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Impact of different treatment methods and timings on soil microbial communities with transgenic maize straw return

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Understanding the impact of genetically modified (GM) crop straw return on soil ecosystems is crucial as GM crops become more prevalent. This study assesses the effects of straw mulching and deep tillage on soil microbial communities from GM and non-GM maize, highlighting potential ecological impacts. Shotgun metagenomic sequencing was utilized to analyze the microbial community structure and functional genes in soil samples collected at different times (30, 180, and 270 days) after straw mulching and deep tillage treatments. The study included insect-resistant transgenic maize varieties 2A-7 and CM8101 and their non-transgenic counterparts B73 and Zheng58. Different treatment methods significantly affect soil microbial alpha-diversity and beta-diversity, with deep tillage resulting in higher alpha-diversity compared to mulching, and the 180-day mark exhibiting the highest alpha-diversity across all sampling times. Early straw treatment prompted a rapid microbial response to nutrient availability, with notable changes in diversity and function over time. Straw treatments notably altered soil microbial functions, especially in carbon cycling and nutrient metabolism. Interestingly, the microbial effects of GM versus non-GM maize straw were similar, suggesting crop residue type under consistent soil management practices might not significantly alter microbial community structures. The methods and timing of straw treatments have a significant impact on soil microbial communities, surpassing the differences between GM and non-GM straw. These findings highlight the importance of straw management practices for sustainable agricultural ecosystem management.

Keywords Soil microbiology, Genetically modified crops, Straw decomposition, Metagenomic sequencing, Agricultural sustainability

Soil, being a critical component of terrestrial ecosystems on Earth, plays a pivotal role in crop growth, ecological equilibrium, and environmental quality, as its health directly influences these factors[1]. As a crucial component of the soil ecosystem, soil microorganisms play a key role in nutrient cycling and plant health, owing to their diversity and functional capabilities[2]. In recent years, with the advancement and application of genetic engineering technologies, genetically modified (GM) crops have been widely cultivated due to their advantages in increasing yield and resistance to pests and diseases[3]. However, the potential impacts of incorporating the residual biomass of GM crops into soil on the structure and function of soil microbial communities remain unclear.

The treatment of GM crop residues may exert an important impact on soil microbial communities. Mulching or deep tillage are prevalent methods for incorporating crop residues, which can alter soil structure and enhance soil organic matter, potentially impacting the composition and functionality of soil microbial communities[4,5]. Although previous studies have suggested that GM crops may affect soil microbial communities, most have focused on the crop growth period[6], and studies on the long-term effects of residue incorporation into soil remain relatively limited.

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Among various crop types, maize warrants particular attention. Given its global cultivation scale and central role in food systems, maize is a key focus in studies concerning agricultural sustainability and soil health[7]. Due to its large planting scale and high biomass, maize residues returned to the soil significantly affect the structure and function of soil microbial communities[8]. GM maize has been widely adopted worldwide. According to the ISAAA database[9], over 300 GM maize transformation events have been developed, many of which have received regulatory approvals for food, feed, or cultivation in multiple countries. These events involve various target traits, including insect resistance, herbicide tolerance, quality improvement, and altered growth or yield. In 2019, the global planting area of GM maize reached approximately 60.9 million hectares, accounting for about 32% of total maize production. The adoption of GM crops has led to substantial economic benefits[10]. Therefore, systematically evaluating the effects of GM maize straw incorporation on the composition and function of soil microbial communities is of great scientific importance and provides critical evidence for environmental safety assessments of GM crops.

Transgenic insect-resistant maize CM8101, developed by the Institute of Crop Science, Chinese Academy of Agricultural Sciences, using Zheng58 as the genetic background, has shown significant insecticidal effects against the Asian corn borer[11]. Studies indicate that CM8101 does not significantly affect the main physicochemical properties and enzyme activities of rhizosphere soil[12], nor does it impact the springtails, earthworms[13], or the rhizosphere microbial community and its functions[14]. Similarly, transgenic insect-resistant maize 2A-7, based on the B73 maize genetic background with the introduction of *mcry1Ab* and *mcry2Ab* genes, enables the host plant to produce *Bacillus thuringiensis* (Bt) toxins, thereby effectively resisting various pest attacks. Research demonstrates that this transgenic maize has no significant impact on rhizosphere microorganisms[15].

Transgenic maize 2A-7 and CM8101 have obtained safety certificates in China and have shown promising application prospects. The main objectives of this study are to systematically evaluate the effects of insect-resistant GM maize straw, under two incorporation methods (mulching and deep tillage), on the structure, diversity, and functional potential of soil microbial communities. Utilizing shotgun metagenomic sequencing technology, a comparative analysis was conducted on the community structure, diversity, and functional genes in soil microbiota before and after different treatments. This study provides a deeper understanding of the impacts of GM crop straw and residues on soil microbial communities, which is crucial for assessing the sustainability of GM crops within agricultural ecosystems. This research supports the environmental impact assessments for the large-scale industrialization of GM crops, offering important scientific data with significant prospective and practical relevance. Additionally, these findings will contribute to more comprehensive scientific bases for environmental risk assessments and management strategies for GM crops.

Results

Soil and straw component analysis

This study measured the total nitrogen, phosphorus, and potassium content of soil and powdered straw samples treated differently, and also determined the moisture content of the soil samples (Table 1). Non-transgenic Zheng58 (CM8101_CK) and B73 (2A-7_CK) maize straw samples showed differences in total potassium content, with Zheng58 having higher levels. In comparisons between transgenic and non-transgenic straws, transgenic Zheng58 maize straw carrying the CM8101 gene exhibited higher total nitrogen content than its non-transgenic counterpart, suggesting an enhanced capacity for nitrogen absorption or utilization. Transgenic B73 maize straw with the 2A-7 gene showed lower nitrogen content but higher potassium content, which may indicate changes in the metabolism or absorption mechanisms for nitrogen and potassium. These results provide baseline data for subsequent analyses of the impact of straw return on soil microbial communities.

Microbial alpha diversity analysis

This study assessed the impact of transgenic straw treatment on the Alpha diversity of soil microbial communities, including species richness index, Shannon diversity index, and Simpson diversity index (Fig. 1). The results indicated that soil samples treated with straw exhibited significant differences in Alpha diversity compared to the untreated control (p-values were not indicated in the figure). However, comparisons of soil microbiota treated with GM straw to those treated with non-GM straw revealed no significant statistical differences in species richness, Shannon index, or Simpson index (with p-values ranging from 0.1 to 1.0), indicating that GM does not affect the Alpha diversity of soil microbial communities (Fig. 1A,B).

As time progressed, the Alpha diversity of the soil microbial communities exhibited significant variations (Fig. 1C). Notably, in nearly all time-point comparisons, significant statistical variations were observed in alpha-diversity indexes, with alpha-diversity reaching its peak at the M6 time point. However, a significant decrease in alpha-diversity was noted at the M9 time point. This pattern may reflect the dynamic changes in soil alpha-

Sample	Total nitrogen (%)	Total phosphorus (%)	Total potassium (%)	Moisture (%)
Soil	0.142 ± 0.003	0.066 ± 0.001	2.146 ± 0.035	4.198 ± 1.115
CM8101_CK	0.713 ± 0.029	0.068 ± 0.005	1.150 ± 0.022	–
CM8101_T	0.785 ± 0.024	0.078 ± 0.005	1.135 ± 0.061	–
2A-7_CK	0.600 ± 0.010	0.070 ± 0.000	0.633 ± 0.017	–
2A-7_T	0.340 ± 0.066	0.040 ± 0.000	1.068 ± 0.051	–

Table 1. Total nitrogen, phosphorus, potassium, and moisture content in soil and differently treated straw samples.

