

Article

Studies on the Potential of Beneficial Bacteria in a Biological Input Made from a Fermented Nettle (*Urtica dioica* L.) Slurry

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Abstract

Replacing chemical compounds with biologically derived bioproducts in agriculture can make a comprehensive contribution to achieving the goals of both the Paris Agreement and the Green Deal. Scientific literature contains a significant amount of data on the use of probiotics in agriculture, which facilitate the supply of nutrients to plants, stimulate physiological processes, and inhibit the development of pathogens. These products do not have a negative impact on the surrounding environment and can therefore be used in sustainable land management. This article analyzes the microbiological composition of a bio-input made from fermented nettle extract and the species diversity of the cultivable bacteria. It was found that this input is rich in bacteria beneficial to plants and soil (*Clostridium*, *Paenibacillus*, *Frankia*, *Rhizobium*, *Bradyrhizobium*, *Azospirillum*, *Azotobacter*, and others), some of which can be cultured under artificial conditions. Among the cultivable bacteria grown from the tested input, the majority were representatives of the *Pseudomonadota* (92%), and a small proportion were *Bacillota* (6%). Among them were a great many different bacteria beneficial to the soil ecosystem. Thus, there is considerable potential here for the use of these bacteria in the production of biological products.

Keywords: biofertilizer; microbial input; nettle slurry; *Urtica dioica*; beneficial bacteria

1. Introduction

Modern agricultural policy must be combined with economic development, environmental and social policies, in harmony with the goals of sustainable development. As part of its commitments under the international Paris Climate Agreement (in force since 2016), the EU has committed to ensure climate neutrality [1]. To achieve this goal, not only society, economy, but also agriculture will have to experience fundamental transformations. According to the European Green Deal, the EU commits to reduce the use of chemical plant protection products and mineral fertilizers by one third by 2030 [2]. In the global scientific literature, there is a lot of data on the use of plant-derived probiotics and biological inputs as an alternative source of soil fertilization in agriculture [3–5]. The use of such products improves the supply of plants with more readily available nutrients, suppresses the development of pathogens, and at the same time saves farming management and does not cause a negative impact on the surrounding environment [6,7]. Currently, there are many different fertilizers of biological origin distributed on the market [8–10], but most of them have a targeted and unambiguous composition, i.e., fertilizers contain one or more types of bacteria designed to perform some narrow function: absorbing atmospheric

Academic Editor: Ligang Zhou

Received: 16 June 2026

Revised: 15 July 2026

Accepted: 25 July 2026

Published: 29 July 2026

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nitrogen (nitrogen fixers), breaking down organic matter or creating symbiosis with plants (legumes-rhizobacteria). Our initial data showed that fertilizer made from fermented nettle slurry contains a wide range of bacteria beneficial to plants and soil-organic matter-decomposing-organotrophic, atmospheric nitrogen-fixing-diazotrophic, bean symbiotic rhizobacteria, and plant pathogen-inhibiting bacteria. In addition, it is rich in trace elements necessary for the normal development of plants. Since our fertilizer contains fermented nettle extract, nettle endophytic microorganisms appear in it. Currently, one of the global scientific trends is the use of plant endophytes in various fields. One of them—in plant pathology—is to inhibit the growth of pathogens [11–14]. Cultivated endophytes are the most studied, but in addition to them, there is a large abundance of uncultivated endophytes, the detection of which is enabled by modern methods such as NGS. Careful functional analysis of endophytes can provide valuable insights not only for the development of agriculture, but also for medicine and industry [15]. Another useful group of organisms are microbes capable of dissolving/mineralizing insoluble soil phosphate to release soluble P and make it available to plants. These microorganisms improve the growth and yield of various plants. Thus, seed/crop/soil enrichment with phosphate-solubilizing microorganisms (PSM) is a promising strategy to improve food production without endangering the environment. Despite their great importance in improving soil fertility, phosphorus-solubilizing microorganisms have yet to replace conventional chemical fertilizers in commercial agriculture. A better understanding of recent developments in the functional diversity, colonization capabilities, mode of action and smart adaptation of PSM should facilitate their use as reliable components of sustainable agricultural systems [16]. Nettle bioactive substances have also been intensively studied for some time [17–21]. Scientific studies have shown that the use of nettle extract as a fertilizer significantly increased all aboveground plant characteristics: height, leaf area, chlorophyll synthesis, flower buds, fruit parameters, etc. [22–24] and played a biopesticidal role with antibacterial and antifungal properties [25,26].

