



Microbe-dependent heterosis in maize

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Hybrids account for nearly all commercially planted varieties of maize and many other crop plants because crosses between inbred lines of these species produce first-generation [F₁] offspring that greatly outperform their parents. The mechanisms underlying this phenomenon, called heterosis or hybrid vigor, are not well understood despite over a century of intensive research. The leading hypotheses—which focus on quantitative genetic mechanisms (dominance, overdominance, and epistasis) and molecular mechanisms (gene dosage and transcriptional regulation)—have been able to explain some but not all of the observed patterns of heterosis. Abiotic stressors are known to impact the expression of heterosis; however, the potential role of microbes in heterosis has largely been ignored. Here, we show that heterosis of root biomass and other traits in maize is strongly dependent on the belowground microbial environment. We found that, in some cases, inbred lines perform as well by these criteria as their F₁ offspring under sterile conditions but that heterosis can be restored by inoculation with a simple community of seven bacterial strains. We observed the same pattern for seedlings inoculated with autoclaved versus live soil slurries in a growth chamber and for plants grown in steamed or fumigated versus untreated soil in the field. In a different field site, however, soil steaming increased rather than decreased heterosis, indicating that the direction of the effect depends on community composition, environment, or both. Together, our results demonstrate an ecological phenomenon whereby soil microbes differentially impact the early growth of inbred and hybrid maize.

environment in which all microbial members are known, we developed gnotobiotic growth bags for growing individual maize plants in sterile conditions (see *Methods*). We planted surface-sterilized kernels of two inbred lines (B73 and Mo17) and their F₁ hybrid (B73xMo17) in individual gnotobiotic growth bags containing autoclaved calcined clay hydrated with sterile 0.5× Mura-shige and Skoog (MS) salt solution. The clay in each growth bag was inoculated with either a highly simplified synthetic community (SynCom) of seven bacterial strains known to colonize maize roots (10, 11) (~10⁵ CFU/mL [colony-forming units per milliliter]) or a sterile buffer control. This system effectively eliminated contact between plants and external microbes (*SI Appendix, Fig. 1B*); however, it is possible that some kernels may have contained viable endophytes that could not be removed by surface sterilization (*SI Appendix, Fig. 1C*). Genotypes and treatments were placed in randomized locations in a growth chamber under standard conditions (12-h days, 27 °C/23 °C, ambient humidity). After 4 wk, we harvested plants to investigate root colonization by these seven strains.

Unexpectedly, we observed that the inbred and hybrid plants were indistinguishable with respect to root and shoot fresh weight when grown in uninoculated growth bags, yet they showed the expected heterotic pattern when grown with the synthetic bacterial community (Fig. 1A and *SI Appendix, Tables 1 and 2*). This was due to a negative effect of the bacteria on both inbred genotypes rather than a positive effect on the hybrid. Although the SynCom

hybrid vigor | microbiome | maize | endophytes | rhizosphere

In nature, all plants form close associations with diverse microbial symbionts that comprise a subset of the microbial species with which they share a habitat (1, 2). As part of their host's environment, the host-associated microbial community (microbiome) can cause plasticity of important plant traits such as reproductive phenology, disease resistance, and general vigor (3–6). However, genetic variation within plant species affects not only microbiome assembly but also the phenotypic response to microbes. Disentangling these relationships is a critical step toward understanding how plant–microbiome interactions evolved and how they can be harnessed for use in sustainable agriculture (7). Here, we describe our observation that the soil microbial community mediates heterosis, the strong and pervasive phenotypic superiority of hybrid maize genotypes relative to their inbred parent lines. The strength of heterosis within a given environment has previously been shown to depend on abiotic factors such as water and nutrient availability (8); however, this report shows that microbes also affect plant heterosis, a phenomenon of immense economic value and research interest.

In a previous field experiment, we observed that maize hybrids generally assemble rhizosphere microbiomes that are distinct from those of inbred lines. In addition, many microbiome features in first generation F₁ hybrids are not intermediate to those of their parent lines, suggesting that heterosis of plant traits is associated with heterosis of microbiome composition itself (9). To determine whether the same patterns manifest in a highly controlled

Significance

Almost all grain crops grown on commercial farms are hybrid cultivars because these hybrid plants are reliably healthier, larger, and more productive than their inbred parent lines. The widespread and valuable phenomenon of hybrid superiority is called heterosis. Despite over a century of intensive research into heterosis, it is unclear how or why hybrid genomes give rise to superior phenotypes. Most hypotheses and research thus far have focused on genetic and physiological mechanisms of heterosis. In contrast, this article presents evidence for a microbe-driven mechanism of heterosis, whereby the activity of live soil microbes affects the expression of heterosis. This finding will open lines of research that could advance our understanding of heterosis.

