0.965	0.569	0.778	0.736	0.963	0.663	0.472	0.264	0.972	0.156
time:MO	0.568	0.824	0.452	0.344	0.831	0.495	0.751	0.982	0.184	0.491
fert:MO	0.466	0.712	0.471	0.621	0.644	0.135	0.583	0.782	0.373	0.255
site:time:fert	0.883	0.984	0.333	0.309	0.793	0.960	0.882	0.982	0.646	0.831
site:time:MO	0.952	0.151	0.465	0.509	0.450	0.084	0.070	0.335	0.777	0.588
site:fert:MO	0.642	0.245	0.109	0.120	0.603	0.405	0.693	0.692	0.065	0.232
time:fert:MO	0.525	0.274	0.044	0.108	0.567	0.207	0.261	0.316	0.403	0.278
plot(site:fert:MO)	0.013	0.025	0.003	0.005	0.111	0.027	0.699	0.914	0.120	0.839
site:time:fert:MO	0.469	0.729	0.615	0.455	0.689	0.411	0.351	0.110	0.906	0.777

Factors: site, time, starter fertilization (fert), plant-beneficial microbe inoculation (MO), plot (nested in site, fert, and MO), and their interactions. Microbial groups: Acido—*Acidobacteria*, Actino—*Actinobacteria*, Alpha—*α-Proteobacteria*, Beta—*β-Proteobacteria*, Cya—*Cyanobacteria*, Fusa—*Fusaria*, Firmi—*Firmicutes*, ITS—total fungi. The permANOVAs of single parameters were calculated with Euclidean distance. The values are *p*-values.

3.4. Description of the Soil Microbial Community and Its Spacial and Temporal Differences

3.4.1. Soil Microbial Community Description

Inside the application area (in the maize rows), the microbial community was dominated by *Acidobacteria* and *Actinobacteria*. Total fungi comprised 0.2%, while only 0.0002% of tot-gcn were *Fusaria*. The community composition was relatively stable between the sites and sampling dates (Figure 3).

Figure 3. Soil microbial community composition in the "Wanna Sand without biogas slurry" (WSo) and "Wanna Sand" (WS) sites at the sampling dates t_3 (July) and t_4 (August), bR—between the maize rows (outside the application area of starter fertilizers and MO), iR—in the rows (inside the application area of starter fertilizers and MO); the quantified groups were: Acido—*Acidobacteria*, Actino—*Actinobacteria*, Alpha—*α-Proteobacteria*, Beta—*β-Proteobacteria*, Cya—*Cyanobacteria*, Fusa—*Fusaria*, Firmi—*Firmicutes*, ITS—total fungi.

The absolute abundances of all groups were correlated with the tot-gcn. Using the relative abundances, however, the microbial groups were clearly clustered. While the relative abundance of *Acidobacteria* was positively correlated with tot-gcn and the gcn of *Bacillus atrophaeus* Abi05, it was negatively correlated with the shares of all other studied groups (Figure S3).

3.4.2. Spatial Distribution of Soil Microbial Communities

Both the size and the structure of the soil microbial community differed between the samples taken outside the application area (between the maize rows) and those taken inside the application area (in the maize rows). At sampling date t_3, the community size (tot-gcn) was around 15–40% lower outside the application area compared to inside the application area at both sites. The difference diminished to 0–15% lower tot-gcn outside the application area at sampling date t_4 (Figure S4).

The *Acidobacteria* were enriched inside the application area (in the maize rows) compared to outside the application area (between the maize rows), while all other groups behaved inversely (Figure 3). The differences in community size and structure between the inside and outside of the application area were considerably more pronounced at sampling date t_3. A detailed view of the differences in the relative abundance of all studied microbial groups inside and outside of the application area is provided in the Supplemental Material (Figure S4).

3.4.3. Effects of Site and Sampling Date

The effects of site and sampling date on the soil microbial community size and structure were less pronounced than the effect of sample location but still highly significant (Table 3 and Table S1). These effects were investigated in detail solely using the samples taken inside the application area (in the maize rows). The community size—represented by the sum of all analyzed gene copy numbers (tot-gcn)—was generally higher for the WS site (12.2 ± 2.0 gcn g^{-1} soil) compared to the WSo site (9.6 ± 2.0 gcn g^{-1} soil). At the WS site, the community size significantly increased from sampling date t_3 (July) to t_4 (August), while at the WSo site, it did not (Figure S5).

