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Microbial proliferation activity of biodynamic preparations and biodynamic compost

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Abstract

Background Biodynamic agriculture, different from other agroecological practices, comprises the use of humified preparations to stimulate humus formation in soil and increase plant growth and quality. The biodynamic preparations (BP), undergone a natural biotechnological transformation, were characterized by their C and N elemental content and carbon molecular distribution, and evaluated in vitro, for the first time, for their capability to proliferate some selected microbial strains active in the composting and humification processes of biomasses.

Results Horn-manure preparation 500 showed no microbial bioactivity, meant as the capacity to induce microbial proliferation, thus confirming its role in direct abiotic stimulation of plant physiology. Preparation 500 K stemming from the 500 product after the combined addition of the six humified inflorescences preparations 502–507 revealed a microbial proliferation activity generally larger than each 502–507 preparation. Though the antioxidant activity of the tested BP generally followed their total phenolic content, the correlation was not confirmed by solid-state NMR spectra of the single BP, which rather suggested a role of oxygen- and carboxyl-containing groups. Different biodynamic composts obtained upon treatment of composting biomasses with the 502–507 preparations enabled a significant microbial growth to an ever-increasing extent with progressive compost maturation. The dissolved organic matter extracts from the corresponding biodynamic composts showed an even larger capacity to induce microbial growth, overcoming the bioactivity observed for humic extracts and compost tea obtained from non-biodynamic compost.

Conclusions This study has shown that the humified inflorescences used as 502–507 biodynamic preparations largely increase the microbial proliferation power when added to both biodynamic horn-manure (500 K) and composting biomasses. Our findings provide a solid scientific basis for the application of biodynamic practices aimed at improving soil health and crop quality.

Keywords Biodynamic preparations, Humus, Microbial proliferation, Compost, Composting microbial species, Crop quality

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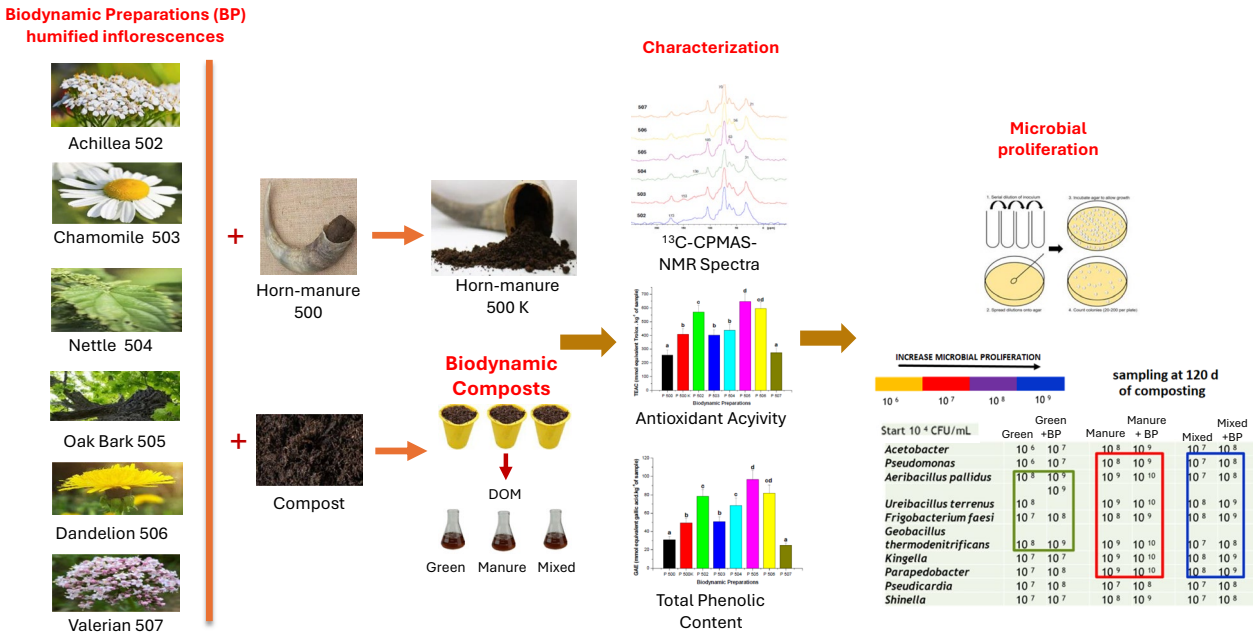
Graphical abstract



Introduction

Since its introduction by Rudolf Steiner in 1924 [1], biodynamic agriculture represented the first systematic example of a holistic and sustainable farming approach that integrates ecological, ethical, and circular economy principles, in contrast to the intensive industrial agriculture that was emerging at the beginning of the twentieth century in Europe. The practices suggested by Steiner were thoroughly experimented with for 15 years and the published results [2] became established guidelines, which promoted an ever-increasing interest in Europe and North America in the potential of biodynamic agriculture to address some of the pressing challenges of modern agriculture [3, 4]. This determined a steady growth of crop land under biodynamic management that reached the actual certified 255,000 hectares in 65 different countries [5], thus certifying the established economic importance of biodynamic agriculture.

The originality of biodynamic cultivation, as compared to other current agroecological practices like organic or regenerative farming [6], relies on the use of natural biotechnological processes to transform local on-farm biomasses into highly bioactive preparations aimed to enhance soil humus, and, hence, overall soil health and plant growth and quality and quality [7]. The peculiarity of these preparations is that they are obtained under anaerobic conditions, thus conferring to the humification process specific molecular and biological properties which cannot be otherwise delivered by aerobic humification [8]. These unique products are applied as solid humified organic matter to composting piles or liquid suspensions or extracts in form of field sprays to soil or as foliar application [9, 10].

The anaerobic humification of horn-manure into preparation 500 is obtained by storing fresh cow manure in cow horns buried in the soil for 6 months during winter. The fine anaerobically produced humus showed a larger phenolic content than in aerobically generated humus in soil and compost [11]. This is well in line with its greater bacterial rather than fungal content [12], that justifies the smaller tendency of bringing phenols to complete degradation, thereby maintaining the phenolic function of plant growth biostimulation [13] and accounting for the concomitant high auxin-like activity found in preparation 500 [12] that it is held responsible for the ultimate enhancement of the yield and quality of crops [14, 15].

Biodynamic compost is obtained by treating manure in composting piles with six biodynamic preparations (BP) made of humified inflorescences to boost microbial activity and increase both the degree of humification and the bioactivity of the resulting compost [16]. BP include yarrow (*Achillea millefolium* L.) blossoms, chamomile (*Matricaria recutita* L.) blossoms, stinging nettle (*Urtica dioica* L.) shoots, oak (*Quercus robur* L.) bark, dandelion (*Taraxacum officinale* L.) flowers, and valerian (*Valeriana officinalis* L.) flower extract, numbered 502 through 507, in the order. Biodynamic preparations were found to improve soil chemical and biological properties in vineyards soils [17, 18], and, in combination with plant-growth-promoting-bacteria (PGPB), to reduce the

adverse effects of salt stress on plants [19]. A 27-years field experiment with six main crops in a yearly rotation provided soil chemical and microbiological data which not only showed significant effects of BP-treated compost over untreated compost but was also able to differentiate between the sole application of the *Achillea millefolium* preparation and the standard application of all compost BP [20]. Similarly, a 45-year field experiment based on five crops in a seven-year crop sequence revealed that field soils added with 500 preparation and BP-treated compost possessed significantly greater physical, chemical, biological and microbiological properties than by organic and conventional management [21]. Nevertheless, a controversy on the capacity of BP and BP-treated compost to affect soil functions is still present in the related literature [22, 23], thus calling for further rigorous scientific investigations.

Biodynamic agriculture also considers the use of a preparation 500 K that is produced anaerobically in the same way as preparation 500 but it is additionally treated with the six compost biodynamic preparations 502–507. Since the 500 K preparation was introduced more recently [24] than the BP originally proposed by Steiner, few relative reports are present in the literature. Biodynamic management of vineyards that included the use of preparation 500 K showed an increase in leaf enzymatic activities that were related to induced plant resistance [25]. Application of preparation 500 K in combination with other biodynamic practices revealed a biofertilization potential since it contributed to increasing the microbial functional diversity of soils, as compared with organically treated soils [17, 18]. Metagenomic techniques were applied to evaluate the microbiota content of preparation 500 K in comparison to common compost and how they both affected the rhizosphere microbiome in orchard soils [26]. This study showed that microbiota abundance, composition, and diversity of biodynamic 500 K were distinct from compost, while rhizosphere application of 500 K extracts reduced a pathogenic fungus, and increased potentially beneficial bacterial genera.