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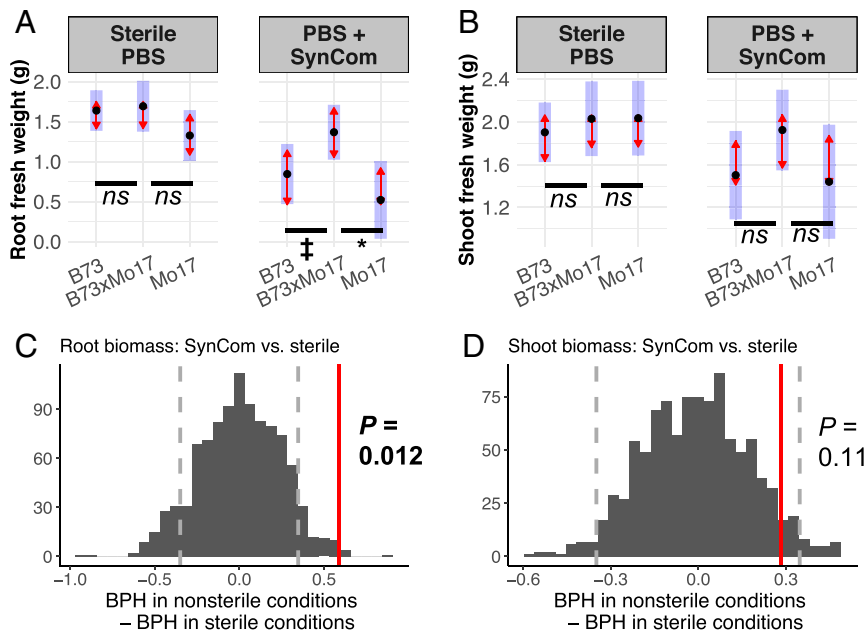


Fig. 1. In Experiment 1, maize kernels were grown in calcined clay inoculated with PBS with or without 10^7 CFU/mL of a SynCom of seven bacterial strains (final concentrations: 0 or 10^5 CFU/mL; see *Methods*). Sterile conditions reduced the strength of heterosis for (A) root biomass but not (B) shoot biomass. (A and B) Black points show the estimated marginal mean (EMM) trait values for each genotype in each treatment; blue rectangles show the 95% CIs for the EMMs. The red arrows show the 95% CIs for pairwise tests between genotypes in each treatment after correction for the familywise error rate using Tukey's procedure; nonoverlapping arrows indicate statistically significant differences ($\alpha = 0.05$). Detailed statistical results are provided in *SI Appendix, Table 1*. $n = 14$ per inbred genotype per treatment; $n = 7$ per hybrid genotype per treatment. $***P < 0.001$; $**P < 0.01$; $*P < 0.05$; \ddagger , $P < 0.1$; ns, $P > 0.1$ (Dunnett's test of contrasts between each inbred line and the hybrid). (C and D) The strength of BPH was calculated for each trait in each treatment using the EMM trait values. Observed Δ BPH is shown as a vertical red line. The histograms show the distributions of Δ BPH for 999 permutations of the data with respect to treatment (i.e., the distribution of Δ BPH if there were no effect of treatment). The vertical gray dashed lines mark the 2.5% tails of the null distribution. P values indicate the proportion of simulated Δ BPH that were at least as extreme as the observed Δ BPH. Permutation test results for MPH are provided in *SI Appendix, Table 2*.

