Identification of Microbial Populations in Blends of Worm Castings or Sugarcane Filter Mud Compost with Biochar

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Abstract: Soil amendments are used to improve soil quality, thereby enhancing plant growth and health. Efforts have been made to replace synthetic chemical enhancers. It is also preferable to not use natural products such as peat moss, the harvesting of which can be harmful to marine ecosystems. Viable replacements include worm castings, which can contribute beneficial microbes, as well as physicochemical amendments. Another potential soil amendment is the compost produced from sugarcane processing byproducts. While the texture of these two materials is not ideal for even dispersal onto fields, the addition of biochar improves the texture. Previous work demonstrated that blending them with biochar from sugarcane byproducts added physicochemical benefits, while not quantitatively reducing the microbial load, even after storage. Microbial populations of the blends in the present study were found to (1) contain taxonomic groups that contribute to plant health and (2) not contain human pathogens. Based on the quantitative and qualitative microbial analyses, it has been determined that 50% or less biochar in a blend will allow maintenance of beneficial microbes in stored samples.

Keywords: microbial population; biochar; worm castings; sugarcane filter mud

1. Introduction

Compost and other organic compounds are potentially beneficial amendments for potting soil mixtures. Organic compounds can contribute to improvements in plant growth and yield due to the nutrients and improved water-holding capacity they provide [1]. Ecological management approaches that use organic amendments are preferred to reach multiple-benefit targets, such as enhancing agricultural production without harming the environment [2]. Organic soil amendments are preferable as efforts are made to reduce the use of inorganic fertilizers [3]. Sphagnum peat moss is one such amendment and has traditionally been used to provide these advantages in soil [4]. However, more recent efforts have been made to replace peat moss due to the destruction of wetland ecosystems by the harvesting process [4].

Vermicompost, worm castings, is an effective soil conditioner because it contributes plant-available nutrients and organic matter [3,5,6]. Vermicompost derives from composted organic waste substrates in the presence of earthworms [7] with a good physical structure, abundant labile resources, and high microbial activities [8]. Farm animal manure is a beneficial material for addition to the soil by enhancing both physical and chemical characteristics [9]: however, to be useful, organic matter, such as manure, must be decomposed to a humus-like state [9]. Humus is the organic material that results from the decomposition of living organisms in soil. It is best to store manure so it can mature in a stable manner, thereby providing the physicochemical characteristics necessary for soil enrichment and crop growth [9].

The introduction of earthworms can accelerate conversion of cow manure to a mature state for enhancement of plant growth [10]. By way of function, earthworms have profound direct and indirect impacts on nutrient availability through increased decomposition of...
plant residues and turnover of soil organic matter [11]. The conversion of manure by worms also addresses animal waste management regulations by stabilizing the waste chemically and reducing the microbial biomass [10]. Vermicompost from cattle manure and paper waste has been shown to benefit growth of peppers (Capsicum annum) var. King Arthur, at levels that are not significantly different than compost and inorganic fertilizers [5].

Soil amendments with biochar have the potential of addressing both soil fertility and carbon sequestration. Wu et al. identified synergistic interactions between vermicompost and biochar in promoting crop yield on rice grown in a paddy soil [2]. Additionally, it was found that, in the presence of vermicompost, the incorporation of biochar amendment significantly decreased the cumulative N₂O emissions.

The sugarcane industry generates large volumes of biological byproducts that are rich in nutrients that can be agriculturally beneficial and may otherwise be wasted [12]. Two of these byproducts generated at the sugarcane factories are filter cake mud and bagasse. Particularly, composting has been used to convert filter cake mud into a humus-like state [12]. A benefit of composting is that it generates heat, which kills microbial plant pathogens and weed seeds [12]. Filter cake mud is a byproduct composed of sugar, fiber, coagulated colloids, albuminoids, inorganic salts, and particles of soil, which can be beneficial for plant growth [12].

Highly weathered and/or used soils have reduced organic matter and fertility [13]. In order to mitigate carbon losses, it is usually necessary to add organic inputs, of which worm castings and sugarcane filter mud compost are good examples. The blending of materials (worm castings or sugarcane filter mud compost) harboring microbes that promote plant growth with biochar was previously shown to have no deleterious effects on the enumerated microbial populations [14]. It was theorized in that work that incorporating biochar, porous carbon clusters with mineral inclusions, into the blends stabilized the moisture and pH levels. It is possible that the release of low-volatile compounds from the biochar may lead to abundance of microbial activity in the soil. These factors might create an environment conducive to microbial survival. It is also possible that the biochar binds harmful bacterial waste products [14]. The biochar may be functioning similarly to a soil aggregate of organic matter and minerals [15].

A study on mycorrhizal responses to biochar in soil [16] proposed several not mutually exclusive mechanisms, from altered nutrient availability and/or soil physicochemical parameters to alterations that are either beneficial or detrimental to other soil microbes, or alterations of signaling processes between plants and mycorrhizal fungi, to biochar serving as refuge from hyphal grazers.