Table 1 Percent elemental C and N, standard deviation and C/N ratio of different biodynamic preparations (BP)

BP	Total C	Total N	C/N ratio
Hornmanure (500)	30.5 ± 0.02	3.9 ± 0.05	7.8
Hornmanure K (500K_1)	30.9 ± 0.03	3.3 ± 0.02	9.3
Hornmanure K (500K_2)	31.5 ± 0.06	3.0 ± 0.08	8.8
Hornmanure K (500K_3)	31.8 ± 0.02	3.6 ± 0.06	10.3
Achillea (502)	35.2 ± 0.01	3.1 ± 0.01	11.3
Chamomile (503)	31.8 ± 0.01	3.2 ± 0.03	9.9
Nettle (504)	30.6 ± 0.02	2.9 ± 0.02	10.5
Oak bark (505)	32.5 ± 0.04	3.1 ± 0.05	10.5
Dandelion (506)	31.4 ± 0.02	3.6 ± 0.04	8.8
Valerian (507)	31.2 ± 0.05	3.8 ± 0.07	8.2

Despite the numerous works of literature describing the effect of BP-treated composts and horn-manure preparations 500 and 500 K on different cropped soils, there are not yet reports of their bioactivity towards the microbiome of the composting and humification process. The objective of this study was hence to assess the capacity of molecularly characterized BP 502–507, 500 and 500 K, as well as different BP-treated composts and their water extracts, on the proliferation of several selected microbial strains. Compost tea isolated from green compost was used as control for the water extracts from BP-treated composts. Our findings may contribute to clarifying the effect of biodynamic preparations in enhancing the microbial activity and biodiversity in soil and their capacity to increase the soil humeome that is essential to maintain soil health and sustain an ecological agricultural production [8].

Materials and methods

Biodynamic preparations

All biodynamic preparations (BP) were provided by the "Carlo Noro-Società Agricola Biodinamica" at Labico, near Rome, Italy [27]. The following BP were used in this study: 500 (horn-manure); 500 K (horn-manure K); 502 (Yarrow blossoms, *Achillea millefoilium* L.); 503 (Chamomile blossoms, *Matricaria recutita* L.); 504 (Stinging nettle shoots, *Urtica dioica* L.); 505 (Oak bark, *Quercus robur* L.); 506 (Dandelion flowers, *Taraxacum officinale* L.); 507 (Valerian flower extract, *Valeriana officinalis* L.). The 500 preparation consisted of manure from a healthy bovine anaerobically humified in a bovine horn that was buried in the soil over winter and recovered after 6 months. Horn-manure 500 K (three different replicates were used here) was developed by Alex Podolinsky in Australia [24] and it is made as horn-manure (500) but it is further added with the six 502–507 preparations, which are used in the making of biodynamic compost. These latter BP consist of inflorescences (except for the oak bark and stinging nettle shoots) which are first dried in the sun and again anaerobically humified inside animal tissues (intestine, bladder, etc.) under the soil for six months. The only liquid BP 507 is made by Valerian inflorescence whose petals are kept in opened water bottles until complete maceration.

Isolation of humic substances and compost tea

A bulk green compost was obtained in the composting facility of the Experimental Farm of University of Napoli Federico II at Castel-Volturno (Caserta, Italy). Coffee husks were mixed with horticultural residual waste at 40/60 w/w ratio and placed on static piles in which air was insufflated from beneath through perforated rubber tubes fed by a rotative air pump. The composting process went through thermophilic and mesophilic phases and

was completed in 100 days. The humified bulk compost was air dried, sieved at 2 mm and stored at 4 °C. Humic substances (HS) were extracted and prepared for further analyses as by conventional extraction procedures [28]. Compost tea (CT) was obtained by weighing 200 g of green compost into a gauze bag and placed in a plastic beaker containing 1 L of distilled water (w/v 1/5). Compost tea was extracted by actively insufflating air into the solution containing the compost bag at regular intervals (5 min every 3 h) with an automatic aeration pump device and under stirring. After seven days of aeration, the CT solution was freeze-dried and stored at 4 °C until redissolution in water for further analysis and experimentation [29].

Biodynamic composts and dissolved organic matter

Three different composts were composted in a climatic chamber at a constant temperature of 25 °C in 10 L plastic containers. 1. Green compost: 6 kg of well-shredded horticultural residual waste were mixed with 2 kg of coffee husks and 400 g of mature green compost as starter; 2. Manure compost: 7 kg of farmyard manure (manure and maize straw) were added with 300 g of mature green compost as starter; 3. Mixed compost: 3 kg of well-shredded horticultural residual waste were mixed with 1 kg of coffee husks, 3 kg of manure, and 300 g of mature green compost as starter. Each compost was prepared in six replicates, three of which were added with all the BP used in this study (502–507) in the proportions recommended by Demeter Biodynamic Federation [30]. Every two days the compost samples were turned upside-down and the moisture controlled by weighing. The composts with and without addition of biodynamic preparations were sampled at 30, 60, and 120 days from start, stored at – 20 °C, and subjected to microbial proliferation experiments. The same compost samples were also used to extract the fraction of organic matter dissolved in water (DOM). 50 g of each compost sampled at different maturation time were added with 500 mL of Milli Q-purified water and shaken for 16 h. Then, the mixture was centrifuged at 3000 rpm for 15 min, and the supernatant filtered through a Whatman 42 filter. The resulting DOM solution was freeze-dried and stored at 4 °C until redissolution in water for further analysis and experimentation.

Elemental analysis

Elemental composition (C, N) of biodynamic preparations, composts and their DOM extracts was determined using 2 mg of each material by an UNICUBE elemental analyzer (Elementar Analysen Systeme GmbH, Germany).

NMR spectroscopy

A 300 MHz Bruker Avance spectrometer, equipped with a 4 mm wide-bore MAS probe, was used to run solid-state spectra of biodynamic preparations (liquid Valerian, 507, was preliminarily freeze-dried). Each fine-powdered sample (150 mg) was packed into a 4 mm zirconium rotor, stopped by a Kel-F cap and spun at a rate of 13,000 ± 1 Hz. ^{13}C NMR spectra were acquired through the Cross-Polarization Magic-Angle-Spinning (CPMAS) technique, by using 2 s of recycle delay, 1 ms of contact time, 30 ms of acquisition time and 4000 scans. All spectra were processed using MestReC NMR Processing Software (v.4.8.6.0, Cambridgesoft, Cambridge, Massachusetts, USA).

Table 2 Relative distribution (percent) of signal area of structural groups over chemical shift regions (ppm) in ^{13}C-CPMAS-NMR spectra of different biodynamic preparations (BP)

BP	Alkyl-C	Methoxyl-/N-Alkyl-C	O-Alkyl-C	Aromatic-C	Phenolic-C	Carboxyl-C	A/OA[a]	ARM[b]	HB[c]	LR[d]
	0–45	45–60	60–110	110–145	145–160	160–190				
Hornmanure (500)	25.0	13.3	37.6	14.4	4.1	5.5	0.7	18.5	0.6	3.2
Hornmanure (500 K_1)	20.0	12.8	44.9	12.7	4.7	4.9	0.4	17.4	0.6	3.1
Hornmanure (500 K_2)	21.4	11.9	45.6	13.0	4.5	3.6	0.4	17.5	0.6	2.6
Hornmanure (500 K_3)	20.9	12.1	45.5	12.9	4.1	4.5	0.4	17.0	0.6	2.9
Achillea (502)	22.4	10.7	44.7	15.8	3.2	3.2	0.5	19.0	0.7	3.3
Chamomile (503)	19.4	11.4	38.1	12.7	5.6	12.7	0.5	18.3	0.6	2.0
Nettle (504)	31.2	11.2	34.7	10.0	4.4	8.4	0.9	14.4	0.8	2.5
Oak bark (505)	18.6	11.8	44.1	16.7	3.8	4.9	0.4	20.5	0.6	3.1
Dandelion (506)	27.3	10.3	43.7	11.4	3.2	4.1	0.6	14.6	0.7	3.2
Valerian (507)	26.2	11.0	42.5	11.2	3.6	5.5	0.6	14.8	0.7	3.1

[a]Alkyl-C/O-AlkylC, A/OA = (0–45) / (60–110)

[b]Aromaticity, ARM = (110–145) + (145–160)

[c]Hydrophobicity, HB = (0–45) + (110–160) / (45–60) + (60–110) + (160–190)

[d]Lignin Ratio, LR = (45–60) / (145–160)

Antioxidant activity and phenolic content of biodynamics preparations

Antioxidant properties of biodynamic preparations were evaluated by ABTS spectrophotometric assay as reported earlier [29]. The method is based on the oxidation of 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) by potassium persulphate to form a radical cation (ABTS+). Briefly a stock solution of ABTS (7 mM) was mixed with 2.45 mM potassium persulphate in the dark at room temperature for 12–16 h before use. Then, the working solution of $ABTS^{\cdot}$ + was prepared by diluting 10 mL of stock solution with 800 mL of water/ethanol solution (1/1, v/v). Then, 4 mg of each sample was dissolved in 2 mL of Milli Q water and 100 µl of this solution was mixed with 1.9 mL of $ABTS^{\cdot}$ + working solution. The mixture was stirred for 2 min in the dark to promote the reaction between the sample and the radical solution and the absorbance was read at 734 nm. Results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC, millimoles.kg^{-1}) based on a linear calibration curve of Trolox standard.