contained no known pathogens (11), it decreased the root weight of B73 and Mo17 seedlings by 48.4% (SEM = 13.6%) and 60.8% (SEM = 21.5%), respectively (Fig. 1). In contrast, the SynCom reduced root weight of hybrids by only 19.2% (SEM = 13.6%). As a result, the strength of better-parent heterosis (BPH) was reduced from 61.9% in nonsterile conditions to 3.3% in sterile conditions (permutation test $P = 0.012$); a similar pattern was observed for shoot weight ($P = 0.11$; Fig. 1 C and D). The SynCom also increased the strength of midparent heterosis (MPH) for root biomass from 14.2% to 100% (permutation test $P = 0.004$). A separate experiment revealed that the SynCom also lowered the germination rates for both inbred lines but not the hybrid (*SI Appendix, Fig. 2*). Germination of B73 after 4 d was 10.7% lower (SEM = 4.3%) in the presence of the SynCom relative to the sterile control; for Mo17, the SynCom decreased germination rates by 32% (SEM = 5.8%) (*SI Appendix, Fig. 2*). To test whether the observed phenotype differences correlated with differences in root colonization by any of the 7 SynCom members, we carried out a separate growth-bag experiment to test root-colonization levels. By 10 d after seedling emergence, total SynCom counts did not differ between genotypes (*SI Appendix, Fig. 3*). We did, however, find that one of the SynCom members (*Stenotrophomonas maltophilia*) was significantly more abundant in B73 than in the hybrid (ANOVA with Tukey post hoc test, $P < 0.05$).

To determine whether natural, complex soil microbial communities also induce heterosis, we conducted a second growth-chamber experiment with surface-sterilized kernels of the same three genotypes and a slightly modified protocol for gnotobiotic growth. We saturated the calcined clay medium in each growth bag with one of three treatments: a slurry derived from filtered farm soil, an autoclaved aliquot of the same slurry, or a sterile buffer control. Genotypes and treatments were arranged into randomized,

replicated blocks in a growth chamber. We recorded the germination success or failure of each kernel and observed that the live soil slurry had a strong negative effect on germination of both inbred lines but not the hybrid (Fig. 2A). The unusually poor germination by Mo17 was likely exacerbated by the humid conditions in the growth bags combined with the microbial activity, because germination success was much higher under more realistic field conditions (see *Microbe-Dependent Heterosis in Two Field Sites*). This aligns with past work demonstrating that the emergence success of Mo17 drops rapidly with excess moisture and other stresses, whereas B73 is less sensitive (12–14). In the two sterile treatments, B73 and B73xMo17 germinated equally well. Mo17 still performed worse than B73xMo17, but the hybrid advantage was much less pronounced than it was in the live treatment. After 1 mo, we harvested all plants and measured fresh weights of roots and shoots. In growth bags that received the autoclaved slurry or sterile buffer treatments, all three genotypes produced root systems of approximately equal biomass on average; in contrast, the hybrid's root biomass was 18.3% higher than the midparent average and 14.3% higher than the better-parent value when grown with the live soil slurry, consistent with the expected pattern of heterosis (Fig. 2 B and C and *SI Appendix, Tables 3 and 4*). Very poor germination of Mo17 prevented statistical comparison of its biomass to the hybrid in the live slurry treatment. Shoot biomass displayed the expected heterotic patterns, with the hybrid outperforming the parental inbred lines under all conditions (*SI Appendix, Fig. 4*).

Microbe-Dependent Heterosis in Two Field Sites

Next, we conducted field experiments in Clayton, NC and Lawrence, KS to assess whether this phenomenon, which we termed "microbiota-dependent heterosis" or MDH, occurs in real soil under

