Microbiological Features and Bioactivity of a Fermented Manure Product (Preparation 500) Used in Biodynamic Agriculture

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The fermented manure derivative known as Preparation 500 is traditionally used as a field spray in biodynamic agriculture for maintaining and increasing soil fertility. This work aimed at characterizing the product from a microbiological standpoint and at assaying its bioactive properties. The approach involved molecular taxonomical characterization of the culturable microbial community; ARISA fingerprints of the total bacteria and fungal communities; chemical elemental macronutrient analysis via a combustion analyzer; activity assays for six key enzymes; bioassays for bacterial quorum sensing and chitooligosaccharide production; and plant hormone-like activity. The material was found to harbor a bacterial community of $2.38 \times 10^8$ CFU/g dw dominated by Gram-positives with minor instances of Actinobacteria and Gammaproteobacteria. ARISA showed a coherence of bacterial assemblages in different preparation lots of the same year in spite of geographic origin. Enzymatic activities showed elevated values of β-glucosidase, alkaline phosphatase, chitinase, and esterase. The preparation had no quorum sensing-detectable signal, and no rhizobial nod gene-inducing properties, but displayed a strong auxin-like effect on plants. Enzymatic analyses indicated a bioactive potential in the fertility and nutrient cycling contexts. The IAA activity and microbial degradation products qualify for a possible activity as soil biostimulants. Quantitative details and possible modes of action are discussed.

Key words: Preparation 500, hornmanure, biodynamic agriculture, biostimulants, auxin-like activity

Biodynamic (BD) agriculture is a form of organic farming that, in addition to ordinary organic farming practices, as soil building, composting, and crop rotations, uses two characteristic preparations as field sprays (referred to as 500 and 501) or additives for manure composting [15, 30]. Such products are included in the list of materials and techniques permitted in organic farming by an EC Regulation (834/2007).

The BD Preparation 500 is a fermented cow manure derivative used to improve the soil fertility and foster the formation of a strong root system, whose chemical features have been previously reported by our group [26]. Results of long-term field trials, such as those published in a Science paper by Mader et al. [17] proved that biodynamic practices, which primarily make use of Preparation 500, improve the overall soil quality; in particular, parameters such as organic matter, and microbial biomass and diversity were significantly higher in the biodynamic farming system in comparison with ordinary organic farming [16, 17]. As the difference between organic and BD farming lies essentially in the use of defined sprayed compounds such as 500 and 501, the basis for its effects can be sought in the features of these preparations.

Short-term field trials have shown that the use of both preparations is correlated with a higher yield of lentils per
unit plant biomass, lower grain carbon and crude protein content, greater NO$_3^-$ content in soft white spring wheat, and greater NH$_4^+$ content in soil [6]. Their application was also found to be associated with higher levels of mineral carbon, which is considered an indicator of microbially available C [8], and differences in soil microbiological fatty acid profiles in the first two years of study [7].

We recently investigated the molecular composition of Preparation 500 by both solid-state nuclear magnetic resonance (NMR) spectroscopy and thermochemolysis, and found the product enriched in bio labile components compared with the starting manure, thus becoming potentially conducive to biostimulation of microorganisms and plants [26]. In comparison with initial manure, earlier reports suggested that Preparation 500 had lower values of pH, CO$_2$ respiration, and C:N ratio, higher nitrate content, and reduced losses of organic matter [3].

The aim of the present work was to perform a microbiological characterization of Preparation 500 and to seize some of its biological activities. The results, along with those of the recently assessed molecular properties, are envisaged to contribute to a clearer understanding of its ways of action in agricultural systems.

MATERIALS AND METHODS

Preparation 500

Different commercial samples of BD Preparation 500 from three leading Italian producers were studied. Samples were produced by “Società Agricola Biodinamica” (Labico, Rome), “La Farnia” (Rolo, Reggio Emilia), and “Biodynamic Agriculture Section” (Bolzano).

Briefly, the routine production comprises the following procedure: in early autumn, hollow cow horns are filled with cow manure from organic farming and buried underneath a biodynamically managed soil. The organic material is left to decompose during winter and cow horns are recovered in the following spring after almost 150–180 days of maturation. The material recovered from cow horns is moderately moist, dark, odorless, and humus-like.

