GLYPHOSATE AND SOIL HEALTH ANALYSIS

SEED2SHIRT B.L.A.C.KOLLECTIVE
CLIMATE BENEFICIAL PROGRAM
BRIDGEFORTH COTTON, TANNER, AL

1. Introduction

Glyphosate, the world's most widely used herbicide, finds extensive application in both agricultural and non-agricultural settings, effectively eliminating broadleaf weeds and grasses without harming genetically modified crops resistant to it. In 2020, agriculture accounted for 81% of all herbicides use in the United States, with glyphosate prominently used on crops like corn, soybeans, and cotton, as well as in non-agricultural settings such as lawns, roadsides, and forestry for weed control.

The intricate relationship between glyphosate and the soil microbiome is a subject of ongoing research, yielding very important findings on its impact on soil health and the microbiome. Studies suggest negative impacts, including a reduction in alpha diversity, affecting soil functions, and leading to shifts in microbial composition, potentially increasing harmful pathogens and decreasing beneficial microbes. Indirect effects involve the lingering residues of glyphosate and its breakdown product, Aminomethylphosphonic Acid (AMPA), potentially harming soil organisms over time.

A deeper exploration into potential impacts reveals that specific microbial groups, such as nitrogen-fixing bacteria and beneficial fungi, may be affected. Shifts in community function could disrupt nutrient cycling, decomposition, and plant-microbe interactions, leading to long-term consequences as residues persist in the soil.

Exploring alternative weed control methods, such as mechanical control and crop rotation, emerges as a strategy to reduce reliance on glyphosate and mitigate potential risks to the soil microbiome. The role of soil health becomes a crucial consideration, with healthy soils potentially exhibiting greater resilience to glyphosate's negative impacts, including alterations in alpha diversity, compared to degraded soils.

Focusing specifically on alpha diversity, denoting the richness and evenness of species within the soil microbiome, research on the impact of glyphosate reveals a reduced alpha diversity, with decreases in observed Operational Taxonomic Units (OTUs) and the Shannon diversity index, particularly in the Proteobacteria phylum.

Factors influencing the impact on alpha diversity include dose-dependency, where higher application rates may lead to more pronounced reductions. Soil characteristics, such as type, organic matter content, and moisture, also play a role, affecting how glyphosate interacts with the microbiome.

Consequences of reduced alpha diversity within the soil microbiome encompass disrupted nutrient cycling, weakened disease resistance due to the loss of beneficial microbes, and reduced resilience to environmental disturbances. Despite growing research, the long-term impacts of glyphosate on alpha diversity and the overall functioning of the soil microbiome remain uncertain, necessitating further studies to investigate chronic exposure, analyze impacts on specific microbial groups, and understand interactions with soil factors and agricultural practices.

As the debate over glyphosate's impact continues, alternative weed control methods like mechanical control, crop rotation, and cover cropping emerge as potential strategies to minimize herbicide use and safeguard the health and diversity of the soil microbiome. These practices are integral to sustainable agriculture, ensuring long-term soil fertility and maintaining crop productivity. In conclusion, a balanced and sustainable future for agricultural practices can be achieved by carefully considering potential risks, ongoing research, and exploring alternative approaches that prioritize the health and diversity of the soil microbiome.

1.1 Study Design

Soil samples were collected from the Bridgeforth Farms during the spring and again, from the same site, in the fall at harvest time to determine the effect of PaleoPower™ application of glyphosate levels and soil health. To this end, the soil samples were submitted to Health Research Institute (HRI) for glyphosate analysis and to EzBiome, Inc. for soil microbiome analysis.

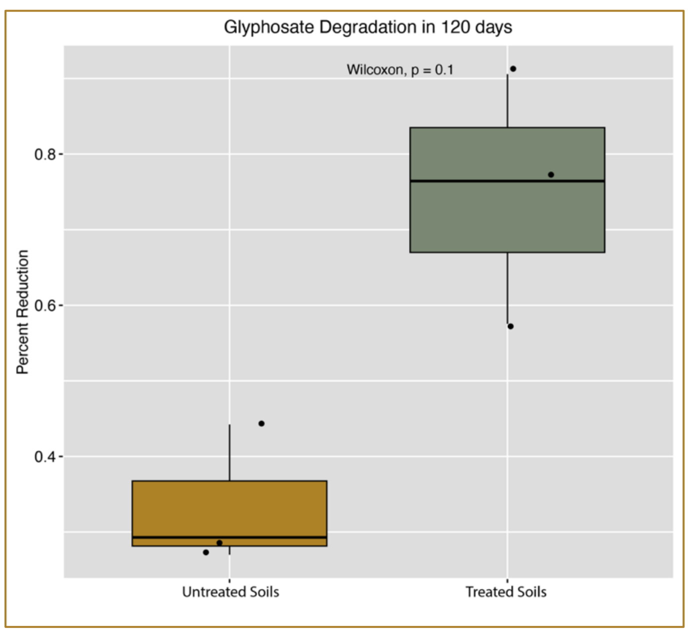
2. Glyphosate Degradation

Glyphosate degradation is primarily carried out by certain microorganisms through microbial processes. Glyphosate, a widely used herbicide, is broken down in the environment through both chemical and microbial pathways. The microbial degradation of glyphosate is particularly important in soil ecosystems.

Many soil-dwelling bacteria are sensitive to glyphosate, and exposure to the herbicide can affect their growth and metabolic processes. Glyphosate disrupts the shikimate pathway, a metabolic pathway present in plants and many soil microorganisms, leading to the inhibition of aromatic amino acid synthesis. Some bacteria have developed adaptations to cope with glyphosate exposure. This adaptation can involve the evolution of specific enzymes or metabolic pathways that allow them to tolerate or utilize glyphosate as a carbon or phosphorus source. Bacterial species in PaleoPower not only have developed resistance mechanisms to glyphosate but also possess specific enzymes that can break down glyphosate into less toxic compounds. As part of the process of degradation, the levels of glyphosate in the soil are reduced sufficiently to permit other autochthonous soil bacteria to proliferate and augment the rate of glyphosate degradation. The results summarized in Table 1 in **Section 3** of this report, reflects such a process and the impact of PaleoPower in such increasing the glyphosate-degrading capabilities of the soil microbiome.

Baseline levels of glyphosate of the control soils at pre-planting (May) averaged 51.63 ± 11.45 ng/g effective glyphosate. At Harvest time (October) the levels of Effective Glyphosate declined an average 35.05 ± 9.36, representing a 33.5 ±5.4% reduction.

Conversely, baseline levels of glyphosate of PaleoPower treated soils at pre-planting (May) averaged 73.12 ± 18.8 ng/g effective glyphosate. At Harvest time (October) the levels of Effective Glyphosate declined an average 19.26 ± 10.92, representing a 74.8 ±9.6% reduction.



The results shown in Figure 1 indicates that the use of PaleoPower as a soil inoculant promotes the degradation of glyphosate by 123%

In our study at Bridgeforth Cotton, Tanner, AL, the application of PaleoPower yielded a noteworthy reduction in glyphosate levels, demonstrating a substantial decrease of 74.8% ± 9.6%. This reduction surpassed the observed rate of decomposition in untreated soils, effectively more than doubling the remediation efficacy. The underlying mechanism for this pronounced effect is attributed to the intrinsic microbial consortium within PaleoPower. Notably, this consortium exhibits a remarkable capability for the breakdown of glyphosate. Furthermore, PaleoPower serves as a stimulant for the native soil microbiome, fostering an environment conducive to its proliferation. This dual action not only facilitates the direct degradation of glyphosate by the introduced microbial consortium but also enhances the bioremediation potential of the indigenous soil microbiome.

3. Ecological Features of the Soil Microbiome

Soil samples were processed for microbiome analysis at EzBiome, Inc. (Gaithersburg, MD) using their standard analytical workflow and the results analyzed to assess the microbiome composition of the soils as well their corresponding alpha diversity of the samples.

3.1 Alpha Diversity Analysis

Alpha diversity, in ecology, refers to the variety of species within a particular community or ecosystem. It's essentially a measure of how many different species are present and how abundant they are relative to each other.