Studies such as those reported in [15] have enumerated microbial populations (but the microbes were not taxonomically characterized) to determine potential blending and storage effects on the numbers of microbes present. It is also necessary to qualitatively determine which microbes are present in the stored blends. Qualitative analysis was undertaken in this study to determine whether known pathogens are present, whether beneficial microbes are detectable after long-term storage, and whether, and in what way, blends with higher concentrations of biochar affect the microbial population. The potential for the presence of pathogens was due to the environmental samples containing unknown microbes, especially those from cow manure.

A microbiome is a community of microbes in an environmental niche [17]. Metagenomics is the identification of the microbial community in environmental samples by direct genetic analysis [18]. This identification of microbes within the population, also called metataxonomics, is made possible by extraction of total DNA from the sample followed by next-generation sequencing (NGS) [17]. Microbes in the rhizosphere, the interface between plants and soil, provide nutritional support for plants and are a source of biochemical reactions that recycle nutrients [19,20]. NGS has become less expensive and more time efficient, enabling broad use of the technology on samples from multiple and varied environments [21]. This work reports the determination of prokaryotic microbial populations in the metagenome of biochar blended with either worm castings or mud compost from
sugarcane processing. These blends have been previously found to have beneficial chemical properties for use as soil amendments [14].

2. Materials and Methods

2.1. Sample Collection and Preparation

Sample collection and materials preparation are fully reported in [14] and are here repeated in an abbreviated form. The same report contains details and results of the physiochemical characterization of the materials.

2.1.1. Biochar

Biochar was produced from surplus sugarcane bagasse and leaf material and provided by American Biocarbon, LLC, at the Cora Texas sugarcane factory (White Castle, LA, USA). A pyrolysis/torrefier unit with an approximate pyrolysis temperature of 316 °C and 10 min residence time in the pyrolysis furnace was used [14].

2.1.2. Worm Castings

Worm castings were harvested at, and provided by, Iverstine Farms (Kentwood, LA, USA) using selective screening based on size from African nightcrawler worms (*Eudrilus eugeniae*). The worms were incubated in a bedding of decomposing cow manure and hay, at ambient temperature and in the dark, for 14 days prior to harvesting, and were used within one week of collection. Screening separated the worm castings from the larger worms and worm eggs [14]. Physical and chemical properties are displayed in Table 1.

| Sample | Total N (%) | Organic N (%) | Ammonium N (%) | Nitrate N (%) |
|--------|-------------|---------------|----------------|--------------|
| Worm Castings | 1.83 | 0.15 | <0.001 | 0.03 |
| Mud Compost | 0.15 | 0.15 | 0.003 | - |
| Biochar | 0.71 | 0.70 | - | - |

| Nitrogen | Worm Castings | Mud Compost | Biochar |
|----------|---------------|-------------|---------|
| Total N (%) | 1.83 | 0.15 | 0.71 |
| Organic N (%) | 1.66 | 0.15 | 0.70 |
| Ammonium N (%) | - | <0.001 | 0.003 |
| Nitrate N (%) | 0.17 | - | - |

| P (%) | 0.67 | 0.30 | 0.07 |
| P as P₂O₅ (%) | 1.54 | 0.70 | 0.17 |
| K (%) | 0.67 | 0.39 | 0.39 |
| K as K₂O (%) | 0.81 | 0.72 | 0.46 |
| S (%) | 0.61 | - | 0.19 |
| Ca (%) | 3.17 | 0.74 | 0.77 |
| Mg (%) | 0.81 | 0.29 | 0.150 |
| Na (%) | 0.145 | 0.048 | 0.043 |

| Macroelements | Worm Castings | Mud Compost | Biochar |
|---------------|---------------|-------------|---------|
| Fe (ppm) | 9863 | 10166 | 4443 |
| Mn (ppm) | 1024 | 301 | 99 |
| Moisture (%) | 65.63 | 37.44 | 6.60 |
| Total solids (%) | 34.37 | 62.56 | 93.4 |
| Organic matter (%) | 39.57 | 7.26 | 89.19 |
| Ash (%) | 60.52 | 92.71 | 10.81 |
| Total Carbon (%) | 20.95 | 5.34 | 45.09 |
| H:C molar ratio | 1.1 | - | - |
| Chloride (%) | 0.12 | <0.01 | - |
| pH | 5.9 | 6.0 | 5.3 |
| Conductivity (mS/cm) | - | 0.22 | - |

| Other properties | Worm Castings | Mud Compost | Biochar |
|------------------|---------------|-------------|---------|
| Bulk density (lb/cu yd) | 0.500 | 0.740 | 0.150 |
| Surface area, m²/g | Negl. | Negl. | 1.52 ± 0.02 |

Table 1. Various physical and chemical properties for worm castings, sugarcane filter mud compost, and biochar samples (taken from Lima et al., 2018).
2.1.3. Sugarcane Filter Mud Compost

Sugarcane filter mud compost was produced and provided by Les Ewing (White Castle, LA, USA). The process of sugarcane raw juice clarification generates filter cake as a byproduct. Sugarcane factories add fly ash to the filter cake to create a soil conditioner. Commercial value is further increased by composting the filter cake/fly ash mixture with 50% sugarcane bagasse for 21 to 30 days. The dimensions of the compost piles were approximately 4 m in diameter and 2 m high. The piles were turned every 3 days with the internal temperature reaching 65 °C [14]. Composting was completed within 30 days and material was utilized within one week of collection. Physical and chemical properties are displayed in Table 1.