Scientific research data show that beneficial bacteria, both used alone and in combination with mineral or organic fertilizers, improve plant productivity and phytosanitary condition of crops [11,27–31].

Another group of microbes—plant growth-promoting bacteria (PGPB) are diverse groups of bacteria that can have beneficial effects on plant growth. PGPB facilitates access to nutrients by secreting phytohormones, degrading or releasing toxic pollutants, attenuating stress responses, stimulating immune defenses, and controlling phytopathogens [32]. These bacteria belong to genera such as *Azospirillum*, *Azotobacter*, *Pseudomonas*, *Bacillus*, *Burkholderia*, *Paenibacillus*, *Pantoea*, *Serratia*, *Streptomyces*, and *Rhizobia*, among others [33]. Specific plant growth-promoting rhizobacteria (PGPR) are characterized by excellent root colonization ability and the production of growth-promoting hormones such as indole-3-acetic acid (IAA) and gibberellic acid (GA3). They are divided into three main categories: (1) free-living bacteria that can interact with plants under appropriate conditions; (2) Rhizobacteria, which live in the rhizosphere soil zones adjacent to the roots or in the phyllosphere zone (plant leaves); and (3) endophytic bacteria, which form associations with specific plant tissues [34]. PGPR also exhibits antagonistic properties against pathogens and synergistic interactions with other soil microorganisms [35].

Since bio-input made from nettle slurry is rich in plant growth-promoting bacteria (PGPB), there should be a positive effect on the germination and growth of plants in the juvenile phase [11]. Thus, this bio-input under development is versatile and has a broad spectrum of action. However, the effectiveness of biological agents, compared to chemical ones, differs in speed, duration and dependence on environmental conditions, especially when it contains a single strain of bacteria. If the signs of activity of chemical measures appear quickly, then biological ones need time, but if the microorganisms of the bio-input

successfully establish themselves in the substrate, then they can ensure a long-term effect. And specifically, the integration of bio-inputs into a sustainable agriculture or Integrated Plant Protection program should be based on prevention and systematic planning, where the ultimate goal is not to completely abandon chemistry, but to gradually reduce its use as much as possible. In this case, it is important to start with soil preparation before sowing and continue to systematically use bio-inputs prophylactically in the early growth phases, which also enables preventive protection. There are also options when the use of bio-inputs is combined with minimal doses of chemistry. And chemical measures can remain as a last resort if the situation in crops becomes uncontrollable.

As we can see, the effects of fermented nettle on plants have been studied quite well in scientific research, and biochemical analysis has also been performed, but the microbiological composition has not been studied.

The main goal of the research was to determine the taxonomic composition of the biological-bacterial input made from fermented nettle extract, and to find out which groups of bacteria beneficial to plants can be cultivated “in vitro”. In this case, culturable bacteria could be used for bio-input production.

2. Materials and Methods

2.1. Preparation of Fermented Nettles Slurry

The pooled mixture for fermentation was prepared from approximately 2–3-month-old nettle (*Urtica dioica* L.) shoots cut from different ecosystems: Ruderal, Segetal and Woodland Edges. The collected shoots were crushed and poured with tap water (300 g of nettles poured 5 L of water). The mixture was fermented at 22 °C for 2 days, then 10% biohumus by volume was added and kept for another 24 h under the same conditions. During fermentation, the mixture was periodically stirred well. After fermentation, the mixture was filtered through gauze and used for further analysis. Three fermentation replicates were performed, from which a final pooled sample was obtained for metagenomic analysis.

