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Carbon starvation raises capacities in bacterial antibiotic resistance and viral auxiliary carbon metabolism in soils

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Organic carbon availability in soil is crucial for shaping microbial communities, yet, uncertainties persist concerning microbial adaptations to carbon levels and the ensuing ecological and evolutionary consequences. We investigated organic carbon metabolism, antibiotic resistance, and virus–host interactions in soils subjected to 40 y of chemical and organic fertilization that led to contrasting carbon availability: carbon-poor and carbon-rich soils, respectively. Carbon-poor soils drove the enrichment of putative genes involved in organic matter decomposition and exhibited specialization in utilizing complex organic compounds, reflecting scramble competition. This specialization confers a competitive advantage of microbial communities in carbon-poor soils but reduces their buffering capacity in terms of organic carbon metabolisms, making them more vulnerable to environmental fluctuations. Additionally, in carbon-poor soils, viral auxiliary metabolic genes linked to organic carbon metabolism increased host competitiveness and environmental adaptability through a strategy akin to “piggyback the winner.” Furthermore, putative antibiotic resistance genes, particularly in low-abundance drug categories, were enriched in carbon-poor soils as an evolutionary consequence of chemical warfare (i.e., interference competition). This raises concerns about the potential dissemination of antibiotic resistance from conventional agriculture that relies on chemical-only fertilization. Consequently, carbon starvation resulting from long-term chemical-only fertilization increases microbial adaptations to competition, underscoring the importance of implementing sustainable agricultural practices to mitigate the emergence and spread of antimicrobial resistance and to increase soil carbon storage.

microbial competition | carbon limitation | organic carbon metabolism | antibiotic resistance | virus–host interactions

Soil microbes actively participate in organic matter decomposition and sequestration, nutrient cycling, and plant growth promotion, making them indispensable for maintaining soil health, ecological balance, and sustainable agriculture (1). As we delve into the microbial world, we encounter an astonishing density and diversity of populations despite limited resources in most soils, creating a battleground, where the survival of one microbial cell can mean carbon starvation and nutrient limitation for others (2). In this context, resource availability becomes a pivotal determinant of microbial growth, metabolism, and overall activities (3), shaping the dynamics of microbial communities in soils.

Fertilization has a profound impact on microbial life by directly providing utilizable resources (4) and indirectly by increasing crop growth and input of organic matter on and into soil. The global agricultural consumption of chemical fertilizers reached 195 million tons in 2021, marking a 44% increase since 2000 (5). During the same period, the global application rate of chemical fertilizers per cropland area rose by 38%, averaging 119 kg of nutrients per hectare (5). Despite the growing trend toward organic farming, only 77 million hectares were either certified as organic or in conversion to organic status in 2021 (5). The predominance of conventional farming over organic practices remains evident, with organic agriculture accounting for merely 1.6% of the total global agricultural area (5). The application of chemical-only fertilizers can satiate the nutrient removal by crops, while the influx of nutrients challenges microbial communities. This challenge stems from the need for microbes to maintain stoichiometric balance during growth (6), the increase of nutrients in soils would heighten the microbial carbon demand. Thus, chemical-only fertilization could inadvertently intensify carbon limitation to soil microbes. Compared to chemical-only fertilization, organic fertilizers provide not only nutrients but also a variety of organic carbon resources, leading to a higher level of soil organic matter content. These distinct patterns of carbon storage prompt microbes to adapt their life strategies to thrive in soil environments. Despite growing recognition of the impact of these microbial adaptations, the related ecological and evolutionary consequences remain an intriguing enigma, calling for further exploration.

Significance

Microbial adaptations to carbon availability are crucial for predicting ecosystem responses to environmental changes. We demonstrated that specialization for organic carbon utilization and genome size reduction in carbon-poor soils decrease functional redundancy and consequently increase vulnerability to environmental changes. The raised antibiotic resistance gene abundance underscores concerns about the spread of antibiotic resistance in chemical-fertilizer-based agriculture. Our results also unveil the role of virus–bacteria interactions in increasing host competitiveness and environmental adaptability. Consequently, carbon limitation common in chemical-fertilizer-based agriculture intensifies microbial competition, driving organic carbon metabolic adaptations, increasing antibiotic resistance, and intensifying virus–host interactions. All these are crucial for microbial competition as driving evolutionary trajectories with implications to increase soil carbon storage, mitigate antibiotic resistance spread, and advance sustainable agriculture.

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When envisioning the ecological outcomes of microbial adaptations to limiting carbon availability, competition emerges as a central concern. Competition is widespread when organisms coexist in a shared environment. In animals and plants, competitive forces play a vital role in determining the distribution and abundance of living organisms (7, 8). For example, plant competition for pollinators weakens plant coexistence by destabilizing interactions and increasing competitive imbalances between plant species (9). A field experiment evaluating plant performance depending on elevations found that species persist better in the presence of competition within their elevation ranges, indicating that competition shapes the spatial distribution of species (10). Resource availability influences the intensity of competition. When carbon limitation increases in soil, microbial competition takes on heightened intensity and significance. Microbes have evolved two main strategies to compete for resources (11). The first is exploitative competition (indirect, passive competition), where one microbial strain outcompetes others by efficiently utilizing available resources (2). This can be achieved through increased nutrient uptake or through the secretion of extracellular enzymes that scavenge and acquire essential nutrients (12). In addition to competition for common nutrients, the acquisition of genetic material resources can serve as another potential exploitative competition strategy. Other than being a source of nutrients, DNA can provide advantageous traits to its hosts (13), enhancing their survival and adaptability in challenging environments. The transfer of DNA is greatly facilitated by viruses (14), which rely on the presence of corresponding hosts for persistence and propagation. Contrary to the traditional notion of viruses as fierce predators, an increasing number of studies have demonstrated a coexistence scenario, known as the “Piggyback the Winner” effect, in which viruses and their hosts mutually benefit each other (15). In this scenario, viruses boost the metabolic capacity and environmental fitness of their hosts by providing auxiliary metabolic genes (AMGs), thereby conferring a competitive advantage compared to cells without viral assistance. For example, in soils contaminated by organochlorine pesticides, viral AMGs have been shown to be more linked to pesticide degradation than those in control soils and to increase bacterial growth at subinhibitory concentrations of pesticide (16). This expands the gene reservoir available to cells, suggesting the formation of an ecological function guild between viruses and their hosts.

In addition to resource consumption, interference competition (direct, active competition) represents the other main strategy type, in which microbial cells engage in chemical warfare to gain an advantage over their competitors (2). The classical example of interference competition is the production of antimicrobial compounds, including bacteriocins, peptides, and antibiotics that can target specific strains versus a broader spectrum (17). In response to these antimicrobial attacks, microorganisms develop defense mechanisms such as antibiotic resistance to counteract the lethal effects and secure their survival (18). While the development of antibiotic resistance offers microbes a survival edge, it simultaneously poses a significant threat to human health. Viewed through the “One Health” concept, which emphasizes the interconnectedness of human, animal, and environmental health, the dissemination of antibiotic resistance genes has emerged as a pressing issue, attracting broad attention and eliciting deep concern.

