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Application of *Jeevamrit* Improves Soil Properties in Zero Budget Natural Farming Fields

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Abstract: Zero Budget Natural Farming (ZBNF), utilizing natural resources, multiple cropping systems, and cow-dung- and urine-based products to improve soil biology, has been practiced by thousands of farmers in India. However, without any scientific proof, this traditional and ancient technique is mocked as a bugged theory in the scientific community. In the current study, we have investigated the effect of *Jeevamrit*—cow-dung- and urine-based formulation—on soil chemical and microbial properties of the ZBNF field coupled with metagenomic analysis and the economics of ZBNF. The percentage increase in soil properties, such as organic carbon, available phosphorus, and available potassium, was recorded up to 46%, 439%, and 142%, respectively, while micronutrients, such as Zn, Fe, Cu, and Mn, also increased up to 98%, 23%, 62%, and 55%, respectively, from 2017 to 2019. Whole genome metagenomic analysis revealed that Proteobacteria were dominantly present, and bacterial phyla including *Bacillus*, *Pseudomonas*, *Rhizobium*, and *Panibacillus*. On the other hand, Ascomycota was the dominating fungal phyla present in the soil sample. Further, functional analysis showed a high representation of genes/enzymes involved in amino acids and carbohydrate metabolism contributing to soil fertility, plant growth, defense, and development. Additionally, the cost–benefit ratio of ZBNF was double the farmer's practice when tested with the rice and wheat cropping system. The results from this study provide a new proof of concept and understanding of the potential of the ZBNF component, i.e., *Jeevamrit*, in improving soil properties.

Keywords: zero budget natural farming (ZBNF); *Jeevamrit*; bacterial community; proteobacteria; functional analysis

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1. Introduction

Green Revolution technology has been proved as a double-edged sword for the Indian agriculture system as well as for the entire globe. Although it intensified Indian agriculture from a food-scarce to a food-surplus country, it has also thrown several challenges in the form of declining factor productivity, depleting natural resources, low water, and nutrients, and adverse impacts on climate change as well as on human health [1–3]. Overuse of chemical fertilizers not only depletes soil nutrients but also reduces the yield and poisons the whole ecosystem [4]. In the past, several management practices, such as biofertilizers/biopesticide application, use of vermicompost, FYM, etc., have been intended

to mitigate the negative impact of chemical fertilizers and pesticides [5]. Despite the continuous efforts of the scientific community, to date there is no proven method that can replace chemical-based fertilizers/pesticides providing sustainable results. Although organic farming seems to ease the above-mentioned effect by cutting greenhouse gas emissions (by ~65%) and comforting climate change, it creates a challenge in the form of low productivity and a difficult certification process [6]. Recently, Zero Budget Natural Farming (ZBNF)—an ancient and traditional farming technique—has been reintroduced as a self-adequate solution that can help to address these concurrent issues.

In ZBNF, the cost of farming activities from external sources (fertilizers/pesticides) is zero as it does not require any credit on purchasing inputs, and crops are cultivated without chemicals exploiting natural resources, such as cow dung/urine, etc. [1,2,6,7]. ZBNF formulations, such as *Jeevamrit, Beejamarit*, and *Panchgavya* induce a multifold increase in microbial population and earthworm activity which enhances nutrient availability in soil, strengthens the resistance mechanism, and increases crop productivity [5,8,9]. Enhanced microbial population diversity index improves the stability and resistance of the soil ecosystem [10–12]. The rich microbial community of soil microorganisms not only transforms the soil organic matter but also acts as source and sink of nutrients that can be used by plants and improve soil fertility as well as crop yield [4]. Recently, studies on ZBNF conducted by Niti Ayog in the Andhra Pradesh, Maharashtra, and Karnataka states of India revealed that the yield, and gross and net income increased in the range of 14.2 to 50%, whereas the cost of cultivation decreased by 23.7% under irrigated and rainfed conditions [13]. This farming technique is now gaining momentum in other states like Himachal Pradesh, Uttar Pradesh, Haryana, Gujarat, Tamil Nadu, and Kerala [7]. With this farming technique, issues of sustainability, conservation of natural resources, global warming, etc., are likely to be addressed in the right perspective [14].

Though ZBNF—as a conventional way of natural farming—is being practiced in different parts of India by thousands of small-scale local farmers, it has not been well accepted by the scientific community and there is reluctance towards it, due to the lack of sufficient proof of concepts. To fill this gap, the present investigation was, thus, undertaken to evaluate the impact of the *Jeevamrit* (the major formulation of ZBNF) on soil health, nutrients, and the taxonomic composition of soil microbial communities to bring new insights and futuristic approaches.

2. Material and Method

2.1. Experimental Site and Preparation of Jeevamrit

The experimental field is situated at Gurukul, Kurukshetra (29.9624° N, 76.8100° E), Haryana, India where ZBNF has been practiced since 2015. The area belongs to the subtropical monsoon climate region with an average annual temperature of 23.9 °C (75.0 °F), and an annual mean precipitation of 808.00 mm per year. The soil was classified as yellow soil and had the maximum water-holding capacity. This field is devoid of any chemical-based fertilizer/pesticide, and instead *Jeevamrit*—the main formulation used as biofertilizer as well as biopesticide agent—is applied from time to time as per requirements. Briefly, *Jeevamrit* was prepared by mixing 10 kg cow dung, 10 L cow urine, 1.5 kg black jaggery, 1.5 kg pulse (chickpea) flour, and a handful of soil (100 g) as microbial inoculum, and the total volume was made up to 200 L by adding water. After incubation for 20 days, the formulation was ready to be used.

2.2. Contribution of Different Components of Jeevamrit on Microbial Growth

Jeevamrit is the main formulation in ZBNF used as an inoculum for triggering the population of microbes in the field. The studies on the contribution of Jaggery and pulse flour added in JA and their combined effect on microbial population were conducted by preparing *Jeevamirt* without its component. The experiment comprised the following details: T1—10 kg cow dung + 10 liter cow urine + 1.5 kg Jaggery + 1.5 kg Pulse flour + 100 g soil; T2—T1 minus Pulse flour; T3—T1 minus Jaggery; and T4—T1 minus (Pulse

flour and Jaggery). The microbial count was studied with each treatment following serial dilution methods as discussed below.

2.3. Sample Collection and Measurement of Soil Chemical Properties

Soil samples from Gurukul farm were randomly collected from the plow layer (1–15 cm) and pooled to form a composite sample (Acharya) from the respective field in May 2017, 2018, and 2019 (from the same fields). During sample collection, 3 samples/sites were collected and pooled from ZBNF or farmer's fields. For example, in wheat soils, the randomly selected sites were fixed, and the sample was taken from the same site, but the crop was different every year in that field as the crop rotation is also an important component of this farming system (ZBNF). Therefore, the soil samples were collected randomly from the same field without caring for the crop grown in that field. These samples were homogenized and spread in trays to be cleaned of extraneous substances (small stems, pieces of roots, leaves, etc.), sieved, followed by storing in plastic bags. The samples were collected from different cropping systems. The soil sample collection procedure, sampling site, and analysis procedure were kept consistent throughout the study. After sample collection, soil pH, organic matter, available phosphorus (P), available potassium (K), micronutrients, such as Zinc (Zn), iron (Fe), copper (Cu), manganese (Mn), and microbial count using standard serial dilution method were studied [4]. In addition, samples were also collected from farmer's fields following the same, above-mentioned procedure, to compare the soil chemical/microbial properties with the ZBNF field.

2.4. Comparison of ZBNF Microbial Load with Farmer's Field

The soil samples collected from the ZBNF and farmer's fields as mentioned above were further assayed for microbial count using the standard serial dilution method [15]. A hundred milligrams of each soil sample were added to 900 mL of sterilized distilled water. After homogenization for 30 min, this solution was decimally diluted (10^{-1} to 10^{-9}) and aliquots of the resulting solution, i.e., 10^{-9}, were used for plating on appropriate culture media (nutrient agar) by pour plate method. After incubation at 30 ± 0.5 °C, the colony-forming units (CFU) were counted. In addition, samples were also collected from farmer's fields to compare the soil microbial properties of the fields.