The total phenolic content (TPC) of biodynamic preparations was measured by a Folin-Ciocalteu colorimetric method [29]. Briefly, 8 mg of each extract was dissolved in 4 mL of methanolic/water solution (50/50). Then, 12.5 µL of each extract was diluted by 50 µL of Milli-Q water and a 12.5 µL Folin-Ciocalteu reagent was added to the solution. After 5 min, 125 µL of a 7% Na_2CO_3 solution was added, and all samples were incubated for 90 min at 25 °C. Then, the absorbance of each sample was read with a Perkin Elmer Lambda 25 UV/Vis spectrometer at 760 nm. A calibration curve for this assay ($R^2 = 0.994$) was based on different concentrations of a gallic acid standard (0.1–100 mg L^{-1}) and results for TPC were expressed as millimoles of gallic acid equivalents (GAE) per kg of dry sample.

Microbial activity of biodynamic preparations

The following microorganisms used in this study were purchased from KAIROSafe srl, Trieste, Italy, as microbial strains active in the composting process, and were employed as received: 1. *Acetobacter aceti* favors the nitrogen metabolism during composting [31]; 2. *Aeribacillus pallidus* vigorous in the thermophilic phase of composting [32]; 3. *Ureibacillus terrenus* isolated during the thermophilic phase of composting [33]; 4. *Frigoribacterium faeni* a generum isolated from spent mushroom compost [34]; 5. *Geobacillus thermodenitrificans* reduces the loss of S^{2-} during composting [35]; *6. Parapedobacter luteus* isolated from cotton waste compost [36]*;* 7. *Pseudomonas aeruginosa,* reduces ammonia volatilization and hasten the humification process [37]; 8. *Pseudonocardia*

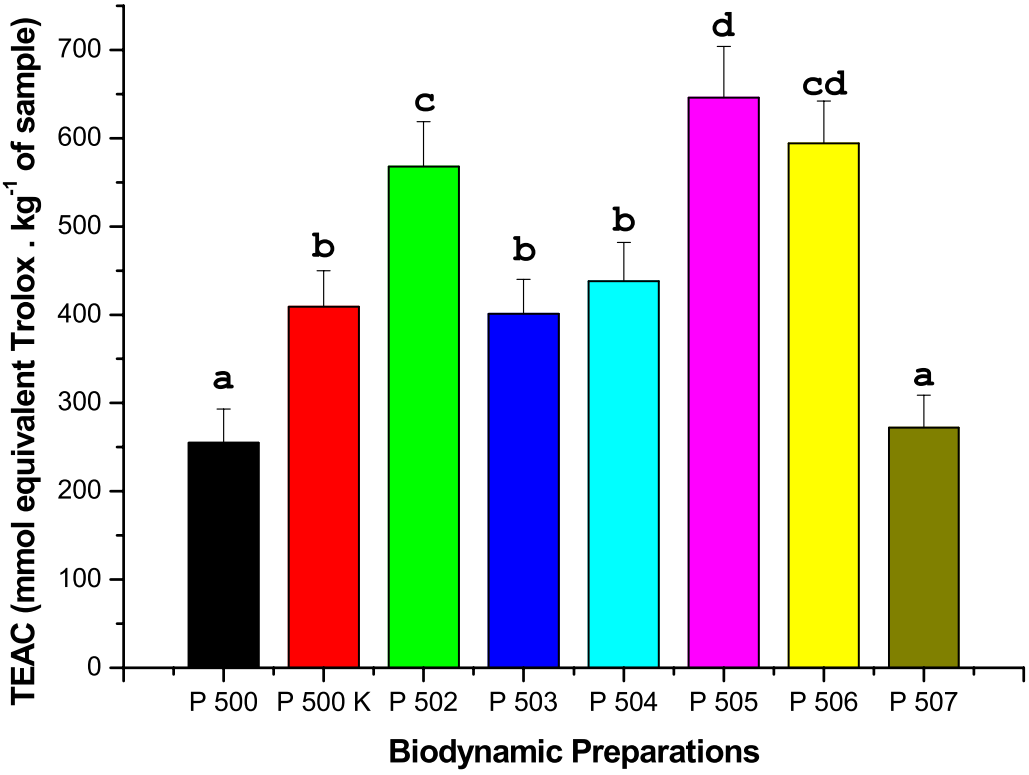


Fig. 1 Antioxidant activity of biodynamic preparations (500, 500 K, 502–507) by the ABTS assay and expressed as mmol equivalent of Trolox kg^{-1} of preparation. Different letters indicate significant differences ($p < 0.05$) between preparations

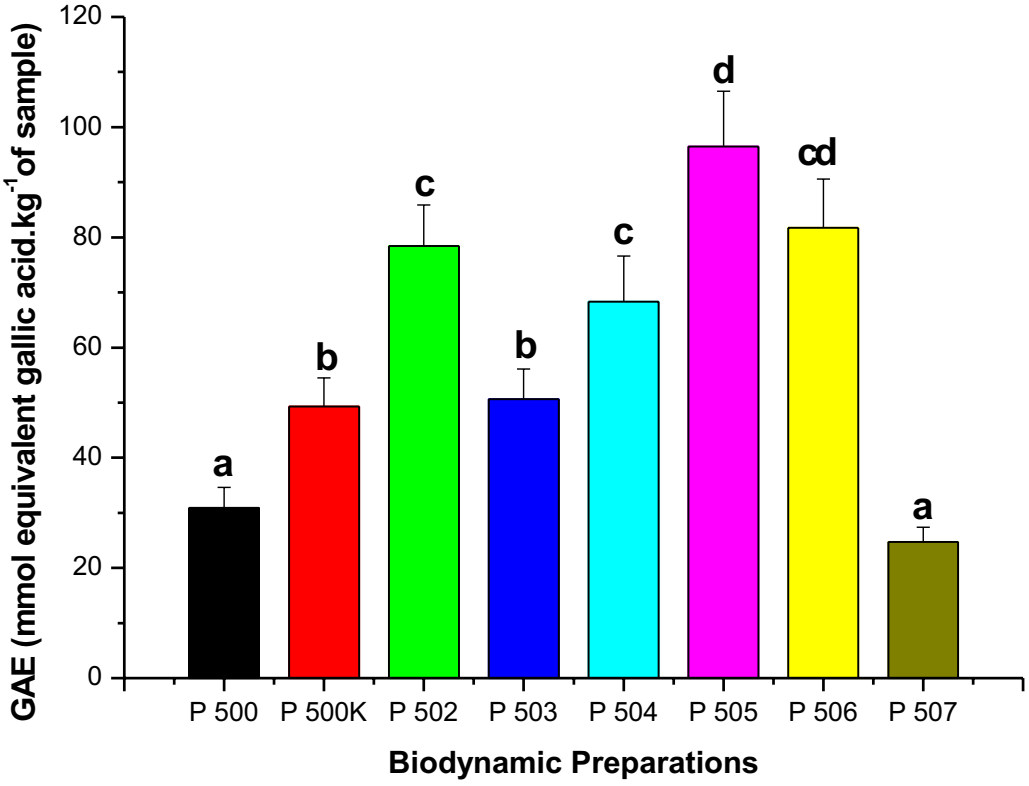


Fig. 2 Total phenolic content of biodynamic preparations (500, 500 K, 502–507) by the Folin–Ciocalteau assay and expressed as mmol equivalent of Gallic acid kg^{-1} of preparation. Vertical bars represent standard deviations of the mean. Different letters indicate significant differences ($p < 0.05$) between preparations

autotrophica produces lignin degrading enzymes [38]; 9. *Shinella zoogloeoides* isolated from activated sludge [39]. Each material to be tested (compost, compost tea and compost-extracted DOM) for the proliferation of the selected microbial strains was placed in vial (50 mL), containing 10 mL of sterile solution of a 0.1% sodium pyrophosphate (w/v) solution at pH 7.0, as modified from an earlier method of [40]. Briefly, the suspensions were shaken for 5 min, centrifuged at 3000 rpm for 5 min and then allowed to stand for 10 min. Furthermore, 1 mL of each supernatant was added into a vial containing 9 mL of sterile 0.1% sodium pyrophosphate solution to reach a final volume of 10 mL. Finally, all plates were incubated at 37 °C for 48 h and the microbial colonies were counted as 1×10^8 CFU mL^{-1} if their diameter was greater than > 0.5 mm. The CFU determination was conducted by a Thermo Scientific™ Multiskan™ FC Microplate Photometer measuring the absorbance of microtiter plates at 570 nm.