Based on this background, we hypothesized that microbial adaptations related to competition are enhanced in response to carbon limitation in soils. In carbon-poor soils, microorganisms may undergo selection and/or adaptive processes to exploit rarer and more recalcitrant organic compounds. This specialization in the use of complex carbon compounds gives microorganisms a

competitive advantage in carbon-limited environments. This adaptation is expected to result in an enrichment of enzymes involved in the breakdown of organic compounds, such as carbohydrate-active enzymes (CAZymes) (19). Moreover, in carbon-poor soils, microbes may tend to form a virus–host guild, with a relatively higher abundance of virus AMGs linked to organic carbon metabolism. This viral association could confer a competitive advantage to the hosts by expanding their metabolic capabilities and allowing them to utilize a wider range of organic carbon sources, ultimately raising their ecological fitness. In the context of agricultural practices, if chemical-only fertilization leads to carbon starvation of microorganisms, intensified competition for carbon resources could also trigger an arms race between microbial cells. This can be characterized by on-going cycles of antibiotic production and antibiotic resistance development.

To address these hypotheses, we sampled soils from four long-term experimental field trials located across China, spanning a latitudinal range of 28°N to 46°N (*SI Appendix, Fig. S1*). These experimental sites, established 40 y ago, involved contrasting fertilization regimes: chemical-only fertilization and organic-only fertilization. This unique and rarely encountered set of long-term experimental designs was established to monitor the ecological and evolutionary impacts of farming practices. Specifically, we examined the ecological and evolutionary consequences of long-term carbon limitations (e.g., resulting from chemical-only fertilization) by investigating bacterial organic carbon metabolism, virus auxiliary carbon metabolism, and antibiotic resistance. This study advances our knowledge of microbial adaptations in response to carbon limitation. Our findings have broader implications for understanding ecosystem processes and will inform sustainable agricultural practices to mitigate antimicrobial resistance and increase carbon sequestration.

Results

Soil Properties and Species Interactions. Long-term fertilization resulted in soils with contrasting chemical properties (*SI Appendix, Tables S1 and S2*). Compared with chemical-only fertilization, organic-only fertilization increased soil organic carbon (SOC) by 38% and total nitrogen (TN) by 31%. Organic-only fertilized soils showed a neutral pH, while chemical-only fertilized soils were acidic (average pH of 5.6). The temperature, precipitation, and light also had impacts on the soil properties (*SI Appendix, Table S3*).

Chemical-only fertilization increased the strength of species interactions, as indicated by the increase in the average degree (e.g., the number of links with other species in the co-occurrence network) from 9.5 to 14 (*SI Appendix, Fig. S2*). Notably, the negative correlations between species abundances were 30% more prevalent in chemical-only fertilized soils (692 negative correlations) than in organic-only fertilized soils (532 negative correlations), indicating potentially stronger species competition in chemical-only fertilized soils.

Organic Carbon Metabolism in Soils at Community Level Revealed by Metagenomics Based on the CAZy Database. The enzyme classes—glycosyl transferases (GT), glycoside hydrolases (GH), carbohydrate esterase (CE), and carbohydrate-binding module (CBM)—collectively constituted ~95% of CAZyme gene abundance (Fig. 1A), thus being the predominant components in both chemical-only and organic-only fertilized soils. Auxiliary activities (AA) and polysaccharide lyase (PL) were at relatively low abundance, accounting for ~5% of total CAZyme gene abundance (Fig. 1A). Compared to organic-only fertilized soils, chemical-only fertilized soils displayed