2.5. DNA Extraction, Library Construction, and Metagenomic Sequencing

DNA for metagenomic analysis was extracted from the soil samples using the Power Soil DNA kit (MoBiol Laboratories, Solana Beach, CA, USA), according to the manufacturer's protocols. The concentration and quality (A260/A280) of extracted DNA were determined using a NanoDrop ND-2000 spectrophotometer (NanoDrop, Wilmington, DE, USA), and evaluated with a 2% agarose gel. To minimize DNA extraction bias, DNA samples from three replicates were pooled. DNA samples were subjected to a random enzymatic fragmentation in which the DNA was simultaneously fragmented and bound to adapters using the QXT SureSelect kit (Agilent Technologies, Santa Clara, CA, USA). The fragmented DNA was purified using AmPure XP beads (Beckman Coulter, Brea, CA, USA) and subjected to an amplification reaction (6 cycles) using primers complementary to the Illumina flow cell adapters. Amplified libraries were again purified using AmPure XP beads (Beckman Coulter), quantified using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), and checked for fragments size in the 2100 Bioanalyzer (Agilent Technologies) using a High Sensitivity DNA kit (Agilent Technologies). Libraries were then pooled in equimolar ratios of 0.7 nM and sequenced paired end (150 bp) for 300 cycles using the Miseq platform (Illumina, San Diego, CA, USA). Sequencing was performed using Illumina Miseq DNA according to the standard protocol (http://www.illumina.com/, (accessed on 1 January 2019)). The bcl data received from the sequencer was de-multiplexed into .fastq raw data.

2.6. Analysis of Illumina Sequencing Reads

The raw data reads received were first analyzed for quality control using FastQC (v0.11.9) and MultiQC and then the low-quality reads, and adapter sequences were trimmed using Trimmomatic (ver 0.40) program with default parameters except for a minimum PHRED score of 30 and minimum length 100 bp [16]. The cleaned reads were further assembled utilizing the MEGAHIT (ver.1.2.9) assembly program [17] and the final contigs in the FASTA format were retrieved which were used further for the downstream processing. The final contigs were then further evaluated using the BBMap tool and then aligned to the NCBI database as discussed by Nisrina et al. [11] using the DIAMOND (ver.0.9) tool resulting in DIAMOND Archive Alignment (DAA) files. These DAA files were then further used for the taxonomic functional analysis using the MEGAN6 program.

2.7. Isolation and Characterization of Plant-Growth-Promoting Activities of the Isolated Microbes

The isolated bacteria were further screened, purified by sub-culturing on the media, and then tested for PGPR properties. Phosphate solubilization, hydrogen cyanide, ammonia, siderophore, indole acetic acid production, biological nitrogen fixation, and antifungal activity were accessed as mentioned previously [2,10]. Enzyme production, such as chitinase, protease, and cellulase was carried out as discussed by Dinesh et al. [18]. Preliminary identification and differentiation of bacterial isolates were performed based on cell morphology, Gram-staining, endospore staining, and utilizing biochemical tests (catalase, indole production, methyl red, VP test, nitrate reduction, sucrose fermentation, starch hydrolysis, citrate utilization, and H_2S production) as outlined in Bergey's Manual of Systematic Bacteriology (Vol. 2).

2.8. Statistical Analysis

The data recorded for the microbial count and soil physiochemical properties were collected in triplicates and analyzed statistically using a *t*-test and Tukey's pairwise comparison test using the GraphPad Prism program (v.5, USA). Results are the mean values of replicates. Small letters represent significant differences according to the Tukey's pairwise comparison test (p value = 0.05). Asterisks indicate significant changes in the values calculated by the student's *t*-test (*** $p < 0.001$).

3. Results

3.1. Effect of Jeevamrit on Soil Properties

The soil properties of the ZBNF farm were monitored for three continuous years and it was noted that with application of *Jeevamrit,* the soil physiochemical properties were boosted. The soil pH was 8.16, 8.14, and 8.12 from 2017, 2018, and 2019, respectively and showed no significant difference. In the year 2017, the analysis of soil samples had revealed that 30% soil samples were in the rich category (>0.75%) with respect to organic carbon (Figure 1a). The 10% soil samples represented a poor status of organic carbon (<0.40%). The remaining samples were categorized in the medium range of organic carbon content (data not shown).

The average organic carbon was 0.61% in 2017 which increased up to 0.78% in the year 2018 and the average organic carbon was recorded at 0.92% in 2019 (Figure 1a). Overall, a total percentage increase in soil organic carbon was 46% from 2017 to 2019. Similarly, available P, available K, and micronutrients (Zn, Fe, Cu, and Mn) have increased significantly from the year 2017 to 2019 (Figure 1a). An increase of 45% in available P was observed in the samples collected in 2018 over those collected in 2017. Interestingly, there was a tremendous increase of 270% in the mean available P status in 2019 when compared with 2017. Additionally, the soil samples were found to be rich in micronutrients (Zn, Fe, Cu, and Mn). An increase of 98%, 23%, 62%, and 55% of Zn, Fe, Cu, and Mn content in the samples were recorded collected in May 2019 while compared with samples of May 2017. Noticeably, the concentration of Fe dropped in the year 2019, which might be due to seasonal variations or the crop type.

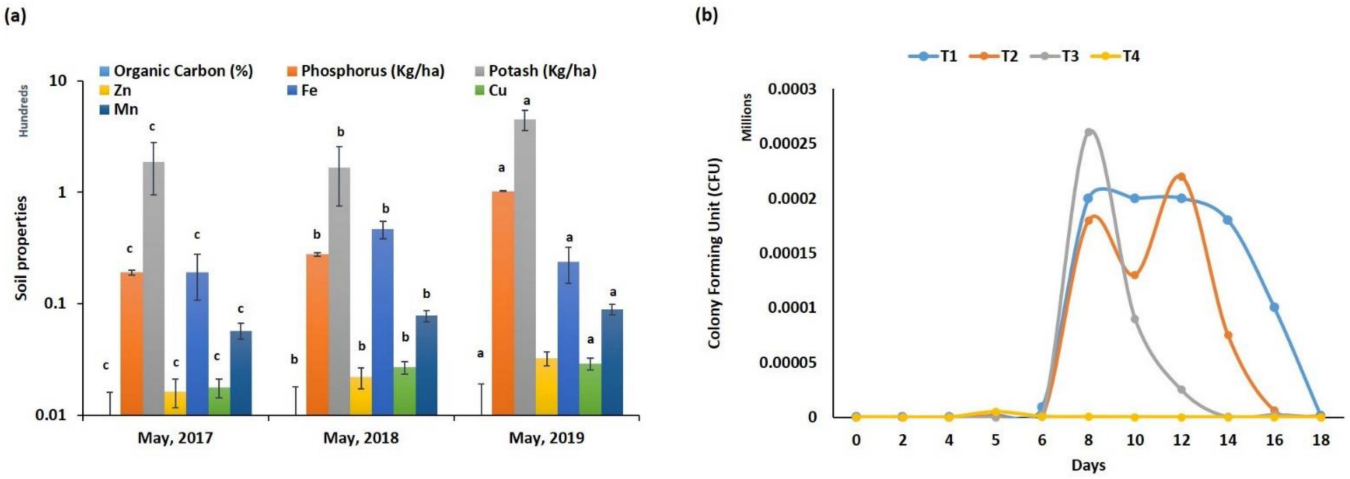

Figure 1. Effect of *Jeevamrit* on soil chemical and microbial counts. (**a**) Chemical properties of soil after Jeevamrit application for 3 successive years. Different letters indicate statistical difference shown in three successive years (2017 to 2019) with a *p*-value = 0.001. (**b**) Effect of different components of *Jeevamrit* on bacterial count in soil. T1 = *Jeevamrit* formulation, T2 = *Jeevamrit* without pulse flour, T3 = *Jeevamrit* without Jaggery, T4 = *Jeevamrit* without pulse flour and Jaggery.

3.2. Effect of Different Jeevamrit Constituents on Bacterial Count

The composition and constituents of *Jeevamrit* can influence the microbial properties of the soil. To investigate it, the effect of *Jeevamrit* constituent on the bacterial count in soil was also studied. It was noticed that the whole *Jeevamrit* formulation (T1) gave the maximum bacterial count when compared with other components, such as T2 (*Jeevamrit* without pulse flour), T3 (*Jeevamrit* minus Jaggery), and T4 (*Jeevamrit* minus Pulse flour and Jaggery) as shown in Figure 1b. These results indicate that the whole *Jeevamrit* is a rich source for microbial inoculation and proliferation.