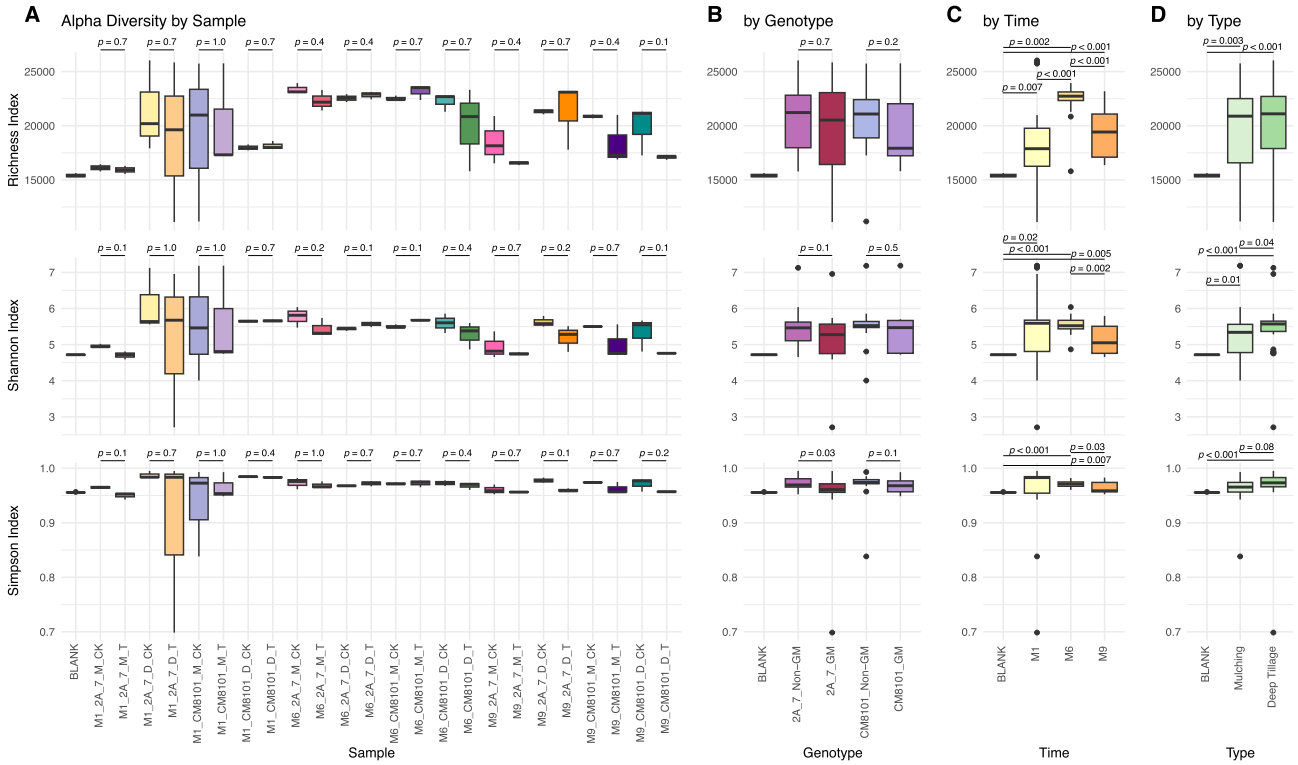

Fig. 1. Comparative analysis of soil microbial alpha diversity across different genotypes, time points, and treatment methods. Alpha diversity indices are presented across different groups: (**A**) individual samples, (**B**) genotypes, (**C**) sampling times, and (**D**) treatment types. Labels:'BLANK'—soil samples without straw;'M'—mulching with straw surface layer;'D'—deep tillage with straw mixed in. Time points:'M1','M6','M9'—months post-treatment. Gene types:'2A_7','CM8101';'CK'—non-transgenic,'T'—transgenic. P-values indicate statistical comparisons.

diversity following straw decomposition. Specifically, the initial phase of straw decomposition might have provided additional nutrients, fostering an increase in microbial diversity, which led to the observed peak in alpha-diversity at the M6 time point. However, over time, these nutrients might have been progressively depleted by the microbial community, leading to a decline in microbial diversity at the M9 time point. These observations underscore the significant impact of sampling time on the alpha-diversity of soil microbial communities.

In assessing the impact of straw treatment methods on soil microbial communities'alpha diversity, comparisons between mulching and deep tillage revealed notable differences (Fig. 1D). The Shannon diversity index showed a p-value of 0.04 and the Simpson index had a p-value of 0.08, indicating that straw treatment methods substantially influence soil microbial Alpha.

In summary, straw treatment notably affected the Alpha diversity of soil microbial communities, yet no significant differences were observed statistically between GM and non-GM straw treatments. Concurrently, sampling time had a significant impact on the Alpha diversity of soil microbial communities, while the specific methods of straw treatment (mulching versus deep tillage) also exhibited notable influence.

Microbial beta diversity analysis

PCoA was utilized to assess beta diversity, revealing the impact of various factors on the distribution patterns of beta-diversity (Fig. 2). In the temporal dimension, the distinct separation between samples indicates that the composition of soil microbiota underwent significant changes over time, reflecting that time is a key factor affecting beta-diversity. The changes in the distribution of samples at different time points reflect the impact of straw decomposition in soil on the composition of microbial communities. In contrast, the treatment methods of mulching and deep tillage showed no significant differences in the microbial community structure on PCo1 and PCo2 axes, indicating that these treatment methods, while different in approach, result in similar impacts on microbial beta-diversity.

Regarding genotypes, the overlap of samples indicates that the straw treatments from different genotypes do not significantly alter the beta-diversity of the soil microbial community. Similarly, no notable distinction in beta-diversity was observed between samples on the level of GM versus non-GM, suggesting that the transgenic traits of the straw do not decisively alter the overall structure of the microbial community. This could be because the microbial communities are not particularly sensitive to the transgenic traits of the straw, or the transgenic traits do not significantly change the overall chemical composition of the straw, thereby exerting a limited impact on the microbial community.