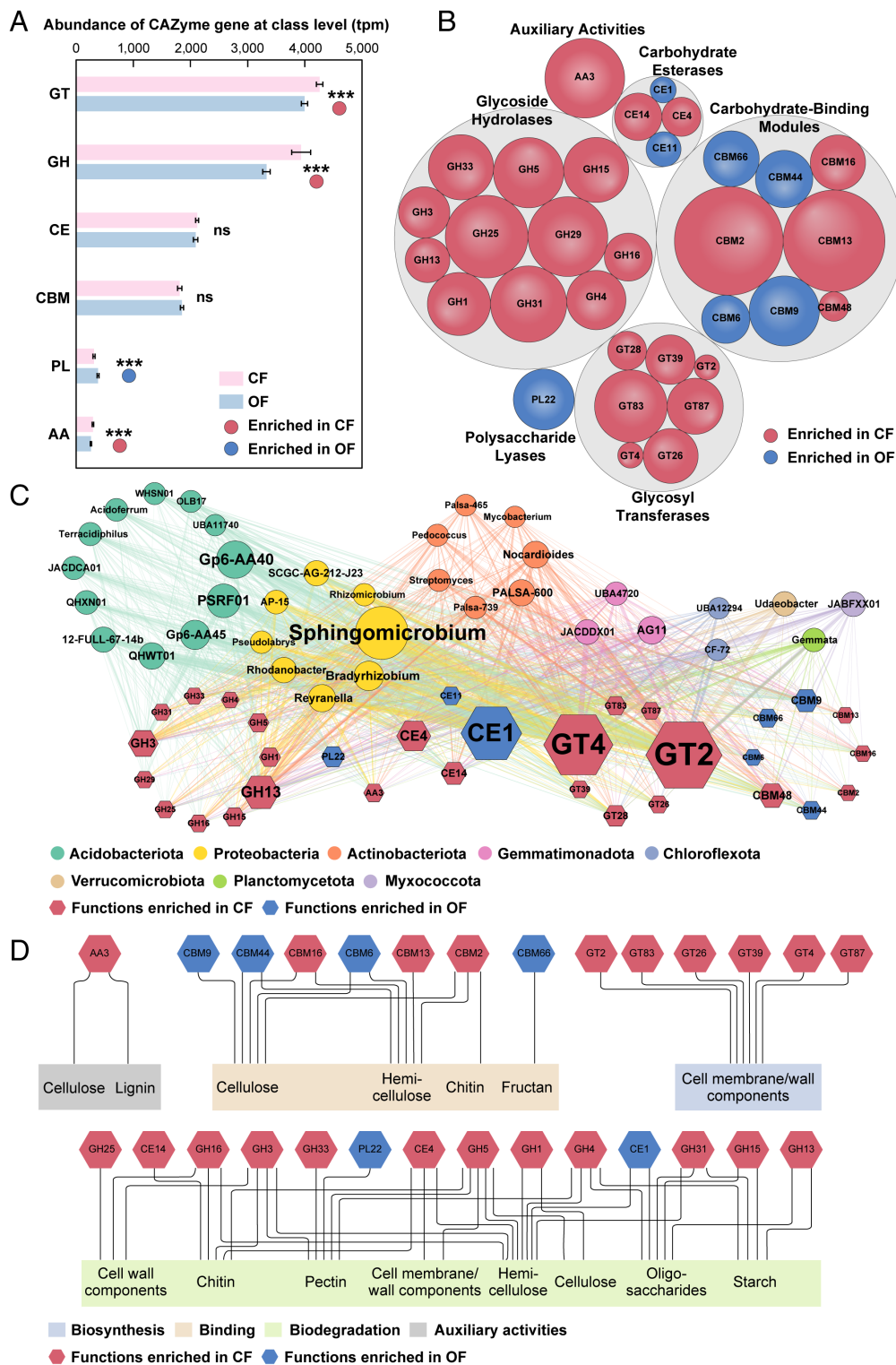


Fig. 1. Summary of putative CAZyme genes in chemical-only fertilized soils (CF) and organic-only fertilized soils (OF). (A) Abundance of CAZyme genes at the class level. Red and blue dots indicate enrichment in CF and OF, respectively. GT: glycosyl transferase; GH: glycoside hydrolase; CE: carbohydrate esterase; CBM, carbohydrate-binding module; PL: polysaccharide lyase; AA, auxiliary activities. The annotation of CAZyme genes was based on the Carbohydrate-Active enZymes database using open reading frames (ORFs) from metagenomics. A linear mixed model was used to compare gene abundance in CF and OF. In the model, the "Fertilization regime" (CF or OF) factor was fixed, and the "Sites" (four locations) factor was considered a random effect. (B) Enriched CAZyme genes in different soils. The size of the circles indicates the response ratio. The response ratio was determined by calculating the natural logarithm of the ratio of the mean gene abundances between CF and OF. (C) Microbes contributing to the enriched CAZyme genes. The size of the circles and octagons indicate the contribution of microbes and gene abundance, respectively. The font size matches the size of the corresponding circles or octagons. The colors of the circles indicate microbial phyla, and the colors of the octagons indicate CAZyme gene enrichment. The relationships between the functions and their contributors were estimated through sequence annotation against both functional and taxonomic databases. (D) Potential substrates of enriched CAZyme genes. The related processes are grouped under biosynthesis, binding, biodegradation, and auxiliary activities. This information was sourced from the Carbohydrate-Active enZymes database.

a 6.6% increase in GT genes (involved in the formation of glycosidic bonds), an 18% increase in GH genes (involved in the hydrolysis and rearrangement of glycosidic bonds), and a 14% increase in AA genes (redox enzymes that act in conjunction with CAZymes, Fig. 1A). PL genes were 22% more frequent in organic-only than in chemical-only fertilized soils (Fig. 1A). Two-way ANOVA of fertilization and experimental site locations along with their interactions revealed that GT, GH, and PL gene abundances were more influenced by fertilization than by the sampling location (SI Appendix, Table S4). This clearly shows the dominance of the fertilization controls over the climatic and soil properties defining CAZyme gene abundances.

At a finer resolution, specifically at the enzyme family level, enrichment of genes from groups GH (11 genes), GT (seven genes), and AA (one gene) was exclusively observed in chemical-only fertilized soils (Fig. 1B). Within the PL group, only PL22 showed differential enrichment and was more abundant in organic-only fertilized soils. In the CE and CBM groups, the number of enriched genes was evenly distributed between chemical-only and organic-only fertilized soils (Fig. 1B).

Twelve genera of Acidobacteriota, eight genera of Proteobacteria, seven genera of Actinobacteriota, three genera of Gemmatimonadota, two genera of Chloroflexota, one genus of Verrucomicrobiota, one genus of Planctomycetota, and one genus of Myxococcota were the main contributors (each accounting for >0.5% of the total abundance of enriched genes) to the enriched CAZyme genes (Fig. 1C).

The CAZyme genes enriched in organic-only fertilized soils were associated with binding fructan, hemicellulose, and cellulose, as well as biodegrading oligosaccharides, pectin, and hemicellulose (Fig. 1D and SI Appendix, Table S5). In chemical-only fertilized

soils, the enriched CAZyme genes were not only associated with the aforementioned compounds but were also involved in the activation, binding, and biodegradation of more complex carbon sources, like cell membrane/wall components, chitin, and lignin. The biosynthesis functions of cell membrane/wall components were also detected in the CAZyme genes enriched in organic-only fertilized soils (Fig. 1D and SI Appendix, Table S5).

Viral AMGs and Their Host. The total abundance of AMGs annotated as CAZyme genes was 1.5 times higher in chemical-only than in organic-only fertilized soils (Fig. 2A). At the CAZyme class level, the AMGs annotated as GT class were enriched in chemical-only fertilized soils. Two-way ANOVA of the fertilization and experimental site locations along with their interactions revealed that the total abundance of AMGs annotated as CAZyme genes, and the abundance of AMGs annotated as GT class, were more influenced by fertilization than by the sampling site (SI Appendix, Table S4). AMGs annotated as GT75 and GT2 were enriched in chemical-only and organic-only fertilized soils, respectively (Fig. 2B). These AMGs were involved in the biosynthesis of cell wall components. AMGs annotated as GT75 were produced from *Reyvirus* (Fig. 2B), while those annotated as GT2 were derived from viruses that remain unclassified.

The AMG K09471, encoding gamma-glutamyl putrescine oxidase involved in GABA biosynthesis, was enriched in chemical-only fertilized soils and produced from *Hollowayvirus*. The identified hosts of *Hollowayvirus* were *Pseudomonas*, *Paracaligenes*, *Citrobacter*, *VBCG01*, and *SCGC-AG-212-J23* (Fig. 2C). The AMG K01939, encoding adenylosuccinate synthase involved in ATP biosynthesis, was enriched in organic-only fertilized soils and produced from