2.2. Quantification of Cultivable Bacteria

Cultivable microbial quantification of bio-input Dx was performed by plate-count techniques in five biological replicates by inoculating the diluted soil suspension on different selective media: meat–peptone agar (ready for use, Liofilchem, Italy) for organotrophic bacteria and Ashby’s mannitol agar (K_2HPO_4 —0.2 g L⁻¹, MgSO_4 —0.2 g L⁻¹, NaCl —0.2 g L⁻¹, K_2SO_4 —0.1 g L⁻¹, CaCO_3 —5.0 g L⁻¹, and agar—20 g L⁻¹, pH 8.0 ± 0.3) for nonsymbiotic diazotrophic bacteria [36]. Rather than observing individual colony identity, community-level composition was assessed. The number of bacterial colony forming units (CFUs) was calculated per gram of dry soil [37]. The pooled sample for metagenomics was formed from all colonies grown in randomly selected 2 Petri dishes with organotrophic and diazotrophic bacteria (total of 4 dishes).

2.3. DNA Extraction and Microbiomic Analysis

2.3.1. DNA Extraction and Targeted Library Preparation

The samples of fermented nettle mixture and cultivated bacteria mixture were processed and analyzed with the ZymoBIOMICS® Targeted Sequencing Service (Zymo Research, Irvine, CA, USA).

The “ZymoBIOMICS®-96 MagBead” DNA kit (Zymo Research, Irvine, CA, USA) was used on an automated platform to extract total microbiome DNA. The entire 16S sequencing library was prepared following the protocol from PacBio (Pacific Biosciences of California, Menlo Park, CA, USA) [38]. The 16S gene was amplified using the 27f (AGRGTTYGATYMTGGCTCAG) and 1492r (RGYTACCTTGTTACGACTT) primers, us-

ing 2 ng of DNA as template. After 25 PCR cycles, the amplified products were purified using the “Select-a-Size DNA Clean & Concentrator MagBead” kit (“Zymo Research,” Irvine, CA, USA). The library from each reaction was quantified using a “NanoDrop” spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and pooled with the same mass of DNA. The pooled library was prepared for PacBio (third generation, long-read DNA technology known as Single Molecule Real-Time (SMRT)) sequencing using the “SMRTbell® Prep Kit 3.0.” (Pacific Biosciences of California, Menlo Park, CA, USA). For each DNA extraction batch, the “ZymoBIOMICS®” microbial community standard (Zymo Research, Irvine, CA, USA) was used as a positive control, including a negative (blank extraction) control. The library was sequenced on a single 8M SMRT cell using the “Sequel IIe” system (Pacific Biosciences of California, Menlo Park, CA, USA).

2.3.2. Statistical and Bioinformatic Analysis

The results of the abundance of culturable bacteria were evaluated by Student’s test using Microsoft Excel for Microsoft 365 software.

Unique amplicon sequences variants were inferred from raw reads using the DADA2 pipeline 1.38.0 [39]. Potential sequencing errors and chimeric sequences were also removed with the Dada2 pipeline. Taxonomy assignment was performed using Uclust from Qiime v.1.9.1 with the Zymo Research Database, a 16S database that is internally designed and curated, as reference. Composition visualization, alpha diversity was performed with Qiime v.1.9.1 [40]. Microbial hierarchical data was visualized with KronaTools v2.8.1 [41].

3. Results

3.1. Abundance of Cultivable Bacteria

Although the number of culturable bacteria (CB) in a soil or other biome may be about 1%, the number of colony-forming units has a positive correlation with enzymatic and respiratory activity [42] and is still used to describe the relative abundance of active and potentially active microbial groups that perform certain functions and trophic requirements [43–45]. Despite this small fraction of microbes determined by plate counting methods, the contribution to nutrient cycles can be quite large, as they constitute 80–90% of the bacterial biological volume in the soil [44]. The amount of culturable organotrophic bacteria in the nutrient media was determined to be $6.3 \pm 0.06 \times 10^5$ CFU ml⁻¹ and the amount of diazotrophic bacteria was determined to be $1.31 \pm 0.03 \times 10^5$ CFU ml⁻¹ (Figure 1).

