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Comparative Metagenomic Profiling of Seed-Borne Microbiomes in a Landrace and a Hybrid Maize Variety

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Abstract: The plant seed-borne microbiome comprises microorganisms vertically inherited from the mother plant. This microbiome is often linked to early-life protection and seedling growth promotion. Herein, we compare the seed-borne bacteriomes of a commercial hybrid (Santa Helena) and a landrace maize variety (Sol da Manhã). The landrace variety displays a more diverse seed-borne microbiome, featuring a variety of taxa across samples with an average Shannon's diversity index of 1.12 compared to 0.45 in the hybrid variety. The landrace variety also showed a greater alpha diversity of 165.8, in contrast to 144.1 in the hybrid. Although both microbiomes lack a functional nitrogen fixation apparatus, we found a remarkably distinct presence of genes associated with phytohormone production and phosphate solubilization, particularly in the landrace variety. In addition, we recovered 18 metagenome-assembled genomes (MAGs), including four from potentially novel species. Collectively, our results allow for a better understanding of the contrasting diversity between maize varieties. The higher potential for phytohormone production in landraces, the absence of nif genes in both varieties, and the identification of core microbiome taxa offer valuable insights into how microbial communities impact plant health and development. This knowledge could pave the way for more sustainable and innovative agricultural practices in crop management.

Keywords: metagenome; bacteriome; plant growth-promoting bacteria; metagenome-assembled genomes; microbial diversity

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1. Introduction

When examining the intricate world of microscopic ecosystems, plants are dynamic habitats in their own right. Plants rely heavily on the microbial communities within and around them [1], engaging in a myriad of complex interactions that can be beneficial, antagonistic, or neutral [2]. The plant bacteriome comprises bacteria acquired from the surrounding soil and those vertically transmitted during seed development. These vertically inherited bacteria comprise the resident or seed-borne microbiome. The seed-borne microbiome is often associated with seedling growth and protection [3].

Factors such as plant genotype and environmental conditions can influence plant microbiome composition and functions, emphasizing the dynamic nature of these bacterial communities [4]. Different plant genotypes are likely to diverge in their seed-borne microbiomes due to artificial selection and recruitment of other bacteria from the soil [5]. In addition, neighboring plants [6] and long-term processes such as global warming [7] can influence a plant microbiome. Maize is a suitable model for studying seed-borne microbiomes. Besides its great economic relevance as the second most produced agricultural commodity in the world [8], maize has a well documented evolutionary and domestication history, valuable germplasm collections, and other genetic resources [9,10].

Although previous works have shown a difference in microbiome composition between hybrid and landrace maize varieties [11], the roles of seed-borne bacteria and their implications for plant development remain unclear. Some studies suggest that plant domestication may lead to a decrease in the transmission capability of the seed-borne microbiome [12], which would gradually compromise its composition and diversity in crop varieties used in intensive agriculture systems. Such a reduction in seed-borne microbial diversity may be linked to negative outcomes, like increased disease susceptibility [13].

Metagenomic studies have also shown a strong correlation between reduced microbiome diversity and declines in both crop productivity and disease resistance [7,14]. Within a plant core microbiome, several taxa play pivotal roles, such as *Pantoea*, renowned for their diverse plant-growth-promoting traits [15,16]. Furthermore, specific bacterial genes as observed in Pseudomonads [17], can also enhance the capability of plant–microbe interactions [18]. Notably, the maize microbiome exhibits a prevalence of families like Rhizobiaceae, Burkholderiaceae, and Microbacteriaceae, particularly during early plant development [19]. These families are usually associated with traits such as phosphate solubilization and nitrogen metabolism [20–23].