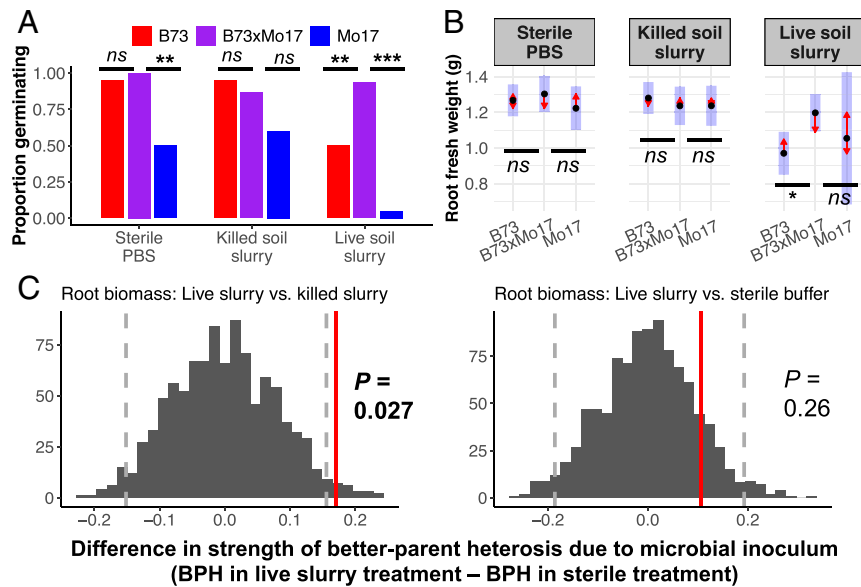


Fig. 2. In Experiment 2, maize kernels were grown in calcined clay inoculated with sterile PBS, a live soil slurry in PBS, or an autoclaved (killed) aliquot of the same soil slurry. Sterile conditions reduced the strength of heterosis for germination success and root biomass. (A) Fisher's exact test was used for statistical inference of germination proportions. (B) A linear mixed-effects model was used for statistical inference of root biomass. Black points show the estimated marginal mean (EMM) trait values for each genotype in each treatment; blue rectangles show the 95% CIs for the EMMs. The red arrows show the 95% CIs for pairwise tests between genotypes in each treatment after correction for the familywise error rate using Tukey's procedure; nonoverlapping arrows indicate statistically significant differences ($\alpha = 0.05$). Detailed statistical results are provided in *SI Appendix, Table 3*. $n = 20$ per inbred genotype per treatment, $n = 15$ per hybrid per treatment. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, $P > 0.1$ (Dunnnett's test of contrasts between each inbred line and the hybrid). (C) The strength of BPH was calculated for each treatment using the EMM trait values. Observed Δ BPH is shown as a vertical red line. The histograms show the distributions of Δ BPH for 999 permutations of the data with respect to treatment (i.e., the distribution of Δ BPH if there were no effect of treatment). The vertical gray dashed lines mark the 2.5% tails of the null distribution. P values indicate the proportion of simulated Δ BPH that were at least as extreme as the observed Δ BPH. Permutation test results for MPH are provided in *SI Appendix, Table 4*.

field conditions. In the North Carolina experiment, we planted surface-sterilized kernels of the same three genotypes into adjacent rows with four soil pretreatments to perturb soil microbial-community composition: 1) steamed, 2) fumigated with the mustard oil allyl

isothiocyanate (AITC), 3) steamed and fumigated with AITC, 4) fumigated with chloropicrin, and 5) untreated control (*SI Appendix, Fig. 5*). All four treatments reduced the density of *Pythium* spp., a common phytopathogenic oomycete, relative to the untreated control

