

Soil substrate source drives the microbes involved in the degradation of gelatin used as a biostimulant

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ABSTRACT

Plant biostimulants improve crop yield and quality by stimulating plant nutrition processes and enhancing nutrient uptake efficiency, likely reflecting indirect effects mediated by beneficial soil microbes. Gelatin is an emerging plant biostimulant. Culture-dependent studies have identified several species of gelatin-degrading microbes, but the effect of gelatin on soil microbial communities in the absence of the plant microbiome has not been investigated. The objectives of this work were to evaluate changes in the microbial community induced by granulated gelatin amendments in different soils and substrates using high-throughput sequencing and to identify gelatin-hydrolyzing microbes for further application. Sandy soil, potting soil, paper plugs, black peat soil and pH-neutralized black peat soil were amended with gelatin and incubated at room temperature. After 7 and 15 days, samples were collected, DNA was extracted, and the bacterial and fungal communities were assessed by high-throughput sequencing of the 16S rRNA gene and the internal transcribed spacer (ITS) region, respectively. In parallel, microbes were isolated in culture medium. Regression analysis of shifts in the microbial communities demonstrated that the microbes positively impacted by gelatin amendment varied among the substrates, whereas few variations occurred between timepoints. The fungal genera *Penicillium*, *Mortierella*, *Fusarium* and *Trichoderma* and the bacterial genera *Burkholderia*, *Pseudomonas* and *Rhodanobacter* were among the microbes that increased in relative abundance in response to gelatin amendment. These microbes are efficient enzyme producers and are potential candidates for formulating beneficial microbial consortia that can be applied in tandem with gelatin to enhance its biostimulant activity.

1. Introduction

Biostimulants are biological substances that stimulate plant physiological processes and functions, increase nutrient uptake efficiency and tolerance to abiotic stresses, and improve crop quality without directly affecting pathogens (Woo and Pepe, 2018). Biostimulants can act as metabolic enhancers, phyto-stimulators, biofertilizers, biogenic stimulants, plant growth regulators, elicitors, plant strengtheners and plant conditioners (Ayed et al., 2022; Yakhin et al., 2017). In agriculture, plant biostimulants are applied to improve crop yield and nutritional quality. The application of biostimulants in combination with fertilizer optimizes the efficiency of fertilizer use and reduces fertilizer application rates (European Parliament and European Council, 2019).

Protein hydrolysates (PHs) are biostimulants produced from the

partial hydrolysis of amino acids, poly- and oligopeptides derived from animal waste and/or plant biomass (Colla et al., 2017; du Jardin, 2015; Schaafsma, 2009). Animal sources include leather byproducts such as gelatin, blood meal, fish byproducts, chicken feathers and casein, while plant sources include legume seeds, alfalfa hay, corn wet-milling products and vegetable byproducts (Colla et al., 2015, 2017). PH application can alleviate salinity, temperature, nutrient and water stresses in diverse crops (Boselli et al., 2019; Botta, 2013; Ertani et al., 2013; Trevisan et al., 2019), in addition to directly impacting plant growth and nutrition (Amirkhani et al., 2016; Caruso et al., 2018; Ertani et al., 2014). PHs stimulate carbon and nitrogen uptake, assimilation and metabolism and can also interfere with hormonal pathways due to the presence of bioactive peptides similar to auxins and gibberellins (Colla et al., 2017). The beneficial effects of PH may be at least partially attributable to

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stimulation of the plant and soil microbiomes (Colla et al., 2017), as microbes can enhance plant development by modifying plant physiological processes and acting against environmental stressors (Labanca et al., 2020; Moretti et al., 2021).

Gelatin, a mixture of peptides and proteins resulting from the partial hydrolysis of collagen (Schrieber and Gareis, 2007), is among the PHs with positive effects on plant growth. Gelatin is also a primary ingredient in hard capsules employed in the pharmaceutical industry and is widely used in the food, cosmetic and photographic industries (Duconseille et al., 2015). As a biostimulant, it can be added to soil as an organic amendment to stimulate microbial activity, improve microbial diversity and facilitate soil restoration (Tejada et al., 2011). Studies of the direct impact of gelatin as a crop biostimulant are scarce (Wilson et al., 2015, 2018), but when applied adjacent to cucumber seeds, gelatin enhances plant growth and nitrogen accumulation (Wilson et al., 2018).

Most studies of biostimulants focus on plant growth and yield responses. However, gelatin amendment might stimulate the growth of beneficial soil microbes that improve plant growth through nutrient release and hormone production. Culture-dependent studies have identified the microbes involved in the degradation of gelatin-based photographic materials, but the effect of gelatin on soil microbial communities in the absence of the plant microbiome has not been explored (Abrusci et al., 2005, 2007). Accordingly, the objectives of this study were to evaluate the microbial community induced by granulated gelatin amendment in different substrates and a sandy soil via high-throughput sequencing of fungal and bacterial phylogenetic markers and to isolate and identify potential microbial fungal gelatin degraders for further agricultural applications.

2. Methods

2.1. Soils and soil incubation with gelatin

Five different soils/substrates were selected as substrates for incubations with gelatin, 1) sandy soil, 2) potting soil, 3) paper plug (plugs with a paper cover filled with KCT Coco Peat), 4) black peat soil, and 5) neutralized black peat soil with $\text{Ca}(\text{OH})_2$ to pH 7.0. Soil and substrates were chosen due to their diversity of characteristics and origin. Sandy soil was used as a natural grassland soil control; potting soil and black peat/neutralized black peat have a high organic matter content and are used for plantlet production; paper plug is rich in cellulose and also used for plantlet production.

Samples were incubated in sterile Petri dishes. 100 g of soil were used for each Petri dish and three spots of 45 g of gelatin (bovine gelatin, 220 Bloom, Van Beekum Specerijen, Harderwijk, NL) per dish were inoculated. For each substrate, six replicates were prepared. The plates were kept at room temperature (20 °C), soil moisture kept at 70 % Water Holding Capacity (WHC) with the addition of distilled water on a periodic basis. As controls, 6 replicates of each substrate without gelatin were incubated under the same conditions. Fig. S1 shows the incubation of the soil and substrates in petri dishes.