With a rising emphasis on sustainable agriculture, bioinoculants have been adopted over the last four decades, a market that grows at an annual rate of 10% worldwide [24]. By integrating environmentally compatible inoculants with insights from large-scale microbiome analyses, we can modify the "stage 0" of the seed microbiome. This approach aims to enhance crop productivity and protection [3] while also compensating for any potential reduction in seed-borne microbial diversity in intensive crop varieties. Currently, the most effective way to increase crop production is through the widespread use of chemical pesticides and fertilizers. However, this demand for chemical inputs increases dependency on imported products that compromise the sovereignty of several countries for food, fiber, and energy production [25,26]. Furthermore, the intensive use of agrichemicals is often associated with groundwater contamination [27], human diseases [28], and the death of pollinating insects [29]. In this scenario, the adoption of sustainable practices based on plant growth-promoting bacteria (PGPB) constitutes an attractive alternative [30]. PGPB typically promote plant growth through diverse mechanisms of action such as biofertilization, bioprotection, or biostimulation [31].

In the present work, we investigate the seed-borne bacteriomes of maize varieties derived from two distinct breeding strategies. We evaluated the microbiome of a commercial hybrid and a landrace maize variety, the latter originating from organic production in an agroforestry ecosystem. We analyzed the structure and diversity of these microbiomes, with particular emphasis on their differences and potential to promote plant growth.

2. Methods

2.1. Maize Varieties

The varieties used were the "SHS 5050" double hybrid commercial variety (SH) and the "Sol da Manhã" homozygous landrace variety (SOL). SH is a hybrid variety from a local Santa Helena retailer with production in northwest Minas Gerais, Brazil. This company works to improve maize yield and uniformity, dealing with a large-scale production area irrigated under a central pivot. The SOL variety was donated by the family of the agribusiness farmer Jamil Bráz Corinto to Prof. Samuel Kamphorst (UNILA, Brazil). This variety originated from agricultural growth corridors in Santo Antônio do Rio Verde, Goiás, Brazil. SOL was initially cultivated in indigenous lands and has been cultivated in agroforestry systems for over 15 years, a period during which it has undergone meticulous participatory mass selection. The cultivation of SOL is a collaborative effort supported by EMBRAPA and in collaboration with the Movimento Camponês Popular (MCP).

2.2. Planting

Maize seeds from both varieties were rinsed with sterile distilled water five times and soaked for five hours to break dormancy and standardize germination. The seeds

were carefully distributed into sterile 2 L glass jars filled with 700 g of Basaplant substrate sieved through a 2 mm mesh previously autoclaved for four one-hour cycles for complete sterilization. We irrigated the substrate with 250 mL of autoclaved distilled water and divided it into three distinct sectors, each containing 10 seeds. We sealed the glass jars with cotton to allow gas exchange while maintaining the seedlings under axenic conditions throughout the incubation period. We conducted the experiment in a Biological Oxygen Demand (BOD) incubator (100 µmol/m^2/s), maintaining the temperature at 28 °C "day" and 25 °C "night", following a 12 h light/12 h dark cycle for 7 days. The choice to work with germinated seeds was so that the microbiome would mimic the real scenario of plant development in the substrate instead of studying only the quiescent seed microbiome [15,32].

2.3. 16S and Metagenomic Sequencing

The rhizospheric substrate was collected by brushing it from the seedlings and DNA extraction conducted using the DNeasy PowerSoil Pro Kit (Qiagen—Hilden, Germany) following the manufacturer's instructions. DNA was sequenced using an Illumina NextSeq instrument at NGS (Piracicaba, Brazil). We also sequenced a DNA sample from the autoclaved substrate to serve as a negative control ("C1") for contamination in shotgun sequencing. The raw sequencing reads were submitted to NCBI SRA under BioProject PRJNA1069023. For 16S rRNA sequencing, we used the Wizard Genomic DNA Purification Kit (Promega – Madison, Wisconsin, United States) followed by sequencing using the Sanger method using the 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) universal primers [33].

QIIME2 v. 2023.5.1 [34] was used to process 16S metataxonomic data and to remove reads from mitochondrial and chloroplast 16S rRNA. The SILVA database Release 138 [35] was used to infer taxonomic classification.