2.2. Storage of Blends of Worm Castings with Biochar or Sugarcane Filter Mud Compost with Biochar

Blends are based on volume, due to the large difference in bulk density of the components. Mixtures with a total volume of 30 mL, and with a total weight varying between 3.75 to 18.21 g, were blended prior to incubation at ambient temperature, in the dark, in 125 mL sterile Nalgene (Rochester, New York, USA) bottles [14]. There were two sets of experiments. The first study included blends of worm castings and biochar at the following ratios: 100/0; 95/5; 90/10; 75/25; 0/100 WC/BC. Based on results from the first study [14], the second study included both blends of worm castings and biochar, and blends of filter mud and biochar, at the following ratios: 100/0; 90/10; 75/25; 50/50; 25/75; 10/90; 0/100 WC/BC. Samples in the first study were stored for 26 months, while second-study blends were stored for 24 months. Samples from the first experiment were stored in 50 mL sterile centrifuge tubes which limited complete blending [14]. The differences in microbial identifications, with some microbes found in samples of second-experiment blends with higher percentages of biochar, are attributed to lack of even dispersion due to restriction at blending.

2.3. Qualitative Microbial Analysis

Microbial identification was performed on subsamples of worm castings alone, worm castings blended with biochar, mud compost alone, and mud compost blended with biochar. The conditions for pyrolysis to produce biochar, including a temperature of 316 °C, incinerates microbes in the plant substrate material. Prior quantitative analysis of 100% biochar samples confirmed no microbial growth [14]; thus, subsamples of 100% biochar were not qualitatively analyzed for microbes in this study. Subsamples of the stored material, 2 g each, underwent microbial identification by ID Genomics (Seattle, WA, USA) [22]. For 4 of the 16 total subsamples (25%), duplicates were submitted for identification, with no means of tracing which samples were replicated. Briefly, inhibitor-free DNA from mixed samples of Gram-positive and Gram-negative bacteria was isolated using the Meta-G-Nome™ DNA Isolation kit. The DNA then underwent 16S metagenomics sequencing, targeting the variable V3 and V4 regions of the 16S rRNA gene. Paired-end sequencing was performed, and data were analyzed in the BaseSpace® analysis environment [22]. Resulting data provided classification at the kingdom, phylum, class, order, family, genus, and species levels. Order, family, genus and species are reported here.

3. Results and Discussion

Blends of biochar with either worm castings or filter mud from sugarcane processing were previously shown to have chemical properties that are beneficial for plant growth [14]. Wu et al. [2] determined the existence of synergistic interactions between vermicompost and biochar in promoting crop yield using only 1% biochar amendments. Using a soil column experiment, it was determined that biochar combined with vermicompost increased rice yields by up to 35%. In our previous study, microbes in the blends were enumerated, but were not taxonomically characterized [14]. The blends are novel, so it was of use to determine whether the microbes also offered benefits for future plant growth, and to ensure that they did not potentially introduce foodborne pathogens to soil and plants. No bacteria were found in samples of biochar alone in the previous study [14], so biochar samples
were not taxonomically characterized. The results show that bacterial genera known to cause foodborne illness were not identified in any of the blends. These genera, listed by the Centers for Disease Control and Prevention (CDC), include *Escherichia*, *Listeria*, *Salmonella*, and *Vibrio* [23], though other nonpathogenic members of the phylum Proteobacteria were identified in some samples. Orders, families, genera, and species that were found in all samples are discussed and indicated in blue in Tables 1–8, those that were affected by higher ratios of biochar are discussed and indicated in red, and those that were found inconsistently in samples within an experiment are not discussed and are indicated in black. All data are reported as percent of total nucleic acids in the sample metagenome. In all cases, there were microbes with fractional percentages as well as unknown DNA. The percentages on the tables therefore do not equal 100%. In all cases, the predominant microbes are included.