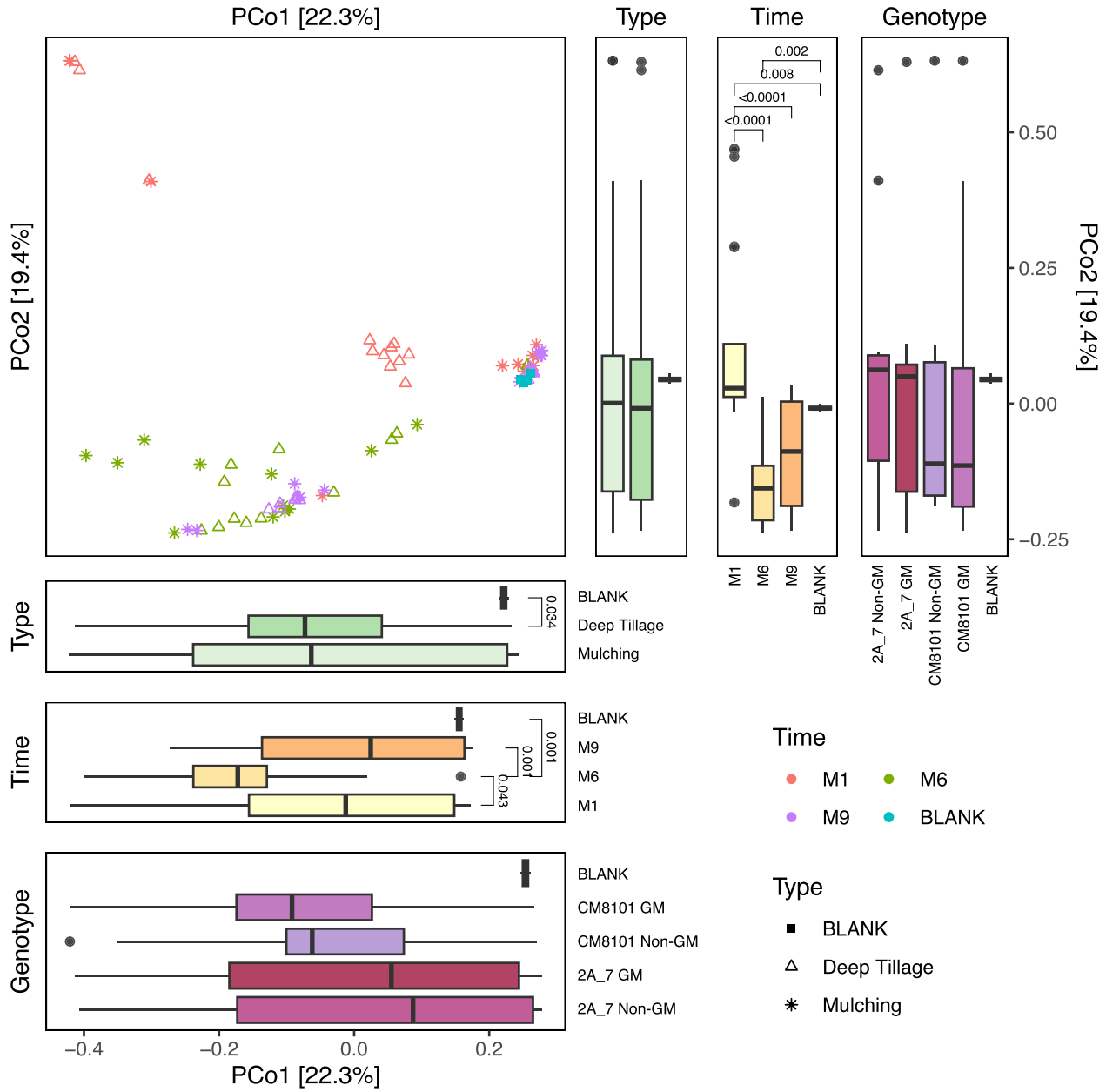

Fig. 2. Principal coordinates analysis of soil microbial communities differentiated by genotypes, time points, and treatment methods. The PCoA plot (top left) shows the distribution of samples, with colors representing sampling times and shapes representing treatment types. Each point represents one sample. The boxplots (right and bottom) display the distribution of PCoA scores along the first and second axes (PCo1 and PCo2) across Type, Time, and Genotype groups, with associated p-values indicating statistically significant differences between groups. Labels: 'M1', 'M6', 'M9' – months post-treatment; 'BLANK' – no straw; 'M' – mulching; 'D' – deep tillage. Statistical significance was assessed using pairwise Wilcoxon tests.

Overall, these results accentuate the significant effect of temporal changes related to straw decomposition on soil microbial beta-diversity and also suggest the potential influences of treatment methods, genotypes, and GM status on the beta-diversity of soil microbial communities.

Differences in microbial community composition

The phylum-level stacked bar chart represents the relative abundance of various microbial phylum across different samples (Fig. 3A). One month into the experiment (at sampling point M1), considerable differences in the composition of microbial phyla were observed among groups based on different genotypes and treatment methods. This indicates that GM straw and soil management strategies have important initial impacts on soil microbial diversity. Specifically, deep tillage treatment in Zheng58 (CM8101_CK/T) significantly decreased

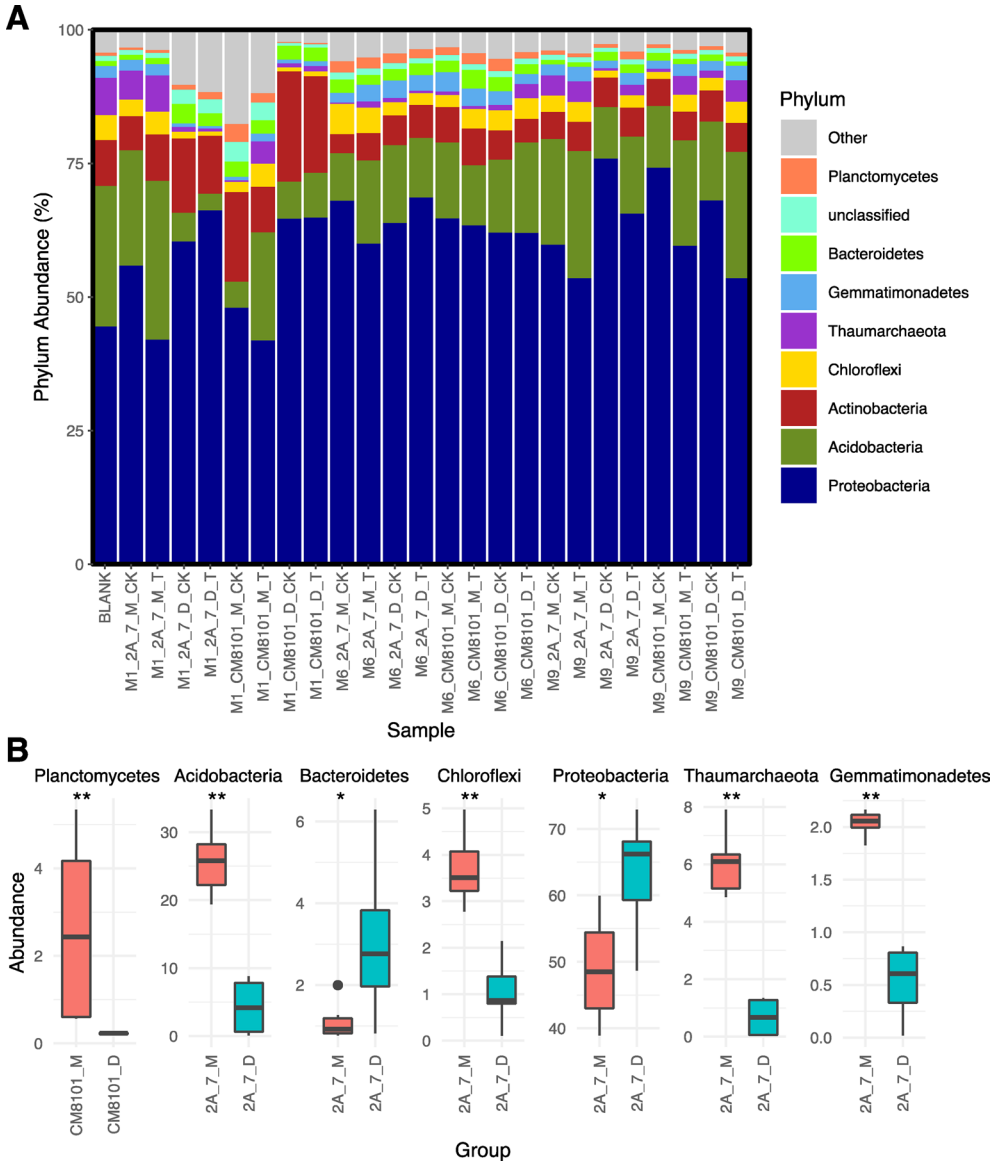

Fig. 3. Microbial community composition at the phylum level and phylum abundance across different treatment groups. (**A**) Relative abundance of major bacterial phyla in soil samples. (**B**) Boxplots displaying the abundance of select bacterial phyla across different groups. * for $p < 0.05$ and ** for $p < 0.01$. Labels:'BLANK'—soil samples without straw;'M'—mulching with straw surface layer;'D'—deep tillage with straw mixed in. Time points:'M1','M6','M9'—months post-treatment. Gene types:'2A_7','CM8101';'CK'—non-transgenic,'T'—transgenic. P-values indicate statistical comparisons.

the abundance of Planctomycetes, whereas the same treatment in B73 (2A-7_CK/T) markedly decreased the abundance of Acidobacteria, Chloroflexi, Thaumarchaeota, and Gemmatimonadetes, and increased the abundance of Bacteroidetes and Proteobacteria, indicating the distinct impacts of maize straw from different cultivars on the soil microbial community structure (Fig. 3B).