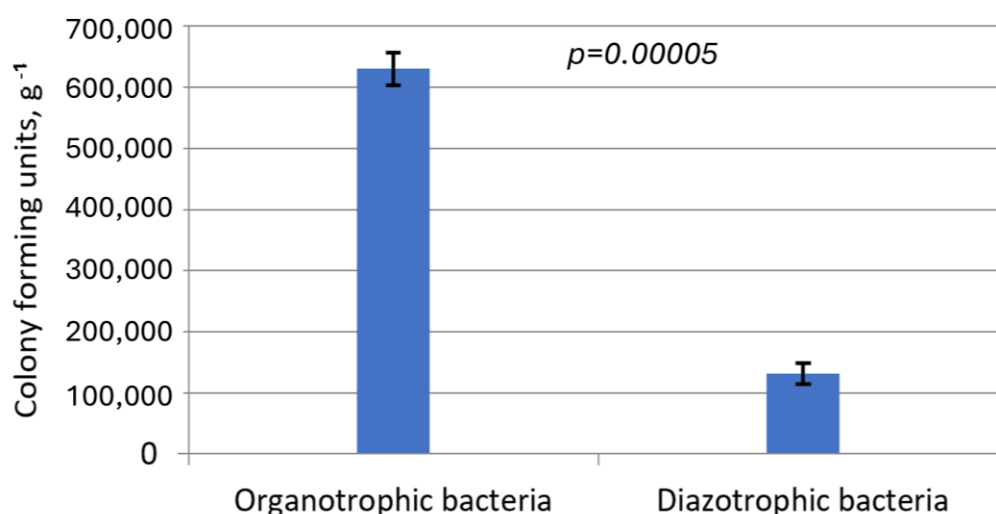


Figure 1. The abundance of cultivable organotrophic and non-symbiotic diazotrophic bacteria in bio-input Dx.

After 48 h of incubation at 25 °C, colonies were harvested from the agar, and a pooled 2 × (O + D) sample was prepared for taxonomic analysis by metagenomic sequencing.

3.2. Microbiomic Analysis

A total of 26,733 high-quality bacterial sequences were obtained from the fermented nettle bio-input (Dx) sample and 8551 from the cultured bacteria (CB) sample, which were grouped into appropriate rank taxonomic units (ASV) (Figure 2). The Chao1 index indicating species richness was 1833.5 ± 25.3 for the bio-input Dx sample and 46.6 ± 3.6 for the cultured bacteria sample. The Shannon index of species diversity was 9.49 ± 0.02 and 2.37 ± 0.01 , respectively. This indicates that the species richness and diversity of the cultured bacterial sample was quite low, which was to be expected considering that only about 1–5% of the bacteria in the biome can be cultured “in vitro”. In the Dx biome, 12 phyla of bacteria were detected, dominated by *Pseudomonadota* (32%) and *Bacillota* (26%), while others were present in 2–4%, except for *Bacteroidota* and *Elusimicrobiota*, which were only present in trace amounts.