3.3. Validation of Jeevamrit Application by Comparing ZBNF Soil Properties with Farmer's Field

To further authenticate the effect of the *Jeevamrit* application, soil properties of ZBNF fields were compared with farmer's fields from the surrounding villages (more than 225 villages) of four blocks (Shahabad, Ladwa, Babain, and Thanesar) of the district Kurukshetra (Haryana). Out of 1701 soil samples tested in four blocks, only 4 and 2 samples of Ladwa block were rich in organic carbon and available P, respectively (Table 1), whereas soil samples of ZBNF were rich in organic carbon and available P in May 2017. These respective figures subsequently reached more than 90% (organic carbon) and 80% (available P) in May 2018 and May 2019. However, the soils of farmers' fields of surrounding villages were comparatively richer in available K as compared to the soils of ZBNF. This difference in available K might be due to the application of NPK in the field.

Table 1. Soil properties of the samples collected from farmer's field in year 2019. Different letters states significant differences among different blocks for each parameter.

Block	Organic Carbon (%)	Available Phosphorus (kg/ha)	Available Potash (kg/ha)	Zn	Cu	Fe	Mn
Shahabad	0.34 ± 0.21 b	6.74 ± 2.64 a	295 ± 68.92 b	2.61 ± 0.93 a	2.56 ± 0.64 a	44.74 ± 8.11 a	6.24 ± 0.62 b
Babain	0.37 ± 0.18 b	7.71 ± 1.98 a	269 ± 46.67 b	2.15 ± 0.80 a	2.26 ± 0.68 a	42.06 ± 11.14 a	6.9 ± 1.66 b
Ladwa	0.46 ± 0.26 a	5.67 ± 3.32 b	439 ± 98.95 a	2.49 ± 1.30 a	2.6 ± 0.53 a	22.90 ± 11.68 c	7.33 ± 1.39 a
Thanesar	0.43 ± 0.10 a	5.94 ± 2.85 b	479 ± 78.88 a	1.98 ± 1.45 b	2.06 ± 0.41 a	40.44 ± 9.15 b	6.11 ± 2.10 b

3.4. Metagenomics Analysis of ZBNF Field Soil

A total of 12.42 Mbp sequence reads were obtained from the rhizosphere samples of the ZBNF farm, out of which 12.01 high-quality sequence reads were clustered into 4520 OTUs (Supplementary Figure S1). The statistical information for the contigs was exhibited in Supplementary Table S1. The rarefication of counts to 1126 reads per sample was sufficient for explaining differences in bacterial diversity. The Shannon–Weaver index of the sample was 4.978. Soil microbial biodiversity is critical for the soil ecosystem and results from our studies (Figure 2) showed that the microbial richness index was quite high in the ZBNF soil.

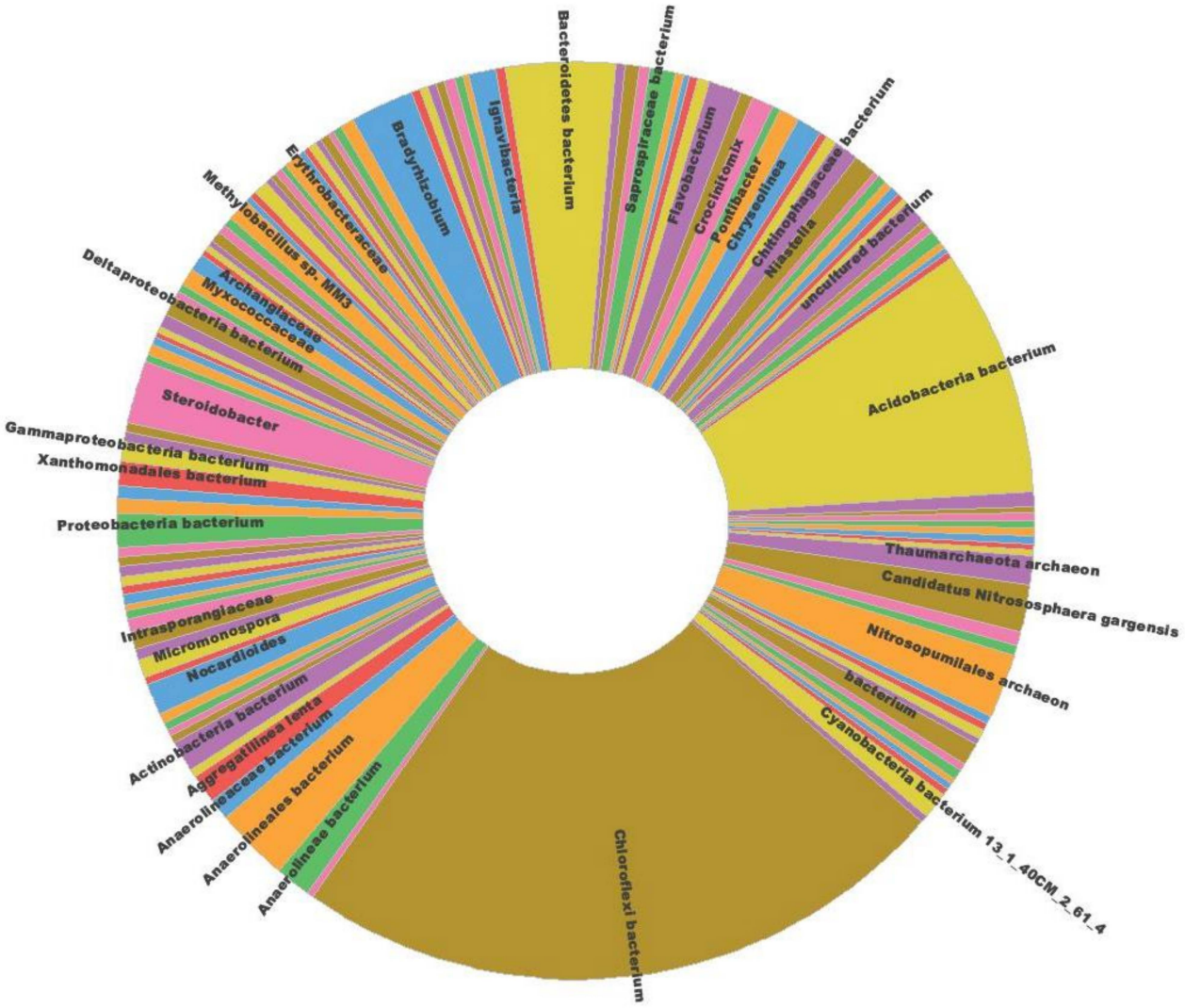

Figure 2. Major microbes identified in the ZBNF soil after *Jeevamrit* application. Application of *Jeevamrit* improved the microbial diversity and load in the ZBNF field. The rich bacterial and fungal diversity shown in the figure indicates the contribution of these microbes to the soil fertility and properties.

3.5. Taxonomic Composition of Bacteria in ZBNF Soil

The bacterial composition in the ZBNF field was found to be highly diverse, comprising more than 35 bacterial phyla with at least 1% abundance in the samples (Figure 3). Proteobacteria (40%) was the most abundant phylum followed by Actinobacteria (21%), Firmicutes (7%), and Cyanobacteria (5%), and other phyla with relative abundance > 1% are presented in Figure 3. Among these bacterial phyla, Actinobacteria were the dominating bacterial class, followed by Alphaproteobacteria (18%), Gammaproteobacteria (16%) and

Betaporteobacteria (11%), Bacilli (5%), Deltaproteobacteria (3%), and remailing bacterial classes constituted in others (18%). Burkhoderales (8%), Rhizobiales (7%), Spterptomycetales (5%), Bacillales (5%), Pseudomnadales (5%), and Sphingomonadeles (4%) were the differentially abundant order reported in the soil samples. At the family taxonomic level, 80.2% of the species were successfully classified, where Streptomycetace (21%), Bacilliaceae (16%), Pseudomonadeace (16%), Rhizobiaece (12%), Sphingomonadeace (12%), and Rhodobacterace (12%) were the major bacterial families. The major genera which formed the microbial community in the rhizospheric region were Streptomyces (5%), Bacillus (4%), Pseudomonas (4%), and Rhizobium (4%). Apart from these bacterial genera, Paenibacillus, Sphingominas, Microbacterium, Mesorhizonium, and Bradyrhizobium were also present in a significant amount. Other genera whose abundance was less than 1% were classified as others (78%). These bacterial genera have been known to play an important role in the nitrogen cycle, organic matter degradation, and carbon cycling, and reshape the soil microbial community [19].