For metagenome analysis, we used Bowtie2 v. 2.3.4.3 [36] and Samtools v. 1.9 [37] to remove fragments of maize DNA. Sequencing quality was evaluated with FastQC v. 0.12.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed on 7 August 2023) and low-quality fragments trimmed using Trimmomatic v. 0.39 [38]. The taxonomic distribution of the reads was conducted with Kaiju v. 1.6.2 [39]. Microbiome diversity analyses were conducted with VEGAN [40].

For optimizing the screening of plant growth promotion capacity in the metagenomes, the microbiome biotechnological potential was inferred using an in-house database of genes associated with different plant growth promotion traits (i.e., nitrogen fixation, phosphate solubilization, phytases, ACC deaminase, and auxin production) (Pedrosa–Silva, Henaut–Jacobs, Venancio, in preparation). This database was constructed by selecting genes widely known for their plant growth promotion capabilities. The database also contains copies of each gene from every different genera that are available in UniProt. These genes were compared with the metagenomes using Usearch [41] with a 60% identity threshold.

2.4. Metagenome-Assembled Genomes (MAGs)

Metagenome assembly was conducted using SPAdes v. 3.15.5 [42] with the "*--meta*" parameter. Assembly quality was inferred using QUAST v. 5.0.2 [43] to help choose the best assembly parameters. Only contigs longer than 1750 base pairs were retained. MAGs were binned with MetaBAT2 v. 2.2.15 [44] (Kang et al., 2019). The GTDB toolkit Release 08-RS214 [45] was used to infer the taxonomy and BUSCO v. 5.4.2 [46] to assess the completeness and duplication levels of the MAGs. The redundancy between the MAGs was estimated with pyani v. 0.2.12 and their genes were predicted with Prokka v. 1.13 [47]. To categorize the MAGs, the MIMAG methodology was used [48], and we retained only non-redundant MAGs that were classified as High-quality or Finished. These were then submitted to GenBank for further analysis.

3. Results and Discussion

3.1. Metagenomics Sequencing Depth Satisfactorily Captured Sample Diversity

We started the analysis with the C1 control sample, which had 1881 operational taxonomic units (OTUs) (Tables S1 and S2). Most of these OTUs are exclusive to C1, suggesting that they result from residual DNA from bacteria killed during sterilization. Conversely, the OTUs from SH and SOL are remarkably more abundant than those of C1, most likely because of living bacteria in these samples. These results support the observation that the DNA reads sequenced from the SH and SOL samples were indeed from the seed-borne microbiome, with virtually no substrate DNA contamination.

To assess the coverage of our data, we estimated the taxa per sample (Figure 1) and computed the Good's coverage index for each sample (Table 1). These analyses demonstrated that our sequencing data achieved robust taxonomic coverage, capturing 80% of the microbial diversity at 10 million reads in both varieties. Notably, the Good's index is in line with the rarefaction curves, with only a slight variation where SH exhibits marginally lower Good's indexes in comparison to SOL. SH samples also exhibited a more delayed plateau phase (Figure 1), which is consistent with their greater number of singletons (Table 1) that probably originated from low-abundance bacteria that are unlikely to significantly contribute to the community structure. Nevertheless, we kept these singletons for downstream analysis because of the overall low bacterial DNA amounts in our samples.