2.2. Sample collection

One gelatin spot was harvested from each sample replicate with a sterile spatula after 8 (timepoint 1) and 16 days (timepoint 2) of incubation. Each individual gelatin sample was used for microbial isolation and then immediately stored at −20 °C until further DNA extraction. DNA was extracted from 250 mg of soil with the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc.) according to the manufacturer's instructions. DNA was quantified by a spectrophotometer (NanoDrop™ 2000, Thermo Fisher Scientific, Massachusetts, USA). The extracted DNA was used for the amplification of the V3–V4 region of the 16S rRNA gene and the internal transcribed spacer 1 (ITS1) region for bacteria and fungi, respectively. For bacteria, the forward primer 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and the reverse primer 806R (5'-

GGACTACHVGGGTWTCTAAT-3') (Bergmann et al., 2011) and for fungi, the forward primer ITS1F (5'-CTTGGTCATTAGAGGAAGTAA-3') and reverse primer ITS2 (5'-GCTGCGTTCATCGATGC-3') (White et al., 1990) were used. Dual-index and Illumina sequencing adapters were attached to the amplicons. After library quantification, normalization and pooling, MiSeq V3 reagent kits were used to prepare the samples for Illumina MiSeq PE250 sequencing. The amplification steps and sequencing were performed at Genome Québec, Montréal, Canada.

2.3. Isolation and identification of fungi

Small pieces of material from the gelatin spots was collected with a microbiological loop of 1 µL (Thermo Scientific™ Sterilin™, Fischer Scientific, Landsmeer, NL) and transferred on Petri dishes containing the agar medium Potato Dextrose Agar (PDA; Oxoid, Hampshire, UK). The plates were incubated at 25 °C and twice a week checked for fungal growth. Colonies were picked and transferred to new PDA plates for purification and identification. We chose to focus on fungal isolates due to wide range of enzyme production and potential for further biotechnological uses.

The isolates were identified by Sanger sequencing of the preferred identification barcode(s) (Samson et al., 2019). In short, the total genomic DNA of the isolates was extracted using the DNeasy UltraClean Microbial Kit (Qiagen) using the manufacturer's instructions. An ITS sequence was generated for all isolates, and additionally, partial β -tubulin (*BenA*) sequences for the *Aspergillus* and *Penicillium* isolates and partial translation elongation factor 1- α (*tef1*) sequences for the *Trichoderma* isolates (Samson et al., 2019) were acquired. Homology searches with the obtained sequences was performed in the NCBI database (NCBI Resource Coordinators, 2016) and internal curated databases of the Westerdijk Fungal Biodiversity Institute.

Isolates were tested for gelatin degradation and plant growth promoting (PGP) traits: siderophore production and phosphate solubilization. For gelatin degradation capacity, 2 % gelatin was prepared: 2 g of previously gamma-irradiated gelatin were added to 100 mL of sterile demi water and microwaved (WL) until completely dissolved. The material was then poured in sterile petri dishes and transferred to the fridge (4 °C) to solidify. Later, each fungal strain was inoculated in the center of the gelatin plates and incubated at room temperature (25 °C). The plates were visually checked every day for evidence of gelatin degradation. The strains were considered positive when gelatin liquified. Phosphate solubilization assay was performed using the solid National Botanical Research Institute's phosphate growth medium (NBRI-P), described in Nautiyal (1999). Siderophore production was tested using the chrome azurol S(CAS) solution (Louden et al., 2011) in PDA medium (Merck KGaA, Darmstadt, Germany) pH 6.8.

2.4. High-throughput sequencing reads processing and data analysis

Adapter and primers were removed with Cutadapt v2020.11.1 (Martin, 2011). In order to identify amplicon variant sequences (AVSs), forward and reverse reads were processed using DADA2 as implemented in qiime2 v2019-08 (Bolyen et al., 2019; Callahan et al., 2016). Quality trimming, denoising, merging, and chimera detection were performed using the qiime2 v2020.11.1 plugin "qiime dada2 denoise-paired" with default settings except for "–p-trunc-len-f" and "–p-trunc-len-r" which were set at 200 and 180 nucleotides, respectively for ITS regions, and 200 and 190 for 16S rRNA gene. The plugin classify-sklearn (Pedregosa et al., 2011) was used to classify the taxonomic lineage of the representative sequences of the resulting ASVs based using SILVA database (v. 138) (Quast et al., 2012) for bacterial and Unite (v. 8.2) (Köljal et al., 2013) for fungal profiles. The ASV tables were converted into tab separated values (tsv) format and exported using the BIOM package (McMurdie and Paulson, 2020).

2.5. Statistical analyses

All statistical analyses were conducted in R Studio version 1.3.959 running R version 4.02 (R Core Team, 2015) using different packages. Generalized Joint Attribute Modeling (GJAM) package (Clark et al., 2017) was used to estimate the effect of gelatin amendment. From the model, we extracted the regression coefficients for each treatment to identify shifts in the microbial community (bacteria and fungi) in different treatments. Model diagnosis evaluated the Markov Chain Monte Carlo (MCMC) to check when the estimated coefficients reached a stable value (after 10,000 simulations). Same coefficients were used on PCA plot analysis to explore communities' similarities between treatments plot to highlight shifts as induced by the gelatin amendment. The significance of the clustering was analyzed by HCPC (Hierarchical Clustering on Principal Components) procedure via FactoMineR package (Lê et al., 2008). Besides the intensity of the shifts in the relative abundance of the soil microbiome provided by the GJAM regression coefficients, the relevance of the different microbes for the soil community was evaluated in terms of the centered log-ratio (CLR) transformed abundance (Leite and Kuramae, 2020). CLR transformation informs the relevance of the different microbial groups (in our case both bacteria and fungi) as a proportion of the sample's average. We used this transformation to classify the soil microbes as originally highly abundant or lowly abundant based on the log-fold differences in relation to the average, which in the CLR-transformed data corresponds to the zero value.