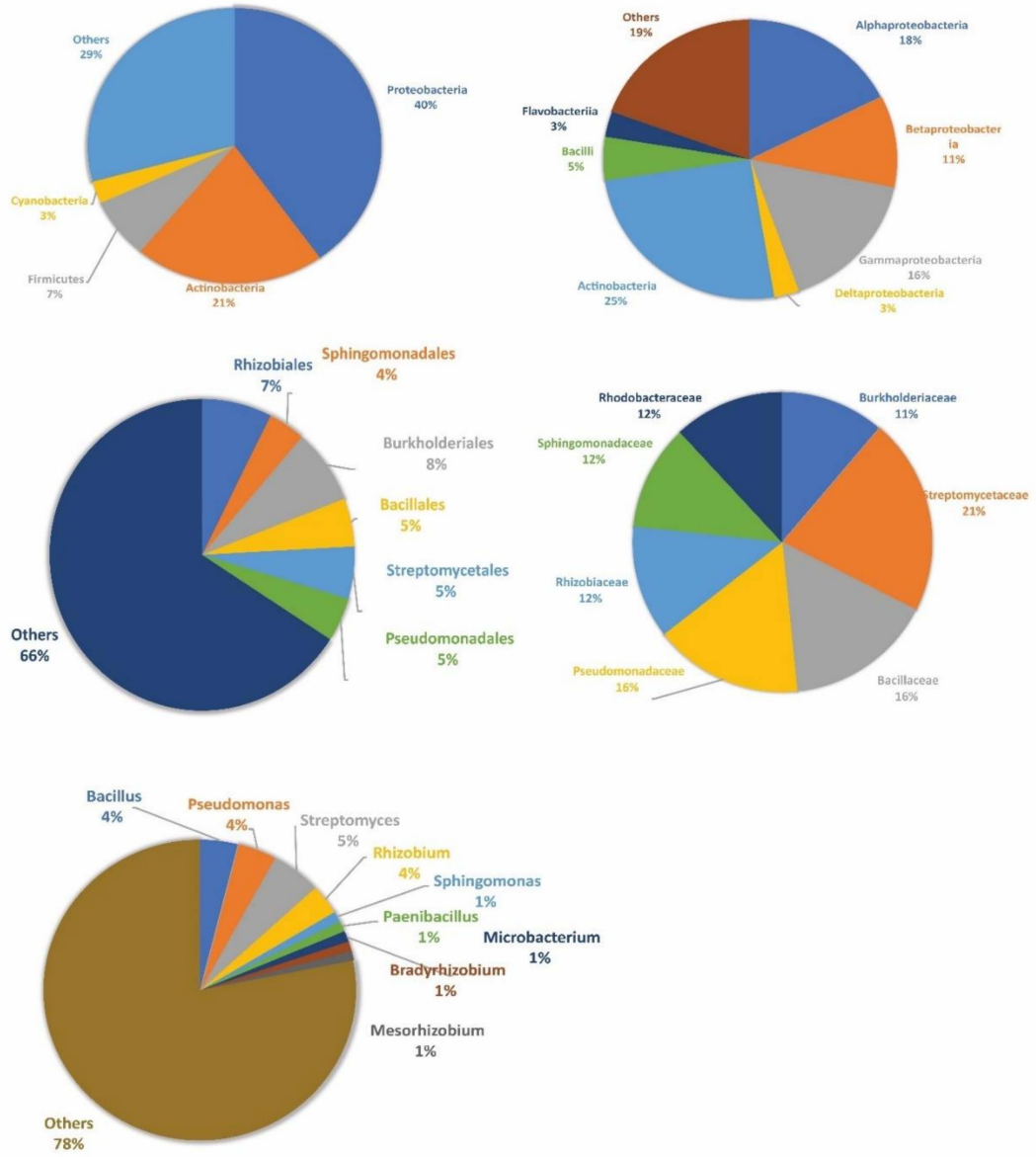

Figure 3. Abundance of various bacterial phyla, order, group, genus, and family present in ZBNF soil. Genera such as *Bacillus*, *Pseudomonas*, *Streptomyces*, and *Rhizobium* which produce various bioactive metabolites and enzymes are known. PGPR were recorded as the major bacterial community found in the ZBNF soil.

3.6. Taxonomic Composition of Fungi in ZBNF Soil

Similar to bacteria, fungi also significantly contribute to maintaining the soil structure, health, and ecosystem [20,21]. Fungal community structure in the ZBNF soil was also studied to figure out what were the dominant fungal groups present in the soil after *Jeevamrit* application. Additionally, it was noted that the abundance of the fungi in the soil ZBNF soil sample was less than the bacteria, revealing that the microbial community in the rhizosphere sample was dominated by bacteria. The most abundant fungal phylum was Ascomycota (88%) followed by Basidiomycota (9%) while other phyla which were lying in an abundance less of than 0.5% constituted the others (3%) (Figure 4). At the class taxonomy level, *Saccharomycetes* which constituted 37%, and Sordiomycetes which occupy 34% of the total population were the most abundant, followed by Eurotiomycetes (7%), Dothideomycetes (5%), Ustilaginomycetes (5%), and 12% others.

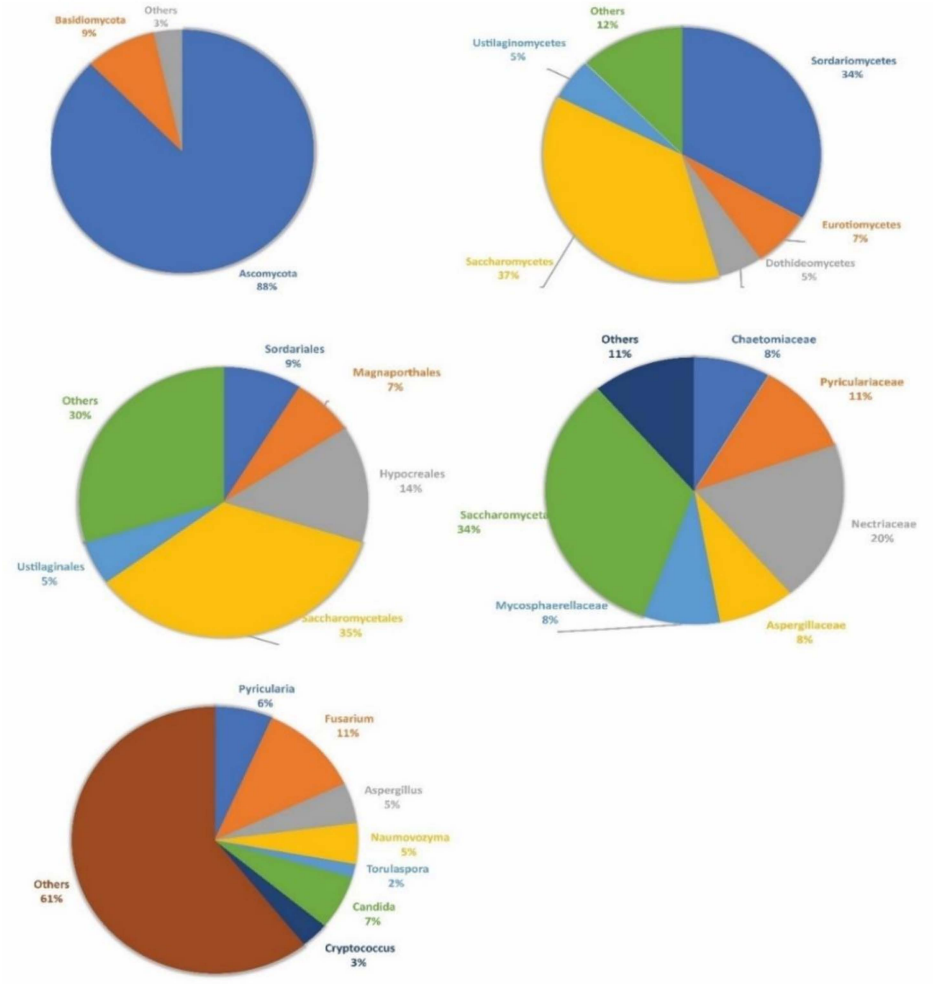

Figure 4. Abundance of various fungal phyla, order, group, genus, and family present in ZBNF soil. *Fusarium, Aspergillus, Pyricularia*, etc., were observed as major genera contributing to the ZBNF soil. All these genera are known fungal decomposers, which decompose the dead decaying matter and improve the soil fertility.