Table 2. Order classification for worm castings (W) blended with biochar (B) by percent of total nucleic acids in the sample metagenome.

|                     | EXPERIMENT 1 |          | EXPERIMENT 2 |          |
|---------------------|--------------|----------|--------------|----------|
|                     | W100         | W95B5    | W90B10       | W75B25   | W100     | W90B10   | W75B25   | W50B50   | W25B75   | W10B90   |
| Actinomycetales     | 8.68         | 13.75    | 11.23        | 7.35     | 7.03     | 6.40     | 7.35     | 7.98     | 11.19    | 19.41    |
| Anaerolineales      | 4.71         | 4.01     | 4.48         | 3.77     | 3.55     | 4.20     | 5.78     | 5.37     |          |          |
| Bacillales          | 4.42         | 3.25     |              | 14.75    | 13.69    | 10.21    | 8.66     | 7.22     | 9.23     |          |
| Chromatiales        | 3.05         | 3.17     | 3.00         | 4.62     | 3.16     |          |          |          |          |          |
| Clostridiales       | 6.64         | 5.90     | 5.38         | 4.66     | 7.98     | 8.24     | 7.90     | 6.31     | 7.96     | 5.49     |
| Rhizobiales         | 10.40        | 6.99     | 11.40        | 18.25    | 7.95     | 7.75     | 8.44     | 9.63     | 10.52    | 10.09    |
| Rhodospirillales    |              |          |              | 3.73     | 7.30     | 5.40     | 5.70     | 5.29     | 3.56     | 5.42     | 5.75     |
| Sphingobacteriales  | 3.24         | 4.19     | 4.28         | 4.97     | 3.73     | 3.94     | 7.44     |          |          | 2.75     |
| Sphingomonadales    |              |          |              |          |          |          |          |          |          | 4.54     |

Legend: Blue text indicates microbes found in all experimental samples; red text indicates microbes found in all samples below the specified concentration of biochar; black text indicates microbes found inconsistently among samples.

Table 3. Family classification for worm castings (W) blended with biochar (B) by percent of total nucleic acids in the sample metagenome.

|                     | EXPERIMENT 1 |          | EXPERIMENT 2 |          |
|---------------------|--------------|----------|--------------|----------|
|                     | W100         | W95B5    | W90B10       | W75B25   | W100     | W90B10   | W75B25   | W50B50   | W25B75   | W10B90   |
| Anaerolinaceae      | 4.71         | 4.01     | 4.48         | 3.77     | 3.55     | 4.20     | 5.78     | 3.57     |          |          |
| Bacillaceae         | 2.26         |          |              | 5.69     | 4.44     | 3.76     | 4.52     | 3.59     | 6.02     |          |
| Brocadiaceae        | 2.26         | 2.95     |              |          |          |          |          |          |          |          |
| Chitinophagaceae    |              |          |              | 2.48     |          |          |          |          |          |          |
| Chromatiaceae       | 2.26         | 2.33     | 2.08         | 3.46     |          |          |          |          |          |          |
| Clostridiaceae      | 3.06         | 2.91     | 2.51         | 2.59     | 3.69     | 3.60     | 3.34     | 2.40     | 3.85     | 2.93     |
| Flexibacteraceae    |              |          |              |          |          |          |          |          |          | 5.07     |
| Gemmatimonadaceae   |              |          |              |          |          | 2.24     |          |          |          |          |
| Hyphomicrobiaceae   | 6.69         | 3.71     | 7.59         | 13.28    | 4.72     | 4.42     | 4.69     | 4.82     | 6.57     | 6.70     |
| Micrococaceae       |              |          |              |          |          | 2.28     | 2.47     | 2.25     |          | 2.99     |
| Peptococcaceae      |              |          |              |          |          | 6.13     | 7.27     | 4.39     |          |          |
| Planococcaceae      |              |          |              |          |          |          |          |          |          | 2.36     |
| Polyangiaceae       |              |          |              |          |          |          |          |          |          |          |
| Propionibacteriaceae|              |          |              |          |          |          |          |          |          |          |
| Pseudonocardiae     |              |          |              |          |          |          |          |          |          |          |
| Rhodospirillaceae   | 2.44         |          |              | 3.30     | 6.93     |          |          |          |          |          |
| Sphingomonadaceae   |              |          |              |          |          |          |          |          |          | 2.99     |
| Streptomycesaceae   |              |          |              |          |          |          |          |          |          | 3.47     |

Legend: Blue text indicates microbes found in all experimental samples; red text indicates microbes found in all samples below the specified concentration of biochar; black text indicates microbes found inconsistently among samples.
### Table 4. Genus classification for worm castings (W) blended with biochar (B) by percent of total nucleic acids in the sample metagenome.