3. Results

3.1. Fungal community

Among all treatments and timepoints, a total of 1955 fungal ASVs distributed among 10 phyla were obtained. The majority of the ASVs belonged to the phylum Ascomycota (69 genera, 67–70 % of the ASVs), followed by Basidiomycota (23 genera, 19–23 % of the ASVs) and Mortierellomycota (2 genera, 6–7 % of the ASVs). Principal component analysis (PCA) of the regression coefficients explained 50.4 % (p -value < 0.05) and 50.6 % (p -value < 0.05) of the differences among the treatments at timepoint 1 and timepoint 2, respectively. At both timepoints, the majority of the control treatments were paired with their gelatin-amended counterparts; however, the black peat was distant from all other treatments. The gelatin-amended black peat was closer to neutral gelatin-amended black peat, while the black peat

control was closer to the potting soil treatments, showing that the black peat fungal community structure was the most affected by the gelatin amendment. The sandy soil treatments were far from the other treatments, while the paper plug treatments were closer to the black peat/neutral gelatin-amended black peat treatments (Fig. 1a). A similar distribution of treatments was observed at timepoint 2, except that the gelatin-amended black peat and neutral black peat treatments were closer to the black peat control treatments and more distant from the paper plug and potting soil treatments. In addition, the paper plug and potting soil treatments were closer to each other at timepoint 2 than at timepoint 1 (Fig. 1b).

The analysis of the most influential ASVs for the dispersion of the treatments in the PCA graph (Fig. 1a) showed that, at timepoint 1, unclassified Fungi, *Fusarium equiseti*, and *Apiotrichum laibachii* had the strongest influence on the sandy soil treatments. The dispersion of the paper plug, gelatin-amended neutral black peat and black peat treatments was highly influenced by *Trichoderma*, *Penicillium* sp. and *Penicillium lapidosum*. Unclassified *Auriculariales* contributed strongly to the dispersion of the black peat control treatments, while *Saitozyma podzolica* was the main contributor to the dispersion of the neutral black peat control treatments. The potting soil treatments were most influenced by decreases in ASVs such as *Apiotrichum laibachii* and *Penicillium lapidosum*.

The treatment dispersion and most influential ASVs were similar at timepoint 2 (Fig. 1b). However, the potting soil and paper plug treatments were closer and were strongly influenced by *Oidiodendron periconioides*, *O. rhodogenum*, *Penicillium lapidosum* and *Trichoderma*. The neutral black peat and black peat treatments formed a second group that was influenced by *Saitozyma podzolica*, *Penicillium* sp. and *Penicillium guabinese*, while *Neocosmospora solani* was important for the dispersion of the sandy soil treatments.

We also identified the relevance of the fungi affected by gelatin amendment in the different substrates compared with the unamended controls by examining the strength of the shifts in relative abundance in the fungal community. At timepoint 1, gelatin amendment significantly influenced the relative abundances of 154 groups at the species level, while at timepoint 2, 161 species-level groups were affected, across all substrates; 110 groups were shared between timepoints. On average, 17 species were positively impacted and 38 were negatively impacted at both timepoints (Table S1).

Figs. 2 and S2 show the top 10 fungal taxa that were most positively (5) and negatively (5) affected by gelatin amendment in all substrates at timepoints 1 and 2, respectively. Overall, at timepoint 1, *Mortierellaceae*

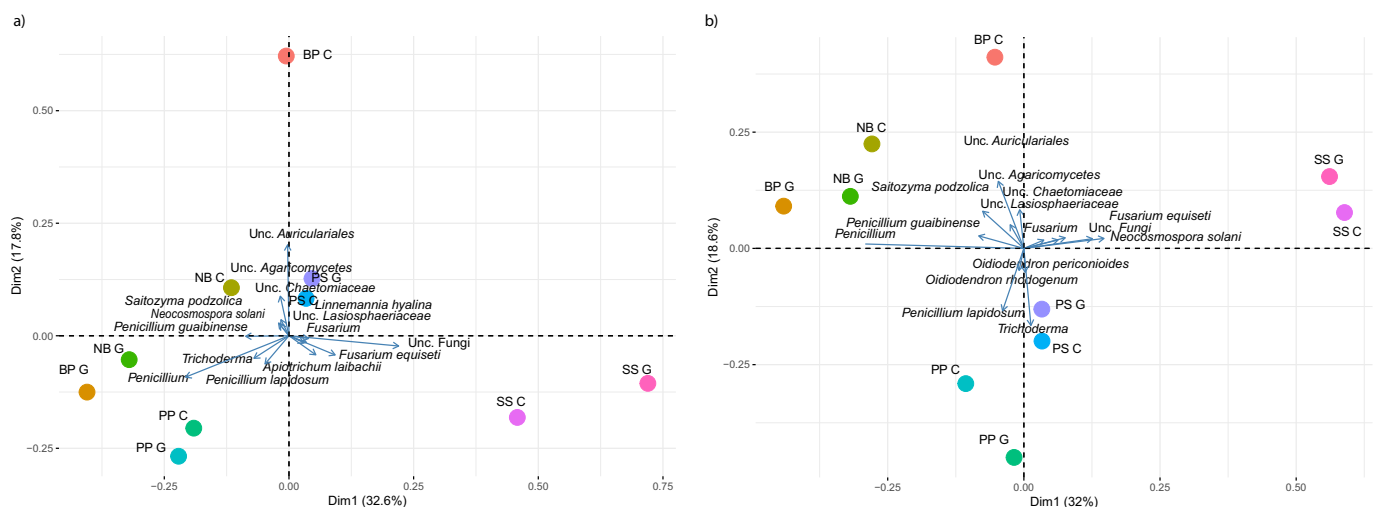


Fig. 1. PCA of regression coefficients of fungal amplicon sequence variants at a) timepoint (8 days) and b) timepoint 2 (16 days). The arrows indicate the 15 main ASVs influencing the dispersion of the samples in the PCA. BP — black peat; NB — neutral black peat; PP — paper plug; PS — potting soil; SS — sandy soil; C — control; G — gelatin-amended; Unc. — unclassified.