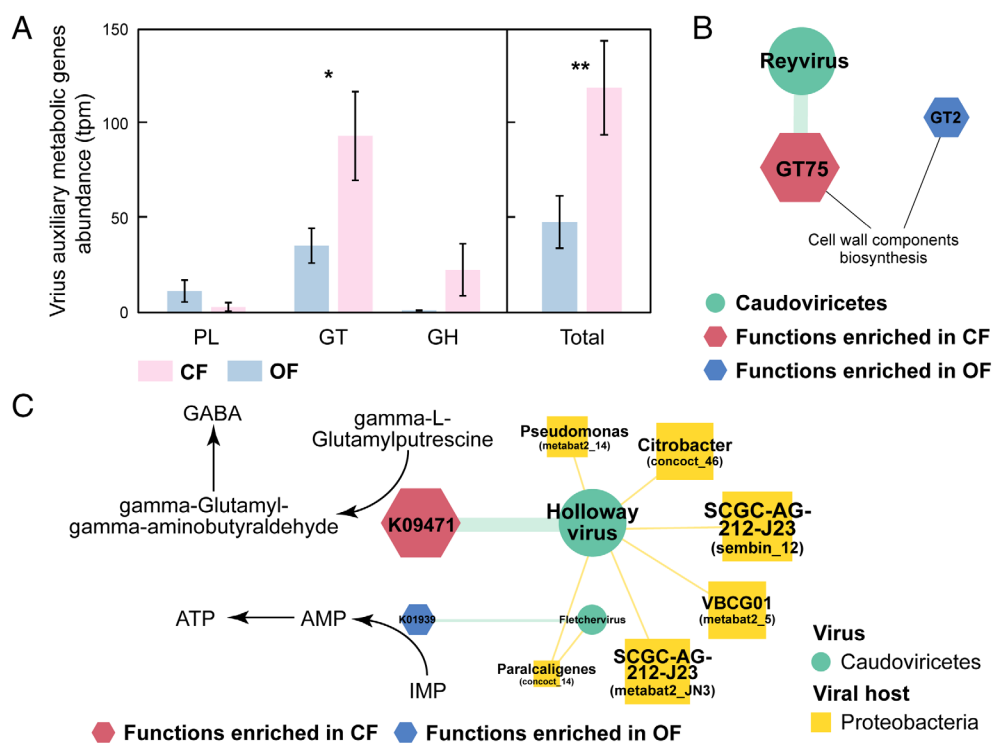


Fig. 2. Summary of viral AMGs in chemical-only fertilized soils (CF) and organic-only fertilized soils (OF). (A) Abundance of viral AMGs annotated as CAZyme genes at the class level. The annotation of CAZyme genes was based on the Carbohydrate-Active enZYmes database using AMGs identified from viral sequences. A linear mixed model was used to compare gene abundance in CF and OF. In the model, the “Fertilization regime” (CF or OF) factor was fixed, and the “Sites” (four locations) factor was considered a random effect. (B) Viruses contributing to the enriched AMGs annotated as CAZyme genes. The size of the circles and octagons indicate the contribution of microbes and AMG abundance, respectively. These AMGs were involved in the biosynthesis of cell wall components. The relationships between the functions and their contributors were estimated based on viral sequences containing both AMGs and taxonomic information. (C) The functions and origins of enriched AMGs, as well as the viral hosts. The virus contribution to AMGs (size of the circle), AMG abundance (size of the octagon), and host abundance (size of the square) are shown. The viral hosts were identified using three methods: homology matches, tRNAs similarity, and CRISPR spacer similarity, based on the high-quality MAGs recovered in this study. The functional information was sourced from KEGG Database.

Fletchervirus. *Fletchervirus* was identified to infect *Paralcaligenes* (Fig. 2C).

The viral richness in both chemical- and organic-only fertilized soils exceeded 2000, yet no significant effect of fertilization on viral diversity was observed. Nearly 90% of the viruses remained unclassified at the family level (*SI Appendix, Table S6*), highlighting the limited current knowledge of soil virome. Among the identified viral families, *Siphoviridae* and *Schitoviridae* were prevalent, each exhibiting relative abundances over 1% (*SI Appendix, Table S6*). Specifically, *Siphoviridae* was more abundant in chemical-only fertilized soils, while *Casjensviridae*, *Mesyanzhinovviridae*, and *Straboviridae* showed higher abundances in organic-only fertilized soils (*SI Appendix, Table S6*).

The functions associated with DNA binding and modification in the process of the integration of viral DNA into host genomes were widespread among the viruses, for example, helix-turn-helix domain, DNA methylase, and HNH endonuclease (*SI Appendix, Table S7*). Chemical-only fertilized soils showed a higher abundance of viral functions related to the helix-turn-helix domain and AAA domain (*SI Appendix, Table S7*), indicating a potentially heightened level of host–virus interaction in carbon-limited soils.

Antibiotic Resistance in Soils at Community Level Revealed by Metagenomics Based on the Comprehensive Antibiotic Resistance Database (CARD Database). Antibiotic resistance genes (ARGs) were 25% more abundant in chemical-only than in organic-only fertilized soils (Fig. 3A). The predominant ARG classes included glycopeptide, cephamycin, macrolide, aminoglycoside, fluoroquinolone, rifamycin, tetracycline, phenicol, fosfomycin, pleuromutilin, and multidrug resistance. Each of these classes accounted for more than 1% of the total abundance, contributing to a combined total of 96% (Fig. 3A). On the other hand, lincosamide, peptide, and penam antibiotic resistance was less common, the relative contribution of each class being less than 1%. In organic-only fertilized soils, the enrichment of ARGs occurred in the abundant class of aminoglycoside antibiotic resistance, whereas in chemical-only fertilized soils, ARG enrichment was observed in the rare classes of peptide and penam antibiotic resistance, as well as in other low-abundance classes (labeled “Others” in Fig. 3A). Two-way ANOVA of fertilization and experimental site locations along with their interactions revealed that total abundance of ARGs, as well as the abundance of ARGs related to aminoglycoside antibiotic resistance, peptide antibiotic resistance, penam antibiotic resistance, and other low-abundance classes (i.e., the “Others” group), were equally or more influenced by fertilization than by sampling location (*SI Appendix, Table S4*).

At the gene level, 8 ARGs related to antibiotic efflux (i.e., they pump antibiotics out of cells), 5 ARGs related to antibiotic inactivation, 2 ARGs related to antibiotic target protection, and 1 ARG related to antibiotic target alteration were enriched in organic-only fertilized soils (Fig. 3B and *SI Appendix, Table S8*). On the other hand, 12 ARGs related to antibiotic efflux, 1 ARG related to antibiotic inactivation, 3 ARGs related to antibiotic target protection, 1 ARG related to antibiotic target replacement, and 2 ARGs related to antibiotic target alteration were enriched in chemical-only fertilized soils (Fig. 3B and *SI Appendix, Table S8*).

The members of Proteobacteria, Actinobacteriota, and Acidobacteriota mainly contributed to the ARG enrichment (Fig. 3C). The average abundance of enriched genes in organic-only fertilized soils was 71% higher than that in chemical-only fertilized soils, as indicated by the size of the octagons in Fig. 3C. This suggests that chemical-only fertilized soils tend to enrich low abundance ARGs. Furthermore, 13 species were identified as the main contributors to both enriched CAZyme genes and enriched

ARGs (*SI Appendix, Fig. S3*). *SCGC-AG-212-J23* was the main contributor to both enriched CAZyme genes and enriched ARGs, as well as being involved in virus auxiliary metabolic processes (*SI Appendix, Fig. S3*).

The ARGs enriched in organic-only fertilized soils were associated with aminoglycoside, macrolide, lincosamide, glycopeptide, phenicol, and multidrug antibiotic resistance (Fig. 3A and *SI Appendix, Table S8*). In chemical-only fertilized soils, the enriched ARGs were involved in peptide, aminocoumarin, aminoglycoside, macrolide, rifamycin, diaminopyrimidine, fluoroquinolone, and multidrug antibiotic resistance (Fig. 3A and *SI Appendix, Table S8*).

We further validated our ARG findings using the ARGs-OAP v3.0 workflow based on metagenomic data. This read-based approach, which normalizes ARG gene copy numbers against estimated cell counts, aligned with our observation of elevated total ARG abundance in chemical-only fertilized soils (*SI Appendix, Table S9*). Moreover, ARGs-OAP identified ARG enrichments associated with novobiocin, vancomycin, macrolide-lincosamide-streptogramin, and mupirocin in chemical-only fertilized soils (*SI Appendix, Table S9*). These drug classes are not recognized as standard categories of the CARD database or were not detected in the analyses using the CARD database. The abundance of multidrug-related ARGs was higher in chemical-only fertilized soils (*SI Appendix, Table S9*), a finding consistent with the results based on the CARD database, but ARGs-OAP highlighted its significance. The beta-lactam-related ARG abundance was higher in organic-only fertilized soils (*SI Appendix, Table S9*), a drug class also not standard in the CARD database. Furthermore, a comparative analysis of individual ARG abundance trends across different treatments revealed over 76% consistency between the two methods, with significant correlations in more than 43% of these ARGs (*SI Appendix, Table S10*). The consistency of findings from the read-based ARGs-OAP approach with those derived from open reading frames (ORFs) assembly and CARD database annotation validates the robustness of our findings.