Statistical analyses

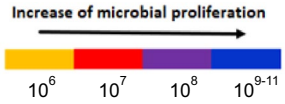
The significant difference between mean values was determined by the one-way analysis of variance, while application of a *t*-test to differentiate among results was used at the $p < 0.05$ probability level by using the Statgrafics Centurion software version XV. Data were checked with the Shapiro–Wilk and Levene test for normality and homogeneity of variance, respectively.

Results and discussion

Elemental composition of biodynamic preparations

The elemental C and N content and C/N ratio of biodynamic 500, 500 K, and 502–507 preparations are shown in Table 1. While a large literature describes the variations in C and N content in soils treated with biodynamic preparations [7], the information is very poor in the elemental composition of the biodynamic preparation themselves. The only other study in which the C and N constituents of three preparations 500 are reported in literature [12] showed a smaller carbon content (24.8–27.1) and larger C/N ratio (9.1–11.9) than observed here, thereby indicating relatively less stable humified materials. Similarly, greater C/N ratios but larger contents of elemental carbon were measured here in the three 500 K products (Table 1). This may be interpreted with lesser biochemical stability of the further processed 500 K preparations. Even larger C/N ratios were shown for the biodynamic 502–505 preparations, whereas the C/N ratios of the biodynamic products prepared from the Dandelion (506) and Valerian (507) inflorescences were smaller. The significant greater biochemical instability shown by the 500 K and 502–507 preparations as compared to the

Table 3 Effect of biodynamic preparations 500 and 500 K and of humic substances and compost tea extracted from compost, on the microbial proliferation (CFU mL^{-1}) of some bacterial species active in the composting process.



	Hornmanure (500)	Hornmanure K (500 K1)	Hornmanure K (500 K2)	Hornmanure K (500 K3)	Humic Substances	Compost Tea
Acetobacter aceti	10^4 Ea	10^6 Bb	10^6 Bc	10^5 Cd	10^6 Bd	10^7 Ad
Aeribacillus pallidus	10^4 Ea	10^6 Db	10^7 Cb	10^7 Cb	10^8 Ab	10^8 Bc
Ureibacillus terrenus	10^3 Eb	10^6 Cb	10^8 Ba	10^9 Aa	10^5 De	10^6 Ce
Frigoribacterium faeni	10^3 Eb	10^5 Dc	10^7 Bb	10^7 Bb	10^6 Cd	10^9 Ab
Geobacillus thermodenitrificans	10^4 Ea	10^7 Ca	10^7 Cb	10^6 Dc	10^9 Ba	10^{11} Aa
Parapedobacter luteus	10^4 Ea	10^5 Dc	10^7 Cb	10^7 Cb	10^8 Bb	10^9 Ab
Pseudomonas aeruginosa	10^3 Db	10^6 Bb	10^5 Cd	10^6 Bc	10^5 Ce	10^7 Ad
Pseudonocardia autotrophica	10^3 Db	10^{7B} a	10^7 Bb	10^6 Cb	10^7 Bc	10^{11} Aa

The initial standardized inoculum was equal to 10^4 CFU mL^{-1}. The coefficients of variation were invariably smaller than 6%. Significant difference ($p < 0.05$) is indicated among rows by capital letters and among columns by small letters

500 product may be attributed to the increased amount of amino acids and other nitrogen hydrophilic metabolites produced by the bioactivity of the humified inflorescences [41], which are reckoned to stabilize nitrogen during decomposition and reduce its loss through volatilization and leaching [42].

The C/N ratio is a key factor in soil health and composting processes [43]. A large ratio in humified organic matter retards nitrogen release, while a small ratio leads to rapid nitrogen mineralization and bioavailable organic carbon, thus enabling humified biodynamic preparations to exert a potential impact on the carbon and nitrogen balance, by promoting a steady microbial activity without excessive depletion of nitrogen [44].

Solid-state ^{13}C-NMR spectra of biodynamic preparations

The ^{13}C-CPMAS-NMR spectroscopy is a useful non-destructive analytical technique for evaluating the carbon distribution in humified organic matter [45]. The ^{13}C-CPMAS-NMR spectra of 500 and 500 K preparations and those of 502–507 are shown in Supporting Information in Figs. S1 and S2, respectively, while the percentage distribution of carbonaceous species in different spectral signal areas are reported in Table 2.

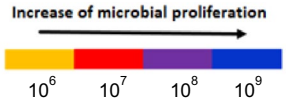
The NMR spectra revealed that the 500 preparation of this study resulted larger in aromaticity (ARM), alkyl/O-alkyl (A/OA) and lignin (LR) ratios, but smaller in hydrophobicity (HB) than the 500 preparations reported earlier [11]. As compared to the 500 product, the three 500 K preparations showed smaller ARM, A/OA, and LR (Table 2), thus suggesting that the treatment with biodynamic humified inflorescences has led to an advanced transformation of the 500 alkyl and aromatic components towards greater potential bioavailability [46].

The spectra of the 502–507 preparations revealed a predominance of O-alkyl carbons (60–110 ppm), whose spectral integration spectra obtained in this region accounted for 44.7, 38.1, 34.7, 44.1, 43.7, and 42.5 percent of total signal area for Achillea (502), Chamomile (503), Nettle (504), Oak Bark (505), Dandelion (506), and Valerian (507), respectively (Table 2). This spectral region can be assigned to oxygen-containing metabolites produced by enhanced microbial activity during the humification process [47, 48] and can be regarded as an index of bioactivity of humified organic matter. Except for Chamomile (503) and Nettle (504), the rest of humified biodynamic inflorescences, together with preparations 500 K, showed larger percentages for the O-alkyl carbon region than preparation 500 (Table 2), thus suggesting their greater capacity of inducing microbial activity [49].

The most aromatic BP were Oak Bark (505) and Achillea (502), followed by preparation 500, with ARM values larger than those of preparations 500 K and of the rest of humified inflorescences. Nevertheless, such aromaticity of preparations 502 and 505 was compensated by their relatively large content of O-alkyl C, that enabled them to maintain their HB values similar to the other biodynamic preparations (Table 2).

On the other hand, Chamomile (503) and Nettle (504) revealed the largest content of hydrophilic carboxyl C (160–190 ppm), followed by BP Valerian (507), Oak Bark (505), Dandelion (506) and Achillea (502). In the case of Nettle (504), the polarity conferred by its relatively large carboxyl composition balanced its greatest signal

Table 4 Effect of biodynamic preparations 502-507 on microbial proliferation (CFU mL^{-1}) of some bacterial species active in the composting process.



	Achillea (502)	Chamomile (503)	Nettle (504)	Oak Bark (505)	Dandelion (506)	Valerian (507)
Acetobacter aceti	10^4 Bc	10^4 Ba	10^4 Be	10^6 Ab	10^3 Ce	10^3 Ce
Aeribacillus pallidus	10^5 Cb	10^4 Da	10^5 Cd	10^7 Aa	10^6 Bc	10^3 Ee
Ureibacillus terrenus	10^5 Db	10^4 Ea	10^8 Aa	10^6 Cb	10^7 Bb	10^7 Bb
Frigoribacterium faeni	10^6 Ba	10^4 Da	10^5 Cd	10^5 Cc	10^7 Ab	10^5 Cc
Geobacillus thermodenitrificans	10^6 Ca	10^4 Da	10^7 Bb	10^7 Ba	10^8 Aa	10^8 Aa
Parapedobacter luteus	10^4 Bc	10^4 Ba	10^6 Ac	10^4 Bd	10^4 Bd	10^4 Bd
Pseudomonas aeruginosa	10^4 Bc	10^4 Ba	10^5 Ad	10^4 Bd	10^4 Bd	10^4 Bd
Pseudonocardia autotrophica	10^5 Ab	10^4 Ba	10^5 Ad	10^4 Bd	10^4 Bd	10^4 Bd
Shinella zoofloeoides	10^5 Ab	10^4 Ba	10^5 Bd	10^4 Bd	10^4 Bd	10^4 Bd

The initial standardized inoculum was equal to 10^4 CFU mL^{-1}. The coefficients of variation were invariably smaller than 6%. Capital letters indicate significant difference among rows, small letters indicate significant difference among columns

Table 5 Percent elemental content (C and N), standard deviation and C/N ratio of different composts treated with and without biodynamic preparations (BP) 502–507 and its dissolved organic matter (DOM) extract after 120 days of compost maturation

	Total C	Total N	C/N ratio
COMPOST			
Green compost—BP	40.7 ± 0.02	3.7 ± 0.06	10.8
Green compost + BP	39.5 ± 0.01	3.6 ± 0.04	10.9
Manure compost—BP	32.0 ± 0.03	2.5 ± 0.01	12.8
Manure compost + BP	32.0 ± 0.02	2.5 ± 0.05	12.8
Mixed compost—BP	36.1 ± 0.07	3.1 ± 0.01	11.6
Mixed compost + BP	35.4 ± 0.02	3.3 ± 0.02	10.7
DOM			
Green compost—BP	28.9 ± 0.03	5.3 ± 0.06	5.4
Green compost + BP	32.6 ± 0.05	4.8 ± 0.02	6.8
Manure compost—BP	28.2 ± 0.02	3.8 ± 0.05	7.4
Manure compost + BP	28.5 ± 0.01	3.4 ± 0.09	8.4
Mixed compost—BP	25.0 ± 0.04	4.5 ± 0.07	5.6
Mixed compost + BP	28.9 ± 0.01	5.3 ± 0.05	5.3

The coefficients of variation were invariably smaller than 5%

intensity in the alkyl-C region (0–45 ppm) and mitigated to only 0.8 an otherwise larger hydrophobicity (Table 2). The content of carboxyl groups in humified matter has been correlated to the control of nitrate uptake by plants roots [50], thereby suggesting that their contribution to the hydrophilicity of humus enhances its potential bioactive capacity [51].