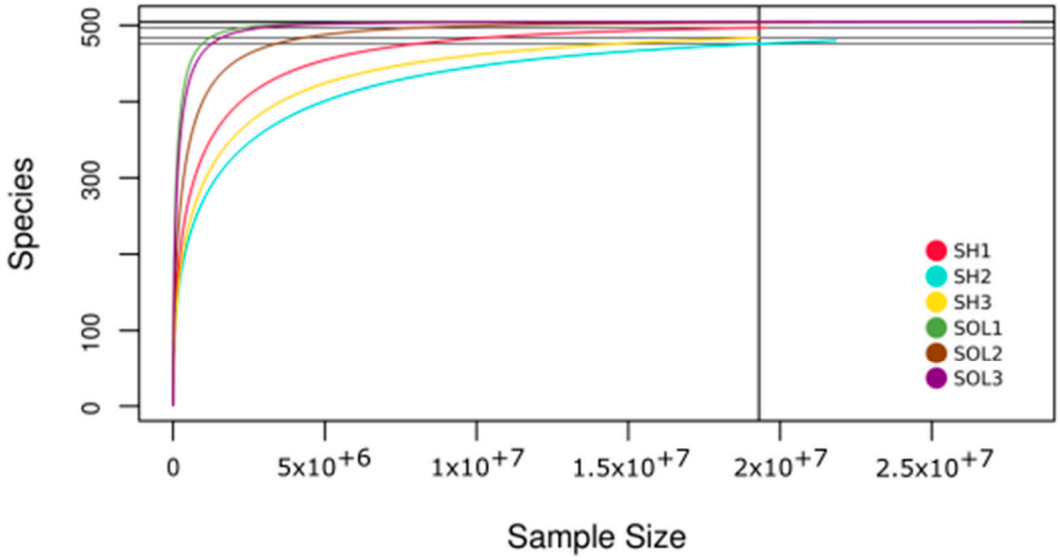

Figure 1. Saturation curve of OTUs found in each sample. Horizontal lines indicate the final number of species from each sample.

Table 1. Complete sample information with sample identification based on the description in the Methods section (Santa Helena variety = SH; Sol da Manhã variety = SOL).

Sample	Variety	Number of OTUs in Metagenomic Data	Total Read Count	16S Read Count	Singletons	Good's Index	Shannon's Diversity Index	Alpha Diversity
SH1	SHS-5050	1741	17,853,454	40,046	123	99.99924	0.46	150.28
SH2	SHS-5050	1637	20,265,869	43,507	225	99.99879	0.44	138.62
SH3	SHS-5050	1670	17,844,958	41,191	209	99.99872	0.45	143.42
SOL1	Sol da Manhã	1899	18,881,650	41,568	40	99.99963	1.56	172.00
SOL2	Sol da Manhã	1821	18,250,328	41,631	69	99.99955	0.82	158.52
SOL3	Sol da Manhã	1888	20,837,848	37,682	38	99.99972	0.99	166.88

3.2. The Hybrid Maize Genotype Harbors a Homogeneous Seed-Borne Bacteriome

We sequenced 124,744 and 120,881 16S rRNA partial gene sequences in the SH and SOL samples, respectively (Table 1). In our 16S metataxonomic data, we observed a substantial abundance of the Burkholderiaceae family in SH and SOL, mainly from the *Burkholderia* and *Paraburkholderia* genera (Figure 2B). Previous studies reported the prevalence of the Burkholderiaceae family in maize rhizosphere microbiomes [11], which could suggest a higher likelihood of these bacteria being inherited across plant generations. Importantly, *Burkholderia* species have been associated with nitrogen fixation and stress resistance in maize [49,50]. Another possible explanation for this persistence is the biological profile of the Burkholderiaceae family. Known for their endophytic lifestyle, they possess a highly developed motility apparatus, which could enhance their ability to colonize seeds [51]. One of the most frequent species in both maize varieties was *Burkholderia cenocepacia* (Table S1), which is part of the *Burkholderia cepacia* complex that has been widely reported as associated with plant growth promotion [52]. The inoculation of *B. cenocepacia* improved maize production (cob length, number of grains per cob, grain weight, and 100-grain weight) in the field, with better results when combined with *Alcaligenes aquatilis* [53].