3.7. Taxonomic Composition of Other Microbes in ZBNF Soil

Archaea have been reported to improve soil properties by excreting several secondary metabolites, such as volatile organic compounds (VOCs), phytohormones, etc. Interestingly, it was noticed that the abundance of Archaea was quite high in the ZBNF soil samples. Halobacteria contributed 32% of the total population while Methanomicrobia contributed 13%, followed by Methanobactria with 5% of the total population (Supplementary Figure S2). Methanocarsina was the richest archeal species among all,

with a total share of 7%. In addition to this, the taxonomic compositions of plasmodium and viruses were also investigated. Noticeably, plasmodium and the viral population were less abundant in the ZBNF soil when compared with bacteria, fungi, and archaea. Among the plasmodium population, Apicomplexa (52%), Aconoidasida (37%), Trypansomatida (19%), Trypansomatideae (23%), Plasmodium (21%) were the major phylum, class, order, family, genus, and species, respectively (Supplementary Figure S3). In the case of viral diversity, in the viral population Uroviricota (80%), Caudoviricetes (82%), Caudovirales (82%), Sipoviridae (48%), Andhravirus (6%), and Pandoravirus (6%) were the major phylum, class, order, family, genus, and species, respectively (Supplementary Figure S4).

3.8. COG Functional Annotation and Analysis

In soil or rhizosphere, microbes interact with each other or associate plants by secreting several metabolites or bioactive compounds. Generally, the genes identified in the soil system are those which interact with the plants or microbes [22,23]. To understand the functions of the microbes present in the soil, the contigs retrieved after assembly were further used for the COG and SEED annotation to predict the putative function of the genes present. The results from functional annotation were majorly summarized into four different clusters (I–IV). Cluster I included the gene functions related to information storage and processing, Cluster II had the genes with cellular processes and signaling pathways, Cluster III comprised metabolism-related functional genes, while the fourth (IV) cluster was assigned to the genes with poorly characterized function. Among these clusters, amino acid transport and metabolism (E), carbohydrate transport and metabolism (G), inorganic ion transport and metabolism (P), replication recombination and repair (L), cell wall, membrane, biogenesis (M) energy production, and conversion (C) were the dominant functions among all COG categories (Figure 5).

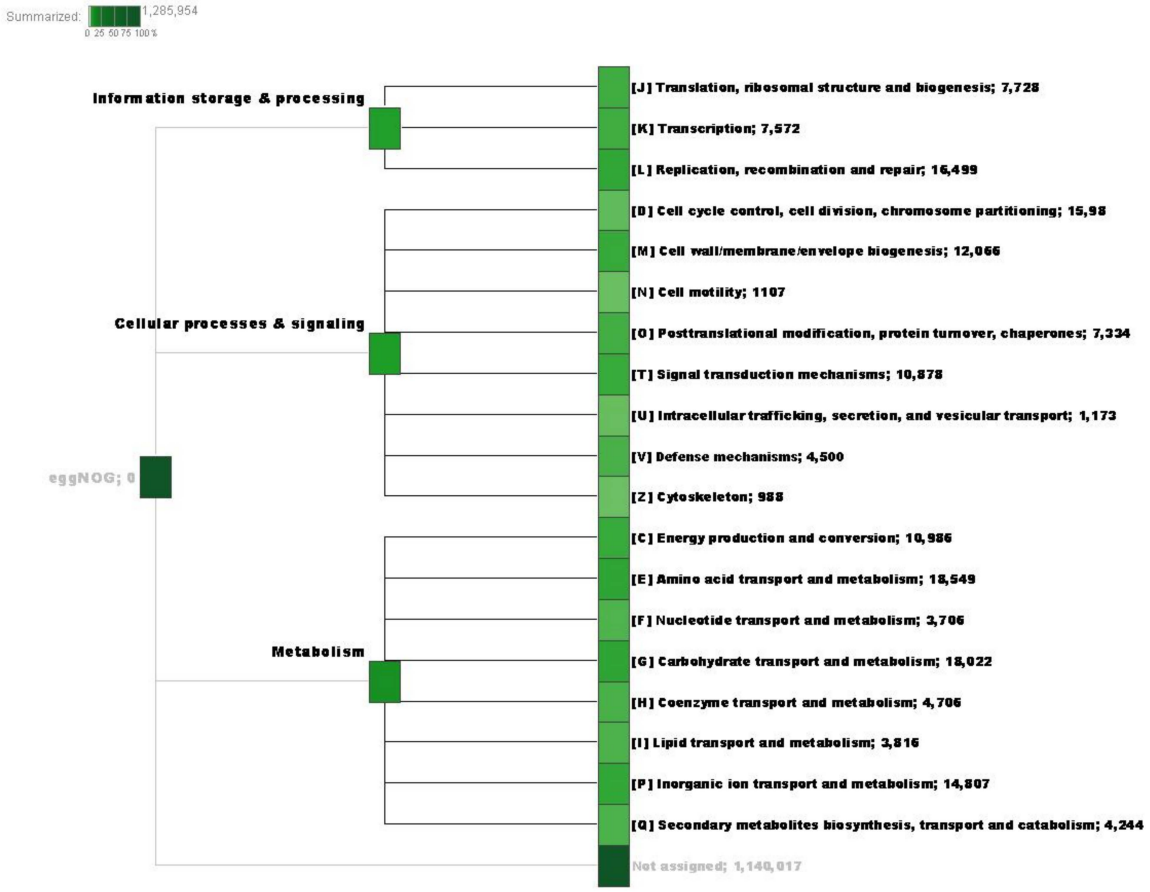

Figure 5. Functional domain analysis showing the major functional domains contributing to growth and metabolism.

It is interesting to note that Cluster III was predominant which is related to the growth of the microbial community. Cluster IV, which had genes with poorly characterized functions, was the second most abundant cluster, followed by Clusters I and II. These results indicate that the application of *Jeevamrit* positively influenced the growth and metabolism of the microbes in the soil as supported by the increase in the relative abundance of metabolic genes detected in the COG analysis (Figure 5). It is interesting to note that these putative functions are associated with microbial growth, metabolism, and antagonism. Our results revealed that soil microbial communities present at ZBNF had a potential function in plant growth promotion, phytopathogen suppression, and establishment of symbiotic relations with plants.

3.9. SEED Analysis and Annotation

Apart from phylogenic insights, the metagenomic analysis also provided an opportunity to assess the functional potentials associated with the soil microbial community. We conducted the functional annotation of genes via SEED. Results indicated that the ZBNF field has abundant genes, pathways, modules, orthologues, and enzymes involved in the metabolic pathways of microbial communities showing the potential of *Jeevamrit* in microbial growth enhancement (Figure 6). The results from the SEED analysis also revealed that the relative abundance of metabolism-related reads was quite high. To completely understand the metabolism in the *Jeevamrit* soil samples, we summarized the enzymes involved in all the identified 230 pathways; then, we achieved 1581 enzymes from the metagenomic sequencing. According to the International Enzymatic Commission classification system, the enzymes associated with these soil samples were classified into six categories: oxidoreductase (I), transferase (II), hydrolase (III), lyase (IV), isomerase (V), ligase or synthetase (VI), and translocases (VII). Transferase was the most abundant enzymatic category, followed by oxidoreductases. Isomerase was the least abundant enzyme observed in the samples. The relative abundance of hydrolase, isomerase, and ligase indicated that these enzymes had a function potential of decomposition, growth, pathogen suppression, and nutrient recycling, and could help the soil microorganisms improve soil fertility [8,24,25].