Antioxidant activity and phenolic content of biodynamics preparations

As biodynamic preparations are thought to promote plant growth and soil health, attention has been devoted to assessing the effects on the antioxidant activity of plants treated with biodynamic preparations [52, 53], but no evaluation of antioxidant activity of the preparations themselves has ever been reported, to the best our knowledge. While an increased antioxidant activity in plants is linked to their potential resistance to oxidative stresses caused by pests, pathogens, or abiotic factors, such as drought and excessive UV exposure [54], enhancement of antioxidant activity in soil introduced by biodynamic compost or BP 500 and 500 K, may improve soil biological functions and overall soil health [55–57]. Antioxidant activity is largely due to bioactive compounds, such as polyphenols, flavonoids, tannins, terpenes, and other secondary metabolites, which are naturally found in the inflorescences used to make biodynamic preparations [58–62].

The results of the ABTS assay on BP are shown in Fig. 1. TEAC values indicate that the most antioxidant preparation was Oak Bark (505), followed by Dandelion (506) and Achillea (502), while the one with the least antioxidant activity was Valerian (507). Conversely, the antioxidant activity of preparation 500 was smaller than all other BP, whereas preparation 500 K showed values similar to those of Chamomile (503) and Nettle (504), being most probably enhanced by the treatment with all the humified inflorescences.

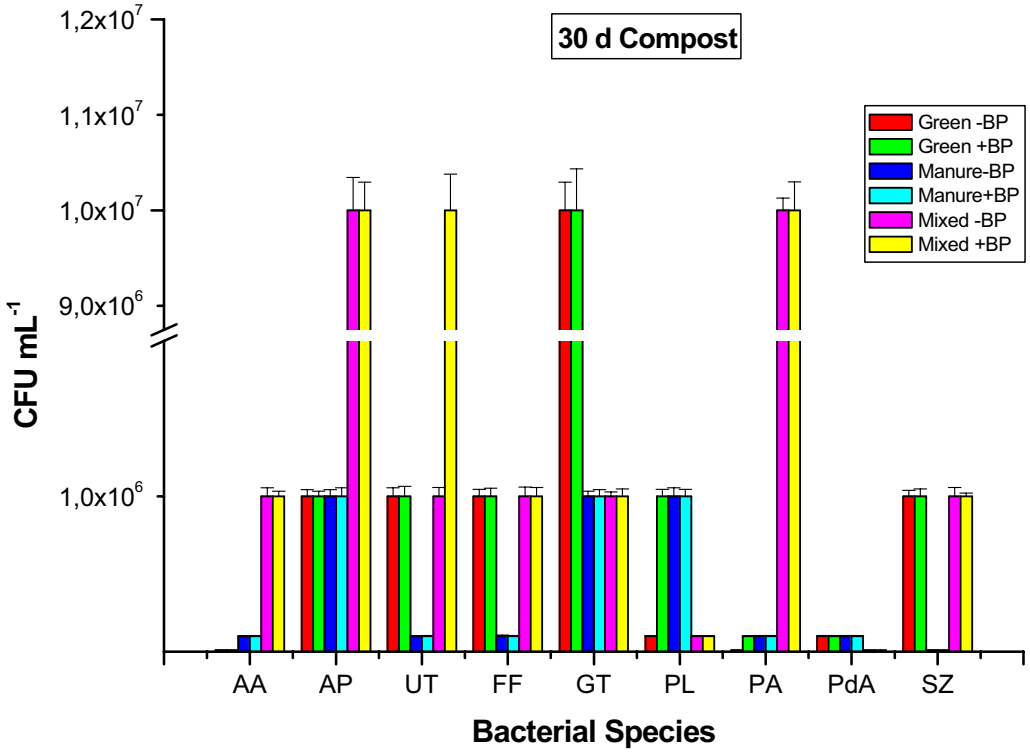


Fig. 3 Effect of different composts (green, manure, mixed) before and after treatment with biodynamic preparations (BP) 502–507 on microbial proliferation (CFU.mL^{-1}) of some bacterial species (AA = *Acetobacter aceti;* AP = *Aeribacillus pallidus;* UT = *Ureibacillus terrenus;* FF = *Frigoribacterium faeni;* GT = *Geobacillus thermodenitrificans;* PA = *Pseudomonas aeruginos;* PdA = *Pseudonocardia autotrophica;* SF = *Shinella zoofloeoides*) at 30 days of compost sampling. The initial standardized inoculum was equal to 10^4 CFU.mL^{-1}. Bars over columns indicate standard deviation

It is interesting to note that the TEAC values followed the same trend as the GAE values for total phenolic content (TPC) in BP as measured by the Folin–Ciocalteau method (Fig. 2), suggesting that the antioxidant activity mainly correlates to the content of phenolic moieties in BP [29]. In turn, the amount of phenols in humified matter is believed to have an impact on plant growth and quality [13, 63]. However, the same relation with antioxidant activity is not observed by the BP percentage of phenols as calculated from the ^{13}C-CPMAS NMR spectra (Table 2). In fact, the spectra indicate the largest quantity of phenolic compounds, 5.6%, was in the less antioxidant Chamomile (503), whereas the most active Oak Bark (505) had only 3.8%. It is instead the spectral aromaticity that can partially justify the large GAE values shown for BP 505 and 502, although the large and small ARM values for preparation 500 and 500 K, respectively, do not yet appear to reflect their values found for TPC (Fig. 2), and consequently, their antioxidant activity (Fig. 1).

These findings suggest that the antioxidant activity of BP does not directly correlate to the sole phenolic content but must also be due to other secondary aromatic compounds possibly glycosylated or carboxylated during microbial transformation in humus [47, 48, 64, 65]. This hypothesis seems to be supported by the evaluation of the O-alkyl moieties in BP, as revealed by the carbon distribution in NMR spectra (Table 2), whose lowest percentage for the preparation 500, justifies, despite a large aromaticity, the smallest antioxidant activity of this preparation (Fig. 1). Moreover, the antioxidant activity of Nettle (503), rather than for the NMR percent of ARM (14,4%) or O-alkyl-C (37%), should be accounted for the additional contribution of its large content of carboxyl carbon (8.4%) (Table 2).

Microbial proliferation induced by biodynamic preparations

Biodynamic preparations are recognized to activate microbial activity in soil and substantially contribute to agroecology by fostering soil biodiversity and fertility, and healthy plant growth [56]. The bioactivity of both preparations 500 and 500 K was reported to exert a beneficial effect on soil microbial communities [17, 18, 25], while the biomasses composted with the addition of humified BP (502–507) showed a large intrinsic microbial diversity [26], and the ability to stimulate a long-term microbial diversity in soils amended with BP-treated compost [20]. Nevertheless, all these BP had never been so far evaluated for their in vitro capability to enhance proliferation of selected bacteria involved in the