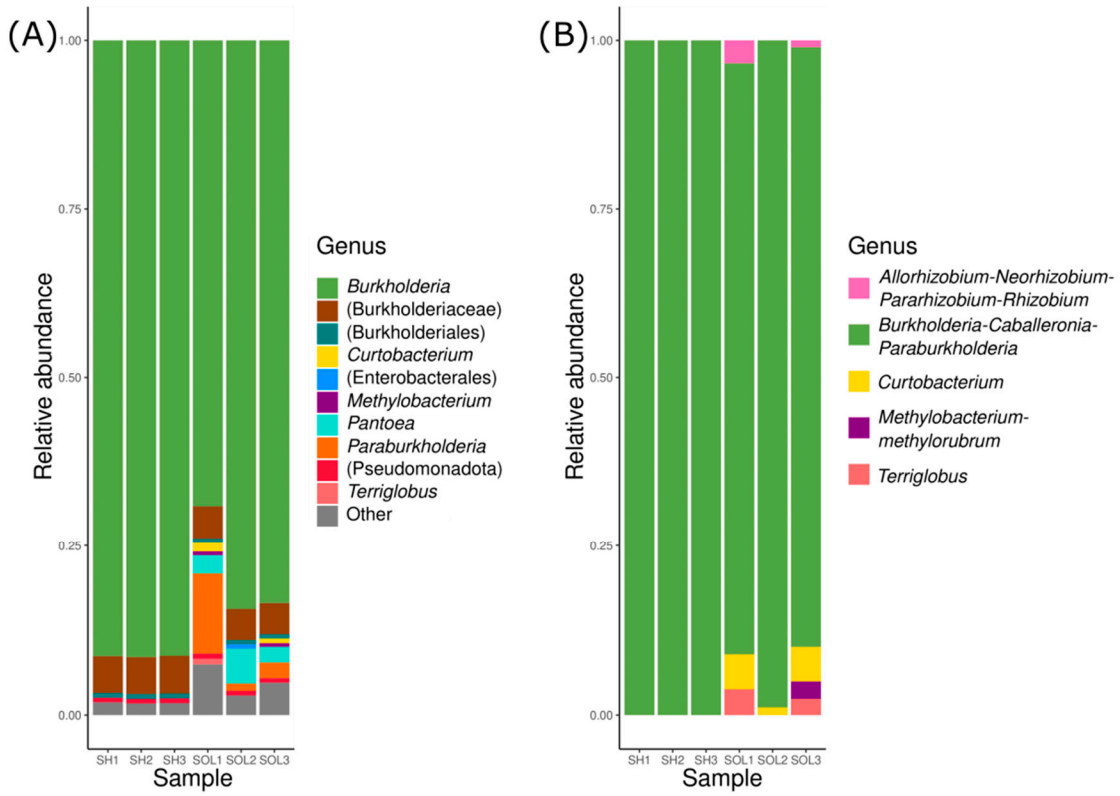

Figure 2. Relative abundance of OTUs. (**A**) Metagenome shotgun sequencing data (higher taxonomic levels are presented between parentheses). OTUs with less than 1% relative abundance across all samples and reads without taxonomic assignment were collectively classified as "Other"; (**B**) 16S rRNA data.

It is important to acknowledge that some *B. cenocepacia* strains are pathogenic to humans, particularly to immunocompromised individuals [54]. Conversely, other *B. cenocepacia* strains are non-pathogenic and deemed safe [55,56]. Hence, it is feasible to strategically incorporate non-pathogenic strains in inoculants or synthetic microbiomes to harness the beneficial features of *B. cenocepacia* as a PGPB while mitigating potential risks to human health.

Burkholderiaceae accounted for over 90% of the SH microbiome. In addition, all classified reads from the SH 16S metataxonomic data were assigned to the *Burkholderia*–

Caballeronia–Paraburkholderia genera (Figure 2B). In contrast, the SOL samples displayed a substantial presence of various taxa and variable proportions of Burkholderiaceae in their composition (Table S1). Principal Component Analysis (PCA) further highlighted this distinction, with the SH samples almost completely overlapping, while the SOL samples were more dispersed (Figure 3). We can also see a greater separation of SOL1 from the other samples on the X-axis, which might reflect the lower prevalence of *Burkholderia* in this specific sample based on the contribution of this taxa to the PCA.

Figure 3. Principal Component Analysis performed with the OTU diversity and abundance across samples.