The differences in community structure are visualized using a principal coordinate analysis (PCO) in Figure 4. While the differences between the sites and sampling dates are visible, there is also a considerable overlap between all groups.

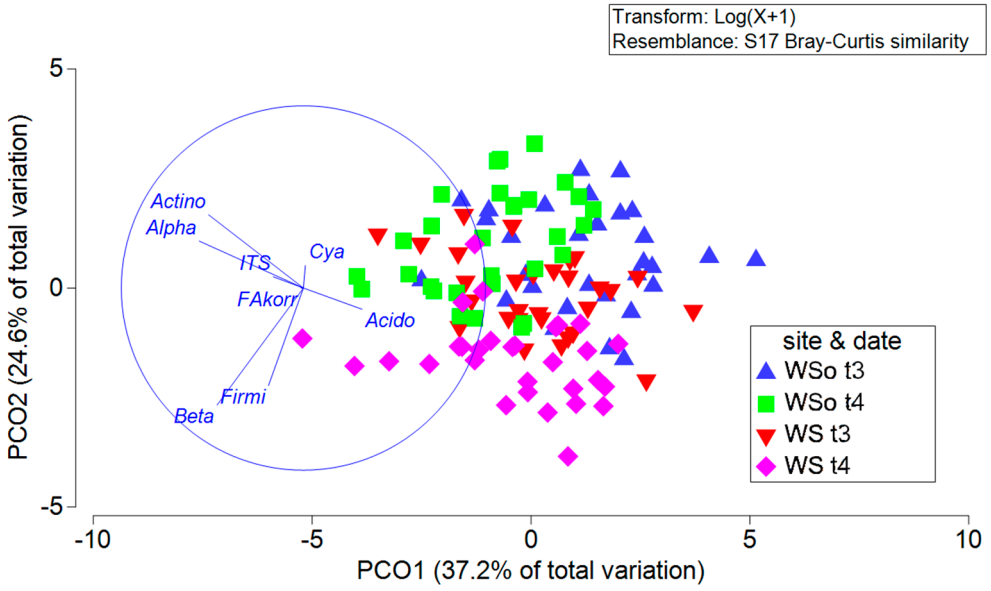


Figure 4. Principal coordinate analysis of microbial community composition between the "Wanna Sand without biogas slurry" (WSo) and "Wanna Sand" (WS) sites at the sampling dates t_3 (July) and t_4 (August). For each investigated microbial group, the lines and group names inside the blue circle indicate the associated direction in the ordination (Acido—*Acidobacteria*, Actino—*Actinobacteria*, Alpha—*α-Proteobacteria*, Beta—*β-Proteobacteria*, Cya—*Cyanobacteria*, FAkorr—*Fusaria*, Firmi—*Firmicutes*, ITS—total fungi). The length of the line indicates the strength of this association.

The differentiation between the sites mainly occurs along the PCO2 axis. The microbial groups driving the differentiation between the sites were *β-Proteobacteria* and *Firmicutes* (Figure 3). The relative abundance of *Fusaria* and total fungi also significantly differed between the sites (Table 3), which is not shown by the PCO due to their low overall abundance. All studied microbial groups except for *Fusaria* exhibited a significant influence from the sampling date and/or the interaction between the site and sampling date (Table 3). The differentiation of sampling dates took place along the PCO2 axis at the WS site and along the PCO1 axis at the WSo site. This indicates that, at the WS site, *Firmicutes* and *β-Proteobacteria* were important drivers for the differences between the sampling dates, while at the WSo site, *Actinobacteria*, *α-Proteobacteria,* and *Acidobacteria* were more important. A detailed view of the differences in the relative abundance of all studied microbial groups between sites and sampling dates is provided in the Supplemental Material (Figure S5).

3.5. Correlations of the Maize Yield with Soil Nutrient Concentrations, Dry Mass Content, and Microbial Relative Abundances

At the WS site, there was a positive correlation between the P_{DL} concentration at sampling date t_2 and the maize yield. At sampling date t_3, the correlation was still detectable at a significance level of 10%. Additionally, there was a positive correlation with the share of *Firmicutes* (t_4) (Table 4). This stands in contrast to the finding that, at the WSo site, the share of *Firmicutes* was diminished by MO application, which, in turn, had a positive effect on the dry mass yield.