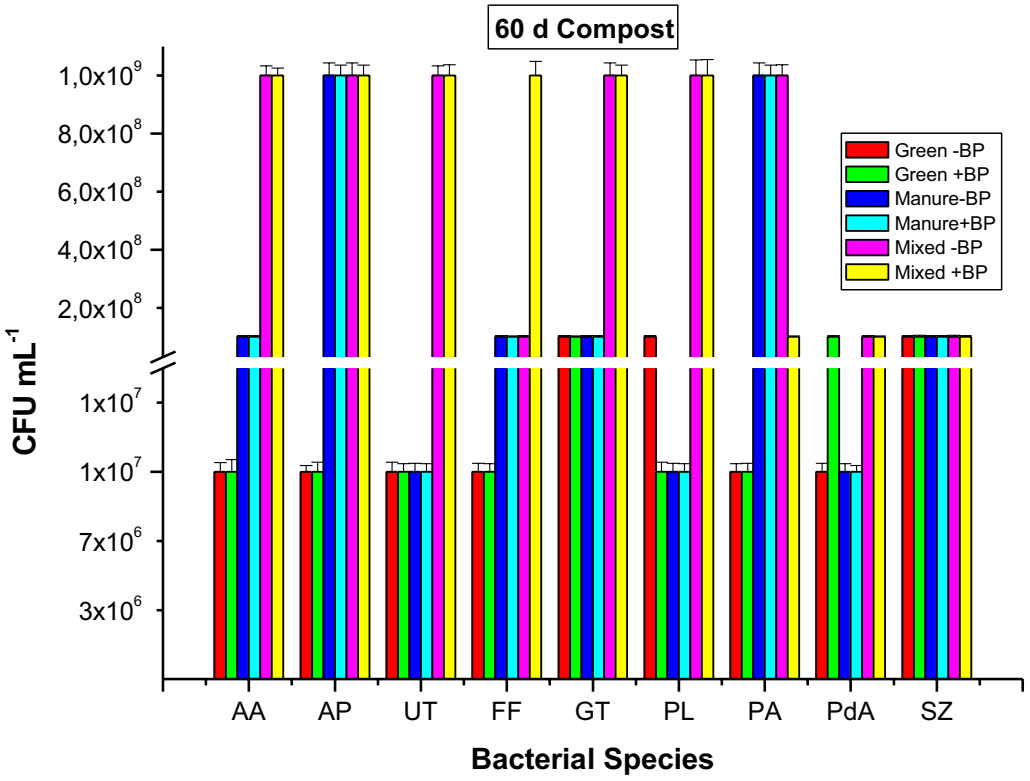


Fig. 4 Effect of different composts (green, manure, mixed) before and after treatment with biodynamic preparations (BP) 502–507 on microbial proliferation (CFU.mL^{-1}) of some bacterial species (AA = *Acetobacter aceti;* AP = *Aeribacillus pallidus;* UT = *Ureibacillus terrenus;* FF = *Frigoribacterium faeni;* GT = *Geobacillus thermodenitrificans;* PA = *Pseudomonas aeruginos;* PdA = *Pseudonocardia autotrophica;* SF = *Shinella zoofloeoides*) at 60 days of compost sampling. The initial standardized inoculum was equal to 10^4 CFU mL^{-1}. Bars over columns indicate standard deviation

composting and, concomitantly, the humification processes of organic matter.

The proliferation of several compost activating bacteria by preparation 500 and three replicates of preparations 500 K, as compared to humic substances and compost tea extracted from a green compost, is shown in Table 3. The horn-manure 500 was the least bioactive preparation, whereas the three preparations 500 K showed a significantly larger proliferation activity than the initial standardized inoculum (ISI) of all tested bacteria. *Ureibacillus terrenus* revealed proliferation by all 500 K preparations from two to five orders of magnitude greater than ISI (reaching 10^8 and 10^9 CFU mL^{-1} for $500K_2$ and $500K_3$, respectively), while the other bacteria proliferated from one to three orders of magnitude more than ISI. However, all 500 K preparations were less bioactive towards bacterial proliferation than both humic substances and compost tea extracted from a non-biodynamic compost (Table 3). The fact that a liquid humified extract from a common compost like compost tea resulted highly active in the proliferation of all bacterial species (up to 10^{11} CFU mL^{-1} for *Geobacillus thermodenitrificans* and *Pseudonocardia autotrophica*) suggests that water-soluble components from horn-manure 500 K, once amended to soils, may potentially exert a greater bioactivity than either a simple 500 preparation or a non-BP-treated compost, thereby significantly contributing to the microbial transformation of organic matter into soil humus [8].

The effect of each single 502–507 preparation on proliferation of some but not all selected bacteria is shown in Table 4. Except for Chamomile (503) that was unable, like horn manure 500 (Table 3), to stimulate the growth of any microbial species more than ISI, the rest of the tested BP showed enhanced proliferation for some but not all the selected bacteria. *Ureibacillus terrenus* and *Geobacillus thermodenitrificans* were significantly proliferated, in order, by Nettle (504) > Dandelion (506) = Valerian (507) > Oak Bark (505), and by Dandelion (506) = Valerian (507) > Nettle (504) = Dandelion (506) > Achillea (502), respectively. The growth of *Aeribacillus pallidus* was stimulated only by both Oak Bark (505) (10^7 CFU mL^{-1}) and Dandelion (506) (10^6 CFU mL^{-1}), whereas proliferation of *Frigoribacterium faeni* was induced by Achillea (502) (10^6 CFU mL^{-1}) and Dandelion (506) (10^7 CFU mL^{-1}), and *Parapedobacter luteus* grew significantly only upon treatment with Nettle (504) (Table 4).

It is interesting to note that the Oak Bark and Dandelion preparations, which are the BP showing the generally largest proliferation capacity (Table 4), are also those revealing the greatest antioxidant activity, followed in

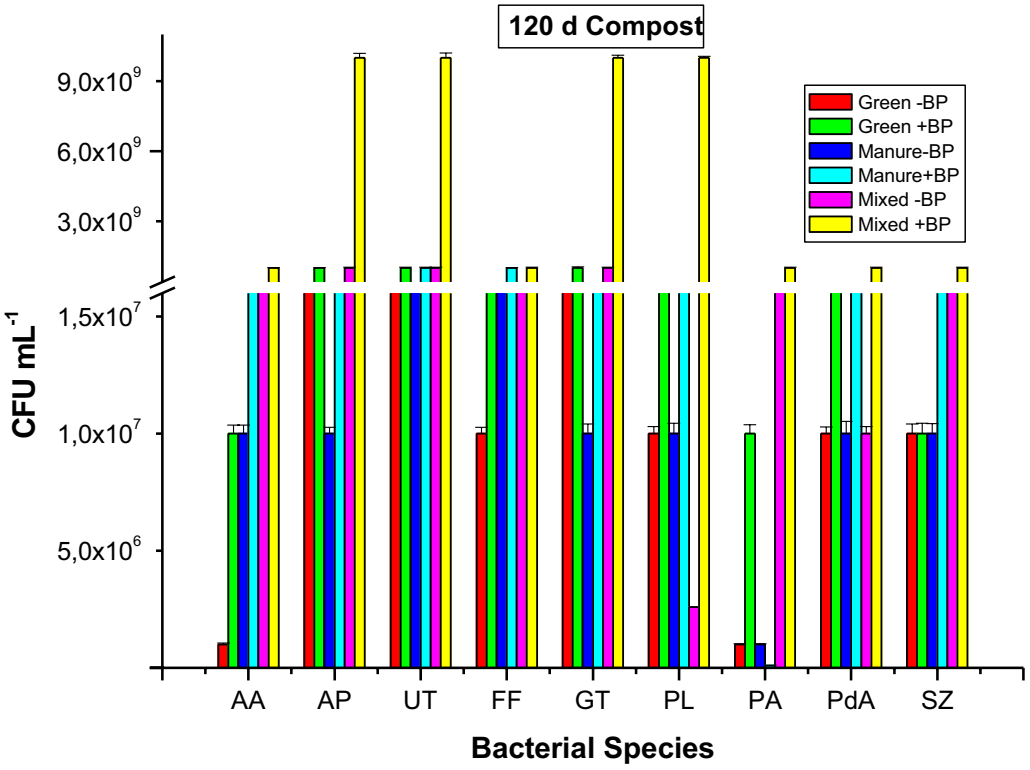


Fig. 5 Effect of different composts (green, manure, mixed) before and after treatment with biodynamic preparations (BP) 502–507 on microbial proliferation (CFU.mL^{-1}) of some bacterial species (AA = *Acetobacter aceti;* AP = *Aeribacillus pallidus;* UT = *Ureibacillus terrenus;* FF = *Frigoribacterium faeni;* GT = *Geobacillus thermodenitrificans;* PA = *Pseudomonas aeruginos;* PdA = *Pseudonocardia autotrophica;* SF = *Shinella zoofloeoides*) at 120 days of compost sampling. The initial standardized inoculum was equal to 10^4 CFU mL^{-1}. Bars over columns indicate standard deviation

microbial stimulation by Achillea (502) and Nettle (504) with a lesser antioxidant activity (Fig. 1). However, it is also true that Valerian (507) revealed a significant proliferation induction (Table 4), despite an antioxidant activity that was even smaller than the biological inactive Chamomile (503) (Fig. 1). These inconsistencies may be attributed, as reminded above, to the highly heterogeneous biomolecular composition of such humified materials, which resulted from a random biological transformation, thus failing to give a net structure–activity relationship.

The proliferation potential of each single preparation, shown here for the first time, indicates that biodynamic humified inflorescences have the capability to activate the processes leading to the formation of bioactive humified compost, thereby confirming their significant role in improving soil health within the practice es recommended by biodynamic agriculture. However, the different chemical and biological compositions of these BP [66, 67], are also responsible for their observed variable bioactivity towards bacterial proliferation as single BP (Table 3). These findings may open to future perspectives by designing experiments in which BP should be tested in their growth stimulation of co- and mixed cultures directly in soil.