The homogeneity found in the SH samples mirrors the inherent genetic uniformity of the hybrid variety. It is well established that the plant genotype profoundly influences the microbiome composition and that intensive agriculture can reduce microbiome diversity [57]. Previous research has shown that inbred maize varieties exhibit changes in microbiome recruitment over time, with more recently developed germplasms recruiting fewer microbial taxa capable of nitrogen fixation and favoring larger populations of microorganisms that contribute to nitrogen loss [58]. It is reasonable to assume that SH experienced a loss of microbiome diversity due to its long-term use in intensive agriculture when compared to SOL, which is also reflected in the high occurrence of singletons in those samples. Another critical aspect to consider is that the intensive use of chemical fertilizers and pesticides reduces the selective pressure on recruiting a supportive microbiome by plants [59], which might also be the case in commercial varieties like SH.

3.3. The Seed-Borne Microbiome Harbors Multiple Phosphate Solubilization Genes but Is Likely Unable to Fix Nitrogen

Bacteria can promote plant growth through various mechanisms, either directly or indirectly. Here we used a list of manually curated genes to investigate the presence of direct plant-growth promotion mechanisms, including nitrogen fixation, phosphate solubilization, ACC deaminase, and auxin production.

We expected a greater abundance of nitrogen fixation genes in SOL because of the gene loss driven by extensive breeding and chemical nitrogen fertilization in varieties like SH [58,60]. Contrary to our hypothesis, the *nifHDK* core nitrogenase genes were absent in both varieties. Nevertheless, we found *nifB* and *nifUS*, which are involved in the biosynthesis of the FeMo nitrogenase cofactor [61,62] and in providing the essential

Fe-S clusters for the biosynthesis of FeMo-Co [63], respectively. In the SH samples, we found *nifQ*, responsible for molybdenum incorporation, which acts in conjunction with other genes (*nifB*, *nifNV*, and *nifE*) in the biosynthesis of the nitrogenase iron-molybdenum cofactor [64,65] (Figure 4). The relevance of these *nif* genes in the absence of the core nitrogenase subunits remains unclear. However, it is important to emphasize that even non-intensive agricultural management involves nitrogen fertilization, which could reduce the selective pressure for retaining diazotrophic microorganisms in the seed-borne microbiome.

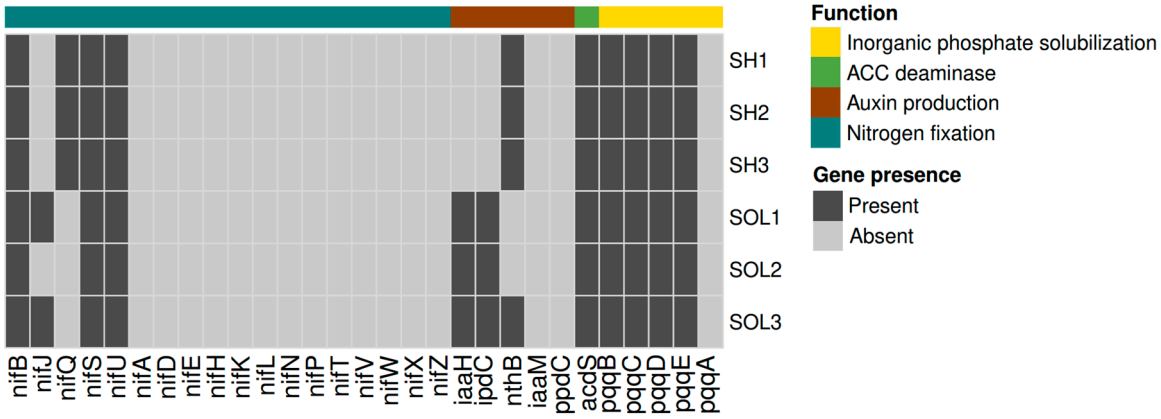

Figure 4. Functional heatmap of plant-growth promotion genes in each sample.