Key aspects of alpha diversity include:

1. Species richness: This is simply the number of different species present in a community. It's the most basic measure of alpha diversity, but it does not consider how abundant each species is.
2. Evenness: This refers to how equally the individuals are distributed among the different species. A community with high evenness has no dominant species, while a community with low evenness has one or a few species that are much more abundant than the others.
3. Composition: This refers to the specific identity of the species present in a community. Two communities can have the same richness and evenness, but they can be very different if they have different species.

3.1.1 Interpretation of results

The alpha diversity of the soils was assessed using a panel of metrics that assess microbial species richness, evenness, composition, and phylogenetic diversity. The metrics used consisted of Chao1, Operational Taxonomic Unit (OTUs), Shannon Diversity, and Phylogenetic Diversity. Alpha diversity is important because it can tell us much about the health and functioning of an ecosystem. A high alpha diversity indicates a higher microbial diversity, which reflects improved soil health and resilience.

Chao1 is a nonparametric estimator of species richness within a community. It is particularly useful in ecological studies because: (a) estimates total species number: Unlike simply counting observed species, Chao1 attempts to account for undetected species, especially those present in low abundance. This is crucial because many species, particularly in microbial communities, may be rare or difficult to detect, leading to underestimation of true richness. (b) It is sensitive to rare species as it specifically focuses on singletons and doubletons (species observed only once or twice), using their presence to estimate the number of undetected species. This makes it valuable for communities where rare species contribute significantly to overall diversity. (c) It is robust to sample size. Chao1 is less sensitive to variations in sample size compared to other richness estimators. This is important because real-world ecological data often comes from samples of varying size, and Chao1 helps to adjust for these differences.

OTU diversity essentially reflects the richness and evenness of different microbial or organismal groups within a sample or community based on their genetic similarity. This information is valuable for understanding various ecological and biological processes, including: (a) community structure and function. Different OTUs often represent different functional groups within an ecosystem, and understanding their diversity can provide insights into how the ecosystem functions. (b) Environmental changes and disturbances. Changes in OTU diversity can be used as indicators of environmental stress or disturbance. (c) Disease states and microbiome analysis: Studying OTU diversity in human microbiomes can help us understand how different microbial communities contribute to health and disease.

The Shannon Diversity Index, also known as the Shannon-Wiener index, is a cornerstone metric in ecology, quantifying the species diversity within a community. It goes beyond simply counting species richness, incorporating the crucial aspect of abundance distribution. It integrates richness and evenness into a single metric provides valuable insights into community structure and function. The Shannon Diversity Index is a biodiversity indicator. A diverse community, as assessed by a high Diversity Index, indicates a healthy soil ecosystem with strong nutrient cycling and resilience to disturbances. The Shannon index, by measuring the diversity of these microbial populations, can be a valuable indicator of soil health. The Shannon index is sensitive to changes in the environment, including soil pollution, land-use practices, and climate change. A decline in the index value over time could be an early warning sign of soil degradation. Conversely, an increase in the Shannon Index reflects a healthy soil ecosystem.

Phylogenetic Diversity Index (PDI) is a measure of the total evolutionary history captured by the members of a community or ecosystem. It goes beyond simply counting the number of species (species richness) and instead considers the unique evolutionary lineages represented by each species. In simpler terms, PDI tells us how much evolutionary diversity is packed into a community. PDI can offer valuable insights into soil health by capturing the evolutionary diversity of microbial communities.

All four methods of assessing alpha diversity showed mark increases after treatment with PaleoPower, indicating that reflected a significant improvement of soil health. The results are summarized in Figure 2.

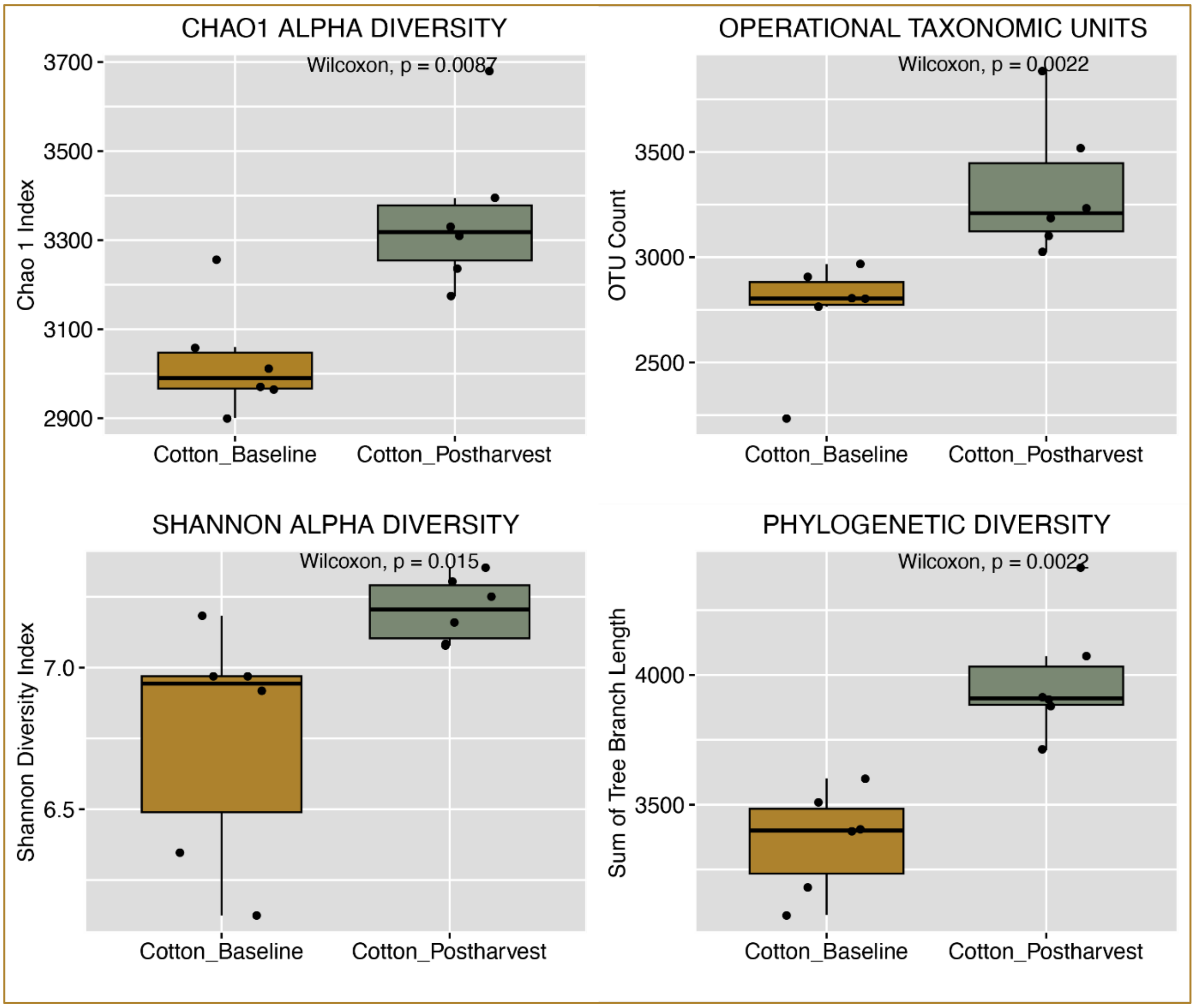


Figure 1. Alpha Diversity Determinations of the Microbiome of Alabama Soils Planted with Cotton and Treated with PaleoPower

3.2 Taxonomic classification of the soil microbiomes

In the taxonomic classification of a microbiome from 16S data, a systematic methodology comprising multiple procedural steps was employed. The initial phase involved preprocessing and quality control procedures. Subsequently, reads were filtered to eliminate low-quality reads, adapters, and contaminants present in the sequencing data. Additionally, chimera checking was executed to discern and eliminate chimeric sequences resulting from the erroneous pairing of DNA fragments during PCR. Further, a clustering process was implemented to group akin reads into operational taxonomic units (OTUs) based on sequence similarity.

Following the preprocessing stage, taxonomic assignment procedures were executed. This involved aligning OTU sequences with the SILVA reference database, housing known bacterial and archaeal 16S sequences. A similarity search was conducted utilizing the RDP Classifier to identify the most closely matching sequences in the reference database. Subsequent taxonomic assignment was performed, allocating the OTU to specific taxonomic levels (e.g., phylum, class, genus, species) based on the closest matching sequence in the reference database.