As time progressed to six months (sampling point M6), the microbial composition of the various treatment groups began to converge, suggesting that the soil microbial community composition is moving towards a new equilibrium after the initial disturbance. This convergence may indicate a certain resilience within the soil microbial communities, which are capable of adapting to environmental changes brought about by the cultivation of GM straw. By the nine-month mark of the experiment (sampling point M9), a reduction in the relative abundance of Proteobacteria was observed across all GM straw sample groups compared to non-GM groups, with proportions more closely aligning with initial conditions. These results reflect the impact of long-term straw return on soil ecological changes, which may be associated with factors such as the type of crops, GM traits, farming practices, and sampling time points (Fig. 3A).

The composite volcano plot (Fig. 4) illustrates the significant variations in microbial community composition at the genus level due to different treatment methods and genetic modification. Additionally, a supplementary

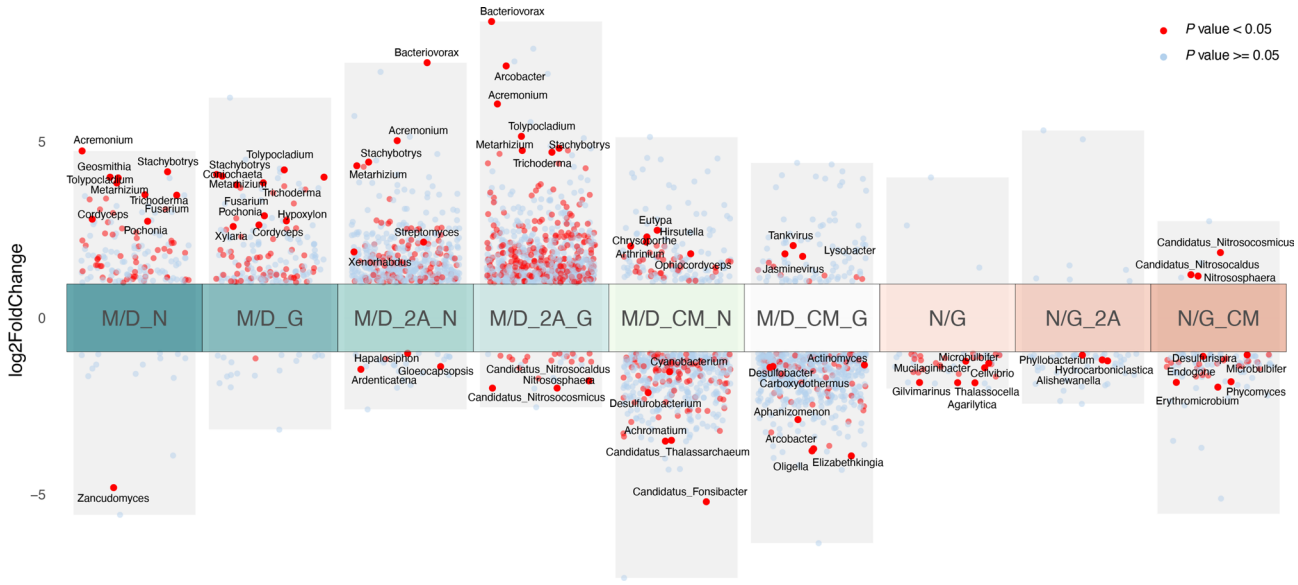

Fig. 4. Significant microbial community changes in response to treatment and genetic modification. The composite volcano plot illustrates changes in microbial community composition at the genus level across different treatment methods (deep tillage vs. mulching, represented in top and bottom rows of the M/D panels) and genetic modifications (GM vs. non-GM, shown in top and bottom rows of the G/N panels). Each data point marks a genus, with significant changes highlighted in red (P value < 0.05) and non-significant changes in blue (P value ≥ 0.05). Key microbial genera are annotated to denote increased or decreased abundance due to treatments.

table is provided to display variations at the species level (Supplementary Material 1). Compared to mulching, deep-tillage samples exhibited significant abundance changes across numerous genera. In all non-transgenic samples, mulching significantly enriched *Zancudomyces* (*Zancudomyces culisetae*), while deep tillage led to an enrichment of *Acremonium* (*Acremonium chrysogenum*), *Stachybotrys* (*Stachybotrys chartarum*, *Stachybotrys chlorohalonata*), *Geosmithia* (*Geosmithia morbida*), *Tolypocladium* (*Tolypocladium ophioglossoides*, *Tolypocladium paradoxum*), *Metarhizium* (*Metarhizium robertsii*), *Trichoderma* (*Trichoderma harzianum*, *Trichoderma gamsii*), *Cordyceps*, and *Pochonia*, among others. In transgenic samples, the genera enriched in deep tillage were similar to those found in non-transgenic deep-tilled samples, although differences may exist at the species level (such as *Metarhizium anisopliae*). For the non-transgenic 2A-7 B73 maize straw soil samples, mulching treatment resulted in an enrichment of *Hapalosiphon*, *Gloeocapsopsis*, and *Ardenticatena*, while deep tillage led to an enrichment of genera such as *Bacteriovorax*, *Acremonium* (*Acremonium chrysogenum*), *Stachybotrys* (*Stachybotrys chartarum*), *Metarhizium* (*Metarhizium robertsii*), *Streptomyces* (*Streptomyces fulvorobeus*), and *Xenorhabdus*. In the 2A-7 transgenic B73 maize straw soil samples, mulching treatment enriched genera including *Candidatus Nitrosocaldus*, *Candidatus Nitrosocosmicus*, and *Nitrososphaera*, whereas deep tillage exhibited a similar enrichment of genera to that found in non-transgenic samples with some differences at the species level (such as *Stachybotrys chlorohalonata*, *Metarhizium anisopliae*). In the non-transgenic CM8101 Zheng58 maize straw soil samples, the mulching treatment enriched genera such as *Cyanobacterium*, *Desulfurobacterium*, *Candidatus Fonsibacter* (*Candidatus Fonsibacter lacus*), *Candidatus Thalassarchaeum*, and *Achromatium*, whereas deep tillage led to an enrichment of *Eutypa* (*Eutypa lata*), *Hirsutella*, *Chrysoporthe*, *Arthrinium*, and *Ophiocordyceps*. In the CM8101 transgenic Zheng58 maize straw soil samples, the mulching treatment resulted in the enrichment of genera including *Actinomyces*, *Desulfobacter*, *Carboxydothermus*, *Aphanizomenon*, *Arcobacter*, *Elizabethkingia*, and *Oligella*, while deep tillage enriched *Tankvirus*, *Lysobacter*, and *Jasminevirus* among others. Comparisons between non-transgenic and transgenic samples revealed significant enrichment of *Microbulbifer*, *Mucilaginibacter*, *Cellvibrio*, *Gilvimarinus*, *Thalassocella*, and *Agarilytica* in non-transgenic samples, with no significant enrichment observed in the transgenic counterparts. In the non-transgenic versus transgenic comparison of 2A-7 maize, non-transgenic samples were notably enriched with *Phyllobacterium*, *Hydrocarboniclastica*, and *Alishewanella*, while no significant enrichment of any genus was detected in transgenic samples. Lastly, in the comparison between non-transgenic and transgenic CM8101 maize, non-transgenic samples showed significant enrichment of *Desulfurispira*, *Microbulbifer*, *Endogone*, *Phycomyces*, and *Erythromicrobium*, whereas transgenic samples were enriched with *Candidatus Nitrosocosmicus*, *Candidatus Nitrosocaldus*, and *Nitrososphaera*. Overall, both transgenic and non-transgenic samples demonstrated similar shifts in microbial communities under the same treatment conditions. Moreover, the number of microbial changes induced by different management practices exceeded those caused by genetic modification. These findings suggest that management practices have a more significant impact on microbial diversity than genetic engineering.