The Genomic Properties and Functional Potential of Metagenome-Assembled Genomes (MAGs). A total of 69 MAGs (high-quality bins with >70% completeness and <10% contamination) were recovered from the metagenomic data (Fig. 4). These MAGs belonged primarily to Proteobacteria, Actinobacteriota, Acidobacteriota, Gemmatimonadota, Dormibacterota, Chloroflexota, and Nitrospirota. At the genome level, MAGs enriched in organic-only fertilized soils had 35% more CAZyme gene sequences, a 5.0% higher likelihood of containing ARGs, a 23% larger genome size, and 14% more coding sequences than MAGs enriched in chemical-only fertilized soils (Fig. 4). Furthermore, MAGs belonging to the same genus but enriched in different soils showed that organic-only fertilization led to species characterized by larger genome sizes, more coding sequences, and more CAZyme genes in the genome (*SI Appendix, Fig. S4*).

Discussion

Carbon availability is a pivotal point in determining the microbial competitive interactions in soil. Therefore, microbial processes associated with competition would undergo changes during microbial adaptations to carbon availability. Species competition is crucial in microbial evolution and assembly as it drives the selection of traits and genetic adaptations that enhance competitive fitness (20). This process can lead to niche specialization and diversification of microbial populations (21, 22). These competitive dynamics profoundly shape the composition, diversity, and functional potential of microbial communities, influencing ecosystem processes and stability (23). Despite

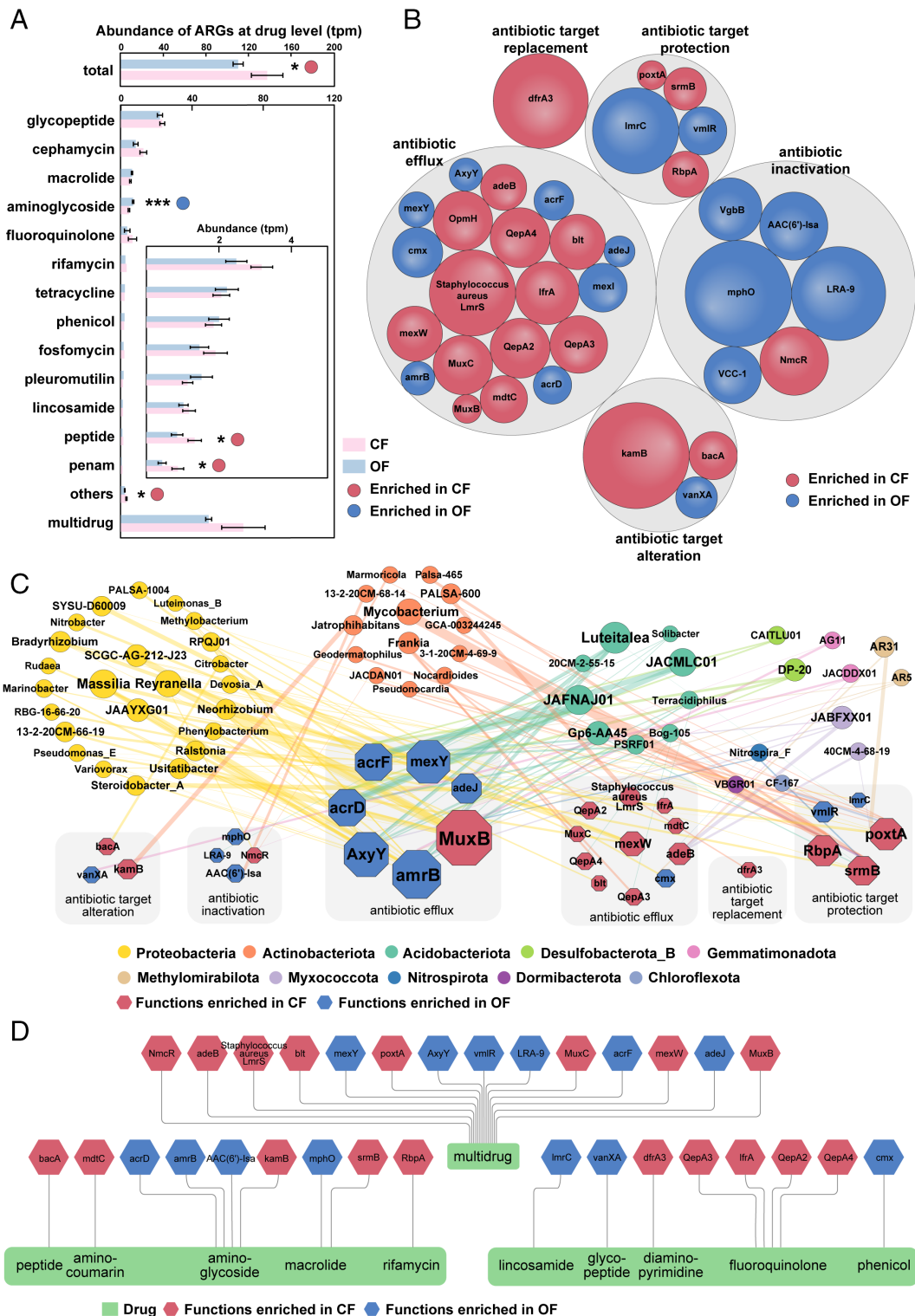


Fig. 3. Summary of putative ARGs in chemical-only fertilized soils (CF) and organic-only soils (OF). (A) Abundance of ARGs at the drug level. Red and blue dots indicate enrichment in CF and OF, respectively. The annotation of antibiotic resistance genes was based on the Comprehensive Antibiotic Resistance Database using open reading frames (ORFs) from metagenomics. A linear mixed model was used to compare gene abundance in CF and OF. In the model, the “Fertilization regime” (CF or OF) factor was fixed, and the “Sites” (four locations) factor was considered a random effect. (B) Enriched ARGs in different soils. The size of the circle indicates the response ratio. The response ratio was determined by calculating the natural logarithm of the ratio of the mean gene abundances between CF and OF. (C) Microbes contributing to the enriched ARGs. The sizes of the circles and octagons indicate the contribution of microbes and gene abundance, respectively. The font size matches the size of the corresponding circles or octagons. The color of the circles indicates microbial phyla, and the color of the octagons indicates ARG enrichment. The relationships between the functions and their contributors were estimated through sequence annotation against both functional and taxonomic databases. (D) Associated antibiotics of enriched ARGs. This information was sourced from the Comprehensive Antibiotic Resistance Database.

growing recognition of the significance of the interplay among carbon availability, microbial adaptations, and competition processes, our understanding of their ecological and evolutionary

consequences remains preliminary (2). Herein, we investigated the ecological implications of microbial adaptations by comparing soils with low and high carbon availability induced by