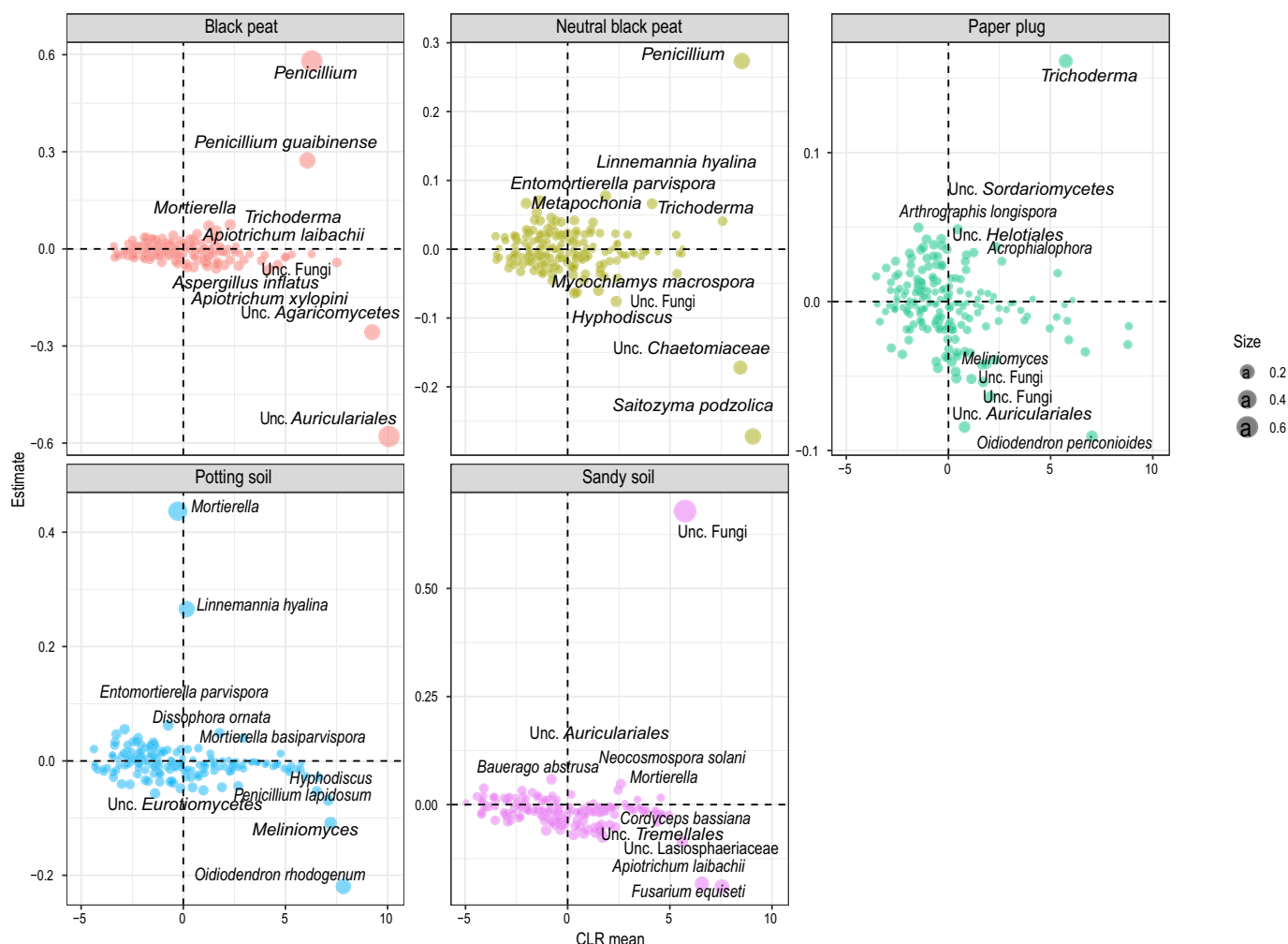


Fig. 2. Relationship between the regression coefficient and the abundance (CLR-transformed). Identification of the significant 5 most negative and most positive shifts from fungal taxa relatively to the control treatments at timepoint 1 (8 days); Unc. — unclassified. Coefficients are significant when their 95 % confidence interval does not overlap with 0.

(*Mortierella*, *Linnemannia*, *Entomortierella*, and *Dissophora*) were positively impacted in 4 of 5 substrates, the genus *Penicillium* increased in black peat and neutral black peat, and *Trichoderma* sp. increased in black peat, neutral black peat and paper plugs. Unclassified *Auriculariales* and *Meliniomyces* were among the most negatively impacted in at least two substrates. At timepoint 2, *Linnemannia* and *Penicillium* were among the genera with the greatest increases in abundance in 3 substrates. Interestingly, *Goidanichiella barronii* and *Trichoderma* increased in abundance in black peat and neutral black peat, while *G. barronii* and *Trichoderma* decreased in abundance in paper plugs and sandy soil.

3.2. Fungal isolates

In total, we obtained 18 different fungal isolates representing 16 species. Among these isolates, 13 were able to degrade gelatin, 12 were able to produce siderophores, and one was able to solubilize phosphate. These fungi are candidates for further assessment of plant growth-promoting (PGP) ability in plants. Table 1 presents the details of the isolates and their activities in the plate assays. Plate activities are shown in Fig. S3. Apart from *Mucor* and *Peziza*, all genera were detected in the ITS amplicon sequencing samples. The species observed in the ITS dataset are highlighted in bold. Among those, only *Clonostachys rosea* had its relevance significantly impacted by gelatin, increasing in sandy soil and potting soil at both timepoints, and decreasing in black peat at timepoint 2.