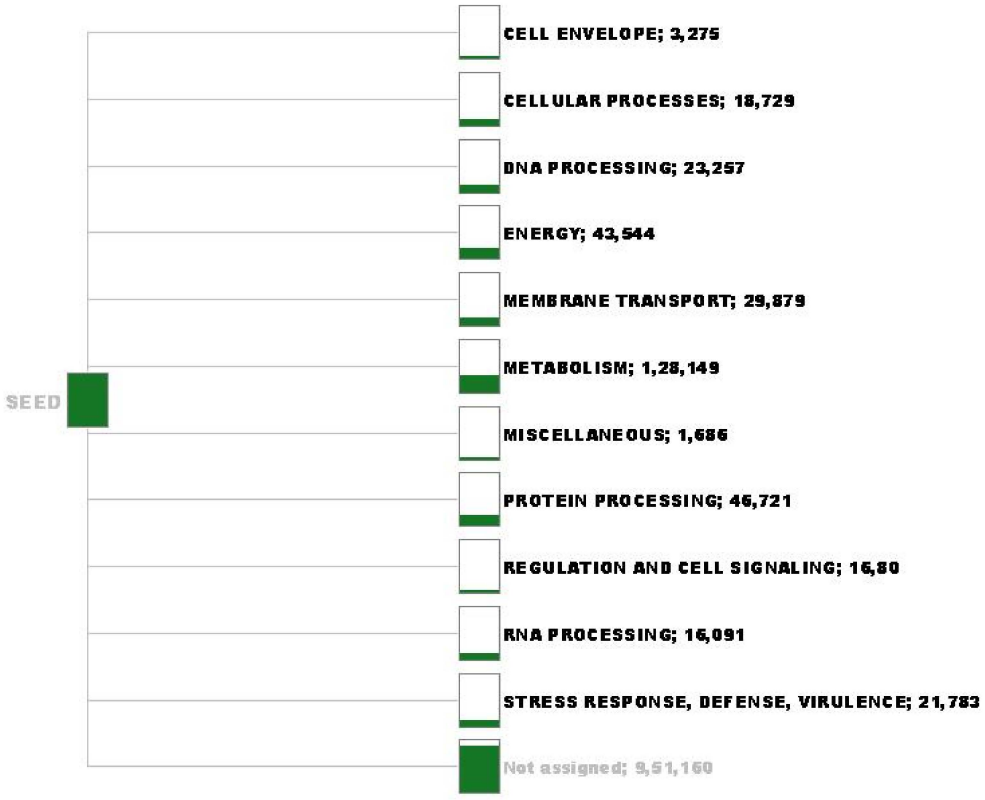

Figure 6. SEED analysis for categorization and identification of major enzyme groups that appear in ZBNF soil.

3.10. Comparison of the ZBNF Field's Microbial Abundance and Diversity with the Farmer's Field

To further validate the metagenomic analysis results, we compared the microbial load of ZBNF soil with the farmer's field. The results of microbial studies revealed that the soils from ZBNF were exuberantly loaded with the bacterial population in comparison to that recorded in the samples collected from farmers' fields. On average, irrespective of cropping systems, the total bacterial count in the soils of Gurukul Farm was 528 times more than that recorded in the soil from farmers' fields (Table 2). On average, the respective colony-forming units (CFU) per gram soil in Gurukul fields were 1610 million as compared to 3.05 million in the soil from farmers' fields. These results indicate the efficiency of ZBNF practices in the manifold multiplication of microbes in the soil, which might have contributed to enriching the nutritional status of the soil. Previous studies have shown that the application of organic manure improves the microbial load and diversity, and contributes to reshaping the microbial community [26].

Table 2. Comparison of microbial load in ZBNF farm and farmer's field. Different letters indicate the significance difference at a *p*-value.

Copping System	Year	Microbial Count
ZBNF		
Moong-sugarcane	2017	$76 \pm 0.23 \times 10^7$ b
Sugarcane	2018	$66 \pm 0.70 \times 10^8$ a
Hybrid Rice	2019	$37 \pm 0.15 \times 10^7$ c
Wheat	2019	$63 \pm 0.23 \times 10^7$ b
Local farmer field		
Sorghum	2017	$12 \pm 0.90 \times 10^5$ d
Rice	2018	$10 \pm 0.73 \times 10^5$ d
Rice	2019	$12 \pm 0.32 \times 10^5$ d
Wheat	2019	$21 \pm 0.23 \times 10^5$ e

3.11. Identification and Characterization of Bacteria/Fungi in the ZBNF Field Soil

Results from metagenomic analysis revealed the high abundance of plant growth promoting bacteria (PGPR) and fungi (PGPF) in the ZBNF soil samples. Further, to validate the results of metagenomic studies, we randomly isolated the bacteria and fungi and characterized them for their plant-growth-promoting activities. PGPR facilitates the plant growth directly or indirectly and increase soil fertility, which ultimately results in the overall improved crop productivity [5,10,12,13,23,27]. It was witnessed that the plant-growth-promoting attributes, such as phosphate solubilization, indole acetic acid (IAA) production, ammonia, hydrogen cyanide, siderophore, and antifungal metabolites, enzyme production, ACC deaminase, chitinase, and protease, antifungal activities, and in vitro biological nitrogen fixation were present in the isolated fungi and bacteria (Table 3; Supplementary Figure S2). Phosphate solubilizing activity was observed by growing the isolates on Pikovskaya's agar plates and a positive reaction was recorded when a clear halo surrounding the inoculated bacterial colonies was observed [28–30]. Further ammonia production was tested by growing the isolates on peptone broth and adding Nessler's reagent, which resulted in a brown to yellow color development, indicating ammonia production. Protease activity of the bacterial isolates was determined using the skim milk agar medium, where development of a halo zone indicates positive activity. Clear zone produced by growing bacteria/fungi on l−1 carboxymethyl cellulose (CMC) agar gave a positive cellulase activity. Antifungal activity was screened in the presence of several phytopathogens, such as *Aspergillus niger*, Rhizopus sp., Fusarium sp., etc. The clear zone around the bacterial isolates confirmed the secretion of antibiotics contributing to antifungal activity. Most of the bacterial isolates were indole and catalase positive (confirmed by color change and bubble formation, respectively), sucrose fermenting, nitrate reducing, endospore forming,

Gram-negative bacilli and cocci, while the fungi showed bactericidal activity, with cellulase producing fungi belonging to the Ascomycota group. Plant-growth-promoting bacteria play an important role in plant growth and development [2,28–32]. Our results supported previous studies which show the production of these above-mentioned metabolites, enzymes, phytohormones, etc., boost the plant growth and development process, as well as induce the resistance against various phytopathogens.

3.12. Economics of ZBNF Field and Crop Yield

Rice, wheat, and sugarcane are the most exhaustive crops and require high doses of fertilizers. The average yield level of non-scented rice varieties including hybrids generally ranges between 70–83 q/ha in the farmer's field with the heavy application of chemical fertilizers and pesticides, which not only increase the production cost but also leave chemical residue, often making it toxic to consume. To understand the production cost and economics of ZBNF, rice and wheat cropping systems were studied from 2017–2019. The rice varieties PR114 (non-scented) and CSR30 (scented) were grown in the rice cropping system, while Bansi and HD2967 were the varieties grown in the wheat cropping system. The economics of rice and wheat crops grown at Gurukul farm and farmers' fields are given in Table 4. After considering all the aspects including weed control in rice and wheat crops grown at ZBNF during 2017–18 and 2018–19, it was found that the average net returns from ZBNF were 1.45 to 2.71 times higher in comparison to the farmers. In non-scented high-yielding rice varieties, this increase was the minimum, i.e., 1.45 times as the produce was sold at minimum support price (MSP). The benefit–cost ratio was 2.59 and 4.33 for non-scented high-yielding rice varieties, 2.10 and 4.25 for scented rice variety (CSR 30), and 2.39 and 4.82 for the wheat crop (variety Bansi at Gurukul farm and HD 2967 at Farmer's field), respectively (Table 4). The produce of varieties CSR 30 (rice) and Bansi (wheat) was sold at a premium price due to the increased demand of consumers for natural products grown at Gurukul farm. PR114 gave an average yield of 65 q/ha when grown in 6 ha for the first time on a newly acquired area under ZBNF in Kharif 2017. This area where PR114 was grown for the first time was having organic carbon in the range of 0.31 to 0.45%, poor to medium in available P and medium in available K content.