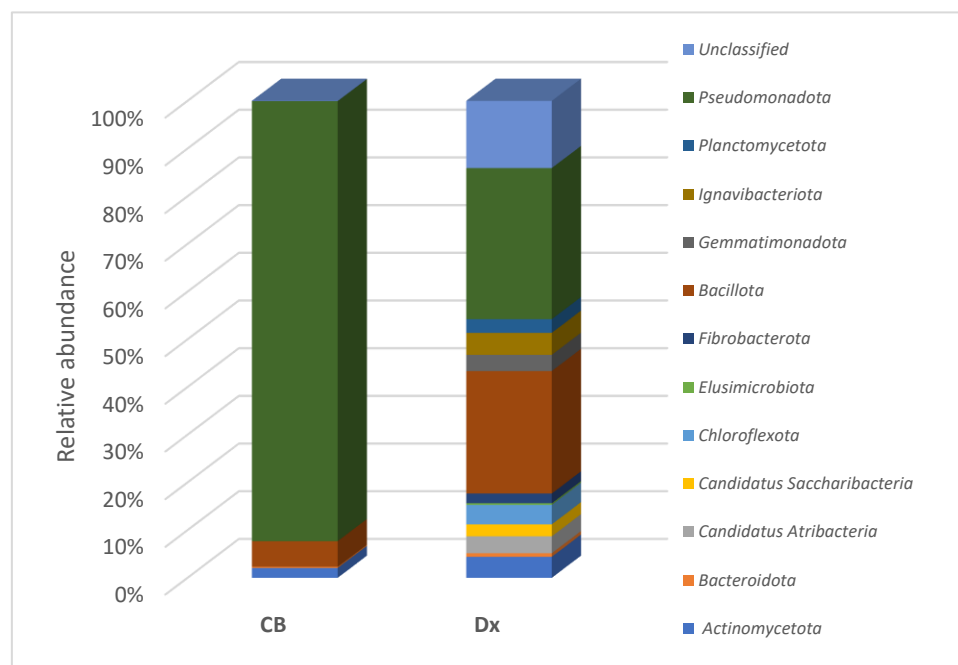


Figure 2. Relative abundance of most common bacteria phyla (CB—cultivable bacteria, Dx—fermented nettle input).

When examining lower-ranking taxa, it becomes clear that the most abundant genera of the *Bacillota* phylum were *Bacillus* (5% of all) and *Clostridium* (8% of all) (Figure 3), while in the *Pseudomonadota* phylum there were no significant differences between individual lower taxonomic units (*Hydrogenophaga*, *Hyphomicrobium*, *Geobacter*, *Methylobacter*, *Azotobacter* and other genera), their amount was 1–3% of all bacteria (Figure 3).

A completely different view is found among culturable bacteria. In this case, all ASVs were assigned to four bacterial phyla (Figure 2). The largest number was *Pseudomonadota*—92%, *Bacillota* 6%, *Actinomycetota* 2% and the smallest number was *Bacteroidota* phyla—0.23%. In the context of the lower hierarchy, 14 genera were distinguished in the *Pseudomonadota* phyla (Figure 4), and 5 genera in *Bacillota* (Figure 4).

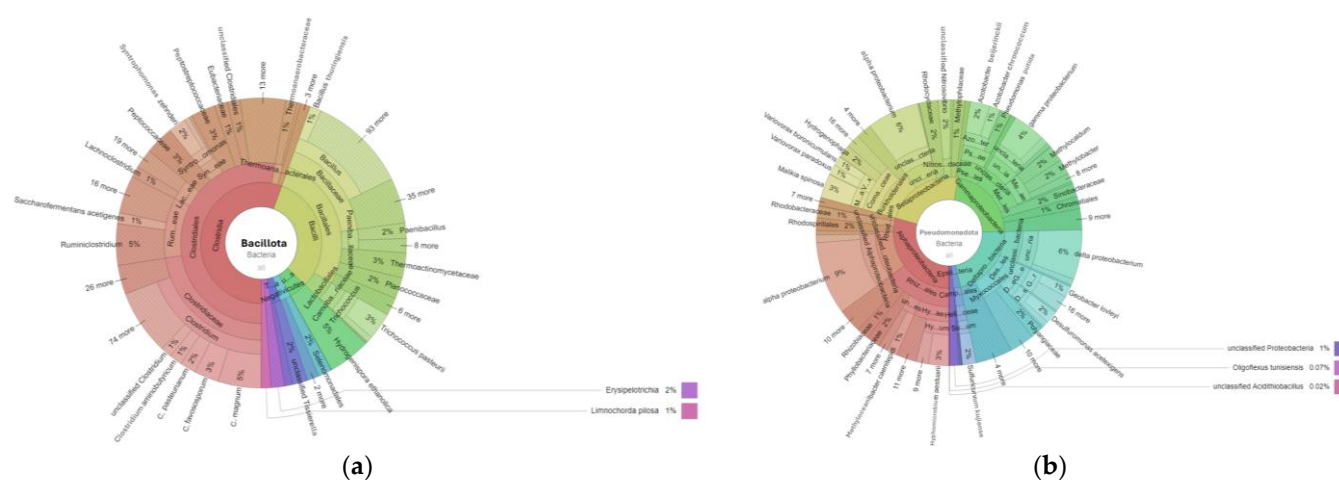


Figure 3. Hierarchical analysis of the input Dx *Bacillota* (a) and *Pseudomonadota* (b) phylum.