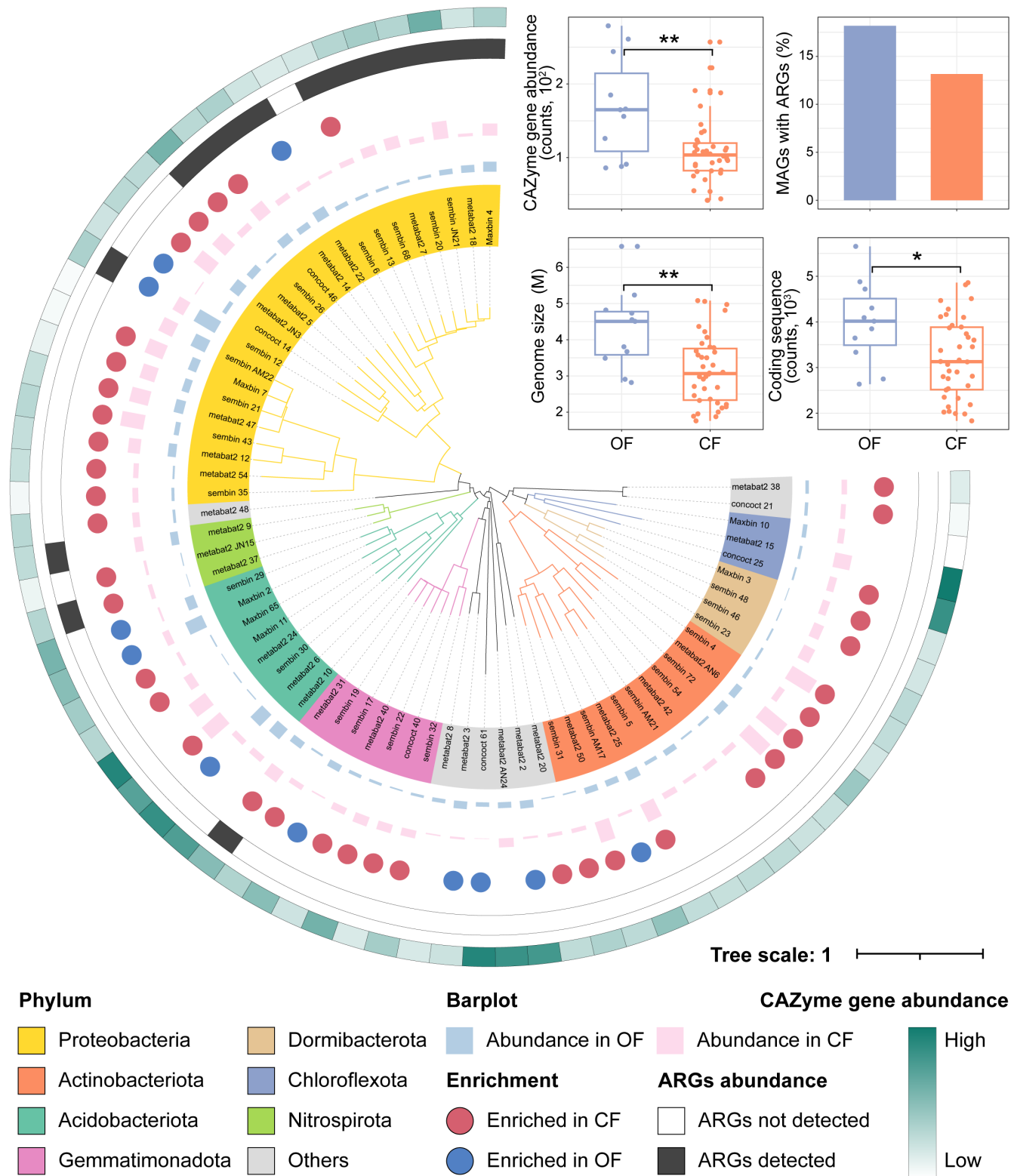


Fig. 4. Summary of metagenome-assembled genomes (MAGs) in chemical-only fertilized soils (CF) and organic-only fertilized soils (OF). Phylogenetic tree of MAGs, taxonomic information, MAG abundances in OF and CF, MAG enrichment, detection of ARGs in MAGs, and the abundance of CAZyme genes are displayed from the inner to outer circles. The graphs in the upper right corner of the figure show CAZyme gene abundance, percentage of MAGs with ARGs, genome size, and coding sequences in MAGs enriched in CF or OF soils. The taxonomic annotation of MAGs and phylogenetic tree were constructed based on the Genome Taxonomy database Release 207 (GTDB). A Wilcoxon rank-sum test was used to compare the parameters in MAGs enriched in CF and OF soils.

long-term chemical- or organic-only fertilization, focusing on the microbial properties related to competition.

Long-term chemical- and organic-only fertilization resulted in distinct carbon storage patterns: carbon-poor and carbon-rich soils, respectively (*SI Appendix, Tables S1 and S2*). As expected, negative

correlations between species abundances, commonly used as indicators of microbial competition (24), were more pronounced in carbon-poor soils (*SI Appendix, Fig. S2*). These results provided a coevolutionary context for our investigation of microbial adaptations concerning competition for carbon resources and their

subsequent consequences. The duration of the field experiments (~40 y) with continuous chemical or organic fertilization is sufficient for changes in microbial communities (4) including viruses to occur and for the development and genetic conservation of genes responsible for adaptation (25) to low/high C availability.

Regarding organic carbon metabolism, carbon-rich soils showed enrichment of CAZyme genes from the class Polysaccharide Lyases (Fig. 1 A and B) with a preference for easily available carbon sources such as oligosaccharides and pectin (Fig. 1D). In contrast, carbon-poor soils had a higher total abundance of CAZyme genes involved in organic matter decomposition (Fig. 1A). This suggests that when faced with carbon limitation, microbes invest more energy to produce diverse extracellular enzymes to depolymerize organic compounds for energy and nutrient acquisition (26). This phenomenon reflects scramble competition (exploitation competition) (11), characterized by the rapid utilization of limited resources through the secretion of nutrient-harvesting enzymes. More specifically, there was enrichment in the family of Glycoside Hydrolases (Fig. 1 A and B), enabling the decomposition of complex carbon sources like cell membrane/wall components, chitin, and lignin (Fig. 1D). Such specialization to use rare or complex organic compounds can increase the competitive advantage of microorganisms in carbon-poor soils (27). This niche differentiation, in which each species specializes in a different type of resource, is the classical evolutionary consequence of competition (28). Such chemical niche differentiation can potentially increase overall productivity and facilitate coexistence among microbial communities (29). Overall, the observed enrichment in Glycoside Hydrolases genes and the ability to use rare or complex organic compounds in carbon-poor soils allow microbes to obtain the necessary energy for survival. This reflects the consequences of competition and selection processes in response to resource depletion—low carbon availability. However, such specialization in organic carbon utilization may come at a cost. The reduced functional buffering capacity of these soils due to reliance on specific carbon sources poses a higher risk of reducing ecosystem functions in the face of environmental changes.