Table 4. Correlation coefficients (Kendall–Tau) of the maize dry mass yield at the "Wanna Sand without biohas slurry" (WSo) and "Wanna Sand" (WS) sites with soil-available phosphorous (P_{DL}) and mineral nitrogen (N_{min}) concentrations, the soil dry mass content (soil DM), the total gene copy number (tot-gcn, sum of the gcn of all investigated microbial groups), and the relative abundances of the investigated microbial groups at sampling dates t_2 (June), t_3 (July), and t_4 (August). The R^2 and significance level of the correlations are displayed in brackets (** $p < 0.01$, * $p < 0.05$, and . $p < 0.1$). Only correlations with p-values $\leq$ 10% are displayed.

	Maize Dry Mass Yield Correlated with:	
Time	**WSo**	**WS**
t_2	Soil DM (−0.27, .)	P_{DL} (0.43, **)
t_3	*B. atrophaeus* Abi05 (0.25, .)	P_{DL} (0.27, .) *α-Proteobacteria* (0.23, .)
t_4	*B. atrophaeus* Abi05 (0.30, *) tot-gcn (0.25, .) soil DM (−0.25, .)	*Firmicutes* (0.26, *)

At the WSo site, there was a significant positive correlation with the gcn of *B. atrophaeus* Abi05 at sampling date t_4. At sampling date t_3, the same correlation was detected at a significance level of 10%. This is in line with the MO application, leading to a higher number of *B. atrophaeus* Abi05 gcn and a higher dry mass yield. Additionally, there were negative correlations at a significance level of 10% between the soil DM content and the maize yield at sampling dates t_2 and t_4, showing that water availability influenced the yield at this site.

4. Discussion

4.1. Drivers of Yield and Plant-Available Nutrient Concentrations

The high N_{min} and P_{DL} concentrations in the 30–60 cm layer at the "plaggic anthrosol" WSo site can be attributed to centuries of nutrient accumulation by plaggen agriculture leading to a 70 cm deep A/E horizon [58]. The differences in maize yield between the sites were likely due to the lower nutrient and water availability during the growing season at the WSo site compared to the WS site.

Although starter fertilization is common in this area, with relatively high nutrient concentrations in the soil, our results showed that it may be ineffective. The P_{DL} concentrations corresponded to the Lower Saxony soil test classes D (high P availability) and E (very high P availability), depending on the sampling date [59]. The Chamber of Agriculture for Lower Saxony recommends farmers with fields in test class E try to omit the starter fertilization in test patches on the field to check if their specific fields show any yield decreases [60]. The lack of yield reaction to P starter fertilization with DAP and MG in this study supports the notion that starter fertilization should be omitted in high P-testing soils. The fact that starter fertilization did not alter the soil-available P concentrations underlines the low effectiveness of fertilization measures in soils with high nutrient concentrations.

However, the (insignificant) yield difference between the fertilized plots and no-fertilization control at the WSo site in this study is in a range that is relevant for practitioners. Similar patterns have been observed before where no significant effect of starter fertilization was observed, although, on single fields, there was a yield difference that is relevant in practice (up to 11% yield increase) [10]. This discrepancy might impair the acceptance of the results by practitioners. Future studies investigating the effect of P starter fertilization should choose the study design carefully to ensure sufficient statistical power [61]. Also, more attention needs to be paid to factors like soil organic carbon content and pH, which have been identified as important mediators of P fertilization's effectiveness [12,62].

It is remarkable that despite the high P availability in the studied fields, the in-furrow MO application led to a significant yield increase. This increase is comparable to the yield increase expected for P starter fertilization [11]. Nonetheless, the yield effect of MO application in this study was low compared to the results of meta-analyses on the effects of AMF and PGPR inoculation [17,18]. This could be related to the high availability of nutrients in the soil at the studied sites. Both meta-analyses showed that yield increases following inoculation tended to be lower at high soil-available P levels [17,18]. Furthermore, the soil pH plays an important role in mycorrhiza establishment, which prefers a neutral pH [18,63,64]. The soil pH of our study sites was relatively low, especially at the WSo site (Table 1). The yield effect of the MO application could be due to the applied mycorrhiza, the applied bacterial strain, or an interaction of both. Previous studies have found positive interactions between the simultaneous application of AMF and PGPR [65,66]. The fact that MO application does not increase soil-available nutrient concentrations and that the yield effect of MO application is independent of the applied starter fertilizer indicates that nutrient mobilization might not be the primary mode of action of the applied AMF and bacterial strain. *Bacillus atrophaeus* is a genus that is native to soils, seas, and even food [67,68]. Many strains have been identified to be plant growth-promoting, to have antimicrobial and antifungal properties, and to be antagonistic to insect pests [69–73]. Further studies should focus on plant nutrient composition, pathogen numbers, and biological microbes in plant messenger molecules to elucidate the pathway of influence. Although the root mycorrhizal colonization rate was not influenced by MO application, the inoculated AMF could have contributed to the observed yield increase. This has been observed previously [74,75] and was attributed to the inoculated mycorrhiza overruling the "legacy effect" of repeated maize cultivation [75].