Figure 5 illustrates the changes in microbial genera over time in GM and non-GM samples compared to the blank control (BLANK). Variations at the species level are detailed in Supplementary Material 2. At time point

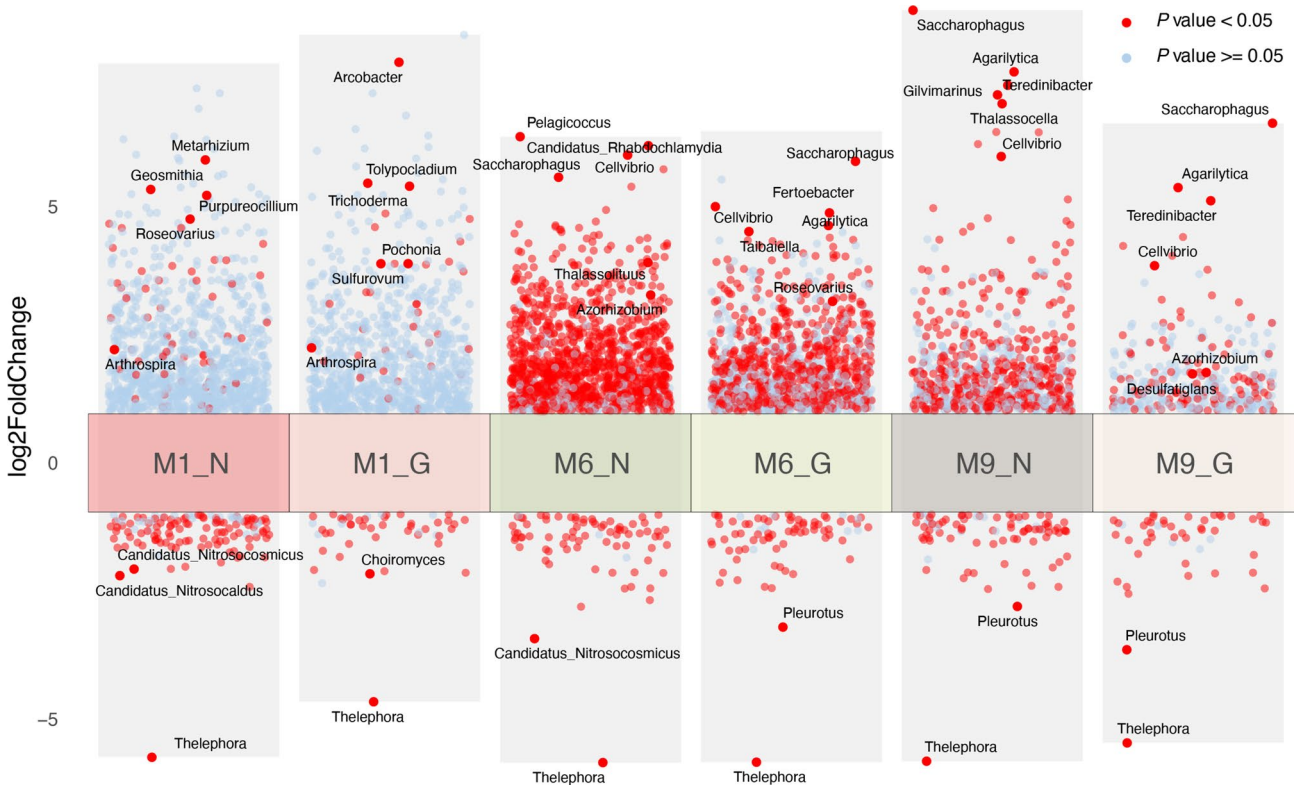

Fig. 5. Significant microbial community changes in response to time. This plot visualizes microbial genus shifts over three time points (1, 6, and 9 months), comparing Non-GM and GM samples across these periods. Each dot represents a genus, with significant abundance changes shown in red (P value < 0.05) and non-significant in blue (P value ≥ 0.05). The arrangement from left to right highlights temporal variations, with a clear distinction between GM and Non-GM conditions. The'BLANK'section at the bottom of each panel serves as a control reference.

M1, non-GM samples exhibited a significant decrease in genera such as *Candidatus Nitrosocosmicus*, *Candidatus Nitrosocaldus*, and *Thelephora* (*Thelephora ganbajun*), while genera such as *Metarhizium* (*Metarhizium anisopliae*), *Geosmithia*, *Purpureocillium* (*Purpureocillium lilacinum*), *Roseovarius*, and *Arthrospira* were significantly enriched. In GM samples, genera like *Choiromyces* and *Thelephora* (*Thelephora ganbajun*) were reduced, with an enrichment of *Arcobacter*, *Tolypocladium* (*Tolypocladium ophioglossoides*), *Trichoderma*, *Pochonia*, *Sulfurovum*, *Arthrospira*, *Flavobacterium akiainvivens*, *Agrobacterium tumefaciens*, etc. At time point M6, non-GM samples showed a notable decrease in genera like *Candidatus Nitrosocosmicus* and *Thelephora* (*Thelephora ganbajun*), with an increase in *Pelagicoccus*, *Candidatus Rhabdochlamydia*, *Saccharophagus*, *Cellvibrio*, *Thalassolituus*, and *Azorhizobium*. GM samples demonstrated a reduction in *Pleurotus* and *Thelephora* (*Thelephora ganbajun*), with an augmentation of *Saccharophagus*, *Fertoebacter*, *Cellvibrio*, *Agarilytica*, *Taibaiella*, and *Roseovarius*. By time point M9, both non-GM and GM samples revealed a marked decline in *Pleurotus* and *Thelephora* (*Thelephora ganbajun*). Non-GM samples were characterized by an enrichment of *Saccharophagus*, *Agarilytica*, *Gilvimarinus*, *Teredinibacter*, *Thalassocella*, and *Cellvibrio*, whereas GM samples were abundant in *Saccharophagus*, *Teredinibacter*, *Agarilytica*, *Cellvibrio*, *Azorhizobium*, and *Desulfatiglans*. Overall, the microbial diversity changes were most abundant at the M6 sampling point. By the M9 sampling point, this diversity had diminished, potentially correlating with the straw decomposition process. Based on the number of significantly changed genera, it can be inferred that straw decomposition might proceed more rapidly in GM straw, or that the soil microbial community shifts in GM straw soils are less pronounced compared to non-GM straw soils relative to blank soil.

Based on the characteristic microbial communities identified by LEfSe analysis, a heatmap was constructed using the average proportions of these microbes in each group of samples. To enhance clarity of the inter-group variations, the data were normalized. The heatmap (see Supplementary Material 3) reveals that at the M1 timepoint, samples from deep tilling treatments of both transgenic and non-transgenic Zheng58 maize were markedly enriched in the Actinobacteria class. Cover treated samples of transgenic CM8101 maize collected at M1 and M6 timepoints showed a significant enrichment of microbes belonging to the classes Cytophagia, Anaerolineae, and Gemmatimonadetes. At the M6 timepoint, the 2A-7 transgenic samples were distinctly enriched in Deltaproteobacteria. Additionally, Gammaproteobacteria and Betaproteobacteria were predominantly distinguished in samples from the M1 timepoint. Overall, samples from the M1 timepoint demonstrated certain degrees of distinction in microbial composition compared to those from M6 and M9 timepoints, which may be

attributed to the differential impacts of treatment methods on soil microbial community structure associated with the timing of sampling.