The final phase encompassed a refinement of the classification process. This involved the application of confidence thresholds predicated on the percentage identity between the OTU sequence and the reference sequence. Lower confidence thresholds were considered, recognizing the potential for more ambiguous classifications. Phylogenetic analysis was undertaken to validate taxonomic assignments and identify potential errors. Manual curation was employed in select cases to rectify misclassified or ambiguous OTUs, leveraging additional contextual information such as known microbial distributions within the sample type.

The results indicated that there was an overall increase in the number of Families, Genera, and Species in the PaleoPower treated samples collected at harvest time. This increase in taxonomic entities reflects soil health. These results are illustrated in Figure 3.

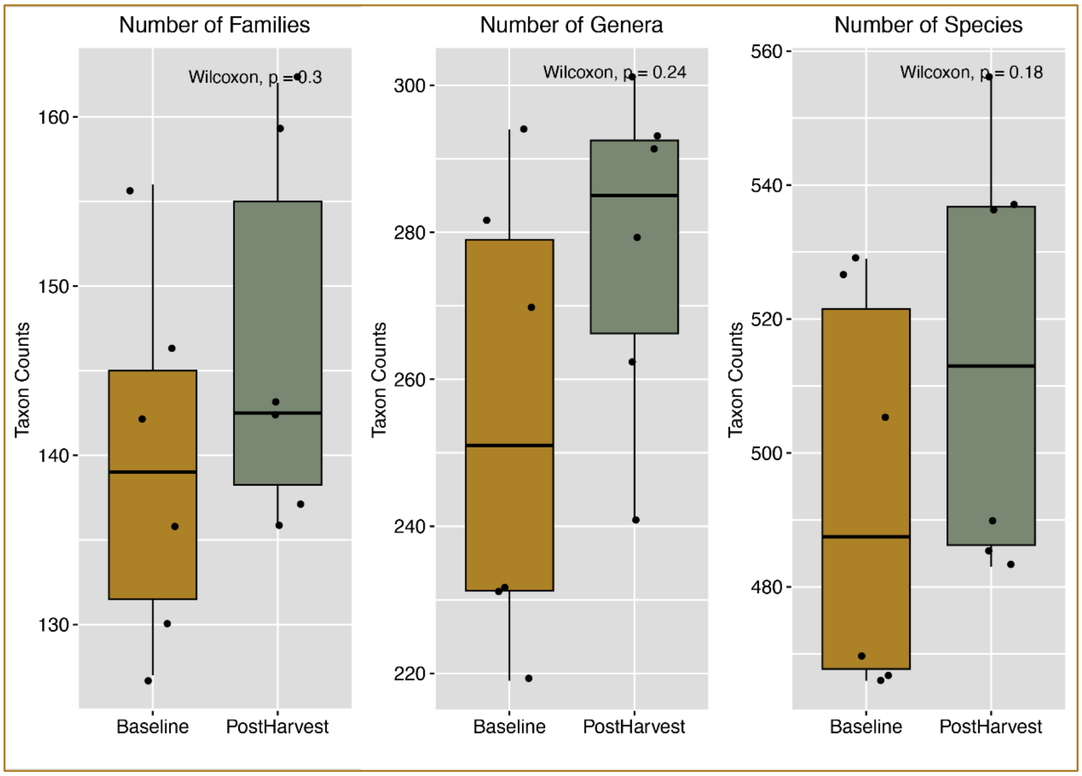


Figure 2. Number of Taxonomic Entities in Baseline and Post-Harvest Soils Treated with PaleoPower

4. Analysis of the Soil Microbiome

4.1 Methods: LDA Effect Size (LEfSe)

LDA Effect Size (LEfSe) is a statistical method used to identify features that are most likely to explain differences between biological groups in high-dimensional datasets. This is particularly useful for analyzing microbiome data, where there are many potential features (organisms, genes, etc.) and it can be difficult to determine which ones are truly relevant.

LEfSe works by combining two statistical tests:

1. Linear discriminant analysis (LDA): This is a supervised learning method that finds linear combinations of features that best separate different classes. The features that have the highest weights in the LDA model are the most discriminatory.
2. Wilcoxon rank-sum test: This is a non-parametric test that compares the distributions of two groups. LEfSe uses this test to determine whether the differences in abundance of a feature between groups are statistically significant.

By combining these two tests, LEfSe can identify features that are both statistically significant and have a large effect size. This makes it a powerful tool for identifying biomarkers that can be used to diagnose or predict disease, or to understand the mechanisms underlying complex biological processes.

4.1.1 Output of LEfSe:

LEfSe outputs a list of features ranked by their LEfSe score. The LEfSe score is a measure of the importance of a feature, considering both its statistical significance and its effect size. Higher LEfSe scores indicate features that are more likely to be biologically relevant.

LEfSe also outputs a cladogram, which is a hierarchical tree that shows the relationships between different features. This can be helpful for understanding the biological context of the results.

4.1.2 Applications of LEfSe:

In this study, we used this LEfSe to assess differences, both in microbial composition and soil metabolism between the baseline soil samples and post-harvest samples from cotton cultivation after treatment with PaleoPower.

Important Taxonomic and Functional Features of the Soil Microbiome

4.2 Taxonomic Features of the Soil Microbiome

LEfSe analysis identified a total of 206 taxonomic biomarkers in 24 bacterial Orders, distinguishing between baseline microbiomes from those from post-harvest soils. Of these, 12 had the highest DA scores. The results, in Percent Change at Post-Harvest, are summarized in Figure 4.

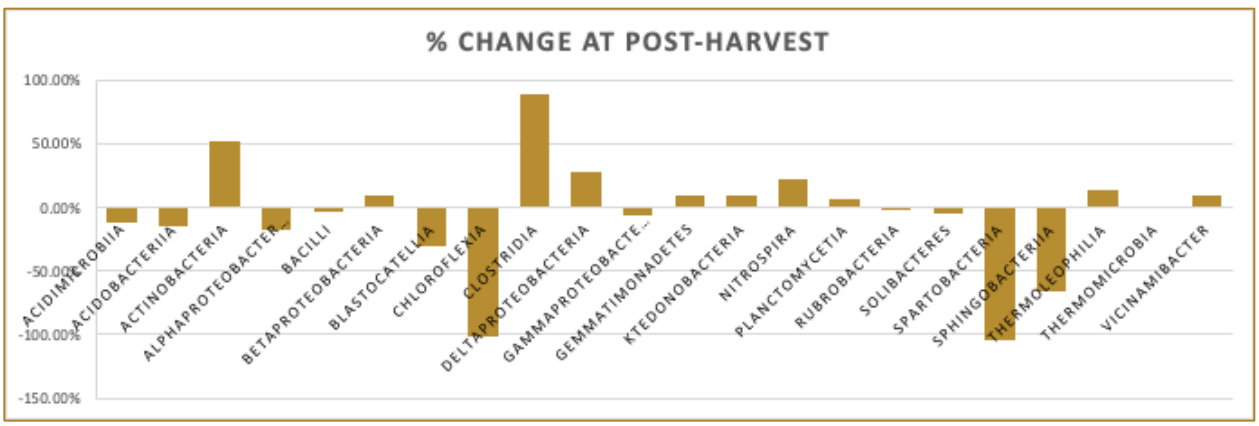
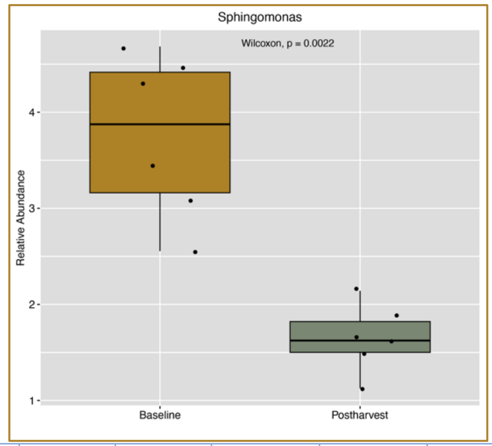


Figure 3. Percent Change of Bacterial Orders from Pre-Planting to Post-Harvest

The principal positive movers, i.e., showed an increase in relative abundance from pre- to post-harvest, included the Actinobacteria (+ 52.4%), Clostridia (+89.5%), Deltaproteobacteria (+28.5%) and the Nitrospira (+22.4%). Conversely, the Chloroflexia (-102.2%), Spartobacteria (-103.7), and the Sphingobacteria (-66.4%) showed a reduction in relative abundance from pre- to post-harvest. Table 1 summarizes some of the functions associated with soil ecosystem health and stability for these taxa.