4.2. Recovery of Inoculated Bacterial Strain and Mycorrhizal Colonisation Rate

The gcn of the inoculated strain could be considered low in relation to the overall community size. It has been stated before that the insufficient establishment of inoculated microbes was a hindrance to the effective application of bioinoculants [22]. The gcn found in this study, however, paired with an increase in dry mass yield, indicates that "low" numbers of inoculated microorganisms can still be effective. The gcn of *B. atrophaeus* Abi05 was increased in plots fertilized with MG that were not inoculated with the bacterial strain. This might be due to an increase in native bacteria that are similar to the strain used for inoculation. Since many *Bacillus atrophaeus* strains have been identified as plant-growth-

and health-promoting [69–73], this indicates that MG fertilizer could have a plant-beneficial influence on the soil microbial community.

The mycorrhizal colonization rate of the maize roots at sampling date t_3 at the WSo site was similar to values that have been found in previous experiments with considerably lower P concentrations in the soil [76,77]. It has been shown that the root mycorrhizal colonization rate negatively depends on P availability [76,78], so the levels found here were surprisingly high.

4.3. Treatment Effects on the Soil Microbial Community

Despite the fertilizer and MO applications, only slight changes in the native soil microbial community occurred. It has been shown that the plant response to PGPR and AMF inoculation does not depend on changes in the native microbial community diversity [40]. Also, most studies investigating the influence of *Bacillus* inoculation on the native soil microbiome showed only slight changes that were mostly limited to particular families or species [41,79,80], despite positive plant growth responses. A lowered share of *Firmicutes* following MO application has not been described before, to the best of our knowledge. The applied bacterial strain belongs to the *Firmicutes* itself, so this reaction could be a sign of competition or even antagonistic interactions between the inoculated bacterial strain *B. atrophaeus* Abi05 and the native *Firmicutes*. *Fusarium* infection has been reported to be promoted by higher fertilization rates [81,82]. This pattern was observed in this study at the WSo site, while at the WS site, the opposite occurred. A *Fusarium* gene copy level's reaction to MO application was expected, as both *B. atrophaeus* and AMF have been reported to hinder *Fusarium* infection [72,83], but this did not occur. It has previously been observed that while *Fusarium* gcn in the roots reacted to treatments and was a good indicator for plant infection levels, *Fusarium* gcn in the soil was not impacted by treatments or related to plant infection levels [84]. No significant change in the relative abundance of *Acidobacteria* was detected after MO application. However, the relative abundance of *Acidobacteria* was positively correlated to the *B. atrophaeus* Abi05 copy number and tot-gcn, which increased at the WSo site after the MO application. An increase in *Acidobacteria* following inoculation with PGPR, including *Bacillus* strains, has been previously reported [85,86].

4.4. Soil Microbial Community: Influence of Site, Sampling Date and Sampling Area

Acidobacteria, Proteobacteria, and *Actinobacteria* make up the largest part of the soil microbial community in both studied fields, which is in line with previous results [34,36,45,48]. The *Acidobacteria* were the most abundant phylum at both sites. This supports the results of Navarrete et al. [87], who found that the share of *Acidobacteria* increases with the soil organic matter content and that, in soil with a high organic matter content, *Acidobacteria* were the largest microbial group. Contrastingly, *Acidobacteria* have been linked to low C-mineralization rates and low nutrient inputs [42,46,48], which is not supported by our results. There was a higher relative abundance of *β-Proteobacteria* at the WS site compared to the WSo site. This is in line with previous research, as *β-Proteobacteria* have been linked to high C-mineralization rates and nutrient inputs [42,46,48], and the WS site exhibits higher total organic carbon contents and nutrient inputs compared to the WSo site.