|               | EXPERIMENT 1 | EXPERIMENT 2 |
|---------------|--------------|--------------|
|               | W100 | W95B5 | W90B10 | W75B25 | W100 | W90B10 | W75B25 | W50B50 | W25B75 | W10B90 |
| **Anaerolinea** | 1.37 |       |        |        |       | 1.93 | 1.82 | 2.97 |        |        |
| **Arthrobacter** |        |       |        |        | 1.64 | 3.95 | 2.16 | 2.48 | 2.08 | 2.48 | 2.02 |
| **Azospirillum** | 1.81 | 3.84 | 2.78 | 2.33 | 3.27 | 2.62 | 4.68 |       |        |        |
| **Bellilinea** | 1.47 |       |        |        |       |       |       |        |        |        |
| **Candidatus** | 2.25 | 2.95 | 1.89 |       |       |       |       |        |        |        |
| **Clostridium** | 3.08 | 2.83 | 2.52 | 2.49 | 3.41 | 3.37 | 3.11 | 2.15 | 3.67 | 2.75 |
| **Conexibacter** | 1.44 | 1.64 |       |       |       |       |       |        |        |        |
| **Gemmatimonas** |       |       | 2.24 |       |       |       |       |        |        |        |
| **Hyphomicrobiium** |       |       | 1.86 |       |       |       |       |        |        |        |
| **Ignavibacterium** | 1.43 | 1.38 |       |       |       |       |       |        |        |        |
| **Kaistobacter** |       |       |       | 2.02 |       |       |       |        |        |        |
| **Longilinea** | 1.65 | 1.35 |       |       |       |       |       |        |        | 3.53 |
| **Paenisporosarcina** |       |       |       | 5.67 | 6.90 | 4.03 | 1.77 | 1.71 |       |       |
| **Pedosporosarcina** |       |       |       | 1.77 |       |       |       |        |        |        |
| **Pseudonocardia** |       |       |       | 1.36 |       |       |       |        |        |        |
| **Rhodoplanes** | 4.28 | 1.93 | 5.01 | 9.69 | 1.69 | 1.60 | 1.72 | 1.89 |       |       |
| **Rhodovibrio** | 1.43 |       |       |       |       |       |       | 2.24 |       |       |
| **Runella** | 1.53 |       |       |       |       |       |       | 4.87 |       |       |
| **Saccharopolyspora** |       |       |       |       |       |       |       | 2.20 |       |       |
| **Streptomyces** | 1.79 | 1.54 |       |       |       |       |       | 3.43 |       |       |
| **Thermodesulfovibrio** | 1.80 |       |       | 1.25 |       |       |       |       |        |        |

Legend: Blue text indicates microbes found in all experimental samples; red text indicates microbes found in all samples below the specified concentration of biochar; black text indicates microbes found inconsistently among samples.

### Table 5. Species classification for worm castings (W) blended with biochar (B) by percent of total nucleic acids in the sample metagenome.

|               | EXPERIMENT 1 | EXPERIMENT 2 |
|---------------|--------------|--------------|
|               | W100 | W95B5 | W90B10 | W75B25 | W100 | W90B10 | W75B25 | W50B50 | W25B75 | W10B90 |
| **Anaerolinea thermolimosa** | 0.98 |       |        |        |       |       |       |        |        |        |
| **Arthrobacter psychrochitiniphilus** | 1.38 | 1.46 | 1.78 | 1.30 | 0.82 | 1.51 | 1.14 |       |       |       |
| **Azospirillum palatum** |       |       |       |       |       |       |       |        | 0.82 | 1.19 | 0.82 |
| **Bacillus arbutinivorans** | 1.13 | 1.14 | 1.04 | 0.92 |       |       |       |        |        |        |        |
| **Bacillus funiculus** | 1.47 | 0.91 | 1.25 | 1.19 | 1.11 | 1.11 | 0.96 | 1.51 | 1.21 |       |       |
| **Bacillus smithii** |       |       |       | 1.11 |       |       |       |        |        |        |        |
| **Bellilinea caldifistulae** | 2.25 | 2.95 |       |       |       |       |       |        |        |        |       |
| **Bifidobacterium bombi** |       |       | 1.89 |       |       |       |       |        |        |        |       |
| **Candidatus scalindua** | 1.06 |       |       |       |       |       |       |        |        |        |       |
| **Candidatus scalindua brodae** |       |       |       | 1.55 |       |       |       |        |        |        |       |
| **Chitinophaga soli** |       |       |       |       | 0.91 | 1.13 | 1.35 | 1.30 |       |       | 1.15 |
| **Chondromyces peliculatus** | 1.36 |       |       |       |       |       |       | 1.15 |       |       | 0.85 |
| **Cycloclastus oligotrophus** |       |       |       |       |       |       |       |       |        |        |       |
| **Desulfitospira thermodiaphila** |       |       |       |       |       |       |       |       |        |        |       |
| **Gemmatimonas aurantiaca** |       |       |       |       |       |       |       |       |        |        |       |
| **Hyphomicrobiium vulgare** |       |       |       |       |       |       |       |       |        |        |       |
| **Longilinea arvoryzae** |       |       |       |       |       |       |       |       |        |        |       |
| **Nitrosococcus watsoni** |       |       |       |       |       |       |       |       |        |        |       |
| **Pedobacter kwangyangensis** |       |       |       |       |       |       |       |       | 1.05 |       |       |
| **Pseudomonas alcaligenes** | 1.58 |       |       |       |       |       |       |       |        |        |       |
| **Rhodothermus clarus** | 1.34 | 0.85 | 1.15 | 1.20 | 1.22 | 1.03 | 0.88 | 1.35 | 2.09 |       |       |
| **Rhodovibrio sodomensis** | 0.87 | 1.15 |       |       |       |       |       |       |        |        |       |
| **Runella limosa** | 0.85 | 1.33 | 1.24 | 1.53 | 1.54 | 4.87 | 1.57 | 1.91 |       |       |       |
| **Steroidobacter denitrificans** |       |       |       |       |       |       |       |       |        |        |       |
| **Thermodesulfovibrio aggregans** |       |       |       |       |       |       |       |       |        | 0.74 |       |
| **Thermodesulfovibrio thiophilus** |       |       |       |       |       |       |       |       |        | 0.96 | 0.93 |