3.3. Bacterial community

The analysis of the 16S rRNA amplicon sequences for all timepoints and treatments yielded 7406 bacterial ASVs belonging to 422 groups at genus level and 30 phyla. The majority of the ASVs belonged to the phylum *Proteobacteria* (144 genera, 33–34 % of the ASVs), followed by *Actinobacteriota* (76 genera, 17–18 % of the ASVs), *Bacteroidota* (33 genera, 7.6–7.8 % of the ASVs), *Acidobacteriota* (31 genera, 7–7.3 % of the ASVs) and *Planctomycetota* (26 genera, 5.7–6.2 % of the ASVs). Principal component analysis (PCA) of the regression coefficients explained 69.1 % (p -value < 0.05) and 67.3 % (p -value < 0.05) of the differences among the treatments at timepoint 1 and timepoint 2, respectively (Fig. 3). The distribution of the treatments was similar at the two timepoints. The sandy soil treatments grouped together, far from the other treatments. The potting soil and black peat treatments grouped in the same quadrant, while the neutral black peat treatments grouped together and separately from the other groups. The paper plug treatments were the most affected by gelatin amendment, as the control is situated far from all other treatments, while the gelatin-amended paper plug treatments were close to the black peat and potting soil treatments. The potting soil and neutral black peat treatments were more distant from each other at timepoint 2 than at timepoint 1 (Fig. 3b).

At both timepoints, the dispersion of the treatments was primarily influenced by a few bacterial groups. *Burkholderia* affected the dispersion of the paper plug treatments amended with gelatin, the black peat

Table 1

Overview of the isolated fungal species and their activities in culture medium.

Isolate number	Identification	Gelatin degradation	Phosphate solubilization	Siderophore production
EEK_D7	<i>Aspergillus westerdijkiae</i>	Yes	No	No
EEK_D3	<i>Clonostachys rosea</i>	Yes	No	No
EEK_D9	<i>Mortierella sossauensis</i>	Yes	No	Yes
EEK_D2	<i>Mucor circinelloides</i>	No	No	Yes
EEK_I1	<i>Mucor moelleri</i>	Yes	No	No
EEK_E1	<i>Mucor</i> species	Yes	No	Yes
EEK_D1	<i>Penicillium aurantiogriseum</i>	Yes	Yes	Yes
EEK_E2	<i>Penicillium cosmopolitanum</i>	Yes	No	No
EEK_A4	<i>Penicillium glabrum</i>	No	No	Yes
EEK_C7	<i>Penicillium pulvillum</i>	No	No	Yes
EEK_G5	<i>Penicillium pulvillum</i>	No	No	Yes
EEK_I4	<i>Penicillium pulvillum</i>	No	No	Yes
EEK_B7	<i>Peziza ostracoderma</i>	Yes	No	Yes
EEK_C9	<i>Trichoderma asperellum</i>	Yes	No	Yes
EEK_D8	<i>Trichoderma hamatum</i>	Yes	No	No
EEK_I2	<i>Trichoderma harzianum</i>	Yes	No	Yes
EEK_C4	<i>Trichoderma simmonsii</i>	Yes	No	Yes
EEK_C6	<i>Trichoderma virens</i>	Yes	No	No

treatments, and the potting soil treatments. *Stenotrophomonas* impacted the dispersion of the paper plug control treatments and neutral black peat treatments. The dispersion of the sandy soil treatments was mostly influenced by unclassified KD-496 and unclassified *Vicinamibacterales*. The abundances of genera such as *Rhodanobacter*, *Streptomyces*, *Pseudomonas*, and *Lysobacter*, among others, also strongly influenced the dispersion of the treatments.

Similar to the fungal dataset, the shifts in the abundances of relevant

bacterial groups varied depending on the substrate but were similar between timepoints. Gelatin amendment significantly influenced the abundances of 308 groups at the species level at timepoint 1 and 297 groups at the species level at timepoint 2; 234 groups overlapped between timepoints. On average, at timepoint 1, 41 species were positively impacted by gelatin amendment, while 102 species were negatively affected across all substrates. At timepoint 2, an average of 38 species were positively affected, while 88 species were negatively affected across all substrates. Gelatin amendment significantly impacted the greatest number of species in the neutral black peat and paper plug treatments at timepoint 1 and the paper plug and potting soil treatments at timepoint 2 (Table S2).

Figs. 4 and S4 show the top 10 bacterial taxa that were most positively (5) or negatively (5) impacted by gelatin amendment in all samples at both timepoints. Overall, at timepoint 1, gelatin amendment increased the abundance of *Burkholderia* in black peat and potting soil but decreased it in paper plugs. *Rhodanobacter* was positively impacted in black peat and potting soil, and *Pseudomonas* increased in abundance in black peat, paper plugs and potting soil. Interestingly, gelatin amendment impacted *Stenotrophomonas* positively in paper plugs but negatively in black peat. Gelatin amendment decreased the relevance of *Aquasphaera* in both black peat and neutral black peat. At timepoint 2, *Stenotrophomonas* was among the most positively impacted in 4 substrates but was negatively impacted by gelatin amendment in neutral black peat. *Pseudomonas* increased in abundance in 3 substrates, whereas the abundance of unclassified *Gaiellales* decreased in both black peat and potting soil.