It has to be noted that the relative abundance of gcn belonging to each microbial group cannot be interpreted as the relative abundance of microbial cells in the sample. First, in this study, the shares were calculated as shares of the sum of all detected microbial groups, not as shares of all bacteria and fungi that were present. Second, there could be considerable errors when microbiome composition is deducted from gene copy composition due to differences in the 16S rRNA and ITS copy numbers in the genome [88–90]. For example, *Acidobacteria* only have a median 16S rRNA copy number of one, compared to a median copy number of three for *Actinobacteria* and six for *Firmicutes*, according to the rrnDB database [91]. With the qPCR method employed in this study, there is an inherent "correction" with the 16S rRNA copy numbers of the standard strains. However, these most likely differ from the group-specific average of the 16S rRNA copy numbers present in the

soil samples. These average copy numbers can only be determined by sequencing [92] and are thus not available for correction in this study.

The absolute abundances of all microbial groups correlating with the tot-gcn are in line with the results of Philippot et al. [46] and confirm the choice to analyze the relative abundances. When analyzing the relative abundances of the microbial groups, the correlations between the phyla in this study differed from correlations found by Philippot et al. [46], underscoring the multifactorial nature of microbial community composition.

Remarkably, the difference in the soil microbial community between the two sampling dates, which were only four weeks apart, was similar in magnitude to the difference between the two sites, underscoring the importance of the influence of environmental parameters and soil parameters on the soil microbial community. Changes in the rhizosphere microbial community structure over time have been well documented and could be attributed to both changes in the environmental conditions and the influence of the plant development stage [33–35,37]. For root-influenced bulk soil, the importance of plant exudates was found to be low compared to changes in soil properties [93]. The change in bulk soil microbial community composition between the sampling dates found in this study is thus likely mainly due to changes in environmental conditions like soil water content and temperature and is less affected by plant development.

The differences between the samples inside and outside the application area might be due to the difference in root influence. An enrichment of *Acidobacteria* in the root-penetrated bulk soil has been published previously [94], whereas in the rhizosphere, the *Acidobacteria* were shown to be depleted [36,39,48]. Subsequently, it can be assumed that the *Acidobacteria* are especially enriched in the bulk soil inside the root-penetrated soil compartment and that they might interact with the plant roots within a short distance. It has been shown that subdivisions of *Acidobacteria* are "keystone taxa" with important roles in soil organic matter's decomposition and denitrification [95,96] and that some *Acidobacteria* have plant growth-promoting properties [97,98]. More research about this phenomenon should thus be conducted. The differentiation of the soil microbiome between inside and between the maize rows was more pronounced in t_3 (July) compared to t_4 (August). This has been shown before [99] and could be due to the root zone expanding into the space between the rows, thus rendering both soil compartments "root-influenced" bulk soil.

4.5. Correlations of Maize Yield with Soil's Physical and Microbial Parameters

Surprisingly, while no influence of P starter fertilization on the maize yield was recorded, the maize yield correlated with soil P_{DL} at sampling date t_2 at the WS site. This shows that the existing differences in P availability between the plots had a larger impact on plant growth than the additional P availability obtained through starter fertilization. It is also interesting that no such correlation was detected at the WSo site, although it had a similar variation in P_{DL} and lower overall nutrient levels compared to the WS site. Instead, at the WSo site, the dry mass yield correlated with the gcn of the applied bacterial strain *B. atrophaeus* Abi05 and the soil DM, which was higher than that at the WS site. Thus, at the WSo site, water shortages and MO application effects might have overruled the possible effect of P availability. It was shown before that water availability potentially impacts maize yield more strongly than soil P levels [100] and plays an important role in P fertilization success [8]. The behavior of *Firmicutes* contrasting between the sites cannot be explained, but it points to a differing role of *Firmicutes* on the two sites. This is supported by the importace of *Firmicutes* in separating the two studied sites.

5. Conclusions

Phosphorous starter fertilization application on sites with high soil-available P levels should be avoided as, according to our study results, it has no relevant yield effect and may result in nutrient losses. Instead of P starter fertilization, the application of MO can be suggested for sites with high soil-available P levels, as a higher maize yield was achieved compared to standard starter fertilization.