Table 1. Role of Representative Taxonomic Orders in the Soil Microbiome Pre- and Post-Harvest

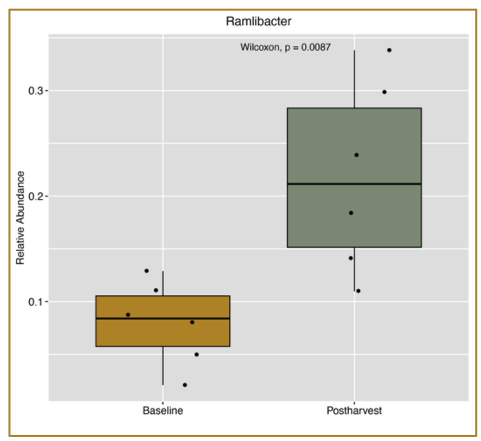
	Actinobacteria	Clostridia	Nitrospira	Proteobacteria (Delta)	Chloroflexia	Spartobacteria	Sphingobacteria
Relative Abundance Change (%)	+52.4	+89.5	+22.4	+28.5	-102.2	-103.7	-66.4
Nutrient Recycling							
Decomposition and mineralization	X	X		X			X
Decomposition of complex organic matter						X	
Nitrogen Fixation	X	X		X		X	X
Nitrate production			X			X	
Phototrophic metabolism					X		
Anaerobic decomposition					X		
Trace gas oxidation					X		
Plant Growth Promotion							
Phytohormone production	X			X			X
Siderophore production	X			X			
Promotes soil fertility			X	X			
Promotes healthy soil microbiome				X			
Biocontrol and Disease Suppression							
Antibiotic production	X	X				X	X
Competition for resources	X	X					
Siderophore production	X	X					X
Ecosystem Stability and Soil Structure							
Humus formation	X						
Aggregation	X					X	X
Glomalin production		X					
Nitrous oxide production			X				
Community Interactions			X				
Ecosystem specialization						X	
Bioremediation and Pollutant Degradation							
Hydrocarbon degradation		X			X		X



Anaerobic digestion		X					X
Pesticide degradation		X		X	X		X
Herbicide degradation				X	X		X

LEfSe analysis revealed a panel of species that were highly representative of a healthy soil microbiome and their abundance showed recovery from the use of herbicides and other toxic chemicals.

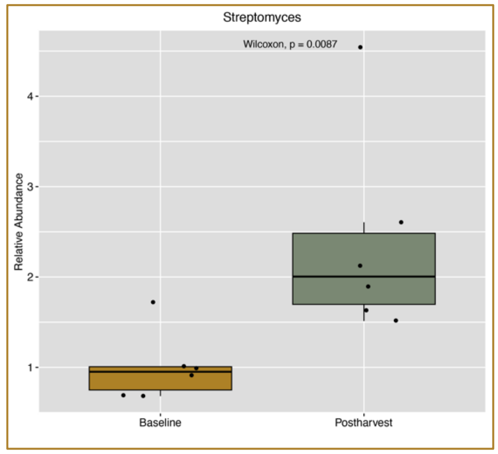
4.2.1 *Sphingomonas*



Sphingomonas is a genus of bacteria that includes several species known for their ability to degrade a variety of organic compounds, including some environmental pollutants. These bacteria are often found in soil and water environments and play a role in the biodegradation of complex organic molecules. Some species of *Sphingomonas*, some of which are represented in the soil microbiome of the Cotton Fields, possess enzymes that can break down glyphosate into simpler compounds, such as aminomethylphosphonic acid (AMPA) and glyoxylate. This degradation process is important for the environmental fate of glyphosate and helps reduce its persistence in soil and water. The relative abundance (RA) of these species is directly proportional to the levels of glyphosate in the soils. Their RA increases as the levels of the substrate glyphosate increases and decreases with lower levels of glyphosate, and as such, they can be considered indicator of soil glyphosate levels and soil health. The results indicate a significant decline in *Sphingomonas* spp. levels from May to October, reflecting the biodegradation process by the microbial community in the soils after treatment with PaleoPower.

4.2.2 *Ramlibacter*

Ramlibacter is a genus Gram-positive, rod-shaped bacteria belonging to the family Streptomycetaceae, in the Phylum Actinobacteria. *Ramlibacter* species are commonly found in diverse soil types, including rhizospheres (the zone surrounding plant roots) and forest soils. Some Ramlibacter species exhibit noteworthy properties, such as nitrogen fixation, converting atmospheric nitrogen into a usable form for plants and potentially promoting plant growth. Additionally, these bacteria are known to produce bioactive compounds, which may possess antibacterial, antifungal, or antiviral properties, thereby protecting plants from pathogens. *Ramlibacter* also contributes to soil health through the degradation of organic matter, supporting nutrient cycling and soil fertility.



Ramlibacter's potential contributions to soil health are significant. Firstly, nitrogen fixation by certain species enhances soil nitrogen content, particularly beneficial in nitrogen-deficient soils to support plant growth. Secondly, the bioactive compounds produced by some *Ramlibacter* species could act as natural antibiotics, potentially reducing plant disease incidence. Lastly, certain *Ramlibacter* species may contribute to improved soil structure by producing substances that bind soil particles, promoting soil aggregation and stability. These bacteria showed a pronounced increase in RA from May to October reflecting the improvement of soil health after treatment with PaleoPower.

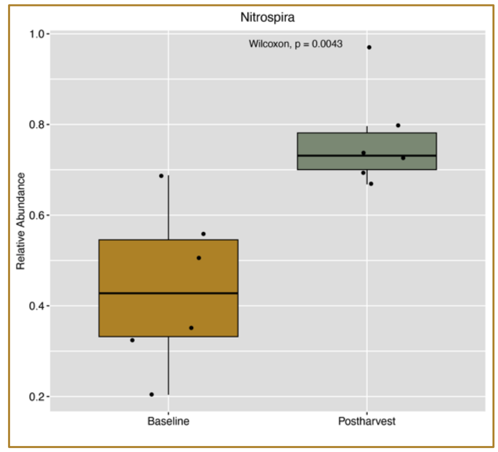
4.2.2.1 Streptomyces

Streptomyces bacteria assume a pivotal role as essential contributors to soil fertility and ecosystem health. Operating through the intricate architecture of their filamentous mycelial networks, *Streptomyces* play a multifaceted array of indispensable functions:

- Nutrient Dynamics:
  - Nitrogen Fixation: Demonstrating proficiency in nitrogen fixation, Streptomyces effectively assimilate atmospheric nitrogen, catalyzing its conversion into bioavailable nutrients conducive to plant growth and heightened productivity.
  - Decomposition Expertise: Leveraging robust enzymatic capabilities, Streptomyces adeptly decompose organic matter, liberating crucial nutrients, notably phosphorus and potassium, thereby sustaining the intricate dynamics of the soil food web.
- Plant health
  - Antibiotic Production: Streptomyces exhibits a formidable repertoire of natural antibiotics, actively engaging in combat against pathogenic bacteria and fungi. This chemical defense mechanism ensures the robust health and productivity of plants.
  - Immunomodulatory Effects: Certain strains of Streptomyces synthesize compounds with immunomodulatory properties, imparting resilience to plants against various infections and environmental stressors.
- Environmental health:
  - Glyphosate Degradation: Noteworthy is the remarkable ability of select *Streptomyces* strains to degrade glyphosate, a widely used herbicide. This capability positions them as potential agents for bioremediation, addressing concerns related to the persistence of glyphosate in soil and its implications for biodiversity and ecosystem health.
- Microbial Diversity:
  - Through the creation of specialized niches within their mycelial networks, Streptomyces foster a diverse community of beneficial microbes, thereby enhancing overall soil health and resilience.

Beyond these distinctive accomplishments, Streptomyces contribute to a cascade of ecosystem benefits including:

- Enhanced Soil Structure: The mycelial network established by *Streptomyces* functions as a stabilizing agent, binding soil particles and mitigating erosion, while concurrently improving water retention—a critical determinant for plant health and drought resilience.
- Carbon Sequestration: By sequestering carbon within their cellular structures, *Streptomyces* play a role in mitigating climate change, underscoring their significance as pivotal environmental actors on a global scale.