KEGG and eggNOG-based functional characterization

Upon analyzing the impact of straw treatment on soil microbial communities, significant differences in the presence of KEGG metabolic pathways among treatment groups were observed (Fig. 6). Compared to the untreated soil, straw treatment predominantly influenced pathways associated with carbon fixation and cycling,

Fig. 6. KEGG pathway analysis across different maize straw soil treatments. KEGG pathway enrichment analysis showing significantly enriched microbial metabolic pathways identified by LEfSe analysis in each sample group. Circle size represents Linear Discriminant Analysis (LDA) scores, reflecting the significance of pathway enrichment. Color gradient indicates the logarithmic scale (Logarithm Value) of pathway abundance. Sample labels indicate sampling times (M1: 1 month, M6: 6 months, M9: 9 months), Gene types (2A_7, CM8101), treatment methods (M: mulching, D: deep tillage), and genetic modification status (CK: non-transgenic, T: transgenic)."BLANK"represents untreated soil without straw addition.

such as carbon fixation pathways in prokaryotes, metabolism of 2-oxocarboxylic acids, and metabolism of acetic acid and dicarboxylic acids, as well as pathways related to protein synthesis and degradation, including RNA polymerase, ribosome, and protein export. At the M1 time point, the samples demonstrated significant presence in energy and nutrient metabolism, such as metabolism of fructose and mannose, galactose metabolism, and pyruvate metabolism, indicating that straw provides a rich carbon source for soil microbes. Additionally, the cycling of sulfur and nitrogen was significantly affected, including sulfur metabolism and amino acid metabolism, showcasing the microbial transformation and utilization of nitrogen and sulfur sources during straw decomposition. The presence of functions in the synthesis and breakdown of secondary metabolites (e.g., tryptophan metabolism, porphyrin, and chlorophyll metabolism), as well as nucleic acid and energy metabolism (e.g., purine metabolism, pyrimidine metabolism, one carbon pool by folate), reflects the rapid microbial response to straw addition, involving key biological processes such as biosynthesis, energy metabolism, and environmental adaptation. As time progressed to the M6 and M9 sampling points, the differences in metabolic pathway presence among the samples began to diminish. At the M6 time point, samples showed an enrichment in pathways related to amino acid biosynthesis, fatty acid biosynthesis, and starch and sucrose metabolism, indicating that the microbial community had undergone certain adaptive adjustments to the environmental conditions. By the M9 time point, samples exhibited enrichment in pathways such as butyrate metabolism, fatty acid degradation, nitrogen metabolism, and pyruvate metabolism, suggesting that the microbial metabolic activities had gradually shifted toward the degradation of complex organic matter, energy recovery, and nitrogen transformation and reutilization during prolonged straw decomposition. These changes reflect the community's adaptive response to alterations in nutrient availability. Furthermore, differences in KEGG functional presence were observed between the mulching and deep tillage treatment samples. Although the deep tillage treatment resulted in a significantly richer microbial community and higher alpha diversity compared to the mulching treatment, the KEGG functional diversity was richer in the mulching samples. This discrepancy may be attributed to the distinct microenvironments provided by the mulching and deep tillage treatments. While deep tillage enhances microbial species richness by thoroughly mixing straw with the soil, mulching may lead to a specialization in metabolic functions, as reflected in the enrichment of KEGG functions. Overall, straw treatment significantly affected the presence of KEGG metabolic pathways in soil microbial communities, revealing the facilitative role of straw decomposition on soil microbial functional diversity and ecosystem stability. It also demonstrated the varying impacts of different treatment methods and time on the functional potential of soil microbial communities.

The results of the eggNOG Pathway analysis (Fig. 7) indicated that at the M1 sampling time point, a high level of potential functionality was noted in multiple metabolic pathways, particularly nucleotide transport and metabolism (F), inorganic ion transport and metabolism (P), and carbohydrate transport and metabolism (G). This heightened presence is likely a response to the increased availability of substrates following the addition of straw. By the M6 time point, while the differential presence of some pathways diminished, an increase in the presence of functions related to cell wall, membrane, and envelope biogenesis (M), as well as cell motility (N), suggested a possible trend toward stabilization of the soil microbial community over time. However, at the M9 time point, enhanced presence of functions was again observed in pathways such as amino acid transport and metabolism (E), lipid transport and metabolism (I), secondary metabolites biosynthesis, transport, and catabolism (Q), and signal transduction mechanisms (T), reflecting the microbial community's adaptation to the prolonged decomposition of straw. In transgenic samples, a particularly pronounced presence of functions was evident in pathways involved in defense mechanisms (V), amino acid transport and metabolism (E), lipid transport and metabolism (I), and secondary metabolites biosynthesis, transport, and catabolism (Q). Collectively, these data reveal the specific impacts of straw management practices on soil microbial functional diversity in both transgenic and non-transgenic crops. Additionally, the findings illustrate the dynamic changes in microbial community functional potential across different time points.

Discussion

The results in this study indicate that the impact of transgenic maize straw on the diversity and community structure of soil microbiota is limited compared to the effects of time progression and treatment methods. Microbial diversity peaked at the M6 time point, while microbial functions were most abundant at the M1 time point. Deep tillage promoted an increase in biodiversity, whereas mulching led to a concentration of microbial functions. These findings underscore that straw return method and time are the primary factors shaping microbial community structure and function, while the impact of transgenic traits is relatively limited.

Previous studies have shown that during straw return processes, different crop straws exhibit unique patterns of microbial diversity and activity at various time points. For instance, research on rice straw return found the highest alpha diversity of microbes at 137 days, with microbial activity being more vigorous in the early phase (0–20 days) and slowing down in the later phase (104–137 days)[16]. Similarly, an oat straw return study observed that microbial diversity was higher in the sixth month than in the first month, although microbial activity measured by soil respiration rate was higher initially and then decreased[17]. Consistent with those findings, this study revealed that microbial diversity at the M6 time point (180 days) was significantly higher than at the M1 time point (30 days), and microbial functional activity peaked at M1 before showing a declining trend. This may be attributed to the release of soluble organic matter and nutrient stimulation during the early stage of straw decomposition, which promotes the activity of microbial communities that utilize fresh organic substrates, particularly certain functional microbes capable of rapidly responding and initiating the breakdown of easily degradable components such as cellulose and pectin[18,19]. At the M1 time point, the enriched pathways were primarily associated with the utilization of readily available nutrients such as soluble sugars, amino acids, and sulfur metabolism, indicating that microbes first responded to the easily degradable components released during the early phase of straw decomposition by activating rapid energy acquisition mechanisms. In addition, the

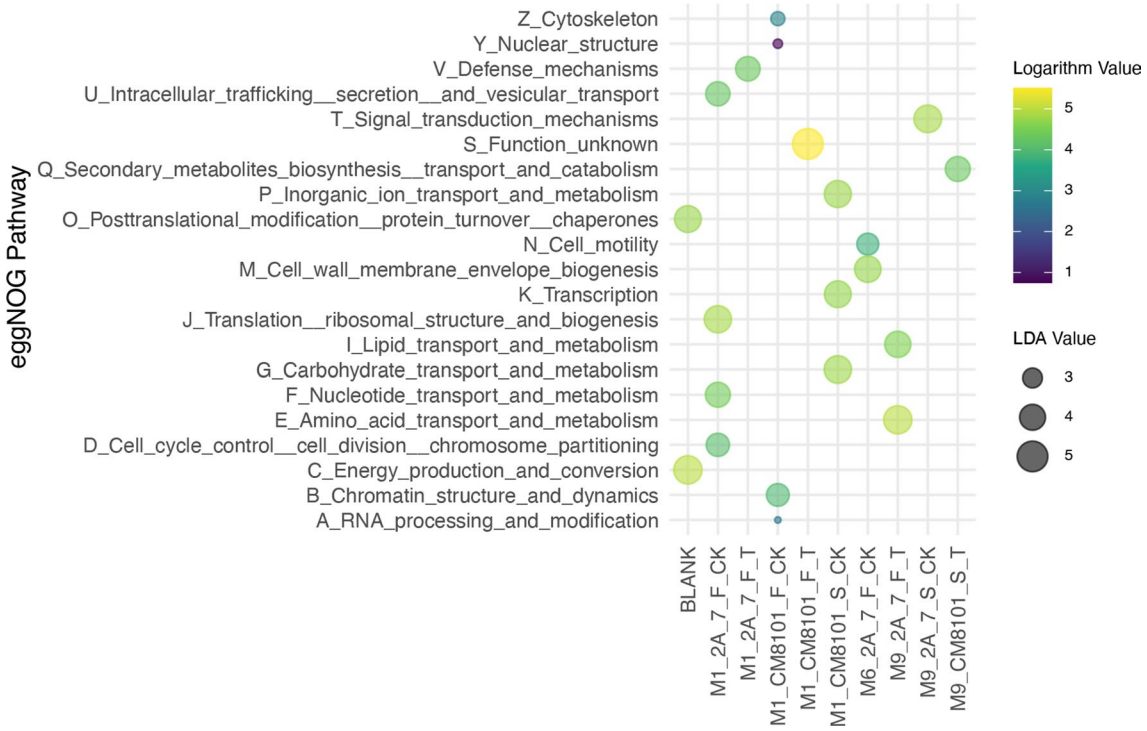

Fig. 7. EggNOG pathway analysis across different maize straw soil treatments. eggNOG functional category enrichment analysis showing significantly enriched functional categories identified by LEfSe analysis in each sample group. Circle size represents Linear Discriminant Analysis (LDA) scores, reflecting the degree of enrichment significance. The color gradient indicates the logarithmic scale (Logarithm Value) of the relative abundance of each functional category. Sample labels specify sampling times (M1: 1 month, M6: 6 months, M9: 9 months), Gene types (2A_7, CM8101), treatment methods (M: mulching, D: deep tillage), and genetic modification status (CK: non-transgenic, T: transgenic).“BLANK”indicates untreated soil without straw addition.