Viruses can contribute to the genetic and phenotypic evolution of their hosts, raising their competitiveness and environmental adaptability through AMGs (30). In carbon-poor soils, viruses established an enhanced beneficial relationship with bacteria, reflected by the elevated total abundance of AMGs annotated as CAZyme genes (Fig. 2A). This “Piggyback the Winner” effect confers population-level benefits to the hosts in their competition for carbon resources (15). When exposed to challenging environments, viruses exhibit a greater inclination to form mutualistic relationships with their hosts (31). They transfer beneficial genes and raise the host fitness under environmental stress, and in return, the hosts provide a stable environment and favorable conditions for viral survival and reproduction. Furthermore, depending on the specific environment, viruses can assist their hosts in various directions. In carbon-poor soils, *Hollowayvirus* was shown to enhance the hosts’ GABA biosynthesis (Fig. 2C), thereby enabling adaptation to stressful conditions, regulation of pH, production of antimicrobials, and the formation of biofilm (32). In carbon-rich soils, *Fletcherivirus* increased the host ATP biosynthesis (Fig. 2C), supplying the energy required for cellular processes, biosynthesis, membrane potential maintenance, and cell motility. Overall, viruses tend to improve the competitiveness and environmental adaptability of their hosts in carbon-poor soils, while in carbon-rich soils, they improve the activity of their hosts. These virus–host interactions highlight the context-dependent dynamics of microbial relationships, with viruses acting as agents of genetic diversification and ecological adaptation.

Furthermore, our study revealed genome-level consequences of microbial competition. MAGs that accumulated in carbon-rich soils had larger genomes than those enriched in carbon-poor soils (Fig. 4). A larger genome size implies increased functional complexity, facilitating regulation in response to environmental disturbances (33). Microorganisms with larger genome sizes are expected to have greater resistance to environmental disturbances and to exploit a broader range of ecological niches, due to their larger pool of candidate genes. In contrast, a smaller genome size suggests specialization and higher resource use efficiency (34), requiring less energy for replication and maintenance (35). In carbon-poor soils, microorganisms face resource limitations and competitive pressures, leading to genome reduction and the elimination of unnecessary functions and metabolic pathways (36). This reduction and specialization help minimize energy and material waste, thus enhancing the competitiveness and survival of microorganisms in stable environmental conditions (22). However, potential ecological risks are associated with smaller genomes. For instance, when dispersing into new environments or encountering environmental disturbances, microorganisms with smaller genomes may be outcompeted by those with larger genomes that possess a wider array of functional genes and metabolic capabilities (37). This is related to the increased reliance of smaller-genome microbes on specific environmental factors or resources resulting from their specialization (22).

In addition to changes in soil carbon functions and genomic characteristics, microbial adaptations to carbon limitation can have broader evolutionary consequences. Our study revealed a classic outcome of microbial competition: the enrichment of ARGs in carbon-poor soils, particularly in low-abundance drug classes (Fig. 2A). This ARG enrichment in rare drug classes has significant implications and highlights the potential limitations of studies that only focus on specific antibiotic resistance genes using quantitative PCR. Antibiotic resistance is ancient and widespread in the natural environment (38), with microbes employing antibiotic production and resistance as strategies to overcome their competitors. To mitigate the antibiotic resistance risk, in addition to efforts toward restricting antibiotic use, it is essential to consider reducing microbial competition as a means to modulate ARGs (18). In our study, carbon starvation drove the enrichment of these ARGs. Starved heterotrophic microbes engaged in an intense struggle for survival, resulting in an arms race. Antibiotic production could eliminate surrounding competitors, and the killed neighbors might also be a source of carbon and nutrients in oligotrophic environments. This strong evolutionary pressure drives mechanisms to evade the toxic effects of antibiotics. Antibiotic resistance genes can thus be seen as the evolutionary trajectory of sensing and evading antibiotic production in chemical warfare.

As carbon starvation is caused by long-term chemical-only fertilization, the implications of these findings for ARG enrichment could extend beyond microbial competition. Soils are increasingly recognized as natural reservoirs of antibiotic resistance, with a potential for dissemination within microbial communities, including clinical pathogens (39). Foods produced on soils subjected to long-term chemical-only fertilization, widely used in conventional agriculture, may contain a higher abundance of antibiotic resistance genes. However, despite its significance to consumers, the fate of microorganisms from soil to food to the human body remains nearly unknown. Our study underlines the importance of a broader perspective to soil biodiversity, encompassing the entire chain from field to fork to human health (40). This, in turn, underscores the need for a more holistic approach that incorporates soil health in the “One Health” concept (41), which was originally based on recognizing the interconnectedness of humans,

animals, and ecosystems to prevent the emergence of zoonotic diseases and promote overall well-being. Further, our study emphasizes the need to understand the impacts of agricultural practices in detail to develop a more sustainable and healthier agriculture. Given the current global threat of antibiotic resistance to public health, agricultural practices that have the least impact on the antibiotic resistome should be prioritized.

We used high-throughput qPCR to quantify genes associated with organic carbon degradation and ARGs, supplementing our metagenomic findings. The quantification of genes associated with organic carbon degradation revealed the hydrolytic capacities of soil microbes for starch, pectin, hemicellulose, cellulose, chitin, and lignin (*SI Appendix, Fig. S5*). Surprisingly, the gene abundance of these categories was generally higher in carbon-rich soils, which appeared at variance with our metagenomic findings based on the CAZy database. This discrepancy may stem from the limited representativeness of the high-throughput qPCR, which targeted only 18 genes (*SI Appendix, Table S11*). For instance, only two genes were considered for chitin hydrolysis and three for cellulose hydrolysis, undersampling the huge functional diversity of soil microbes. In contrast, the metagenomic approach covered nearly 400 CAZy families, comprising ~2.9 million amino acid sequences, and thus offering a broader view. This difference could also be attributed to the specifics of the quantification approaches: absolute quantification in high-throughput qPCR versus relative quantification in metagenomics (42). Regarding ARGs, the high-throughput qPCR findings largely mirrored those of the metagenomic analysis based on the CARD database (*SI Appendix, Fig. S6*). Among the eight drug classes identified by qPCR, the trends in gene abundance of four classes (aminoglycoside, phenicol, sulfonamide, and tetracycline) aligned with the metagenomic findings. One class (multidrug) showed a divergent trend, and the remaining three were not comparable due to their absence as standard categories in the CARD database. Notably, qPCR primarily captured high-abundance drug classes, and the ARG enrichment in rare drug classes identified in the metagenomic analysis was overlooked in the qPCR, underscoring its limited representativeness for the overall community function. The qPCR targeted 243 ARGs (*SI Appendix, Table S12*), while the CARD database contains over 5,000 genes. In addition, we compared the abundance trends of single ARGs detected by both methods (*SI Appendix, Table S13*). More than 80% of the genes exhibited consistent trends, with about 35% of these correlations being significant. This concordance validates our results to an extent. Overall, while high-throughput qPCR provides precise quantification of specific gene groups, metagenomics offers a broader perspective on community-level functions. This study prioritizes the latter, considering our focus on the overarching functionality of microbial communities. Future research could benefit from expanding the range of target genes in high-throughput qPCR or integrating absolute quantification in metagenomics to deepen our understanding of microbial functional dynamics in soil ecosystems. Furthermore, we utilized single-cell techniques based on the cellenONE platform to investigate soil microbes, confirming the results derived from metagenomic MAGs and achieving consistent conclusions (*SI Appendix, Table S14 and Fig. S7*).