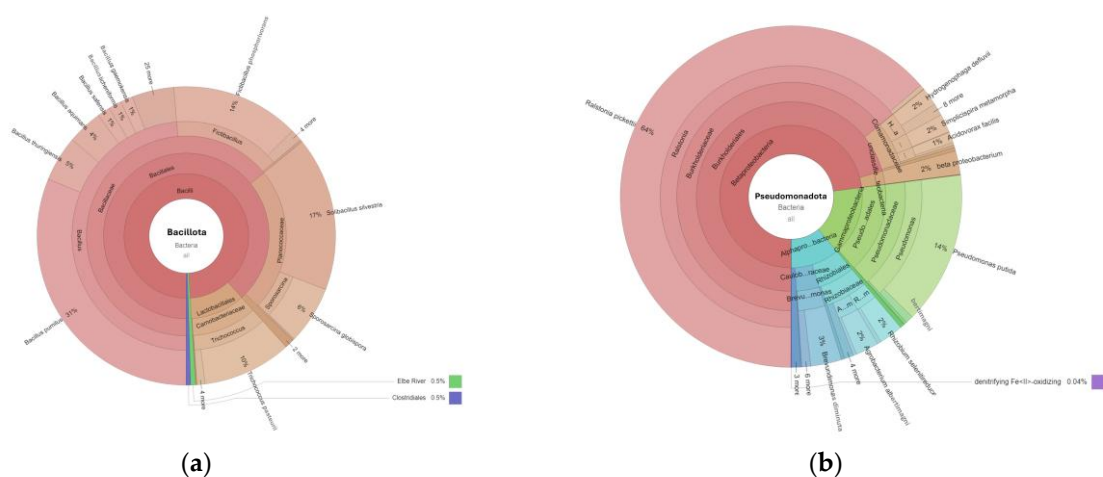


Figure 4. Hierarchical structure of the *Bacillota* (a) and *Pseudomonadota* (b) phylum of culturable bacteria.

4. Discussion

Although various nettle extracts, extracts, and other preparations are being studied, their microbiological composition has been largely overlooked. It has already been established that nettle extracts have a positive effect on plant growth and health, as well as on the soil in which the plants grow [22,23,46–49], but studies that incorporate individual species or species consortia into the development of new bioproducts are also important [50]. This would be the result of research conducted at the intersection of metagenomics and bioinformatics. Therefore, during this study, we attempted not only to determine the microbiological composition of nettle bio-input Dx but also to cultivate the bacteria, identify them, and assess their potential for bioproduct production. As mentioned earlier, bio-input Dx contains a wide variety of bacterial physiological groups: organotrophic, diazotrophic, phosphorus-solubilizing, as well as plant growth-stimulating bacteria. Most of them belong to the *Pseudomonadota* and *Bacillota* phyla. The hierarchical structure of these phyla is shown in Figures 3 and 4. A total of 3653 Amplicon Sequence Variants were obtained in the Dx biological sample, which according to the Chao1 (1833.5 ± 25.3) index probably corresponds to 1834 species. Among this great diversity, there are also representatives important for agroecosystems. These include nitrogen-fixing bacteria belonging to the genera *Clostridium*, *Paenibacillus*, *Frankia*, *Rhizobium*, *Bradyrhizobium*, *Azospirillum*, and *Azotobacter*. Nitrifying bacteria from the genera *Nitrosomonas* and *Nitrobacter* were detected in the bio-input Dx. It also contains plant-growth-promoting bacteria from

the *Bacillus*, *Streptomyces*, and *Micromonospora* genera. Our preliminary research data also indicates that the bio-input Dx has a positive effect on plants (wheat and peas) during the early stages of growth. In 2025, a study was conducted under laboratory conditions and showed that, compared to others, this input increased the root system volume in wheat and the total plant biomass in peas [51]. Initial field studies have also been conducted and have yielded positive results and are therefore being continued and have not yet been published.