enhancement of energy and nutrient related pathways such as nucleotide transport and metabolism, inorganic ion transport and metabolism, and carbohydrate transport and metabolism further supports the microbial community’s rapid response to the influx of fresh organic substrates.

As decomposition progresses, the proportion of recalcitrant components in the straw increases, and microbial metabolic byproducts gradually accumulate in the soil. These changes in soil environment and available substrates may lead to dynamic shifts in the composition and functional potential of the microbial community[19]. At the M6 time point (180 days), the enriched pathways shifted toward fatty acid biosynthesis, amino acid biosynthesis, and starch and sucrose metabolism, indicating that microbes began to utilize more complex organic substrates to sustain their metabolic activity and community growth. At the same time, alpha diversity reached its highest level. By the 180-day mark, a considerable portion of the straw had been decomposed, with other studies indicating a decomposition rate of about 50%[20], leading to a relative decrease in available nutrients. By the M9 time point (270 days), the enriched pathways included butyrate metabolism, fatty acid degradation, nitrogen metabolism, and pyruvate metabolism. During this stage, the microbial community enhanced its capacity to decompose complex organic matter and recover energy to maintain community stability and metabolic balance. Meanwhile, microbial diversity slightly declined, which may also be associated with reduced resource availability.

As time progresses, towards the M6 and M9 time points, the microbial community adapts to the new environment, displaying diverse metabolic activities such as enhanced cellular structure and energy metabolism. This reflects the community’s evolution towards a stable state and its adaptation to the decomposition of more complex organic materials. These changes reveal the dynamic impact of straw return on the functional diversity of soil microbial communities, highlighting the significant role of time in the process of straw decomposition. The observed shifts in microbial functional potential were further corroborated by taxonomic changes at the genus level. At the M6 and M9 time points, several typical cellulose-degrading genera, such as *Saccharophagus*[21] and *Cellvibrio*[22], were consistently enriched, suggesting that straw return enhanced soil cellulose decomposition capability and promoted nutrient cycling and the decomposition of complex organic matter; the increase in *Agarilytica*[23] might reflect the activation of specific organic matter decomposition pathways.

In addition, across all time points when compared to control soil, the reduction of *Thelephora ganbajun* might indicate an increase in competitive pressure during straw decomposition, leading to the loss of survival conditions for this species in the new environment. In non-transgenic samples, the absence of *Candidatus_Nitrosocosmicus* and *Candidatus_Nitrosocaldus* suggests alterations in the ammonia oxidation process and nitrogen cycle pathways[24,25]. The enrichment of known biocontrol fungi, *Metarhizium*[26] and *Purpureocillium*[27],

in non-transgenic M1 samples could imply that the straw itself may offer certain resistance to pests. Meanwhile, in transgenic M1 samples, the enrichment of biocontrol fungi such as *Trichoderma*[28] and *Pochonia*[29] further underscores the positive role of transgenic straw return in enhancing the potential for biological control in soil.

In discussing the impacts of deep tillage and mulching treatments, it is noteworthy to consider the significant influence of deep tillage on the soil microbial communities. Regardless of whether in non-transgenic or transgenic deep tillage treatment samples, a variety of fungi and bacteria such as *Acremonium*, *Stachybotrys*, *Metarhizium*, and *Trichoderma* were enriched. These fungi are commonly associated with the decomposition of organic matter and nutrient cycling[30,31], indicating that deep tillage treatment facilitated the activity of microorganisms closely related to straw decomposition. *Particularly*, *Trichoderma* and *Metarhizium*, which are widely used in biological control[26,28], their enrichment may suggest that deep tillage treatment helps enhance the potential for natural disease management in the soil. Overall, these results demonstrate that deep tillage treatment increased the microbial diversity related to straw decomposition in the soil, increasing the relative abundance of some beneficial microorganisms. This may positively impact soil fertility and plant health. However, further studies are necessary to directly link these microbial changes with specific soil health indicators and plant performance.

Although this study provides important insights, certain limitations remain. For instance, our inferences were primarily based on metagenomic functional annotations and community composition, without simultaneous measurements of soil physicochemical properties such as metabolite concentrations or nutrient levels. As a result, the interpretation of microbial functional changes is limited to potential functions and lacks direct evidence of actual metabolic activities. Future research could integrate soil enzyme activity assays, metabolomic profiling, or monitoring of key elemental cycling rates to better elucidate the relationship between microbial functional changes and real ecological processes in the soil, thereby providing a more comprehensive understanding of the ecological impacts of straw return.

This study investigated the impacts of straw return from GM and non-GM maize through two different management practices, mulching and deep incorporation, on soil microbial community composition and function. These results indicate that, compared to GM traits, the method and timing of treatment played a more significant role in shaping the composition and function of soil microbial communities. Particularly, deep incorporation significantly enhanced microbial diversity and enriched microorganisms closely associated with straw decomposition, potentially benefiting soil fertility and plant health. Additionally, this research revealed dynamic changes in soil microbial communities in response to straw return, adapting to different stages of straw decomposition. Notably, the impact of GM straw on soil microbes was relatively limited, an important finding for assessing the sustainable application of GM crops in agricultural ecosystems. It suggests that returning GM crop straw is unlikely to adversely affect soil microbial ecology, supporting the integration of GM crops into sustainable agricultural practices without compromising soil microbial diversity and function. In summary, this study underscores the importance of considering management practices and timing in optimizing agricultural practices, while also providing scientific evidence for further research into the role of GM crops within agricultural ecosystems.

Methods

Materials and location

Four types of maize straw were utilized in this study: (1) non-transgenic B73 maize straw, (2) B73 maize straw GM with the 2A-7 gene, (3) non-transgenic Zheng58 maize straw, and (4) Zheng58 maize straw GM with the CM8101 gene. Designate the non-transgenic maize straw as CK.

The experimental site was located at the Transgenic Plant Field Testing Base of Jilin Academy of Agricultural Sciences, situated in Gongzhuling City, Jilin Province, which is a proprietary testing ground of the Jilin Academy of Agricultural Sciences.

All experiments were conducted under laboratory-controlled conditions as a soil microcosm study, using beakers to simulate straw return scenarios with precisely adjusted soil and moisture levels.

Treatment details

This experiment was conducted within planting areas of no less than 150 square meters, managed using conventional farming practices and without the use of insecticides throughout the entire growth period. The experiment was replicated six times with the following treatments:

No Straw Added Treatment: Soil samples adjusted to the appropriate moisture level (20%–25%) were placed in beakers without the addition of maize straw, labeled as'BLANK'.

Mulching Treatment: Soil samples adjusted to the appropriate moisture level were placed in beakers, and then a uniform layer of maize straw was spread on the top, labeled as'M'.

Deep tillage Treatment: Soil samples adjusted to the appropriate moisture level were thoroughly mixed with maize straw, labeled as'D'.