Legend: Blue text indicates microbes found in all experimental samples; red text indicates microbes found in all samples below the specified concentration of biochar; black text indicates microbes found inconsistently among samples.
| Table 6. Order classification for mud compost (M) blended with biochar (B) by percent of total nucleic acids in the sample metagenome. |
|--------------------------------------------------|
| **Order** | M100 | M90B10 | M75B25 | M50B50 | M25B75 | M10B90 |
| Actinomycetales | 5.73 | 6.18 | 6.02 | 10.20 | 14.25 | 28.85 |
| Bacillales | 2.76 | 3.64 | 4.43 |
| Calditrichiales | 2.60 |
| Chromatiales | 4.55 | 4.36 | 4.51 | 3.85 |
| Clostridiales | 8.36 | 6.80 | 8.44 | 8.23 | 7.27 | 8.60 |
| Gemmatimonadales | 3.36 | 3.15 |
| Rhizobiales | 7.93 | 8.21 | 8.40 | 8.12 | 7.83 | 4.32 |
| Rhodospirillales | 4.61 | 4.81 | 4.32 | 4.05 | 3.81 |
| Sphingomonadales | 3.81 | 2.79 | 3.23 |
| Thermoanaerobacterales | 3.81 | 3.03 | 3.62 | 3.50 | 2.89 |

Legend: Blue text indicates microbes found in all experimental samples; red text indicates microbes found in all samples below the specified concentration of biochar; black text indicates microbes found inconsistently among samples.

| Table 7. Family classification for mud compost (M) blended with biochar (B) by percent of total nucleic acids in the sample metagenome. |
|--------------------------------------------------|
| **Family** | M100 | M90B10 | M75B25 | M50B50 | M25B75 | M10B90 |
| Anaerolinacea | 2.05 | 2.60 | 2.77 | 2.30 |
| Calditrichaceae | 3.36 | 3.36 | 3.45 | 2.88 |
| Clostridaceae | 5.67 | 4.98 | 3.59 | 3.91 | 3.74 | 4.32 |
| Gemmatimonadaceae | 2.32 | 3.62 | 3.59 | 3.42 | 4.00 |
| Hyphomicrobiaceae | 4.53 | 4.81 | 5.89 | 5.75 | 5.24 |
| Micrococcaeae | 2.32 | 3.36 | 3.15 |
| Nocardioaceae | 5.92 | 3.14 | 3.38 |
| Pseudonocardiaaceae | 3.97 | 4.08 | 3.59 | 3.57 | 3.34 |
| Rhodospirillaceae | 2.70 | 2.07 |
| Sphingomonadaceae | 7.87 | 7.31 |
| Streptomycesaceae | 3.17 |
| Thermoanaerobacteraceae | 3.87 |
| Thermomonomosporaceae | 3.09 |

Legend: Blue text indicates microbes found in all experimental samples; red text indicates microbes found in all samples below the specified concentration of biochar; black text indicates microbes found inconsistently among samples.

| Table 8. Genus classification for mud compost (M) blended with biochar (B) by percent of total nucleic acids in the sample metagenome. |
|--------------------------------------------------|
| **Genus** | M100 | M90B10 | M75B25 | M50B50 | M25B75 | M10B90 |
| Aeromicrobium | 2.19 | 2.22 | 2.06 | 2.18 |
| Actinallomurus | 3.84 | 3.40 |
| Arthrobacter | 3.66 | 3.05 |
| Azospirillum | 2.60 | 2.77 | 2.74 | 2.30 |
| Calditrix | 3.35 | 2.60 | 3.27 | 3.59 | 3.42 | 4.00 |
| Candidatus | 1.40 | 2.17 | 2.99 | 2.05 |
| Clostridium | 1.63 | 1.50 | 1.95 |
| Conexibacter | 2.32 | 3.36 | 3.15 | 1.64 |
| Desulfovibrio | 3.84 |
| Gemmatimonas | 2.05 |
| Kaistobacter | 1.93 |
| Kribella | 4.00 |
| Kluyvera | 2.60 |
| Phyllobacterium | 1.65 |
| Rhodoplanes | 3.64 | 3.17 | 3.57 | 3.58 | 2.81 |
| Saccharopolyspora | 1.37 | 2.00 | 2.48 |
| Streptomyces | 3.06 |

Legend: Blue text indicates microbes found in all experimental samples; red text indicates microbes found in all samples below the specified concentration of biochar; black text indicates microbes found inconsistently among samples.
3.1. Qualitative Analysis of Worm Castings Plus Biochar Blends

The following orders were identified in all samples of both experiments that included blending worm castings with biochar, in order of prevalence: Rhizobiales (10.34%), Actinomycetales (10.04%), Clostridiales (6.65%), and Rhodospirillales (5.19%) (Table 2).