Streptomyces spp. represents a keystone species within a healthy soil microbiome, exerting a profound influence on crucial processes such as soil fertility, nutrient cycling, and overall ecosystem vitality. Their versatile capabilities, encompassing nitrogen fixation, organic matter decomposition, and bioactive compound production, position them as indispensable contributors to plant health and resilience against diseases. *Streptomyces* spp.'s distinctive functions, including glyphosate degradation and the promotion of microbial diversity, underscore their pivotal role in maintaining the equilibrium of a thriving soil ecosystem.

Moreover, the implementation of PaleoPower treatment in soil management practices amplifies the beneficial impact of Streptomyces spp., promoting a healthier soil microbiome. The integration of PaleoPower practices aligns with sustainable and ecologically sensitive approaches to agriculture, further emphasizing the importance of leveraging Streptomyces spp. for enhanced soil health and overall ecosystem sustainability.

4.2.3 Nitrospira

Nitrospira plays a crucial role in the nitrogen cycle within the soil, ultimately impacting soil health and plant growth. This bacterial genus specializes in the second step of nitrification, a process where soil bacteria convert ammonia into nitrate. They act as nitrite-oxidizing bacteria (NOB), consuming nitrite (produced by other bacteria in the microbiome) and transforming it into nitrate, a readily available form of nitrogen for plants.

Nitrospira exhibit remarkable tolerance to low oxygen and nitrite levels, allowing them to thrive in diverse soil environments. This makes them particularly dominant in acidic soils and deeper soil layers where oxygen is limited. Compared to other NOB like *Nitrobacter*, *Nitrospira* possess a more efficient nitrite uptake system, giving them an edge in competitive environments with limited nitrite. They also tend to require less organic carbon, making them suited to nutrient-poor soils. By efficiently converting nitrite, *Nitrospira* help regulate nitrogen availability in the soil. This prevents excessive nitrite accumulation, which can be harmful to both plants and the environment. *Nitrospira's* role in nitrification contributes to global nitrogen cycling and influences several environmental factors. Maintaining healthy *Nitrospira* populations helps mitigate greenhouse gas emissions from excessive soil nitrogen and reduces nitrate leaching into groundwater.

Recent Discoveries: Research has revealed intriguing variations within *Nitrospira*. Some members harbor a unique ability called comammox, short for “complete ammonia oxidation.” Comammox, is a recently discovered metabolic process where a single microorganism, primarily from the *Nitrospira* genus, can transform ammonia all the way into nitrate through the nitrification process. This differs from the traditional understanding of nitrification, which involved two separate steps: (a) Ammonia-oxidizing bacteria (AOB) or archaea convert ammonia to nitrite and (b) Nitrite-oxidizing bacteria (NOB), including *Nitrospira*, then convert nitrite to nitrate. The presence of comammox bacteria can influence the overall rate and regulation of nitrification in various ecosystems, affecting nitrogen availability for plants and potentially reducing ammonia and nitrite losses to the environment.

Overall, *Nitrospira* spp. are essential players in maintaining healthy soil by regulating nitrogen availability, promoting plant growth, and contributing to environmental stability. Understanding their functions and ecological niches is crucial for developing sustainable soil management practices. This genus appears to become an important component of the soil microbiome after treatment with PaleoPower, thus contributing significantly to improved soil health.

4.2.4 Other significant taxa associated with PaleoPower treated soils.

The boxplots illustrated below represent other 8 microbial taxa that are significantly represented in higher RA in PaleoPower treated soils.

4.2.4.1 Vicinamibacter

Vicinamibacter is a genus of bacteria belonging to the Acidobacteria phylum, known for their widespread occurrence in soils across various ecosystems. They participate in the following processes in soils:

- Cellulose Degradation: Like many Acidobacteria, *Vicinamibacter* species possess enzymes that break down complex carbohydrates like cellulose, a major component of plant biomass. This process releases vital nutrients like carbon, nitrogen, and phosphorus back into the soil, making them available for other organisms and plant growth.
- Nitrogen Fixation: Some Vicinamibacter strains have been shown to exhibit nitrogen fixation, a process where atmospheric nitrogen is converted into a usable form for plants. This ability contributes to soil fertility and reduces reliance on synthetic fertilizers.
- Polysaccharide Production: Vicinamibacter can produce extracellular polysaccharides, sticky molecules that bind soil particles together. This promotes soil aggregation, improving water retention, aeration, and erosion resistance.
- Pathogen Suppression: Some Vicinamibacter strains might compete with or even inhibit growth of plant pathogenic bacteria, potentially contributing to plant disease resistance.
- Stress Tolerance: Vicinamibacter often thrive in acidic and nutrient-poor environments, suggesting they might play a role in maintaining soil health under challenging conditions.

4.2.4.2 Myxococcus

- Nutrient cycling: By decomposing other bacteria, Myxococcus releases essential nutrients like nitrogen, phosphorus, and carbon back into the soil, making them available for plants and other organisms.
- Improved soil structure: Myxococcus produces extracellular polysaccharides that act like glue, binding soil particles together. This enhances soil aggregate stability, leading to better water retention, aeration, and resistance to erosion.
- Potential for biocontrol: Myxococcus species are being studied for their ability to control harmful soilborne pathogens. Their predatory nature and antibiotic production capabilities make them promising candidates for sustainable pest management strategies.

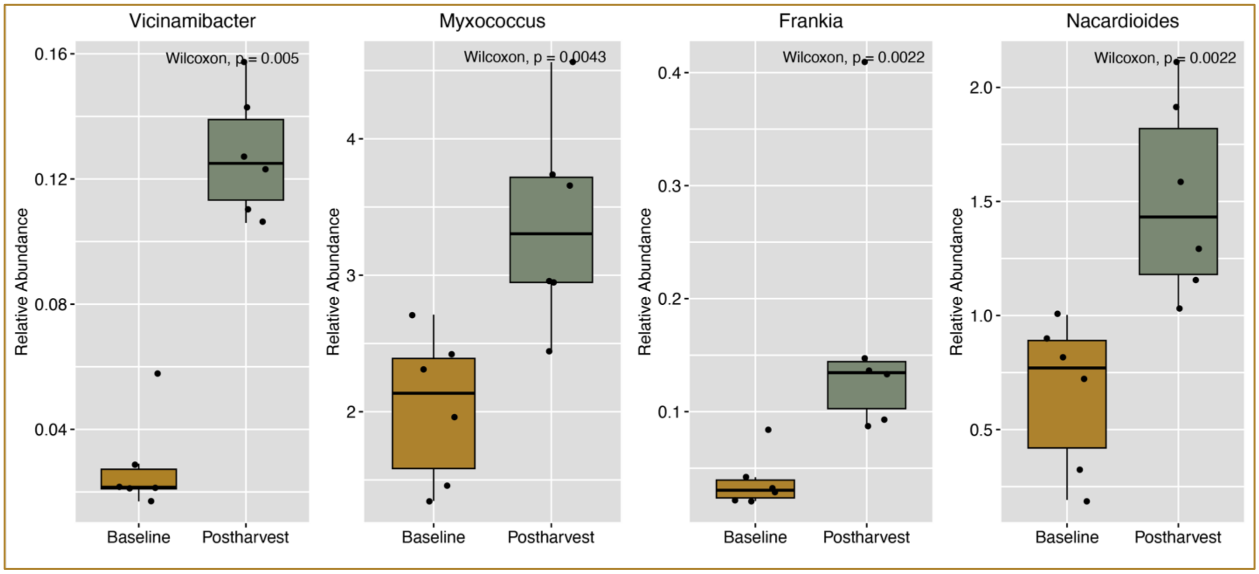
4.2.4.3 Frankia

Frankia's nitrogen-fixing capabilities have significant implications for soil health beyond individual plants. By enriching the soil with nitrogen, they:

- Stimulate microbial activity: Increased nitrogen availability fuels the growth and activity of other beneficial soil microbes, leading to a more diverse and dynamic soil ecosystem.