The mechanisms by which the applied AMF and PGPR influence plant growth should be further investigated by monitoring pathogens and microbe–plant interaction pathways. The *Firmicutes* species that react to the MO application should also be elucidated, and the underlying mechanisms should be discovered. The enrichment of *Acidobacteria* in the root-influenced bulk soil and possible interactions with plants should receive more consideration in future studies.

Finally, further field studies with different soil characteristics should be conducted to test the repeatability of our results. Moreover, additional efforts should be made to increase the knowledge of practitioners about scientific findings concerning starter fertilization.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13122900/s1, Figure S1: Rainfall and temperature at the weather station Steinau, Lower Saxony, in 2021; Figure S2: Reactions of total gene copy number (tot-gcn) and relative abundance of Firmicutes to fertilization and MO application; Figure S3: Intercorrelations of the relative abundances of the investigated microbial groups; Figure S4: Soil microbiome differences between inside and outside of the application area of starter fertilizers and plant-beneficial microorganisms; Figure S5: Influence of site and sampling date on the soil microbiome's size and structure; Table S1: Five-factorial permANOVA analysis of the soil microbial community's composition outside the application area of starter fertilizers and MO.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. qPCR conditions for all assays, including assay name, name of Forward primer, name of Reverse primer, reference the assay was taken from (Ref.), amplicon length (AL), target region in the genome, quantity and dilution of used template DNA (DNA), annealing temperature and time (Annealing), cycle number (Cyc), and standard strain.

Assay	Forward Primer	Reverse Primer	Ref.	AL [bp]	Target Region	DNA	Annealing	Cyc	Standard Strain
Acidobact.	Acido31	Eub518	[45]	500	16S-rRNA	1 µL, 1:10	61 °C, 45 s	35	*Acidobacterium capsulatum*
Actinobact.	S-P-Acti-1154-a-S-19	S-P-Acti-1339-a-A-18	[101]	166	16S-rRNA	1 µL, 1:10	62 °C, 30 s	35	*Microbacterium foliorum*
α-Proteobact.	S-C-aProt-0528-a-S-19	S-C-aProt-0689-a-A-21	[101]	142	16S-rRNA	1 µL, 1:25	58 °C, 25 s	35	*Sphingomonas paucimobilis*
B-Proteobact.	S-C-bProt-0972-a-S-18	S-C-bProt-1221-a-A-17	[101]	231	16S-rRNA	1 µL, 1:10	57 °C, 45 s	35	*Variovorax paradoxus*
Firmicutes	S-P-Firm-0525-a-S-18	1040FirmR	[101]	501	16S-rRNA	1 µL, 1:25	64 °C, 30 s	35	*Bacillus subtilis*
Cyanobact.	CYA361f	CYA785r	[102]	440	16S-rRNA	1 µL, 1:25	62.2 °C, 30 s	40	*Chroococcidiopsis cubana*
Fungi (ITS)	ITS F	5,8S-R	[45]	300	ITS1	1 µL, 1:10	59 °C, 30 s	35	*A. tenuissima GH50t*
Inhibition	T7 F	M13 rev	[56]	210	-	1 µL, und.	58 °C, 30 s	30	*Artificial DNA-Fragment*
	Forward Primer	Reverse Primer	Probe						
Fusarium	FA pl3 forward	Fus pl reverse	S FUS pl	[103]	TEF1	2 µL, und	67 °C, 90 s	45	*F. graminearum 486*

Each 20 µL qPCR reaction (except *Fusarium* assay) contained 4 µL of 5× HOT FirePol® EvaGreen® HRM Mix (ROX) (Solis Biodyne, Tartu, Estonia, 2.5 mM $MgCl_2$), 2.5 pmol of each primer (biomers.net, HPCL purification), and 1 µL of (diluted) DNA sample. The PCR protocol consisted of initial denaturation (95 °C for 10 min), followed by a number of cycles of denaturation (95 °C for 15 s), annealing, and elongation (72 °C for 60 s for ITS, *Cyanobacteria*, and *Firmicutes* and 30 s for all other assays). For the *Fusarium*-specific qPCR assay, each 20 µL reaction contained 4 µL of 5× HOT FirePol® Probe GC qPCR Mix (Solis Biodyne, Tartu, Estonia, 3 mM $MgCl_2$), 2.5 pmol of each primer, 6 pmol of hydrolysis probe, 3 µL of 25 mM $MgCl_2$ (Solis Biodyne, Tartu, Estonia), and 2 µL of DNA sample. The PCR protocol consisted of initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 67 °C for 90 s.

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