The order Bacillales was identified in all samples of the second, expanded experiment, and in blends of the first experiment that contained 5% or no biochar, with an average of 10.34% (Table 1). The order Anaerolineales was found in both experiments in blends that contained less than 90% biochar, with an average of 4.3% (Table 2). The order Actinomycetales was classified by Buchanan [24], and it comprises Gram-positive bacteria that form branched chains, members of which are present in soil and offer the benefit of nitrogen fixation to plants. The order Clostridiales was classified by Prevot [25], and it comprises Gram-positive, anaerobic bacteria that are present in the soil. Members are symbionts, which digest organic matter. Members of the included family Clostridiaceae were identified in all samples of both experiments (Table 3), as were included members of the genus \textit{Clostridium} (Table 4).

The order Rhizobiales was classified by Kuykendall [26], and it comprises Gram-positive, nitrogen-fixing, symbiotic bacteria found in the soil. The order includes members of the family Hyphomicrobiaceae, which were identified in all samples of both experiments (Table 3). The included genus \textit{Rhodoplanes} was identified in all samples except in those where the biochar ratio exceeded 50% (Table 4).

The order Rhodospirillales was classified by Pfennig and Truper [27] in the phylum Proteobacteria, which also includes several foodborne pathogens, none of which were identified in the worm castings plus biochar blends. Rhodospirillales are primarily purple nonsulfur bacteria that undergo photosynthesis. Members of the included family \textit{Rhodospirillaceae} were identified in all samples of the second experiment (Table 3). The order Bacillales was classified by Prevot [25]. The order includes Gram-positive bacteria in the phylum Firmicutes, which includes microbes found in, and beneficial to, the human gut [28], none of which were identified in the worm castings plus biochar samples. Members of the included family \textit{Bacillaceae} were identified in all samples of the expanded second experiment and in samples of the first experiment that did not contain biochar (Table 3). Included members of the genus \textit{Bacillus} were identified in all samples of the second experiment (Table 4). No single species within this genus was found consistently in a range of samples (Table 5).

The genus \textit{Bacillus} includes a broad range of characterizations, making generalizations at this level impossible [29]. The genus \textit{Paenisporosarcina}, which is classified in the family \textit{Planococcaceae}, was identified in all samples from the second experiment which included 50% or less biochar (Table 4). Similar to \textit{Bacillus}, it is an aerobic spore-forming bacterium, but is found in dairies at a much lower frequency [29]. The order Anaerolineales was classified by Yamada [30] and comprises Gram-positive bacteria. Members of the included family \textit{Anaerolineaceae}, anaerobic digesters of organic waste, were also identified in blends with less than 50% biochar (Table 3). Two species of this family, \textit{Bellilinea caldifistulae} and \textit{Longilinea arvoryzae}, were classified after isolation from digester sludge and rice paddy soil, respectively [31]. \textit{B. caldifistulae} was found in samples of the second experiment in blends containing 50% or less biochar (Table 5). \textit{L. arvoryzae} was found in samples of both experiments in blends containing less than 75% or less biochar (Table 5). Both species are syntropic, allowing growth in environments that would otherwise be deleterious to the growth of these bacteria [32]. They reverse the homoacetogenic pathway, thereby preventing accumulation of inhibitory concentrations of acetate and allowing continued conversion of agricultural waste [32].

The species \textit{Runella limosa} and \textit{Bifidobacterium bombi} were also identified in samples of worm castings plus biochar, as was the genus \textit{Rhodoplanes}. \textit{Runella limosa} is classified in the order Cytophagales and family Cytophagaceae. It was isolated from activated sludge [33] and found in all samples of the second experiment and samples of the first experiment that contained 10% or more biochar (Table 5). \textit{Bifidobacterium bombi} is classified in the order
Bifidobacteriales and the family Bifidobacteriaceae and is found in the digestive tract of bumblebees [34]. The genus is characterized in the gastrointestinal system of humans and several other animals. B. bombi is not one of the two Bifidobacterium species that have been detected in both animals and humans [34]. Rhodoplanes is classified in the order Rhizobiales and family Hyphomicrobiaceae [35]. It produces hopanoids, which are characteristic bacterial biomarkers in the biomass of soils and sediments [36].

3.2. Qualitative Analysis of Mud Compost Plus Biochar Blends

The following orders were identified in all samples blending mud compost with biochar, in order of prevalence (Table 5): Actinomycetales (11.87%), Clostridiales (7.95%), Rhizobiales (7.47%). The order Chromatiales (4.32%) was identified in all samples that contained 50% or less biochar. The orders Rhodospirillales (4.32%) and Thermoanaerobacterales (3.37%) were found in samples that contained less than 75% or less biochar (Table 6).