In conclusion, our study probed the ecology and evolutionary consequences of microbial adaptations to carbon limitation from the perspectives of organic carbon metabolism, antibiotic resistance, and virus–host interactions. In response to carbon limitation, microbes invest more energy in producing diverse extracellular enzymes to break down organic compounds for energy and nutrient acquisition, reflecting scramble competition. Microbes also specialize in utilizing more complex organic molecules to broaden

chemical niches, thereby gaining a competitive advantage in carbon-poor soils. The ARG enrichment in carbon-poor soils indicates an ongoing arms race among microbes (i.e., contest competition), raising concerns about the potential spread of antibiotic resistance. In addition to resource consumption and chemical warfare, the virus confers population-level benefits to its hosts by improving both their competitiveness and environmental adaptability through AMGs. These findings point to the enhancement of microbial adaptations related to competition in response to carbon limitation. This study deepens our understanding of microbial strategies to compete for resources and emphasizes the role of viral auxiliary metabolisms in microbial competition. Future efforts should be devoted to quantifying the contribution of microbial competition to microbial community assembly using ground-truthing techniques and direct observation at a finer resolution, such as single-cell technologies (43). These approaches will enable accurate prediction of the responses of microbial communities and their functions in the context of competition dynamics. Harnessing microbial competition offers the potential for managing antibiotic resistance, thereby safeguarding human and ecosystem health.

Materials and Methods

Field Sites and Soil Sample Collection. Four long-term fixed fertilization experiments were selected throughout the major grain-producing areas of China (4), spanning approximately 28°N to 46°N (*SI Appendix, Fig. S1*). Each field experiment has been underway for about 40 y. The soil properties at the sites at the start of the field experiment are listed in *SI Appendix, Table S15*. The two contrasting fertilization treatments were 1) chemical-only fertilization, CF; 2) organic-only fertilization, OF. Three separate plots were used for each treatment at each experimental site, and all the plots are arranged in a randomized block design. In each plot, ten 5-cm diameter soil cores were taken at a depth of 0 to 20 cm and combined to form a composite sample representative of the plot.

DNA Extraction and Metagenomic Sequencing. Soil DNA was extracted using a FastDNA™ SPIN Kit for Soil (MP Biomedicals). Paired-end sequencing was performed on an Illumina sequencing platform, and reads with an average length of 150 bp were generated for each end, yielding ~960 G raw data. The quality of raw data was evaluated by FastQC v0.11.9. Low-quality reads and adapter sequences were filtered out using Trimmomatic v0.38 (adaptor trimming, average quality = 20), yielding ~733 GB of clean data. Megahit v1.2.9 was used to assemble clean reads into contigs with a k-mer list: 21, 29, 39, 49, 59, 69, 79, 89, 99, 109, 119, 129, and 141. Approximately 59.3 million contigs were obtained. Prodigal v2.6.1 was used to predict ORFs, producing ~57 million ORFs. Clean reads were mapped to each ORF using Salmon v1.4.0 to quantify gene abundance (transcripts per million, TPM). To estimate the functions related to organic C metabolisms, the ORFs were annotated against the Carbohydrate-Active enZymes database (CAZy database) using hmmscan-parser.sh (<https://bcbl.unl.edu/dbCAN2/download/Databases/dbCAN-old@UGA/>) with default parameters. Antibiotic resistance genes were annotated based on the CARD database with USEARCH v.11.0 (-id 90, -evaluate 1e⁻¹⁰).

Construction and Classification of MAGs. Contigs were clustered to recover bins using MaxBin2 v2.2.7, CONCOCT v1.0.0 and MetaBAT2 v2.0 with default parameters. After that, the “bin_refinement” module in MetaWRAP v1.2.1 was used to improve the quality of bins. SemiBin v1.0.0 was used to achieve semisupervised binning. Finally, 4,851 bins were obtained. CheckM v1.1.3 was used to evaluate the completeness and contamination of bins. After filtering out low-quality bins (completeness <70% and contamination >10%) (44), 517 high-quality bins were retained and considered MAGs. These MAGs were further dereplicated using dRep v3.3.0 with “-sa 0.95,” resulting in 69 unique MAGs. The taxonomic annotation and phylogenetic tree were constructed based on the Genome Taxonomy database Release 207 (GTDB) using GTDB-Tk v1.5.0 with “de_novo_wf” module. CoverM v0.6.1 was used to estimate the abundance of MAGs across samples. The processes and parameters

for predicting ORFs and functional annotations for MAGs were consistent with those used for contigs.

Identification of Viral Sequences, AMGs, and Their Hosts. Viral sequences were identified using VIBRANT v1.2.1 (default parameters), VirSorter v2.2.3 (max_score ≥ 0.9), and CheckV v0.8.1 based on assembled contigs. Contigs identified by all three tools as viral sequences were retained (45). Viral contigs were binned into viral metagenome-assembled genomes using vRhyme v1.1.0 and then clustered into vOTUs (i.e., representative sequences for virus) along with unbinned contigs using CD-HIT-EST v4.8.1 with the parameters “-c 0.95 -aS 0.85” (46). Further, vOTUs with a length ≤ 2 kb were filtered out to reduce false positives (47), except for those identified as complete genomes by VIBRANT v1.2.1 and CheckV v0.8.1. The abundance of vOTUs was also estimated using CoverM v0.6.1. PhaGCN2.0 based on the built-in database and mmseq2 (e-value $\leq 10^{-5}$ and bit score ≥ 50) based on the NCBI RefSeq virus database was used to classify the vOTUs. VIBRANT was used to identify AMGs in viral sequences (48). The abundance of AMGs was calculated with Salmon v1.10.1.

Three methods, homology matches, tRNAs similarity, and CRISPR spacer similarity, were used to identify the hosts of virus (49). The full sequences, tRNA region, and CRISPR spacer regions of vOTUs were searched against the high-quality MAGs recovered in this study. The blastn v2.5.0 + (coverage = 100, identity = 100), tRNAScan-SE v1.2.3, and CRISPRCasFinder were used to align sequences, identify tRNAs, and search for CRISPR spacers, respectively.

Statistical Analyses. Data normality was tested using the Shapiro–Wilk test. Given the large geographic scale of sampling locations, it was important to consider the potential influence of geographic factors on gene abundance across samples. To specifically investigate the effects of fertilization while minimizing the influence of geographic factors, we used a linear mixed model to compare gene abundance between the chemical-only and organic-only fertilization regimes. In the model, the “Fertilization regime” (CF or OF) factor was fixed, and the “Sites” (four locations) factor was considered a random effect. This approach effectively addressed any potential data nonindependence or within-cluster correlation arising from the sampling locations, thereby

guaranteeing rigorous and accurate analysis. The two-way ANOVA was conducted in R software using the “aov” function. Co-occurrence networks were generated using the Molecular Ecological Network Analyses (MENA) Pipeline (<https://ieq2.ou.edu/MENA/>).

Data, Materials, and Software Availability. The metagenomics sequences obtained in this study have been submitted to NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/Traces/sra/>) under accession number SRP443231 (50).

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