Hence, the speculation and fear of farmers and scientists about the reduction in yield during the first 3–4 years under ZBNF as expressed in organic farming do not seem to hold. There is a basic difference between organic farming and ZBNF. In organic farming, there is a need for bulky organic manures like FYM, vermicompost, and other organic materials for the fulfillment of the nutritional requirement of the crops. It is a slow process of building soil health and hence, crop yields are likely to decrease during the initial few years. In ZBNF, not the manures in the form of FYM or vermicompost, but microbial inoculum in the form of *Jeevamrit* or *Ghanjeevamrit* are applied in the field which promotes the multiplication of earthworms and microbes in the soil. However, green manuring, residue incorporation, mulching, minimum tillage, crop rotation, etc. are other complementary practices that form a composite package in ZBNF. The yield of the Bansi variety of wheat is obtained with an average of 25–35 q/ha. The grain of this variety fetches more than Rs.4000 per quintal with a consistent demand from consumers. Our results indicate that the ZBNF practice is safe, eco-friendly, does not require heavy investment and has a good productivity, making it an economic and sustainable approach to be adopted by the farmers.

Table 3. Plant-growth-promoting activities shown by the isolated bacteria and fungi from ZBNF field soil.

Test Name	* Bacterial Isolates					* Fungal Isolates				
	ZBNF_B1	**ZBNF_B2**	**ZBNF_B3**	**ZBNF_B4**	**ZBNF_B5**	**ZBNF_F1**	**ZBNF_F2**	**ZBNF_F3**	**ZBNF_F4**	**ZBNF_F5**
Phosphate solubilization	+	+++	++	+++	-	+++	+	++	-	+++
HCN production	-	-	++	+++	-	-	-	-	+	-
Ammonia production	+++	++	-	++	-	-	-	++	-	+
IAA production	+++	+++	++	++	+	-	++	++	+	-
Siderophore production	++	++	_	++	+++	-	-	++	+++	-
Starch Hydrolysis	++	+++	+	++	++	++	+	++	-	++
Biological nitrogen fixation	++	+++	++	+	++	-	-	-	-	-
Antifungal activity	++	-	+	++	++	ND	ND	ND	ND	ND
Chitinase production	+++	-	+++	++	+					
Morphology	Cocci	Rods	rods	cocci	spiral	white cottony and dark purple undersurface	blue-green with a suede-like surface consisting of a dense felt of conidiophores	white cottony and dark purple undersurface	blue-green with a suede-like surface consisting of a dense felt of conidiophores	intracellular hyphae with large amounts of electron-dense globules
Gram Staining	-ve	+ve	+ve	-ve	-ve	NA	NA	NA	NA	NA
Endospore staining	-ve	-ve	-ve	+ve	-ve	ND	ND	ND	ND	ND

Table 3. *Cont.*

Test Name	* Bacterial Isolates					* Fungal Isolates				
	ZBNF_B1	**ZBNF_B2**	**ZBNF_B3**	**ZBNF_B4**	**ZBNF_B5**	**ZBNF_F1**	**ZBNF_F2**	**ZBNF_F3**	**ZBNF_F4**	**ZBNF_F5**
Citrate utilization	+ve	-ve	-ve	+ve	-ve	ND	ND	ND	ND	ND
H_2S production	-ve	+ve	+ve	-ve	+ve	ND	ND	ND	ND	ND
Protease activity	+	+++	++	-	+	+	++	ND	-	+
Catalase test	+	++	++	+	+++	++	+	++	+	+
Indole test	-	++	+	-	+	ND	ND	ND	ND	ND
Methyl red test	+	+	++	-	+	ND	ND	ND	ND	ND
Nitrate reduction test	++	-	+	-	+	ND	ND	ND	ND	ND
VP test	+	+	-	-	-	ND	ND	ND	ND	ND
Sucrose fermentation	+	-	-	+	+	+	++	+	-	++

- = negative test; + = positive test; ++ = good; +++ = excellent; ND = Not determined; NA = Not applicable. * random samples were tested for various attributes/activities/parameters.

Table 4. Economics of rice and wheat crops at ZBNF farm (mean of 2017–18 and 2018–19).

Crop/Variety	Yield (q/ha)	Cost of Cultivation (Rs./ha)	Total Return (Rs./ha)	Net Return (Rs./ha)	Benefit–Cost Ratio
Non-scented Rice Varieties (PR114)					
Gurukul Farm	74.45	30,150	130,575	100,425	4.33
Farmer's field	66.75	43,405	112,485	69,080	2.59
Scented Rice Variety (CSR30)					
Gurukul Farm	32.50	35,000	148,700	113,700	4.25
Farmer's field	30.50	48,750	102,550	53,800	2.10
Wheat Varieties					
Gurukul Farm (Var. Bansi)	32.30	27,280	131,500	104,220	4.82
Farmer's Field (Var. HD2967)	48.00	34,760	83,115	48,355	2.39

4. Discussion

4.1. Jeevamrit Application Improved the Soil Properties

ZBNF is a kind of agriculture practice used by Indian farmers where no chemical is used but soil biology is managed by multi-cropping, cow-dung/urine-based formulations to instigate microbial growth [29,33]. Though ZBNF is criticized by scientific communities as an irrational and propaganda practice, farmers across the Indian states use one or other components, such as *Jeevamrit*, as biofertilizers or biopesticides. However, to date, there is no scientific proof that *Jeevamrit* improves soil fertility and contributes to improved crop yield. To test the effect of *Jeevamrit* on soil fertility, we employed the next-generation sequencing approach, i.e., metagenomics, to study the detailed soil microbial community structure and function from soil samples collected from the ZBNF field. *Jeevamrit* contains numerous bacterial and fungal communities which secrete several metabolites and ease the competition among microbial groups.

In the current study, high throughput metagenomic analysis of ZBNF soil was performed to provide the scientific pieces of evidence supporting this ancient Indian agriculture practice. The soil samples were collected from ZBNF and farmer's field, and their physiochemical as well as microbial properties were investigated. It was noticed that the ZBNF field soil was rice in organic carbon, available P, available K, and other micronutrients when compared with the farmer's field. The farmer's field soil was recorded to be rich in available P and available K due to the application of NPK; however, the organic carbon content in farmer's field was quite low, indicating poor soil chemistry. It is reported that soil inhabits a huge and diverse microbial population, including bacteria, fungi, and viruses with different characteristics and functions, which directly or indirectly improve or downgrade the plant health [29,30,33].

In past centuries, studies have proven the role of microbes in plant growth promotion, development, nutrient recycling, and improvement of soil fertility by excreting several primary and secondary metabolites [5]. The species and quantity of soil microorganisms are not only dynamic for the transformation and circulation of soil organic matter and soil nutrients, but also act as reserve storage for the available nutrients of plants in the soil and are closely coupled with soil fertility [15]. Generally, it is assumed that the higher the soil microbial diversity index, the better the stability and resistance of soil ecosystems and the better the plant growth and yield [29,33,34]. Supported by these studies, we noticed that the microbial load in the ZBNF field was significantly higher than in the farmer's field. The *Jeevamrit* application not only improved the soil microbial load but also contributed to the soil chemistry, improving the organic carbon content in the soil. It is interesting to note that at farmer's field where chemical-based fertilizers are used, the microbial load was quite lower, indicating the poor soil biology and properties. Our results agree with the previous studies reporting that organic manure composed of poultry and cattle manure—

analogous to *Jeevamrit*—improved the soil's physical and chemical properties significantly and gave better results than the chemical NPK treatment [34]. Adekiya et al. [20,35] also have reported that cow dung manure increases the soil organic matter significantly when compared with other manures and improved the soil chemical properties, i.e., N, P, K, Ca, and Mg. Similar results were reported by Shang et al. [19] when the organic fertilizers were applied in the field and the application of these fertilizers not only increased the soil nutrients but also shaped the microbial community structure in the soil. More recently, Sharma [26] also showed that the application of farm yard manure and *Jeevamrit* improved the soil structure, water holding capacity, and soil organic carbon significantly.

It should also be notice that ZBNF practices do not rely on heavy machinery or commercial techniques; rather, they use basic components that are easily available and do not hold high cost. Thus, such practices can be easily adopted by farmers from India or worldwide. Cattle farming is common in every country from where cow dung and urine can be collected easily at a very low cost while lentil, jaggary as a source of protein, and sugar can be replaced with locally available products. However, a replacement of these constitutes and their effect should be investigated first.