Analysis of metagenomic diversity of cultured bacteria shows that 272 Amplicon Sequence Variant were obtained, while 47 species should be expected according to the Chao1 index (46.6 ± 3.6). Compared to the total number of species found in the bio-input, this is a very small number. However, the bacteria that grew here were the most active and significant for the soil ecosystem. Representatives of the *Pseudomonadota* phylum constituted the largest portion of this consortium (92%), with the remainder consisting of *Bacillota* (6%), *Actinomycetota* (2%), and *Bacteroidota* (0.3%) (Figure 2). Of particular note are certain species that grew in the agarized medium derived from bio-input Dx. The highest number of reads (64%) was attributed to *Ralstonia pickettii* (Figure 4). Its primary function in the ecosystem is the breakdown of organic pollutants and the cycle of nutrients. It plays an important role in bioremediation (environmental cleanup) because it breaks down toxic compounds and participates in the decomposition of organic matter, converting it into forms accessible to plants and other organisms [52]. So, this bacterium is very important in agrobiotechnology, bioremediation, and soil ecology research. If traces of the *Ralstonia* genus are found in the soil, accurate laboratory identification is necessary, as it can sometimes be confused with another species of this genus—*R. solanacearum*, which causes a dangerous quarantine brown rot of solanaceous plants. Unlike the latter, *R. pickettii* is not dangerous to plants, but rather beneficial, as its effects are multifaceted. First, it is highly resistant to unfavorable environmental conditions and can be used in contaminated soils because it can efficiently metabolize toxic chemical compounds, including pesticide residues. This bacterium can not only survive in soil contaminated with heavy metals but also neutralize their environmental effects [53]. Recent scientific studies show that certain *R. pickettii* strains can colonize the rhizosphere and helping to release insoluble phosphorus, converting it into a form that plants can absorb. Although this bacterium is beneficial in the soil, it is also classified as an opportunistic pathogen. This means that it can cause infections in immunocompromised patients [54]. Therefore, its use in this case is controversial, and it probably should not be included in commercial products, but if it is present in a natural nettle-based input, it could be beneficial.

Another bacterium found in abundance, also belonging to the phylum *Pseudomonadota*, is *Pseudomonas putida* (Figure 4). *P. putida* is a highly beneficial bacterium used in agriculture as a plant growth-promoting rhizobacterium (PGPR). It increases crop yields, mobilizes vital nutrients in the soil, acts as a natural biological plant disease control agent, and helps plants tolerate abiotic stressors such as drought and salinity [55,56]. It is also important to mention diazotrophic microorganisms, which can be classified as free-living and symbiotic. The differences between these groups relate to their lifestyle, nitrogen fixation efficiency, and importance for agriculture. Both types fix atmospheric nitrogen but do so according to different biological and ecological principles. Free-living diazotrophs live freely in the soil without a host, and their connection with plants is indirect. Their energy source is soil organic matter, and the efficiency of nitrogen fixation is low; therefore, their importance in agriculture is rather limited and can be described as complementary. Symbiotic diazotrophs live in symbiosis with plants, maintaining a close, mutually beneficial relationship. In this case, the energy source is the products of plant photosynthesis, and the efficiency of nitrogen fixation is quite high; therefore, their practical significance in agriculture is much greater than that of free-living diazotrophs. However, the