We found all the *pqq* genes for inorganic phosphate solubilization in all samples, except for the *pqqA* gene (not necessary for PQQ cofactor synthesis [66]). Further, phytase genes are absent in metagenomes, supporting the dominance of inorganic phosphate solubilization in the seed-borne microbiome. Seeds are usually rich in stored organic compounds such as phytates, which plants and their microbiomes can access during germination. Furthermore, inorganic phosphate availability in the soil can often be a limiting factor for plant growth [67]. Hence, the evolution of the seed-borne microbiome to prioritize the solubilization of inorganic over organic phosphate is compatible with the general availability of phosphorus in soils used in agriculture [68]. In this scenario, these microbial communities play a crucial role in supporting early seedling growth by making inorganic phosphate available, directly contributing to the seedling's immediate phosphorus demands.

3.4. The Seed-Borne Microbiome May Affect Phytohormone Production

We found distinctive patterns of auxin biosynthesis genes, with SH harboring the *nthB* gene, while SOL showed a dominance of *ipdC* and *iaaH*. These results show much greater auxin biosynthesis potential in the SOL seed-borne microbiome. In this context, we can hypothesize that increased utilization of nitrogen fertilizers could compensate for any deficiency in microbial-produced auxin, and intensive breeding might render auxin-producing microorganisms dispensable, with plant varieties that are more efficient in nitrogen utilization and auxin biosynthesis being selected [69,70].

We also found the ACC deaminase gene *acdS* in all samples. ACC deaminases convert ACC in α-ketobutyrate and ammonia [71,72], lowering ethylene levels in the plant [31]. Reduced ethylene levels can delay senescence [73] and increase plant resistance to stressful environments [74] and pathogen attacks [75]. Hence, the prevalence of *acdS* could be linked to the needs of crop plants under stressful conditions, leading to better crop performance and growth [76].

3.5. Metagenome-Assembled Genomes (MAGs) in Seed-Borne Microbiome

A total of 18 valid bacterial MAGs were recovered from the seed-borne metagenome (Table 2). We identified ten high-quality MAGs that met the stringent criteria of at least 90% genome completeness and up to 5% contamination. There were five medium-quality draft

MAGs, with 50 to 90% completeness and less than 10% contamination, and one low-quality draft MAG, with less than 50% genome completeness and more than 10% contamination. Two MAGs exhibited duplication levels exceeding 10% and were likely contaminated.

Table 2. MAGs' features and metadata. MAGs classified only at the genus level are presented between parentheses.

Assembly	Contigs	Total Length (bp)	GC (%)	N50	L50	Classification	Completeness (%)	Duplication (%)
SH1.2	67	7,326,046	66.84	159,765	12	*Burkholderia cenocepacia_B*	97.8	0.4
SH1.3	107	7,837,774	68.31	126,332	18	*Burkholderia gladioli*	91.9	0.6
SH2.1	71	7,313,029	66.85	179,016	14	*Burkholderia cenocepacia_B*	98.1	0.4
SH2.2	82	7,745,205	68.3	150,959	16	*Burkholderia gladioli*	92.1	0.4
SH3.1	66	7,322,313	66.84	188,414	14	*Burkholderia cenocepacia_B*	97.9	0.4
SH3.3	100	7,780,057	68.26	120,202	20	*Burkholderia gladioli*	93.9	0.4
SOL1.1	784	2,479,334	58	3304	264	(*Terriglobus*)	49.2	0.3
SOL1.2	472	3,628,554	71.42	12,246	96	(*Curtobacterium*)	91.3	0
SOL1.7	504	9,566,513	67.44	35,148	78	*Burkholderia gladioli*	75.5	24
SOL1.9	18	4,819,029	57.46	374,213	5	*Pantoea dispersa*	69.2	0.9
SOL1.10	58	8,220,759	65.06	258,149	10	*Paraburkholderia tropica*	95.8	1.2
SOL1.11	182	5,852,377	68.83	47,277	37	(*Burkholderia*)	51	15
SOL2.3	12	4,843,396	57.44	690,450	2	*Pantoea dispersa*	73.6	0.9
SOL2.4	92	8,328,544	67.93	222,006	12	*Burkholderia gladioli*	99.2	0.7
SOL3.3	20	4,859,991	57.45	416,592	4	*Pantoea dispersa*	74.7	0.9
SOL3.4	661	3,263,738	71.34	5741	171	(*Curtobacterium*)	76.1	0.3
SOL3.6	1435	8,789,041	64.69	8009	303	*Paraburkholderia tropica*	59.2	3.1
SOL3.8	203	7,160,874	68.82	62,203	41	*Burkholderia gladioli*	95.2	0.4