4.2. ZBNF Soil Has Rich Microbial Diversity

Further, a metagenomic analysis of ZBNF soil was performed to investigate the microbial communities' structure and diversity. It was noticed that Proteobacteria was the dominating phyla, followed by Actinobacteria, Firmicutes, and Cyanobacteria. The member of Proteobacteria have been found to play a role in in nutrient cycling [18,21,23,30], their opportunistic use of the additional moisture and nutrients in agricultural soil [10,23,29,30,34], or their ability to survive the selective pressures of agriculture [14]. On the other hand, Actinobacteria help in nitrogen fixation and organic matter decomposition, and produce various metabolites, such as enzymes and antibiotics, which suppress plant pathogens and improve plant growth [19,24,27,36]. The members of Firmicutes are the chitinolytic bacteria exerting resistance against fungal pathogens, while several cyanobacteria have been reported to show plant-growth-promoting properties as well as being a good source of various enzymes contributing as biocontrol agents against phytopathogens [19,24,27,36]. At the genus level, Streptomyces, Bacillus, Pseudomonas, and Rhizobium were the dominating genera. These bacterial genera have been known to play an important role in the nitrogen cycle, organic matter degradation, and carbon cycling, and reshape the soil microbial community [19].

The use of different organic manures has been reported to induce the soil microbial, physical, and chemical properties [19,24,27,32,36] supporting our study. Hartman et al. [37] demonstrated that the use of organic-manure-based products only boosts the bacterial population but also enhances the soil nutrients. Providing the role of proteobacteria/actinobacteria in nitrogen fixation, carbon recycling, diseases, pathogen suppression, soil remediation, phosphate dissolution, and many more biological activities which directly or indirectly improve plant growth [10,13,14,18,26,29], it is confirmed that the *Jeevamrit* application enhanced the bacteria abundance and boosted the soil ecosystem (Figure 4).

Further, the fungal diversity was also studied, which indicated that Ascomycota was the dominating fungal phylum followed by Basidiomycota. The members of Ascomycota and Basidiomycota phylum have known waste decomposers and are involved in nutrient recycling in agri-ecosystem [5,21,38]. This was also interesting to note that the abundance of these fungi was much higher than the diversity in ZBNF soil. It is evident that manure input strongly increased fungal abundance but decreased fungal diversity and the total number of species [8,20,21,35,38]. It has been also confirmed that the application of organic manures not only antagonizes the soil-borne phytopathogens but also improves plant growth [8,20,21,35,38]. The members of *Saccharomycetes, Sordariomycetes, Eurotiomycetes* are reported to be correlated with nitrogen concentration in soil, reflecting that ZBNF soil is rich in N and favored the growth of these families [39]. Wen et al. [39] also noted the same response when they compared the fungal diversity in soil treated with organic and

chemical fertilizers. Conclusively, the *Jeevamrit* application significantly structured the soil microbial communities with the members of bacterial and fungal groups that are reported to plant a direct or indirect role in soil ecosystem and plant growth promotion.

4.3. Microbes Improved the Soil Biology and Chemistry

The bacteria and fungi identified from the ZBNF field have shown strong plant-growth-promoting properties (Figure S5, Table 3) indicating their role in improving soil chemistry and biology. Further, the functional annotation of the reads revealed that the abundance of the metabolism cluster was highest when compared with the information storage and processing cluster and cellular processing and signaling cluster. This indicates the activity of microorganisms in the soil. An increase in the relative abundance of metabolism-related genes, such as hydrolase, isomerase, and ligase indicated that these enzymes had a function potential of decomposition, growth, pathogen suppression, and nutrient recycling, and could help the soil microorganisms improve soil fertility [8,12,24,25]. Microbes present in the rhizosphere or soil produce several metabolites, bioactive compounds, enzymes, and phytohormones (such as cellulase, catalase, siderophores, indole acetic acid, etc.) which not only reshape the adjoining microbial community but also contribute to the plant growth promotion and improve the soil fertility [8,12,24,25]. Additionally, the secretion of several enzymes, antibiotics, and various organic compounds suppresses phytopathogens and neutralizes toxic compounds and microbes [25]. Microbial community analysis of ZBNF soil through metagenomic analysis was enough to give information about abundance, diversity, and metabolic/physiological function. A rich and diverse microbial community is crucial for maintaining soil bioavailability and results from our studies have shown that *Jeevamrit* has the potential to improve the abundance and diversity of soil microflora. It is also evident from our results that the functional pathways involved are related to growth promotion, fertility, and nutrient recycling of the soil microbes. In our study, all the core microorganisms were Gram-negative bacteria, belonging to Proteobacteria, with most of them being related to the nitrogen cycle and organic matter decomposition, antagonism. These bacteria and their physiological functions were important for the soil microbial community to stabilize the physical, chemical, and biological properties of soil.

4.4. Limitation/Weakness

A few studies conducted by the Indian Council of Agriculture Research (ICAR), University of Agricultural Sciences (UAS), Dharwad, from 3-year-long natural farming experiments at several locations, have indicated that these practices reduced the yield (up to 40%) in rice–wheat cropping, soybean–wheat, groundnut–sorghum, and maize–chickpea cropping systems [7,13]. Additionally, it is interesting to note that native cow breeds' dung/urine is recommended for the preparation of *Jeevamrit*, which in many parts of the country has been systematically replaced with crossbreeding from exotic, foreign stock. These highly productive breeds were promoted to increase milk and meat yield. Therefore, the farmers must invest in native cows, lentil powder, jaggery, and labor costs for the preparation of a huge amount of *Jeevamrit* which will add to the cost. Additionally, the produce from such practices will not have an organic produce or pesticide-free label, which again can add challenges to finding a suitable market. In the current study, we have investigated the effect of *Jeevamrit* on soil physiochemical properties, and the economics of the different cropping systems grown in ZBNF is also discussed. However, further plant studies should be carried out to see the impact of such practices on plant growth and development.

5. Conclusions

ZBNF is one of the ancient and traditional approaches used by Indian small/medium-scale farmers; however, due to the lack of scientific evidence it is not well accepted by the scientific community. In our study, it was recorded that the application of *Jeevamirt* not only improves the soil chemical and microbial properties but also reshapes the microbial

community structure, improving the soil biology. To our knowledge, this is the first report providing the proof of concept providing evidence of how the application of *Jeevamrit* improves soil fertility and improves the soil microbial community structure as well as soil physiochemical properties. The current study indicates that *Jeevamrit*, which is the major formulation used in ZBNF, has the potential to increase the taxonomic species of microbes in the soil, and further change the soil microbial composition. The functional pathways involved in the soil are related to the fertility, growth, and development of the soil microbes. *Jeevamrit* application in the soil is a potential resource for improving soil fertility and boosting the plant growth. Such practices, which support the ecosystem, cut down the chemical-based fertilizer/pesticide's cost, and improve the total production economics should be supported and must be adopted, especially by the small and marginal farmers. In addition, the introduction and adaptation of such ecofriendly techniques worldwide will positively contribute to saving the ecosystem and mitigating the adverse effects caused by agrochemicals. However, there is still a need to generate more evidence supporting such natural practices.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13010196/s1, Figure S1: The operational taxonomic units identified after metagenomics analysis. The phyloge-237 netic tree of major microbial groups (OTUs) identified by metagenomic analysis after *Jeevamrit* ap-238 plication in the ZBNF field; Figure S2: Abundance of various Archaea phyla, order, group, genus, and family present in ZBNF soil. Halobacteria, Methanomicrobia, Methanobactria, Methanocarsina were the major phylum, order, group, genus and family in the ZBNF soil; Figure S3 Abundance of various Plasmodium phyla, order, group, genus, and family present in ZBNF soil. Apicomplexa, Aconoidasida, Trypansomatida, Trypansomatideae, Plasmodium was the major phylum, class, order, family, genus, and species respectively; Figure S4: Abundance of various virual phyla, order, group, genus, and family present in ZBNF soil. Uroviricota, Caudoviricetes, Caudovirales, Sipoviridae, Andhravirus and Pandoravirus respectively; Figure S5: Plant growth promoting activities reported in the different bacteria and fungi isolated from ZBNF soil; Table S1: The statistical information of the reads received after metagenomic analysis. Raw reads were filtered from for low quality and adapter sequences.

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