The experimental sampling times were set as follows:'M1','M6', and'M9'represent sampling conducted 1 month (30 days), 6 months (180 days), and 9 months (270 days) after the start of the experiment, respectively.

According to the naming conventions based on sampling time (M1, M6, M9), gene type (2A_7, CM8101), treatment method (M for mulching, D for deep tillage), and sample status (CK for non-transgenic, T for transgenic), a total of 24 experimental groups were established. An additional no-straw control group (BLANK) was also included, resulting in 25 groups in total. The naming convention for each group follows the format: [Timepoint][Gene][Treatment]_[Status], such as M6_CM8101_D_T, indicating samples collected at 6 months, under the CM8101 gene background, with deep tillage treatment, and transgenic straw. The group structure is summarized in Table 2.

Factor	Levels
Timepoint	M1 (1 month), M6 (6 months), M9 (9 months)
Gene types	2A_7, CM8101
Treatment method	M = mulching, D = deep tillage
Transgenic status	CK = non-transgenic straw, T = transgenic straw
Control group	BLANK (no straw added)
Example group name	M6_CM8101_D_T

Table 2. Definition of group names based on experimental factors.

Soil and straw sample component analysis

Soil moisture content was determined using the drying method according to the People's Republic of China national standard (NYT52-1987)[32]. The procedure involved weighing approximately 10 g of soil to determine its wet weight, then drying the sample in an oven at 105 °C until constant weight was achieved. After cooling in a desiccator to room temperature, the dry weight was measured. Moisture content was calculated using the formula: Moisture percentage = [(Wet weight—Dry weight)/Wet weight] × 100%.

Total potassium and phosphorus contents in the soil were determined based on the forestry industry standards LY_T 1234–2015[33] and LY_T 1232–2015[34], respectively, using the alkali fusion method. The procedure included mixing 0.25 g of soil with 2 g of sodium hydroxide, then heating in a high-temperature furnace to 750 °C and holding for 15 min. After cooling, the sample was treated with water and sulfuric acid to adjust the pH of the solution to an appropriate level before filtration. The filtrate was then directly measured using a photometer for potassium or phosphorus content.

Total nitrogen content was determined according to the forestry industry standard LYT1228-2015[35] using the Kjeldahl method. The soil sample was digested with concentrated sulfuric acid to convert all nitrogen to ammonium form, which was then alkalized and distilled. The ammonia distilled was absorbed by boric acid solution and quantified by titration with a standard acid solution. For samples containing nitrate and nitrite nitrogen, pre-treatment included oxidation with potassium permanganate and reduction with iron powder.

Straw samples, prepared as dry powders, were analyzed according to the agricultural industry standard NY525-2012[36]. Total nitrogen was determined using the Kjeldahl method, total phosphorus by the molybdenum blue spectrophotometric method, and total potassium by the flame photometric method. In the molybdenum blue method, samples were digested with a mixture of concentrated nitric and perchloric acids, reacting with ammonium molybdate and ascorbic acid under acidic conditions to form a blue complex, the absorbance of which was measured at 880 nm. For potassium, the digested samples were diluted appropriately and the potassium content was measured at specific wavelengths using a flame photometer.

Treatment and soil sampling

In this experiment, 500 mL beakers were filled with 120 g of soil, which had never been planted with GM maize. All soil was finely ground, sieved, air-dried, and weighed. The amount of straw was calculated based on the typical straw return amount in black soil regions (12,000 kg·hm-2) and the average soil bulk density of the plow layer (1.1 g·cm-3), initially resulting in a soil-to-straw ratio of 281:1. However, as adding only 0.4 g of straw to each 120 g of soil was insufficient for covering or mixing, following recommendations, the ratio was adjusted to 5.6:1. Some studies have used a straw addition ratio of 10%. The corresponding maize straw was dried, crushed, thoroughly mixed, and the remaining powder was stored frozen. Before the experiment began, the air-dried soil samples were analyzed for organic carbon content, various nutrient contents, and pH values, and the sieved soil samples underwent microbial sequencing. Each beaker, containing 120 g of air-dried soil covered with a breathable plastic film, was adjusted to field capacity moisture content (about 20%) with distilled water, and it was determined through gradient experiments to periodically spray each beaker with 20 mL of water to maintain moisture. Dynamic sampling points were set for 30, 90, 180, and 270 days, with straw being removed from the soil prior to sampling. Samples were quickly placed in ice boxes and then transferred to a −80 °C freezer awaiting sequencing analysis.

Microbiome data acquisition

Metagenomic sequencing and preliminary data processing were conducted at Shanghai OE Biotech Co., Ltd. Approximately 0.5 g of fresh soil sample (stored at −80 °C) was used for microbial genomic DNA extraction using the MagPure Soil DNA KF Kit, according to the manufacturer's protocol. After DNA extraction, DNA quality and quantity were assessed by agarose gel electrophoresis and a NanoDrop spectrophotometer.

Sequencing libraries were prepared without PCR amplification steps using the TruSeq Nano DNA LT Sample Preparation Kit (Illumina, Cat. No. FC-121–4001). Shotgun metagenomic sequencing was performed on an Illumina high-throughput sequencing platform (Illumina Inc., USA). Trimmomatic[37] was utilized for quality control of the sequencing data to ensure data integrity. Then, the assembly of metagenomic sequences was carried out using MEGAHIT[38], which is based on the De Bruijn graph principle, resulting in the construction of Contigs. Only Contigs exceeding 500 bp in length were selected for subsequent analysis.

Upon the assembly of genomes, Prodigal[39] was applied to predict Open Reading Frames (ORFs) from the Contigs, and the predicted ORFs were then translated into amino acid sequences. To acquire a non-redundant initial Unigene set, the ORF prediction results from all samples were subjected to redundancy removal using

CD-HIT[40], with an identity threshold of 95% and a coverage threshold of 90%, resulting in a non-redundant gene set. Representative sequences from each cluster were selected as Unigenes.

Bowtie2[41] was used to align the clean reads from each sample to the non-redundant gene set (with 95% identity) to obtain gene abundance information for each sample. The non-redundant gene sequences (amino acid sequences) were aligned to databases including NR, KEGG[42], COG[43], SWISSPROT[44], and GO[45], using DIAMOND (v0.9.7). BLAST alignment was performed with an expectation value (e-value) threshold of 1e-10, and the best-hit result for each gene (alignment score > 60 bits and at least one high-scoring segment pair) was selected for functional annotation. Based on the annotation results and gene abundance profiles, the relative abundances of various functional categories (e.g., KEGG, eggNOG) were calculated. Functional differences between groups were assessed using ANOSIM and LEfSe.

Statistical analysis and visualization

Data analysis and visualization were conducted in the R programming environment, version 4.3.1. Alpha diversity indices, including species richness, Shannon index, and Simpson index, were calculated based on species-level abundance data using the vegan package[46]. Principal Coordinates Analysis (PCoA) was performed based on Bray–Curtis distances calculated using the vegdist function from the vegan package, and ordination was conducted with the pco function from the labdsv package[47]. The EnhancedVolcano package[48] was employed for volcano plot generation, while the pheatmap package

[49] was used for heatmap creation.

P-values were calculated using the Wilcox test. Unless otherwise stated, p-values less than 0.05 were considered statistically significant.

Data availability

The data supporting the findings of this study are provided within the manuscript and its supplementary materials, as well as the NCBI Sequence Read Archive (SRA) repository, under accession number PRJNA1203889 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1203889/), and are available on request from the corresponding author (Feiwu Li, lifeiwu3394@sina.com).

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Author contributions

Yanbo Xie: investigation, experiments, writing—original draft preparation; Jun-Yan Xiang: data analysis, visualization, writing—original draft preparation; Likun Long: validation, writing—review and editing; Yue Ma, Zhenjuan Xing, Ling Wang, and Chunyu Shao: experiments; Na Liu: conceptualization, supervision; Feiwu Li: conceptualization, supervision, project administration, funding acquisition.

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