4. Discussion

PHs have been shown to promote plant growth, increase yield and mitigate abiotic stresses (Colla et al., 2017). Like other protein sources, gelatin can be used as a plant and soil biostimulant. Moreover, since gelatin is a waste byproduct of the leather industry, its application for agricultural purposes represents a sustainable, economical and eco-friendly alternative to waste disposal as part of the circular economy (Colla et al., 2017). The sensitivity of gelatin to biodegradation under favorable environmental conditions makes microorganism contamination a particularly important issue for gelatin production in countries with tropical climates (Abrusci et al., 2004, 2006, 2007). However, few studies have examined the microbial degradation of gelatin, and most have employed cultivation-dependent methods (Abrusci et al., 2004,

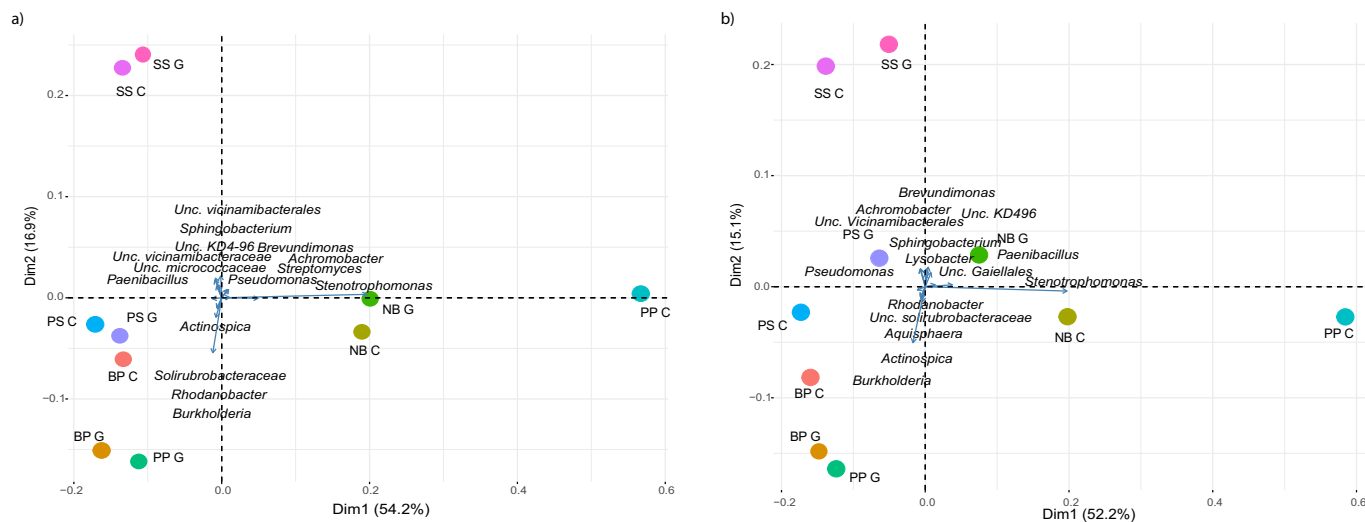


Fig. 3. PCA of regression coefficients of bacterial species at a) timepoint (8 days) and b) timepoint 2 (16 days). The arrows indicate the 15 main species influencing the dispersion of the samples in the PCA. BP — black peat; NB — neutral black peat; PP — paper plug; PS — potting soil; SS — sandy soil; C — control; G — gelatin-amended; Unc. — unclassified.