Remarkably, we discovered four MAGs belonging to novel species, including two from the *Curtobacterium*, one from *Burkholderia*, and one from *Terriglobus*. These four novel MAGs were found in the SOL samples, with SOL1.2 being classified as high quality according to the MIMAG methodology [48]. Furthermore, the two unknown *Curtobacterium* MAGs were 99% identical, supporting their affiliation with the same novel species.

It is reasonable to assume that identifying a high-quality MAG is more common for abundant strains. Thus, the independent recovery of the same MAG in different samples strongly supports its robust occurrence in the seed-borne microbiome. Species such as *Burkholderia gladioli*, found in all six samples, might have an essential role in the seed-borne microbiome. Importantly, given the ANI values greater than 99%, we probably found the same *B. gladioli* strain in all six samples. *B. gladioli* has been previously reported as a beneficial seed-borne bacteria, showing a good repertoire of phosphatases and no nitrogen fixation genes [77], which is consistent with the results presented herein. *B. gladioli* has also been shown to enhance plant resistance to *Fusarium oxysporum* by inducing fungal cell death [78]. We hypothesize that the presence of *B. gladioli* in all samples could represent a seed-borne biocontrol mechanism to counter phytopathogenic fungi during germination and early post-germination development.

Another interesting MAG is that of a *B. cenocepacia* strain found in all SH samples. *B. cenocepacia* is also a known biocontrol agent [79] generally associated with *Fusarium* wilt protection. Furthermore, *B. cenocepacia* has been reported to protect against *Fusarium* root rot by controlling other *Fusarium* species that may interact with *F. oxysporum* [80]. The co-occurrence of *B. cenocepacia* with *B. gladioli* in the SH seed-borne microbiome may indicate an evolving system in which the maize plant recruits biocontrol agents. Our findings also suggest that these two strains are compatible and could work together, either additively or synergistically. With further validation, these strains might be utilized in a novel biocontrol strategy.

4. Concluding Remarks

In this study, we demonstrated that a landrace maize variety has a more diverse and less uniform (between samples) seed-borne microbiome than a hybrid commercial variety. We identified a higher potential for phytohormone production in the landrace microbiome. Our exploration of MAGs revealed that certain taxa are consistently present across different varieties, supporting the existence of a core seed-borne microbiome. Additionally, we observed an unexpected absence of *nif* genes in both varieties, which may be linked to the widespread use of nitrogen fertilization, even in non-extensive agricultural environments. Understanding the dynamics of seed-borne microbiome evolution could guide the development of novel precision agriculture applications.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/seeds3040035/s1, Table S1: Metagenomic classification of reads to their higher classification rank, Table S2: Metataxonomy classification based on 16S rRNA.

Author Contributions: Conceptualization: S.H.-J., F.L.O. and T.M.V.; formal analysis: S.H.-J.; computational methodology: S.H.-J. and F.P.-S.; wet lab methodology; B.E.B.C.; writing—original draft: S.H.-J. and T.M.V.; writing—review and editing: S.H.-J., B.E.B.C., F.L.O. and T.M.V.; funding acquisition: F.L.O. and T.M.V.; project administration: F.L.O. and T.M.V. All authors have read and agreed to the published version of the manuscript.

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