The analyses included three preparations from 2010 (Rome, Bolzano, and Reggio Emilia), one preparation from 2011 (Rome) and its manure of origin (produced in 2010).

Elemental Content Analysis

The percent proportions of carbon, nitrogen, and sulfur of Preparation 500 or soils were determined on material dried for 2 days at 70°C using a CNS Macrovario combustion analyzer (Elementar Analysensysteme GmbH, Germany).

Culturable Microbial Population Analyses

Fresh aliquots of 3 g were resuspended by vigorous shaking for 1 h in 27 ml of physiological solution at room temperature and serial dilutions were plated on plate count agar (PCA, Difco, USA). Plates were incubated in aerobic conditions or within anaerobiosis jars. Colonies were sorted by morphology and confirmed by ARDRA using enzymes HhaI and CfoI.

DNA Extraction

Cells were lysed by resuspending a loopful of a plate-grown isolated colony in 50 µl of lysis buffer (0.25% sodium dodecyl sulphate, 0.05 M NaOH) in a 1.5 ml polypropylene tube, followed by stirring for 60 s on a vortex and heating at 95°C for 15 min. The lysate was centrifuged for 15 min and 10 µl of the supernatant was mixed with 90 µl of sterile water. Lysates were stored at 4°C prior to PCR.

PCR Amplification of the 16S rRNA Gene and ARDRA

One microliter of the lysate containing the total DNA of each bacterial isolate was treated in a PCR BioRad 1-Cycler using the two 16S rRNA gene-targeted universal bacterial primers 63F (5’CAGGCTTAAACATGCAAGTC) [18] and 1389R (5’ACGGGCGGGTGTTGACAAGG) [22] at 1 µM each in a 25 µl reaction volume, using the following program: initial denaturation at 95°C for 2 min; 35 cycles at 95°C for 30 s, 55°C for 1 min, 72°C for 4 min, and a final extension at 72°C for 10 min. The PCR mixture contained 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 1 µM of each primer, and 2.5 U Taq DNA Polymerase, recombiant (Invitrogen Life Technologies, Italy).

Amplicons were digested overnight at 37°C upon mixing 5 µl from the 25 µl reaction volume with 1 µl of CfoI enzyme (Pharmacia, Sweden) and 2 µl of 10× reaction buffer. Digested DNA was loaded on a 1.5% agarose gel, run electrophotically for 3 h at 110 V. The ethidium bromide-stained gel was visualized over an UV transilluminator and photographed by a Kodak DC290 digital camera. Upon ARDRA, the isolates were sorted and selected for sequencing.

DNA Sequencing and Bacterial Molecular Taxonomy Analysis

One microliter of the amplicon resulting from the above-described PCR amplification was mixed with 1 µl containing 6.4 picomoles of the above-described forward primer 63F in a 0.2 ml polypropylene tube and then dried by incubating the tube open for 15 min at 65°C in an I-Cycler thermal cycler. The template and primer mix was directly used for dye-deoxy cycle DNA sequencing with fluorescent terminators (Big Dye; Perkin-Elmer/Applied Biosystems, USA) and run in an Applied Biosystems ABI Prism 3730XL automated DNA sequencer. Chromatograms were analyzed by Chromas 2.23 software (Technelysium Pty Ltd, Australia). BLAST analysis against nucleotide databases was performed via the NCBI website (http://www.ncbi.nlm.nih.gov/).

Estimation of Overall Bacterial and Fungal Species Richness by ARISA

Genomic DNA extraction from Preparation 500 and soils used for comparison was performed upon drying the material and starting from 250–550 mg amounts using a Genomic DNA from Soil kit from Macherey Nagel (Macherey-Nagel Inc., USA) as recommended by the manufacturer. The protocol involves a mechanical lysis with beads and a lysis based on SDS. The lysate was purified by passage through a Nucleospin Inibitor Removal Column, and eluted in 100 µl. Quality was assessed spectrophotometrically upon absorbance ratios 260/280. The PCR amplifiability was verified using three primer pairs on conserved bacterial and fungal targets (16S rRNA, 18S rRNA, and ITS).

Samples were amplified using two primer pairs (forward 6-FAM, labeled blue; reverse VIC, labeled green) and run along with a marker (LIZ1200) on a capillary sequencer ABI3730. The first pair amplifies the region between genes of the bacterial 16S rRNA and microbial 18S rRNA.