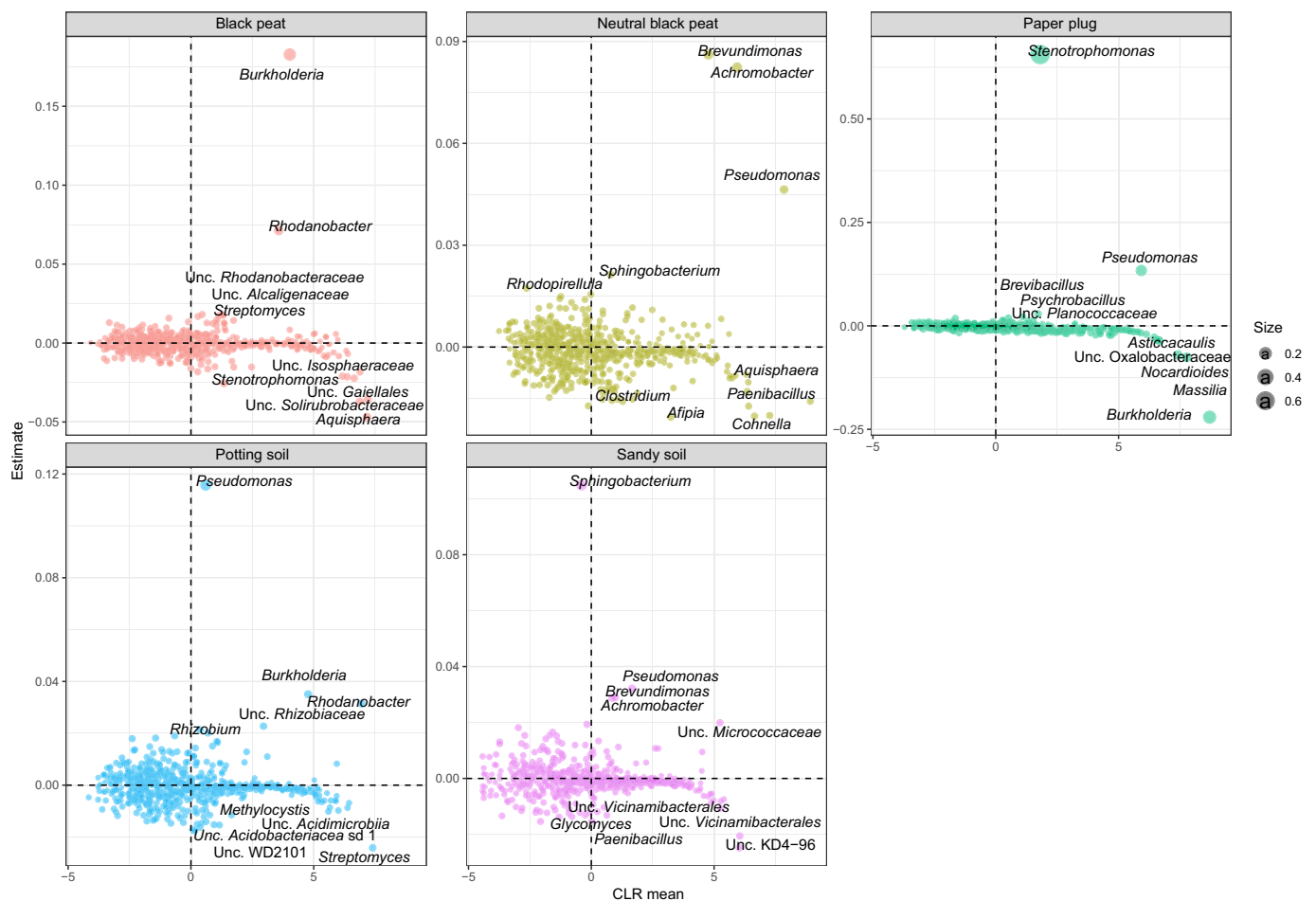


Fig. 4. Relationship between the regression coefficient and the abundance (CLR-transformed). Identification of the significant 5 most negative and most positive shifts from bacterial taxa relatively to the control treatments at timepoint 1 (8 days). Unc. — unclassified. Coefficients are significant when their 95 % confidence interval does not overlap with 0.

2006, 2007). In the present study, we evaluated the changes in whole microbial communities caused by the application of gelatin to different substrates by performing high-throughput sequencing, which can detect microbes that cannot be cultivated using current techniques.

The initial microbial community in each substrate varied, as reflected in the dispersion of the treatments in the PCA analyses of the fungal and bacterial communities. Consequently, there was no core group of microbes that increased in relevance or abundance with gelatin amendment in all substrates. Instead, a variety of microorganisms capable of producing extracellular proteases were detected, and no large changes in microbial communities were observed between timepoints. Previous studies observed a limited range of microbes in gelatin materials, possibly because the source materials were not as rich in microbes as the soil and substrates used in the present study (Abrusci et al., 2006; Kosel and Ropret, 2021; Szulc et al., 2020).

Although there was no core group of microbes, differential abundance analysis did reveal taxonomic groups that were observed more frequently. Overall, *Penicillium*, *Trichoderma*, and *Fusarium* were the fungal genera most positively affected by gelatin amendment and increased in abundance at both timepoints, despite being already dominant in most substrates.

Penicillium, *Aspergillus*, *Trichoderma*, *Metarhizium*, *Humicola*, *Fusarium*, *Cordyceps*, *Alternaria*, *Acremonium*, and *Cladosporium* are all fungi that have been found to produce various enzymes (Souza et al., 2015). *Penicillium* and *Aspergillus* are commonly used in the food and beverage industry for enzyme production and gelatin degradation (Souza et al., 2015). *Trichoderma* and *Fusarium* are known for producing proteases,

with *Trichoderma* also being used for biocontrol and enzyme production (Kredics et al., 2005; Paloheimo et al., 2016), while *Fusarium* is abundant in soil and can produce various extracellular enzymes (Crous et al., 2021; Kwon et al., 2007). Additionally, *Alternaria*, *Acremonium*, *Cladosporium*, and *Penicillium* have the ability to degrade cellulose, hemicelluloses, and chitin (Baldrian et al., 2011).

Cultivation-dependent studies have frequently isolated these genera from gelatin-containing photographic materials. In their review, Kosel and Ropret (2021) concluded that *Aspergillus*, *Penicillium*, *Trichoderma*, *Chaetomium*, *Alternaria*, *Geotrichum*, *Microascus*, *Phoma*, *Pleosporeles*, *Gnomonia*, *Nectria*, *Mucor* and *Rhizopus* are the predominant genera isolated from the surfaces of photographic material. The limited number of culture-independent studies have detected a wide diversity of fungal genera associated with the degradation of gelatin-based materials, such as *Ceriporiopsis*, *Vuilleminia*, *Galactomyces*, *Coprinellus*, *Vishniacozyma* and *Aspergillus* species with a eurotium morph (Bučková et al., 2014; Szulc et al., 2020).

Interestingly, in the potting soil treatments, five fungal species belonging to unclassified *Mortierellaceae* were among the most positively affected by gelatin amendment. The ecology of *Mortierellaceae* species is not well resolved, but there is evidence that members of this family are saprotrophs with roles in soil carbon cycling, P dissolution and mobilization, lipid metabolism, chitin degradation and even soil bioremediation (Phillips et al., 2014; Zhang et al., 2020). Saprotrophs are efficient biopolymer decomposers and produce a wide range of hydrolytic enzymes to break down and mobilize nutrients from organic matter (Baldrian et al., 2011; Phillips et al., 2014). Potting soil is mostly composed

of peat soil, which is formed by the accumulation of partially decomposed and undecomposed plant material and therefore is rich in organic matter (Osman, 2018).

In addition to *Penicillium*, *Aspergillus* and *Trichoderma*, fungal genera such as *Clonostachys*, *Mortierella*, *Peziza* and *Mucor* showed gelatinase activity in plates. Interestingly, several isolates produced siderophores, and one *Penicillium* isolate solubilized phosphate. Siderophore producers and phosphate solubilizers increase the availability of iron and phosphate to plants, respectively (Etesami and Maheshwari, 2018). These capabilities make these fungi interesting candidates for further analysis of their potential growth-promoting abilities or other beneficial effects on crops when inoculated alone or in combination with gelatin bio-stimulant (González-González et al., 2020; Raho et al., 2022).