The order Chromatiales was classified by Imhoff [37], and comprises photosynthetic, purple, sulfur bacteria found in water environments. The included family Chromatiaceae was also identified in samples containing 50% or less biochar (Table 7).

Within the order Clostridiales, Clostridiaceae was the only family identified in all mud compost plus biochar blends (Table 7), and Clostridium was the only genus found in all samples (Table 8). Within the order Rhizobiales, both the family Hyphomicrobiaceae and the genus Rhodoplanes were identified in samples that contained 75% or less biochar (Tables 6 and 7). Within the order Rhodospirillales, the family Rhodospirillaceae was identified in samples containing 75% or less biochar (Table 7). The order Thermoanaerobacterales was classified by Wiegel [38], and it comprises bacteria that share the survival at high temperatures and includes families that produce biohydrogen more efficiently than the families Clostridiaceae and Enterobacteriaceae [39].

The following species were also identified in samples of mud compost plus biochar: Azospirillum, Calditrich, and Gemmatimonas. Azospirillum is among the best-studied rhizobacterial genera, capable of colonizing and enhancing growth of multiple plant species [40] and was identified in samples containing 50% or less biochar (Table 8). Calditrich is a newly recognized genus comprising two species, having been originally isolated from a hydrothermal vent [41]. Calditrich paleochoryensis was isolated from geothermally heated sediment [42], and was the only species identified in all samples of mudcompost + biochar (Table 9). Gemmatimonas is a newly recognized genus found in soil, sludge, and water [43]. It, as well as the included species G. aurantiaca, was identified in samples of mud compost + biochar with 50% or less biochar (Tables 8 and 9).

| Table 9. Species classification for mud compost (M) blended with biochar (B) by percent of total nucleic acids in the sample metagenome. |
|-----------------------------------------------|-----|-----|-----|-----|-----|-----|
| Actinoallomurus caesius                        | M100| M90B10| M75B25| M50B50| M25B75| M10B90|
| Amycolatopsis mediterranei                     |     |     |     |     |     |     |
| Bellilinea caldifistulae                       |     |     |     |     |     |     |
| Calditrich palaeochoryensis                    | 2.37| 2.57| 2.55| 2.15| 1.03| 1.02|
| Candidatus Rhabdoclamydia crassificans         | 0.85| 0.77|     |     |     |     |
| Candidatus scalindua                           |     |     |     |     | 0.95| 1.82|
| Cohnella soli                                 |     |     |     |     | 1.04| 1.84|
| Desulfomonile tiedjei                         | 0.78| 0.88| 0.92| 0.91|     |     |
| Desulfovibrio oryzae                          | 0.93| 0.87|     |     |     |     |
| Gemmatimonas aurantiaca                       | 0.92| 1.40| 0.96|     |     |     |
| Geobacter pickeringii                         |     |     |     |     |     |     |
| Kribbella ginsengisoli                        |     |     |     |     |     |     |
| Longilinea arvoryzae                          |     |     |     |     |     |     |
| Pelomaculum isopthaliticum                    | 0.76|     |     |     |     |     |
| Phyllobacterium catacumbae                    |     |     |     |     |     |     |
| 1.28                                          | 1.04| 1.59|     |     |     |     |
| 1.59                                          |     |     |     |     |     |     |
| 1.31                                          |     |     |     |     |     |     |
| 1.65                                          |     |     |     |     |     |     |
Table 9. Cont.

| M100 | M90B10 | M75B25 | M50B50 | M25B75 | M10B90 |
|------|--------|--------|--------|--------|--------|
| Sphaerisporangium cinnabarinum | 0.82 | | 0.96 | | |
| Streptomycyes chartreusis | | 0.83 | 1.03 | | |
| Symbiobacteria toebii | | | | | 1.03 |
| Thiobacillus sajanensis | | | | 1.11 | |
| Vogesella perlucida | | 0.79 | 1.05 | | 0.76 |

Legend: Blue text indicates microbes found in all experimental samples; red text indicates microbes found in all samples below the specified concentration of biochar; black text indicates microbes found inconsistently among samples.

4. Conclusions

Characterization of soil microbes is a necessary step toward the goals of tracking population changes, their impact on plants, and breeding for genotypes that are conducive to beneficial interactions. In the study presented here, microbial populations were qualitatively identified in blends of worm castings or mud compost with biochar sourced from sugarcane bagasse, with the intention to apply them as soil amendments. As anticipated, microbes most likely to survive long-term storage, including spore formers, were identified in the samples. Identified orders and their potentially beneficial characteristics include Actinomycetales and Rhizobiales, which are involved in nitrogen fixation, Clostridiales, which digest and recycle organic matter, Anaerolineales, which digests organic waste and sequesters compounds that could be harmful to plant growth, and Thermoanaerobacteriales, which produces biohydrogen. Bacterial genera known to cause foodborne illness were not identified in any of the blends.

Based on the microbes identified in this study, the application of worm castings or mud compost blended with 50% biochar would provide beneficial microbes as well as the previously identified physicochemical properties to enhance soil quality and plant growth, even after long-term storage.

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