Microbial proliferation induced by BP treated composts and their water-soluble fractions

The elemental C and N content in the three different composted biomasses (green, manure, mixed), as well as in their dissolved organic matter (DOM) before and after treatment with the 502–507 BP are shown in Table 5. There were no substantial C and N differences in the various composts either treated or untreated with BP, except for a slight decrease in total C in the BP-treated green and mixed composts. DOM samples resulted to have less C but more N than the corresponding composts, while, in contrast, they showed a slight carbon increase when from BP-treated green and mixed composts. The smaller C/N values in DOM than in compost samples (Table 5) suggest a prompter N availability for DOM extracts and their potentially larger microbial bioactivity that the composts of origin [44].

The proliferation of the selected bacterial species by green, manure and mixed composts with or without amendment with the 502–507 preparations, as sampled at 30, 60, and 120 days from composting onset, is shown in Figs. 3, 4 and 5, respectively. The CFU mL^{-1} values at 30 d of sampling revealed a general increase in respect to ISI for all the tested composts. However, there was no preferential increase of proliferation activity between the

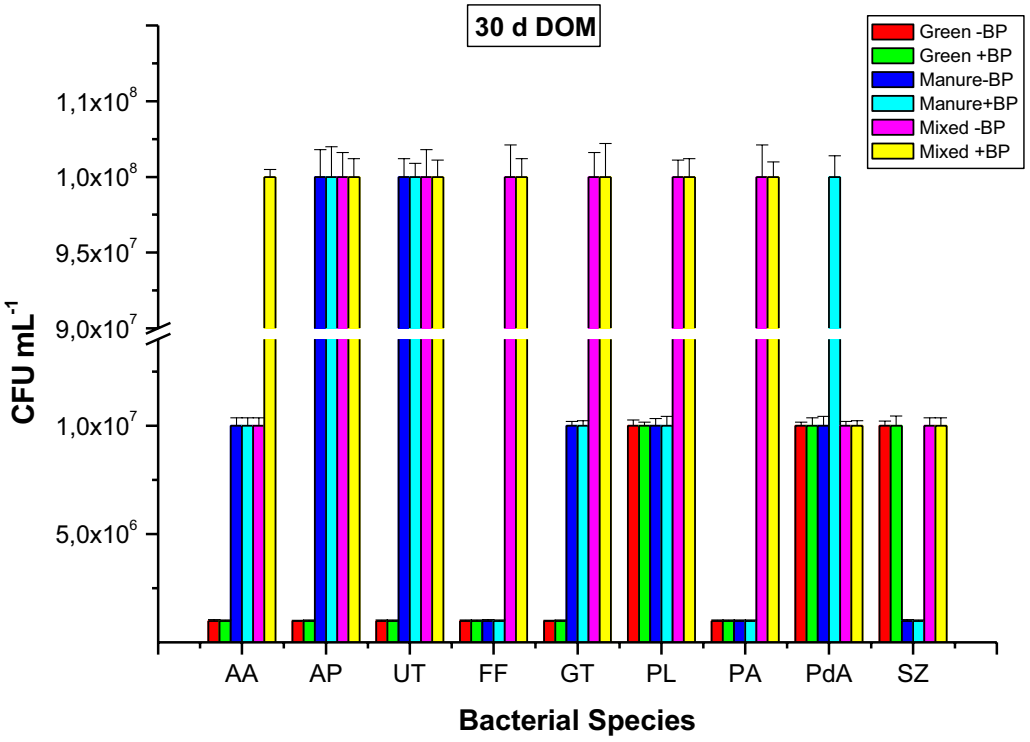


Fig. 6 Effect of dissolved organic matter (DOM) extracted from different composts (green, manure, mixed) before and after treatment with biodynamic preparations (BP) 502–507 on microbial proliferation (CFU mL^{-1}) of some bacterial species (AA = *Acetobacter aceti;* AP = *Aeribacillus pallidus;* UT = *Ureibacillus terrenus;* FF = *Frigoribacterium faeni;* GT = *Geobacillus thermodenitrificans;* PA = *Pseudomonas aeruginos;* PdA = *Pseudonocardia autotrophica;* SF = *Shinella zoofloeoides*) at 30 days of compost sampling. The initial standardized inoculum was equal to 10^4 CFU mL^{-1}. Bars over columns indicate standard deviation

BP-treated and untreated composts for all tested bacteria, except for an order of magnitude larger than ISI for *Parapedobacter luteus* and *Pseudomonas aeroginosa*, and for *Ureibacillus terrenus* in BP-treated green and mixed compost, respectively (Fig. 3).

At 60 d of composting time, all composts either treated or untreated with 502–507 BP showed an increase of proliferation activity from 2 to 4 orders of magnitude in respect to 30 d, with the mixed composts showing the largest increment of bioactivity (Fig. 4). However, there was still no difference in growth of bacterial species between BP-treated and untreated composts, except for an increase of one order of magnitude for *Geobacillus thermodenitrificans*, and even a decrease in bioactivity of some bacterial species after BP-treatment for both manure and mixed composts (Fig. 4).

The proliferation of microbial strains was enhanced significantly at 120 d of sampling in comparison to previous composting times for all three composts and reached a maximum for mixed compost when passing from the untreated to the BP-treated samples (Fig. 5). In particular, while the average microbial growth of the BP-treated manure compost was as high as 10^8–10^9 CFU mL^{-1}, that of the similarly treated mixed-compost achieved values up to 10^9–10^{10} CFU mL^{-1} (Fig. 5).

These results show that even though the proliferation activity of each single 502–507 preparation was moderate, with the exception of Nettle, Dandelion and Valerian (Table 4), the combined addition of all 6 biodynamic preparations resulted in a larger compost bioactivity that increased significantly in mature composts. However, the efficacy of the microbial proliferation depended on the original materials being composted, and was largest for mixed compost containing an equal amount of both manure and green biomass. These findings are in line with a previous report [26] that showed a microbial diversity among biodynamic composts made of different biomasses.

The microbial proliferation of DOM solubilized from the same three different composts revealed much larger CFU mL^{-1} values than by the corresponding composts at all diverse times of sampling (Figs. 6, 7, 8). At 30 d of sampling, DOM microbial growth was already from 2 to 4 orders of magnitude larger than ISI (Fig. 6), but did not show any difference when solubilized from either BP-treated or untreated composts, except for an increase of one order of magnitude in *Acetobacter aceti* for DOM from treated mixed compost.

As observed for composts, at 60 d of sampling, all DOM samples showed a microbial proliferation larger at least one order of magnitude than for 30 d of sampling.

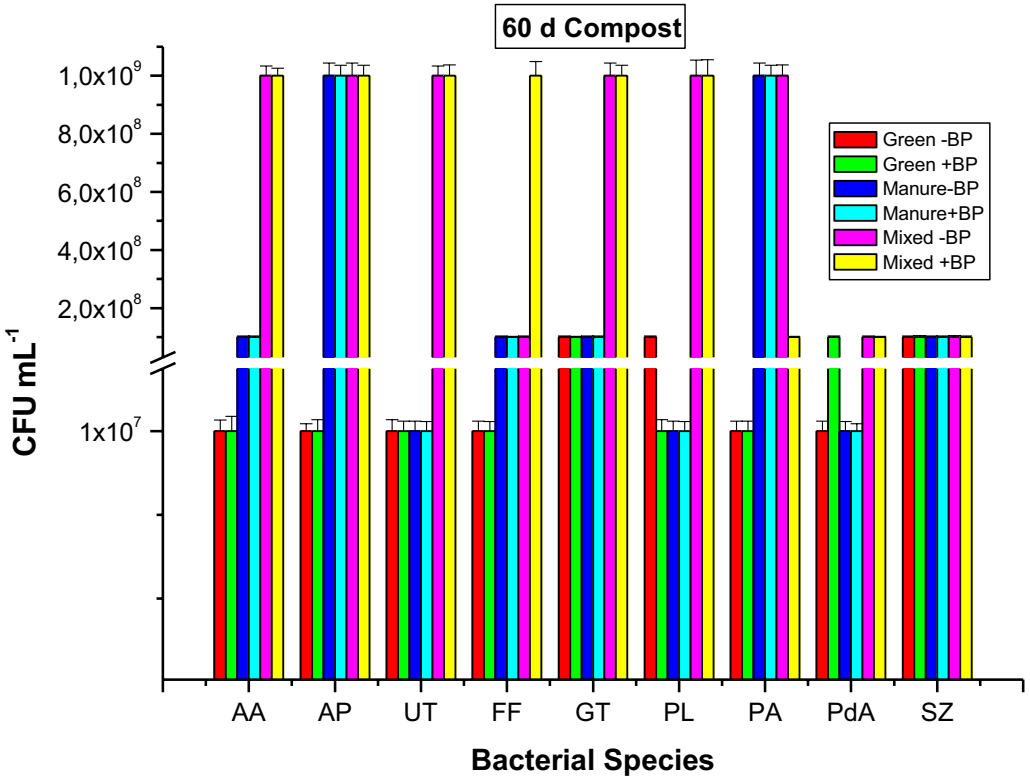


Fig. 7 Effect of dissolved organic matter (DOM) extracted from different composts (green, manure, mixed) before and after treatment with biodynamic preparations (BP) 502–507 on microbial proliferation (CFU.mL^{-1}) of some bacterial species (AA = *Acetobacter aceti;* AP = *Aeribacillus pallidus;* UT = *Ureibacillus terrenus;* FF = *Frigoribacterium faeni;* GT = *Geobacillus thermodenitrificans;* PA = *Pseudomonas aeruginos;* PdA = *Pseudonocardia autotrophica;* SF = *Shinella zoofloeoides*) at 60 days of compost sampling. The initial standardized inoculum was equal to 10^4 CFU mL^{-1}. Bars over columns indicate standard deviation

However, again there was no difference beween BP-treated and untreated DOM samples, except for a decrease from 10^8 to 10^7 CFU mL^{-1} in the growth of *Pseudomonas aeroginosa* and an increase from 10^7 to 10^8 CFU mL^{-1} in the growth of *Parapedobacter luteus* in DOM from BP-treated mixed compost (Fig. 7).