- Improve soil structure: Organic matter produced by both Frankia and the host plant contributes to soil aggregate stability, enhancing water retention, aeration, and erosion resistance.
- Promote land reclamation: *Frankia* can be used to inoculate degraded lands and support the establishment of actinorhizal plants. This helps restore soil fertility and prevent soil erosion, contributing to ecosystem restoration efforts.

Figure 4. Microbial Taxa with Significantly Higher RA in PaleoPower Treated Soils



4.2.4.4 Nocardioides

Nocardioides is a genus of Actinobacteria well represented in soils worldwide. It contributes to soil health in various, important ways:

- Decomposition: Many *Nocardioides* species possess enzymes that degrade complex organic matter like cellulose and lignin, common components of plant debris. This process releases essential nutrients such as nitrogen, phosphorus, and carbon back into the soil, making them available for plant growth and other organisms.
- Nitrogen fixation: Some *Nocardioides* strains have shown the ability to fix atmospheric nitrogen, a process where they convert it into a usable form for plants. This contributes to soil fertility and reduces reliance on synthetic fertilizers.
- Polysaccharide production: *Nocardioides* can produce extracellular polysaccharides, sticky molecules that bind soil particles together. This promotes soil aggregate stability, improving water retention, aeration, and resistance to erosion.
- Mycelium formation: Certain *Nocardioides* species can form filamentous structures called mycelia, which further enhance soil aggregation and stability.
- Antibiotic production: Several *Nocardioides* strains produce antibiotics that can inhibit the growth of harmful plant pathogens. This provides a natural defense mechanism for plants and contributes to disease suppression.
- Competition for resources: *Nocardioides* compete with plant pathogens for space and nutrients, further limiting their ability to harm plants.
- Adaptability: Many *Nocardioides* are adapted to challenging environments, such as acidic soils or drought conditions. This allows them to persist and contribute to soil health even under pressure.

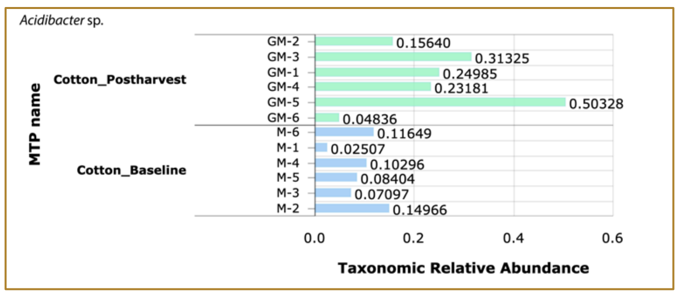
- Bioremediation: *Nocardioides* are being explored for their ability to degrade pollutants and contaminants in soil, aiding in soil remediation efforts.
- Biofertilizers: Strains with desirable capabilities, such as nitrogen fixation or antibiotic production, could be developed into biofertilizers to promote plant growth and soil health.
- Sustainable agriculture: Understanding *Nocardioides'* roles in soil health can inform sustainable agricultural practices that promote beneficial microbial communities and reduce reliance on harmful chemicals.

4.2.4.5 Acidibacter

Acidibacter thrives in naturally acidic or man-made acidic environments where most other organisms struggle. They possess unique mechanisms for pumping out protons and tolerating low pH levels, allowing them to colonize these challenging habitats.

Many *Acidibacter* species are adept at breaking down complex organic matter like cellulose and lignin, found in plant debris and other organic materials. This decomposition process releases vital nutrients like nitrogen, phosphorus, and carbon back into the soil, making them available for other organisms and plant growth.

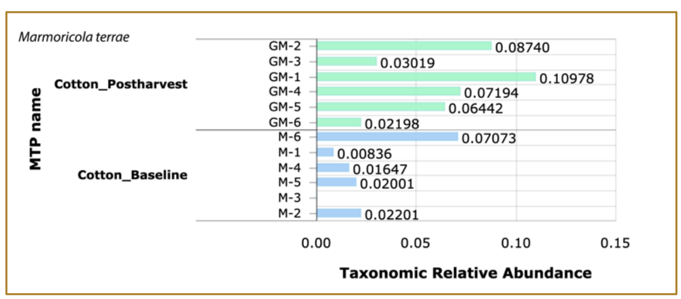
Acidibacter spp. promote soil health by acid soil mitigation. Their acid tolerance and decomposing abilities could help remediate contaminated soils and restore their fertility. This genus of bacteria can potentially promote a more diverse and resilient microbial community, leading to improved soil health and plant growth.



4.2.4.6 Marmoricola and other soil "core" bacteria

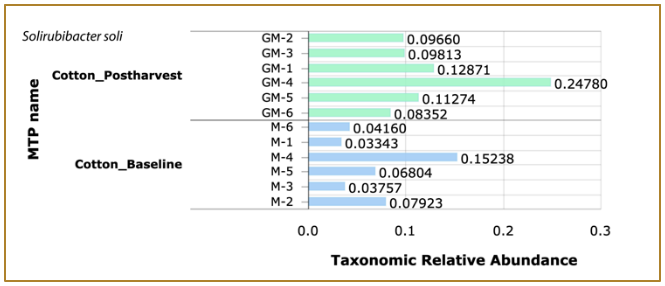
Marmoricola is one of a few genera of bacteria identified as the core bacteria in soils. The relative abundance of these bacteria is impacted adversely using fertilizers and herbicides. This genus has been associated with several processes that are essential for soil health:

- Production organic acids that can solubilize minerals like phosphorus and potassium, making them more readily available for plant uptake. This can improve plant growth and overall soil fertility.
- Fixation of atmospheric nitrogen into a form usable by plants. This reduces the need for synthetic fertilizers and improves soil nitrogen content.
- Hydrolysis of organic matter, releasing nutrients and improving soil structure.
- Competition with harmful soilborne pathogens, reducing plant diseases and promoting overall soil.
- Work synergistically with other soil microbes to accelerate the breakdown of glyphosate and other pollutants. This can be a more sustainable and effective approach to remediating contaminated soils than traditional methods.



4.2.4.7 Solirubibacter

Solirubrobacter soli is another member of the "core bacteria of soils." It is well represented in PaleoPower post-treated soils (see figure below). It plays a principal role in Nutrient Mobilization as it produces organic acids like gluconic acid that can solubilize minerals like phosphorus and potassium, making them more readily available for plant uptake. This enhances plant growth and overall soil fertility.

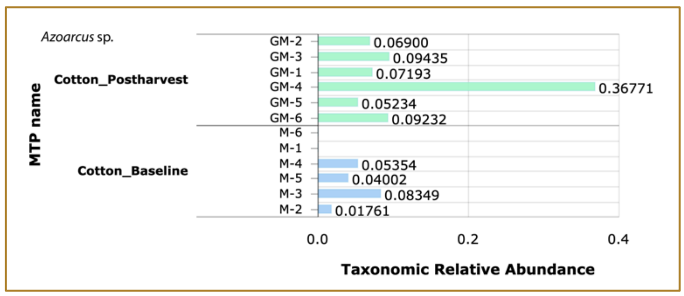


Its ability to break down complex organic matter releases nutrients back into the soil, contributing to nutrient cycling and promoting a healthy soil microbiome. It is noteworthy that certain strains of *S. soli* have been shown to fix atmospheric nitrogen into a form usable by plants, potentially reducing reliance on synthetic fertilizers and improving soil nitrogen content. Similarly, some *S. soli* strains produce compounds with antibiotic activity against plant pathogens, potentially contributing to natural disease suppression in the soil.

S. soli has been shown to degrade glyphosate, thus, offering a sustainable approach to addressing glyphosate contamination in soils and water.

4.2.4.8 Azoarcus

Azoarcus is a genus of Gram-negative, aerobic bacteria known for their diverse metabolic capabilities and potential applications in soil health and bioremediation. *Azoarcus beijerinckii*, harbor the nitrogenase enzyme, enabling them to fix atmospheric nitrogen into a form usable by plants. This enriches soil nitrogen content, reducing reliance on synthetic fertilizers and promoting plant growth. Some *Azoarcus* strains produce organic acids that can solubilize minerals like phosphate, making them more readily available for plant uptake. This improves plant phosphorus nutrition and overall soil fertility.



Azoarcus species can form symbiotic relationships with plants, promoting their growth and enhancing their ability to absorb and degrade pollutants from soil and water. As such, they can detoxify pesticides and herbicides, such as atrazine and 2,4-D, in contaminated soils. This contributes to the natural attenuation of these harmful chemicals and reduces their environmental impact.