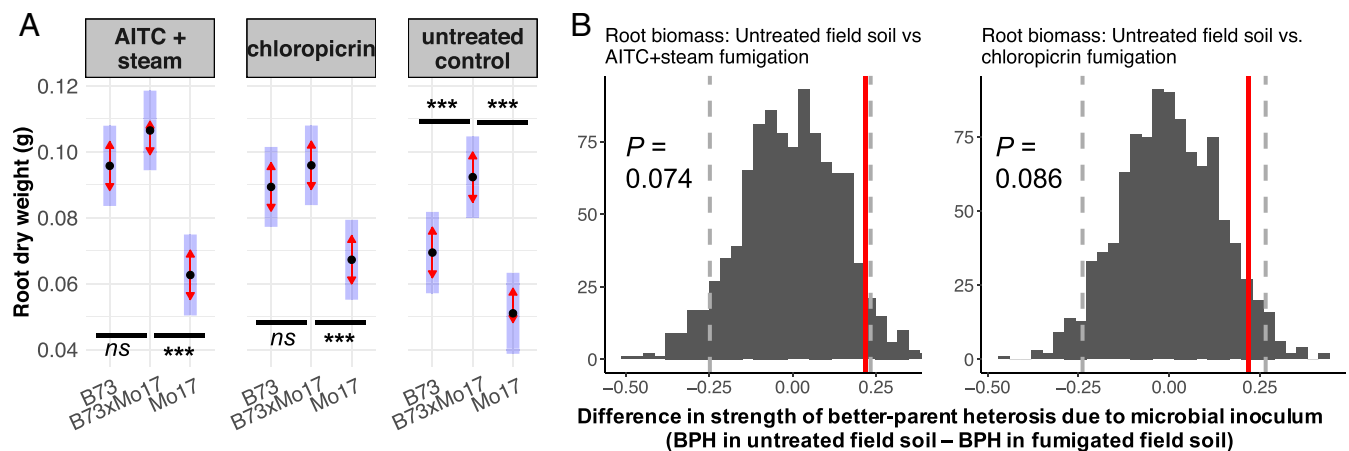


Fig. 3. In Experiment 3, we grew maize in a field in North Carolina from seeds planted into untreated soil, soil fumigated with chloropicrin, or soil fumigated with AITC and steamed. Perturbation of soil microbiomes reduced heterosis for root biomass after 4 wk of growth. (A) Black points show the estimated marginal mean (EMM) trait values for each genotype in each treatment; blue rectangles show the 95% CIs for the EMMs. The red arrows show the 95% CIs for pairwise tests between genotypes in each treatment after correction for the familywise error rate using Tukey's procedure; nonoverlapping arrows indicate statistically significant differences ($\alpha = 0.05$). Detailed statistical results are provided in *SI Appendix, Table 6*. Effects on shoot biomass, plant height, and number of leaves are presented in *SI Appendix, Fig. S9*. $n = 56$ per genotype per treatment. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; †, $P < 0.1$; ns, $P > 0.1$ (Dunnnett's test of contrasts between each inbred line and the hybrid). (B) The strength of BPH was calculated for each treatment using the EMM trait values. Observed Δ BPH is shown as a vertical red line. The histograms show the distributions of Δ BPH for 999 permutations of the data with respect to treatment (i.e., the distribution of Δ BPH if there were no effect of treatment). The vertical gray dashed lines mark the 2.5% tails of the null distribution. P values indicate the proportion of simulated Δ BPH that were at least as extreme as the observed Δ BPH. Permutation test results for MPH are provided in *SI Appendix, Table 7*.

(SI Appendix, Table 5); however, 2 wk after treatment, counts of viable culturable bacteria were temporarily reduced only in the AITC + steam treatment and only in shallow soil (SI Appendix, Fig. 6). Additionally, amplicon sequencing of the 4th variable (V4) region of the 16S ribosomal RNA (rRNA) gene and the fungal internal transcribed spacer 1 (ITS1) confirmed that these treatments shifted the composition of the bacterial and fungal soil microbiomes relative to the control (SI Appendix, Fig. 7). The effects of the fumigation treatments persisted in the bulk soil for at least 6 wk and were also detected in the root microbiomes of juvenile plants at the end of the experiment (SI Appendix, Fig. 8). We monitored seedling emergence and measured leaf number and plant height at 15 d after planting (d.a.p.) and again at 27 d.a.p. After this final in-field measurement, we uprooted all plants in the control, chloropicrin, and AITC + steam treatments and measured their root and shoot biomass. Perturbation of the soil microbial community using chloropicrin or AITC + steam weakened heterosis of root biomass (Fig. 3 and SI Appendix, Tables 6 and 7). Additionally, all fumigation and steaming treatments decreased the strength of BPH and MPH for both height and leaf number (SI Appendix, Fig. 9 and Table 7). In contrast, heterosis of shoot dry weight was not affected. Germination success rates were similar among treatments (SI Appendix, Fig. 10). We note that AITC may influence plant development directly (15); however, the responses of each genotype to treatments involving AITC were generally congruent with responses to the non-AITC treatments.

In the Kansas field experiment, we planted surface-sterilized seeds ($n = 270$) of the same genotypes into shallow open-bottom pots that were filled with either steam-sterilized or nonsteamed soil. Thus, individuals that were planted in steamed soil encountered a normal soil microbiome as soon as their roots grew down beyond the depth of the pot (6 inches), whereas the control plants encountered a normal soil microbiome immediately as seeds. Despite the temporary nature of the microbiome perturbation, its effects on biomass heterosis persisted throughout the 8-wk duration of the experiment (Fig. 4 and SI Appendix, Tables 8 and 9). This indicates that the microbes encountered by very young plants can influence the expression of heterosis well into plant adulthood. Unlike in the previous experiments, however, microbiome perturbation strengthened heterosis rather than weakening it, and the change in heterosis was driven by the stronger phenotypic plasticity of the hybrid relative to the inbreds (Fig. 4 A and B). Soil steaming also increased BPH and MPH of leaf chlorophyll concentration in 4- to 8-wk-old plants (SI Appendix, Fig. 11) but slightly decreased BPH of the developmental stage (SI Appendix, Fig. 12). In contrast, it did not affect heterosis of emergence success or timing, and the increased heterosis of shoot height faded after a few weeks of growth (SI Appendix, Figs. 13 and 14). Interestingly, the soil-steaming treatment also reduced the variation in several quantitative traits including shoot biomass, chlorophyll concentration, and the developmental stage; this effect was more pronounced in the hybrid than in the inbreds (SI Appendix, Fig. 15).