A large and significant increase of proliferation of all selected bacteria was noted for DOM at 120 of sampling regardless of the original compost treatment (Fig. 8). Moreover, at this sampling time, DOM from BP-treated composts invariably showed one order of magnitude larger microbial growths than for untreated ones, reaching up to 10^{13} CFU mL^{-1} for *Pseudonocardia autotrophica* and *Shinella zoofloeoides* upon treatment with DOM from mixed compost (Fig. 8).

As observed earlier for the microbial proliferation activity of the 500 and 500 K preparations which were found less extensive than for the humic extract and compost tea from a non-biodynamic green compost (Table 3), also DOM extracts are more bioactive than the corresponding composts, and even more so (from one to two orders of magnitude larger than for untreated composts) when DOM are solubilized from a BP-treated compost (Figs. 6, 7, 8). These results agree with previous works which showed for various soils microbial responses in bacterial community composition with a relative increase in PGPM when treated with either 500 and 500 K preparations [17, 18], or with biodynamic compost obtained after treating with 502–507 BP [20], thus indicating a possible selective activity of biodynamic products towards soil microbial species. It is thus conceivable that such humified biodynamic preparations are capable to provide to the soil solution some readily accessible bioactive molecular and microbial characteristics that may stimulate the growth of soil microbial communities and improve the soil biological functions.

Conclusions

Our findings revealed that the horn-manure 500 preparation did not show any proliferation of microbial species, thereby suggesting that its role in the plant rhizosphere may be mainly abiotic, due to its relevant content of phenolic molecules [11] which exert an auxin-like stimulation towards plant roots [12]. Conversely, the horn-manures 500 K of this study were conferred by the treatment with the combined humified preparations 502–507 additional abiotic properties which enabled an enhanced proliferation of microbial growth. The difference in bioactivity between the 500 and 500 K preparations was also shown by their antioxidant activity that

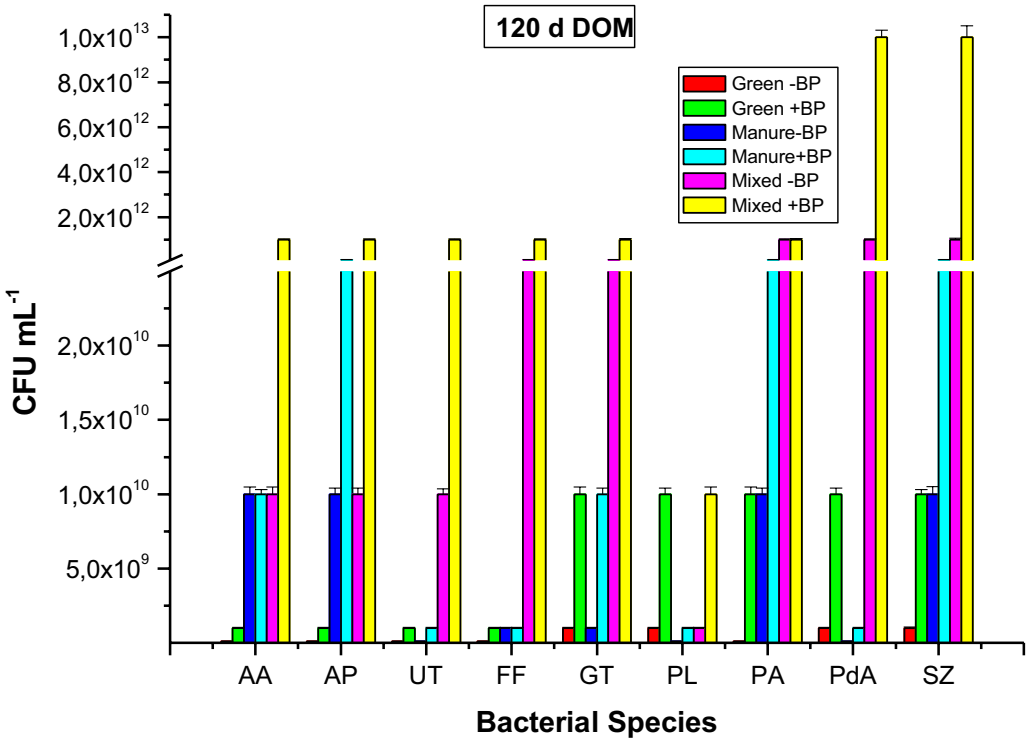


Fig. 8 Effect of dissolved organic matter (DOM) extracted from different composts (green, manure, mixed) before and after treatment with biodynamic preparations (BP) 502–507 on microbial proliferation (CFU.mL^{-1}) of some bacterial species (AA = *Acetobacter aceti;* AP = *Aeribacillus pallidus;* UT = *Ureibacillus terrenus;* FF = *Frigoribacterium faeni;* GT = *Geobacillus thermodenitrificans;* PA = *Pseudomonas aeruginos;* PdA = *Pseudonocardia autotrophica;* SF = *Shinella zoofloeoides*) at 120 days of compost sampling. The initial standardized inoculum was equal to 10^4 CFU mL^{-1}. Bars over columns indicate standard deviation

was significantly larger for the latter, possibly because of the greater content of phenols in 500 K resulting from the impact of the humified inflorescences on the biomolecular transformation of the 500 preparation.

The antioxidant activity of the single 502–507 preparations was either similar to or larger than that of 500 K, and generally in line with their phenolic content. However, the capacity of each humified inflorescence to induce proliferation of microbial strains was not aligned to the corresponding antioxidant activity and phenolic content, often showing a microbial growth capacity smaller than for 500 K. Such a poor correlation was also indicated by their molecular distribution, as revealed by ^{13}C solid-state NMR spectroscopy, that rather suggests an influence of oxygen-containing and carboxyl molecular functions on their activity towards microbial proliferation.

The BP-treated composts induced a proliferation of selected microbial strains that was already significant at 30 d from the start of the composting process. However, while the compost activity towards microbial multiplication grew with composting time, the differentiation between BP-treated and untreated composts was achieved only after 120 d and resulted most extensive for the mixed compost. Significantly larger microbial growth was obtained by the activity of DOM solubilized from the biodynamic composts with CFU values much greater than for the initial inoculum of selected bacterial species. As in the case of biodynamic composts, we observed for DOM the maximum bioactivity for the extracts from the mixed compost after 120 d of composting time.

These in vitro results indicate that mature biodynamic composts are potentially active, when amended to soil, towards the proliferation of a number of microbial strains responsible for the humification of organic matter. Moreover, their water-soluble extracts are shown to be potentially more bioactive in soil than the corresponding biodynamic composts by as much as three orders of magnitude and even overcoming the microbial proliferation activity found for the soluble humic extract and compost tea obtained from a non-biodynamic mature compost.

For the first time we tested here the capability of different humified preparations commonly applied in biodynamic agriculture to boost the growth of microbial species active in the composting processes towards the humification of organic matter. We found that while the 502–507 humified preparations, the horn-manure 500 K, and the mature composts treated with the 502–507 BP as well as their DOM extracts, all exerted significant bioactivity on the proliferation of selected bacterial

species, the horn-manure 500 appeared to limit its effect to an abiotic plant stimulation through its already verified auxin-like activity. Hence, this study indicates that the products prepared after Steiner (1924) [1] by natural biotechnological humification do possess a considerable activity towards bacterial growth and so do the composts treated with such preparations, thereby amply justifying their use in biodynamic agriculture and the previous literature reports on their improvement of general soil health and plant growth sustainability.

Abbreviations

BP	Biodynamic preparations
NMR	Nuclear magnetic resonance
DOM	Dissolved organic matter
CT	Compost tea
A/OA	Alkyl-C/O-AlkylC
ARM	Aromaticity
HB	Hydrophobicity
LR	Lignin ratio

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-025-00891-y.

Additional file1 (DOCX 190 KB)

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Author contributions
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Data availability
All datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
Alessandro Piccolo was the Editor-in-Chief at the time of submission and acceptance.

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