Azoarcus spp promote soil health and bioremediates contaminated soils. In this context, the RA of these beneficial bacteria is directly proportional to improvements in soil health and as seen in the figure above, it is well represented in soils that have been treated with PaleoPower and have a diverse community of bacteria.

4.3 Functional Features of the Soil Microbiome

PICRUSt, which stands for "Phylogenetic Investigation of Communities by Reconstruction of Unobserved States," is a bioinformatics tool designed for predicting the functional capabilities of microbial communities based on 16S rRNA gene sequencing data. This tool is commonly used in microbial ecology and metagenomics research. 16S rRNA Gene Sequencing: Microbiome studies often use 16S rRNA gene sequencing to identify and characterize microbial communities. This method provides information about the diversity and relative abundance of different bacterial taxa in a given sample.

While 16S rRNA gene sequencing can tell us about the types of bacteria present, it does not provide direct information about the functional capabilities of these bacteria. PICRUSt addresses this limitation by using computational approaches to predict the functional gene content of a microbial community based on the known functions of closely related bacteria with sequenced genomes.

PICRUSt relies on reference databases of bacterial genomes and their annotated functional genes. By comparing the 16S rRNA gene data from a sample to this database, PICRUSt can make predictions about the functional potential of the microbial community. Once the functional potential is predicted, researchers can make inferences about the potential metabolic pathways and biological processes that may be active in the microbial community. The output from PICRUSt analysis of the microbiome can then be used as input data for biomarker discovery using LEfSe. Such analysis was conducted on the soil microbiome data of pre- and post- treatment with PaleoPower.

LEfSe analysis of the PICRUSt data revealed a total of 50 orthlog genes. An ortholog is a gene found in different species that originated from a common ancestor through speciation. These genes are homologous, meaning they share a common ancestry and often perform similar functions in their respective organisms, despite potential differences in sequence and regulation. A total of 14 biomarkers were identified by LEfSe as significant and associated with Post-Harvest microbiome of PaleoPower treated soils. All 14 biomarkers were associated with beneficial soil health functions attributed to the microorganisms identified above.

Table 2. Ortholog Genes found in the Soil Microbiome as Biomarkers in Pre- and Post-Treatment with PaleoPower

Definition	Pathway	p-value	Relative Abundance		% Change Post Harvest
			Baseline	Post-Harvest	
FUNDAMENTAL PROCESSES					
Starch Sucrose Metabolism	ko00500	0.02497	0.47470	0.48548	2.3%
Fatty acid biosynthesis	ko00061	0.03737	0.44397	0.45540	2.6%
Oligogalacturonide transport system substrate-binding protein	ko02010	0.02497	0.00006	0.00010	66.7%
N-glycan biosynthesis, high-mannose type	ko00510	0.03737	0.01500	0.01637	9.1%
Ribosome biogenesis GTPase / thiamine phosphate phosphatase	ko00730	0.03737	0.03702	0.04020	7.6%
POSPHATE METABOLISM					
PhoR-PhoB (phosphate starvation response) two-component regulatory system	ko02020	0.02497	0.09049	0.09917	9.6%
Methane metabolism	ko00680	0.01631	0.00823	0.59675	7150.9%
1L-myo-inositol 1-phosphate cytidylyltransferase	ko00562	0.01041	0.00035	0.00058	65.7%
multiple inositol-polyphosphate phosphatase / 2,3-bisphosphoglycerate 3-phosphatase	ko00010	0.01041	0.00035	0.00058	65.7%
3,4-Dideoxy-4-amino-D-arabino-heptulosonate 7-phosphate synthase	ko01051	0.00395	0.00023	0.00041	78.3%

Definition	Pathway	p-value	Relative Abundance		% Change Post Harvest
			Baseline	Post-Harvest	
Phosphate acetyltransferase-acetate kinase pathway, acetyl-CoA => acetate	ko00710	0.03737	0.04483	0.04833	7.8%
HOMEOSTASIS AND OSMOTIC STRESS					
Sulfiredoxin	K12260	0.00395	0.00004	0.00013	225.0%
Manganese/iron transport system	M00243	0.02497	0.02202	0.02295	4.2%
Ectoine hydroxylase	K10674	0.00649	0.00204	0.00241	18.1%
Anaerobic nitric oxide reductase transcription regulator	ko05132	0.02497	0.00895	0.01103	23.2%

4.3.1 Fundamental Processes

There was a mean increase of 17.7% of fundamental processes in the post-harvest soil microbiome from PaleoPower-treated soils as compared to baseline levels. The most significant changes occurred in processes associated with soil health and contaminant degradation.

Starch and sucrose metabolism showed a 2.3% increase in post-harvest as compared to baseline samples. Starch and sucrose metabolism in soils can have significant implications for soil health and ecosystem functioning. The impact is often indirect, as these processes influence the microbial communities, nutrient cycling, and overall soil fertility. Here are some key points regarding how starch and sucrose metabolism can impact soil health. Starch and sucrose serve as energy-rich carbon sources for soil microorganisms. Soil microorganisms, such as bacteria and fungi, utilize these compounds as substrates for metabolism and growth. The breakdown of complex carbohydrates like starch into simpler sugars, such as glucose, is an essential step in the microbial decomposition of organic matter in the soil.

The availability of starch and sucrose influences the activity and diversity of soil microorganisms. Different microorganisms have varying abilities to utilize different carbon sources, and the composition of available carbon can shape the microbial community structure. Microbial diversity is often associated with improved soil health, as diverse microbial communities contribute to various ecosystem functions, including nutrient cycling and disease suppression.

Microbial decomposition of starch and sucrose releases carbon dioxide into the soil, contributing to soil respiration. This process is part of the carbon cycle in soils. The microbial breakdown of organic matter also releases nutrients, such as nitrogen and phosphorus, into the soil, making them available for plant uptake. This nutrient cycling is crucial for maintaining soil fertility.

Microbial activities influenced by starch and sucrose metabolism can contribute to the formation and stabilization of soil aggregates. Soil aggregates are important for soil structure, water infiltration, and root penetration. Improved soil structure enhances water retention, aeration, and nutrient availability, positively influencing overall soil health. Including disease suppression. Microbial communities supported by starch and sucrose metabolism may contribute to disease suppression in soils. Certain microbial populations can act as biocontrol agents, protecting plants from soil-borne pathogens.

Fatty Acid Biosynthesis showed a 2.6% increase in the post-harvest microbiome of treated soils as compared to baseline levels. Fatty acid biosynthesis is a fundamental process in living organisms, including bacteria, and it plays a role in microbial metabolism within soils. The influence of microbial processes, specifically fatty acid biosynthesis, on phytohormone production is a complex and interconnected aspect of plant-microbe interactions. Microbes in the soil, particularly certain beneficial bacteria, and mycorrhizal fungi, can enhance phytohormone production in plants through various mechanisms.

Some soil bacteria, such as certain strains of plant growth-promoting bacteria (PGPB), are known to produce *indole-3-acetic acid (*IAA*),* which is a type of auxin phytohormone. IAA is involved in various plant growth and developmental processes, including cell elongation, root development, and tropic responses. Certain bacteria can stimulate the production of IAA, influencing plant growth.

Certain bacteria can produce *cytokinins*, another class of phytohormones, which play a role in cell division and differentiation. Cytokinins produced by soil microbes affect the balance of phytohormones in plants, influencing processes such as shoot development and nutrient uptake.

Similarly, soil microbes can influence the levels of *abscisic acid (*ABA*),* a phytohormone involved in stress responses, seed development, and stomatal regulation. Microbial interactions with plants, especially under stressful conditions, may lead to changes in ABA production in plants.

Soil bacteria also influence *ethylene* production in plants. Ethylene is involved in various physiological processes, including senescence, fruit ripening, and stress responses. The microbial modulation of ethylene levels can impact plant growth and responses to environmental stimuli.

Fatty acid biosynthesis also can affect the relative abundance of the soil microbiome and the quality of soils by promoting nutrient cycling and organic matter. The efficiency of microbial decomposition and nutrient cycling processes in soils may be influenced by the availability and composition of fatty acids.