importance of the latter should not be underestimated. They improve the biological activity of the soil, stimulate root growth by synthesizing various growth hormones (auxins, gibberellins, cytokinins), and enrich the soil with nitrogen, although not as the main source. Meanwhile, symbiotic nitrogen-fixing bacteria are very active in the field of nitrogen fixation, accumulating from 50 to 300 kg/ha per season [57]. Thus, symbiotic diazotrophs are directly beneficial to plants and are indispensable in agriculture, while free-living diazotrophs can be used as an additional biological resource. Some biological products used in agriculture are based on the use of diazotrophic microorganisms, both free-living and symbiotic. The use of such products allows for a gradual reduction in the total nitrogen balance and helps stabilize crop yields, maintaining high productivity. It is also important to mention the bacteria *Azotobacter beijerinckii* and *A. chroococcum* identified in the bio-input Dx, as well as bacteria of the genus *Rhizobium*, which are important for the soil nitrogen cycle. Most of the isolates in *Rhizobium* genus belong to the taxonomic unit *Rhizobium selenitireducens*, which, unlike most bacteria of the *Rhizobium* genus, was isolated from a laboratory bioreactor, i.e., in an artificial environment rather than a natural one [58]. Scientific research indicates that *R. selenitireducens* can act as an endophyte (a bacterium living within plant tissues). It effectively promotes the development of the root systems of various non-leguminous plants (such as basil, lettuce, or corn) as well as the growth of total biomass [59]. Another interesting member of this genus that grew in the medium was *Rhizobium azooxidifex*. This is a Gram-negative, facultative anaerobic soil bacterium known as an N₂O-producing bacterium, and, unlike many classical rhizobia, it was isolated directly from soil rather than as a nodule symbiont and described 10 years ago [60]. Since *R. azooxidifex*, among other things, is characterized by its ability to produce nitrous oxide (N₂O), it is an important subject for understanding greenhouse gas emissions from agricultural soils. Within the *Bacillota* phylum, the highest number of reads (31%) was assigned to the species *Bacillus pumilus*. This is a spore-forming bacterium widely distributed in soil and plants that performs important functions in agriculture, probiotic production, and environmental protection [61,62]. Since it is a spore-forming bacterium, its potential for promoting plant growth and for disease protection and prevention is promising. *Bacillus thuringiensis*, a Gram-positive bacterium naturally found in soil, water, and on plant leaves, was also grown in an artificial medium; it is the most widely used safe biological insecticide in the world. This microorganism is valued in organic farming because it acts very selectively and does not harm humans, animals, or beneficial insects, such as bees or ladybugs [63,64]. *B. thuringiensis* is generally one of the most important, successfully, and widely used bacteria in agriculture worldwide. It primarily acts as a biological pesticide, protecting crops from pests without harsh chemicals. Products based on these bacteria have been developed and are officially certified for use in organic farms, as they quickly integrate into the soil and leave no toxic residues in the soil or food products [65]. Another important bacterium was subsequently identified: *Paenibacillus macerans*. *Paenibacillus macerans* is valued in agriculture and agrobiology as a plant growth-promoting rhizobacterium. It exhibits multifaceted activity: primarily, it is one of the atmospheric nitrogen fixers, and it is also capable of secreting organic acids that liberate fixed phosphorus and potassium, converting them into soluble compounds more readily accessible to plants. Furthermore, this bacterium demonstrates broad enzymatic activity (secreting amylases, cellulases, and pectinases), which breaks down complex plant carbohydrates, thereby accelerating the mineralization of crop residues. Another beneficial trait is the synthesis of plant hormones, which promotes the development of lateral roots and root hairs, and a larger root surface area enhances plant development [66,67]. Finally, another interesting bacterium was isolated—*Fictibacillus phosphorivorans*. It is a Gram-positive, aerobic or facultatively anaerobic, spore-forming rod-shaped bacterium, which in 2013 became the basis for the creation of a new genus, *Fictibacillus* (meaning “false bacil-

lus”), into which several bacteria previously belonging to the genus *Bacillus* were transferred [68]. These bacteria are characterized by exceptional bioremediation capabilities; furthermore, *F. phosphorivorans* has the ability to eliminate plant root nematodes (worms), which opens opportunities for the development of eco-friendly biological agents for agriculture [69].

5. Conclusions

Thus, after reviewing the composition of the biological input Dx made from fermented nettle slurry, it can be stated that it is rich in bacteria important to soil ecosystems, which actively participate in the processes occurring within them and, when used correctly, can bring significant benefits to agroecosystems where various agricultural crops are grown. Considerable potential can also be seen in the cultivable bacteria derived from the bio-input, as, by appropriately balancing their composition, they can be utilized in the production of biological products. Individual bacterial strains can be used, but it would be more effective to assemble an appropriate consortium of them, which would contribute to improving soil health and well-being through its activity.

Funding: The research was supported by the Private Limited Company “Circulab” (CPPS-22-22).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is available through the NCBI Sequence Read Archive (SRA): BioProject ID PRJNA1479156 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1479156>, Accessed on 22 June 2026).

Acknowledgments: The author thanks the Private Limited Company “Circulab” for cooperation and support for the research.

Conflicts of Interest: The author declares no conflicts of interest.

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