Among the bacterial genera positively impacted by gelatin, the most prominent were protease-producing *Burkholderia*, *Rhodanobacter*, *Pseudomonas* and *Stenotrophomonas*. Similar to the fungal community, several of the bacterial genera were detected in association with gelatin for the first time in this study due to the depth of the sequencing technique and the microbial diversity of the substrates investigated. The *Burkholderia* genus consists of genetically diverse bacteria found in various ecological niches, such as soils, plants, and animals, with the ability to produce proteases, collagenases, lipases, and chitinases (Chiarini et al., 2006; Mannaa et al., 2018; Vial et al., 2007). *Pseudomonas* secretes various enzymes, including proteases, lipases, phosphatases, and chitinases, while *Stenotrophomonas* can degrade cellulose and produce extracellular proteases and gelatinases and promote plant growth (Berendsen et al., 2012; Dumont and Murrell, 2005; Sadida et al., 2020; Tielen et al., 2010). Other gelatin-amendment-influenced genera known for protease production include *Achromobacter*, *Brevundimonas*, *Chitinophaga*, *Ochrobactrum* and *Sphingobacterium* (Chaia et al., 2000; Meliah et al., 2018; Norioka and Sakiyama, 1993; Sirvas et al., 2021; Tomova et al., 2013).

In a study of gelatin-containing photographs, a varied bacterial diversity was observed. *Pseudomonas* was the most abundant genus on cellulosic envelopes of gelatin-containing photographs, while *Geobacillus*, *Lactococcus* and *Aeromonas* were most abundant in albumen-based photographs (Bučková et al., 2014; Puškárová et al., 2016). *Staphylococcus* and *Bacillus* were commonly found in cinematographic films (Abrusci et al., 2005). *Bacillus*, *Anoxybacillus*, *Brevibacillus*, *Paenibacillus*, and *Geobacillus* are common gelatin contaminants that can produce spores resistant to gelatin extraction temperatures (De Clerck et al., 2004). Other genera observed in gelatin include *Burkholderia*, *Salmonella*, *Enterococcus*, *Yersinia*, *Brevundimonas*, *Enterobacter*, *Kluyvera* (De Clerck and De Vos, 2002), as well as *Mesorhizobium*, *Ralstonia*, *Burkholderia*, *Delftia*, and *Paenibacillus* (Szulc et al., 2020).

The variations in the microbes detected in gelatin-containing materials are attributable to the different compositions of the materials. In this study, we observed a wider diversity of microbes positively associated with gelatin than reported previously (Abrusci et al., 2005, 2006), which clearly reflects the rich microbial diversity of soils and substrates compared with gelatin-based materials. Many of the microbes that we detected have been reported in previous studies of gelatin, namely, protease-producing genera that are able to degrade gelatin. However, microbial community assembly is influenced by many factors. Resource concentrations vary between environments, and resource limitations can lead to microbial competition, with impacts on microbial growth and expansion (Ghoul and Mitri, 2016). Nutrients other than gelatin were present in each of the substrates used in this study. Paper plugs, for instance, contain cellulose, which may select for different microbes compared with other substrates, such as the strong cellulase producers *Penicillium* and *Fusarium*. At timepoint 2, these genera surpassed *Trichoderma* in abundance, which may have been due to competition for resources and more efficient enzyme production for gelatin degradation. Microbial community assembly could also be driven by other types of nutrients that were not accounted for in this study, such as the high amount of organic matter in potting soil, peat and black peat.

Consequently, some of the microbes that were positively influenced by the presence of gelatin might be “cheaters”, i.e., microbes that do not produce extracellular enzymes but use the products of degradation, thereby benefiting from the resource investments of other microorganisms (Smith and Schuster, 2019). Such dynamics might also underlie the differences between timepoints in each treatment, since nutrient availability is an important factor for controlling microbial community succession (Allison, 2005; Knelman et al., 2014).

Amending soil and substrates with gelatin might have potential agricultural benefits. For example, the use of gelatin capsules as a plant biostimulant significantly improves cucumber plant growth and nitrogen content (Wilson et al., 2018). Application of a mixture of gelatin, sugarcane bagasse biochar and gum Arabic in organic mulch to soil in Iran improved the physical and mechanical properties of the soil and protected it against wind erosion. However, the microbial community involved in this process was not investigated (Gholamiderami et al., 2020) and further studies of the degradation of gelatin and its potential benefits as a biostimulant are needed. We detected and isolated several genera that are capable of degrading proteins and may produce other types of enzymes and metabolites that are beneficial for the soil microbial community, soil structure and crops. The microbes involved in gelatin degradation, such as *Mortierella*, *Penicillium* and *Trichoderma*, are potential candidates for formulating beneficial microbial consortia that may enhance the biostimulant activity of gelatin.

5. Conclusion

In the present study, we investigated the impact of gelatin amendment in the microbial community of diverse type of soils substrates. We observed that several known beneficial microbial genera had their relevance increased, including fungal genera *Penicillium*, *Aspergillus* and *Trichoderma* and bacterial genera *Burkholderia*, *Rhodanobacter*, *Pseudomonas* and *Stenotrophomonas*. Furthermore, our fungal isolates demonstrated gelatin-degrading activity, as well as phosphate solubilization and siderophore production, traits commonly present in plant growth promoting microorganisms. Overall, our study demonstrated that gelatin is a good candidate for enrichment of a diversity of soil and substrate beneficial microbes, that can increase the efficiency of nutrient uptake in plants. In addition, the microorganisms can be further isolated and applied individually or in combination with biostimulants for potential improvements in crop yield and quality.

CRediT authorship contribution statement

Conceptualization: E.K.; methodology, A.P., O.C., J.H. and E.K.; data analysis, O.C.; Writing – original draft preparation, O.C.; writing—review and editing, O.C., W.v.L., J.H. and E.K. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest. This work was supported by the Top Consortium for Knowledge and Innovation (LWV20.385 TKI).

Data availability

The Illumina sequences were deposited in the European Nucleotide Archive (ENA; <https://www.ebi.ac.uk/ena>) under the accession number PRJEB55048. Sequences from ITS region, *BenA* and *tef1* genes obtained from isolates were deposited in GenBank under numbers OP346765–OP346780, OP688491–OP688497 and OP688486–OP688490, respectively.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.apsoil.2023.104906>.

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