Fatty acid biosynthesis is also essential for bacterial growth and reproduction. Changes in the availability of substrates for fatty acid biosynthesis may impact the growth rates and community structure of soil bacteria. The diversity and composition of microbial communities are key factors in maintaining soil health and functionality.

Fatty acids can play a role in plant-microbe interactions, as they are involved in signaling processes and the establishment of symbiotic relationships. For example, certain fatty acids may act as signaling molecules in the rhizosphere, influencing plant growth and development. The balance between different types of fatty acids produced by soil microbes may impact the establishment of beneficial interactions between plants and microbes.

Certain fatty acids produced by soil microbes may have antimicrobial properties, contributing to biocontrol and disease suppression in soils. Microbial communities with the capacity to produce specific fatty acids may play a role in protecting plants from soil-borne pathogens, positively influencing soil health.

4.3.2 Phosphate Metabolism

Phosphate metabolism plays a crucial role in plant health and is essential for various physiological processes, growth, and development. Phosphorus is a vital nutrient that plants obtain primarily in the form of inorganic phosphate (Pi) from the soil. There was a mean increase of 45.4% in phosphate metabolism-associated genes in the microbiome of post-harvest soils treated with PaleoPower when compared to baseline levels.

Phosphate metabolism is crucial for seed germination and flowering. Phosphorus is a component of nucleic acids, energy transfer molecules, and structural components that are vital for these developmental processes.

The *PhoR-PhoB two-component regulatory system* is a signaling pathway involved in the phosphate starvation response in bacteria. This system typically consists of two proteins: PhoR, which is a membrane-bound sensor kinase, and PhoB, which is a cytoplasmic response regulator. When phosphate availability is limited, this system becomes activated, leading to the regulation of various genes involved in phosphate uptake and utilization. There was an increase of 9.2% in genes in the soil microbiome of treated soils as compared to baseline values.

This regulatory system is associated with prevention of Phosphate Starvation and promoting Plant Growth. Phosphate is an essential nutrient for plant growth, playing a crucial role in various physiological processes, including photosynthesis, energy transfer, and nucleic acid synthesis.

Soil bacteria, including those with the PhoR-PhoB system, can influence the availability of phosphorus in the rhizosphere (the soil region influenced by plant roots). Some bacteria can enhance plant phosphorus acquisition through mechanisms such as phosphate solubilization or the production of organic acids that facilitate phosphorus release from minerals.

4.3.3 Homeostasis mechanisms

Homeostasis refers to the ability of living organisms, including plants and the soil microbiome, to maintain internal stability and balance in the face of changing external conditions. In the context of the soil microbiome and plant health, homeostasis involves a dynamic equilibrium that ensures mutualistic interactions, nutrient cycling, and overall ecosystem stability. Here are key homeostasis processes in the soil microbiome and their implications for plant health and growth promotion. There was a mean increase of 67.7% in phosphate metabolism-associated genes in the microbiome of post-harvest soils treated with PaleoPower when compared to baseline levels.

A healthy soil microbiome plays a crucial role in promoting homeostasis mechanisms within ecosystems. The soil microbiome encompasses a diverse community of microorganisms, including bacteria, fungi, protozoa, and other microbes. These microscopic organisms contribute to the overall health and balance of the soil ecosystem in various ways.

Nutrient Cycling: Microorganisms in the soil break down organic matter into essential nutrients, such as nitrogen, phosphorus, and potassium, making them available for plants. This nutrient cycling ensures a continuous supply of vital elements, supporting plant growth and overall ecosystem stability.

Disease Suppression: Certain beneficial microbes in the soil act as natural antagonists against plant pathogens. They can outcompete harmful microorganisms for resources or produce compounds that inhibit the growth of pathogens, contributing to a natural defense mechanism within the ecosystem.

Water Retention and Filtration: Microbial communities help improve soil structure, enhancing water retention and drainage. This is critical for maintaining proper moisture levels in the soil and preventing erosion. A well-structured soil with a balanced microbiome also filters out pollutants, contributing to water quality.

Carbon Sequestration: Soil microorganisms are essential players in the carbon cycle. They decompose organic matter and contribute to the formation of stable soil organic carbon. This process helps in carbon sequestration, mitigating the impacts of climate change by storing carbon in the soil.

Stability and Resilience: A diverse and healthy soil microbiome promotes ecosystem stability and resilience. It acts as a buffer against environmental stressors, such as extreme weather events or changes in land use. Biodiversity within the microbial community provides a range of functions, ensuring that the ecosystem can adapt to disturbances.

Reducing Dependence on External Inputs: A well-balanced soil microbiome reduces the need for external inputs like synthetic fertilizers and pesticides. This can lead to more sustainable agricultural practices, preserving the long-term health of the soil and minimizing environmental impacts.

In summary, a healthy soil microbiome is essential for maintaining ecological balance and supporting homeostasis mechanisms within ecosystems. Its impact extends beyond the soil itself, influencing plant health, water quality, carbon sequestration, and overall ecosystem resilience. Promoting practices that enhance soil microbial diversity and activity is crucial for sustainable land management and global environmental health.

5. Conclusions

During our studies, the application of PaleoPower revealed a significant reduction in glyphosate levels, showcasing a substantial decline of 74.8% ± 9.6%. This reduction substantially outperformed the observed rate of glyphosate decomposition in untreated soils, effectively more than doubling the remediation efficacy compared to control conditions.

The efficacy of PaleoPower in glyphosate degradation can be attributed to its inherent microbial consortium, which exhibits a demonstrated proficiency in breaking down glyphosate molecules. This consortium, specifically tailored for bioremediation purposes, plays a pivotal role in directly mitigating glyphosate levels in the soil.

Moreover, PaleoPower acts as a potent stimulant for the native soil microbiome. By creating a favorable ecological niche, PaleoPower promotes the thriving of indigenous microorganisms, thereby enhancing the overall bioremediation potential of the soil. The synergy between the introduced microbial consortium and the native microbiome creates a dynamic environment that not only accelerates the breakdown of glyphosate but also fosters a sustained and resilient remediation capacity within the soil ecosystem. This dual-action approach underscores the multifaceted impact of PaleoPower on glyphosate bioremediation, positioning it as a promising and effective tool for addressing pesticide contamination in soil environments.

PaleoPower, a microbial soil inoculant, also serves as a valuable tool in promoting soil health by introducing a diverse array of beneficial microorganisms and their corresponding plant growth promoting substances into the soil ecosystem. These microbes work in synergy to enhance critical soil functions, including nutrient cycling, disease suppression, and the improvement of soil structure. By fostering microbial diversity, PaleoPower contributes to the creation of a resilient soil environment that supports healthier crops and soils.

The nutrient cycling facilitated by the introduced microorganisms leads to improved nutrient availability for plants, promoting optimal growth and development. Moreover, the inoculant contributes to disease suppression by harnessing the antagonistic properties of certain microbes against soil-borne pathogens, thereby creating a more protective and conducive environment for plant roots.

PaleoPower's influence extends to the enhancement of soil structure and aggregation. The microbial activity promotes better water infiltration, root penetration, and nutrient mobility in the soil, resulting in an overall more favorable habitat for plant growth.

Crucially, PaleoPower contributes to the environmental stress tolerance of plants, enabling them to withstand challenging conditions such as drought or salinity. This is achieved through mechanisms like the production of stress-induced hormones by beneficial microbes and the improvement of nutrient availability under stress conditions.

Furthermore, the use of microbial soil inoculants, particularly PaleoPower, aligns with sustainable agriculture practices by potentially reducing the reliance on chemical inputs. By promoting a balanced and resilient agroecosystem, PaleoPower supports long-term soil health and crop productivity, contributing to the broader goals of sustainable and environmentally friendly agricultural practices.

The Transformative Potential of PaleoPower for the Cotton Project entitled “A Soil-Centric Approach to Enhanced Productivity and Sustainability” is measurable and significant. The cultivation of cotton, while economically significant, can present substantial challenges due to its resource-intensive nature and susceptibility to disease. However, advancements in microbial soil inoculants like PaleoPower offer a compelling solution, promoting increased yields, robust plant health, and environmental sustainability.

The benefits of PaleoPower extend beyond the field, aligning seamlessly with the BLACKollective's commitment to sustainable and climate-friendly agricultural practices. By reducing reliance on chemical fertilizers and pesticides, PaleoPower minimizes greenhouse gas emissions, contributing to