Discussion

Our results suggest that interactions with soil-borne microbes are important for the expression of heterosis in maize. We observed MDH in four independent experiments representing very different environmental contexts: in tightly controlled laboratory conditions with an inoculum of only seven bacterial strains (Fig. 1); in a growth chamber with a more complex microbial slurry derived from farm soil (Fig. 2); and in two distinct field sites with or without soil fumigation or steaming (Figs. 3 and 4 and SI Appendix, Figs. S9, S11, and S12). This repeatability suggests that the mechanism could be robust across environments in the sense that all of these microbial communities altered the expression of heterosis in some way. However, the fact that soil steaming increased heterosis in one field site (Kansas) and decreased heterosis in another (North Carolina) indicates that the outcome depends

on the composition of the local microbial community, the abiotic environment, or both.

In the cases in which soil fumigation weakened heterosis, MDH was driven not by microbes selectively boosting the performance of hybrids but by soil-borne microbes selectively reducing the performance of the inbred lines. In the case in which soil steaming strengthened heterosis, however, it was the hybrid that showed the larger phenotypic response to the treatment, while the inbreds stayed relatively stable. These contrasting results suggest two possible, non-mutually exclusive explanations. First, they may indicate that hybrids are more resistant than inbreds to weakly pathogenic soil microbes (the “inbred immunodeficiency hypothesis”). Second, they may reflect a costly defensive overreaction by inbreds, but not hybrids, to innocuous soil microbes (the “inbred immune overreaction hypothesis”).

Multiple previous studies have described how plants that are immunocompromised through either genetic or chemical means can suffer infections that are not apparent in their immunocompetent neighbors. For example, maize mutants deficient in the defense hormone jasmonic acid were unable to grow to maturity in nonsterile soil (16). Similarly, *Arabidopsis* mutant lines lacking three defense hormone signaling systems displayed reduced survival in wild soil (17). Application of glyphosate to bean plants temporarily arrested their growth in sterile soils; in nonsterile soils, however, the plants died quickly due to root infection by *Pythium* and *Fusarium* species (18). Because glyphosate inhibits the biosynthesis of phenylalanine and chorismite—which are precursors of several important components of the defense response including lignin, salicylic acid, and phytoalexins—the study authors suggested that glyphosate predisposes the treated plants to infection by opportunistic pathogens to which they would otherwise be resistant (19). If weak pathogens drive MDH, then this implies that superior disease resistance in hybrids is a key mechanism of heterosis. Somewhat surprisingly, however, the effect of heterosis on plant disease resistance has not been well characterized. In maize, heterosis has been observed for resistance to anthracnose leaf blight and southern leaf blight but not to anthracnose stalk rot (20, 21). Heterosis for late-blight resistance has also been noted in the potato (22). This hypothesis suggests a possible link to overdominance, long proposed as a genetic mechanism of heterosis: if hybrids express more allelic variants for pattern recognition receptors and/or resistance genes than inbreds do, then they may be able to recognize and defend themselves against a wider variety of parasites (23).

In contrast, the inbred immune overreaction hypothesis does not require soil microbes to be pathogenic or parasitic but instead links MDH to the well-documented trade-off between growth and genetic disease resistance (24). For instance, innocuous soil microbes could trigger a costly defensive response in inbreds but not in hybrid maize. The most detailed work on heterosis of disease resistance supports this hypothesis: in the model species *Arabidopsis*, hybrids displaying heterosis for growth and yield also displayed a decreased level of basal defense gene expression and decreased concentrations of the defense signaling hormone salicylic acid (25–28). However, despite their lower investment in constitutive defenses, the hybrids were not compromised in resistance to the biotrophic pathogen *Pseudomonas syringae* nor in the inducible response to infection (28, 29). We note that, in our experiments with the seven-strain SynCom (which contained no known pathogens), total bacterial colonization did not differ between inbreds and hybrids (SI Appendix, Fig. 3). This suggests that, if the inbreds did mount a stronger defense response, it was not effective at preventing colonization.

Soil microbial communities are immensely complex, dynamic mixtures of organisms that are (whether consistently or facultatively) beneficial, parasitic, or neutral. Thus, a plant might express too much resistance to one community member and not enough resistance to another. This tension between evolutionary pressures

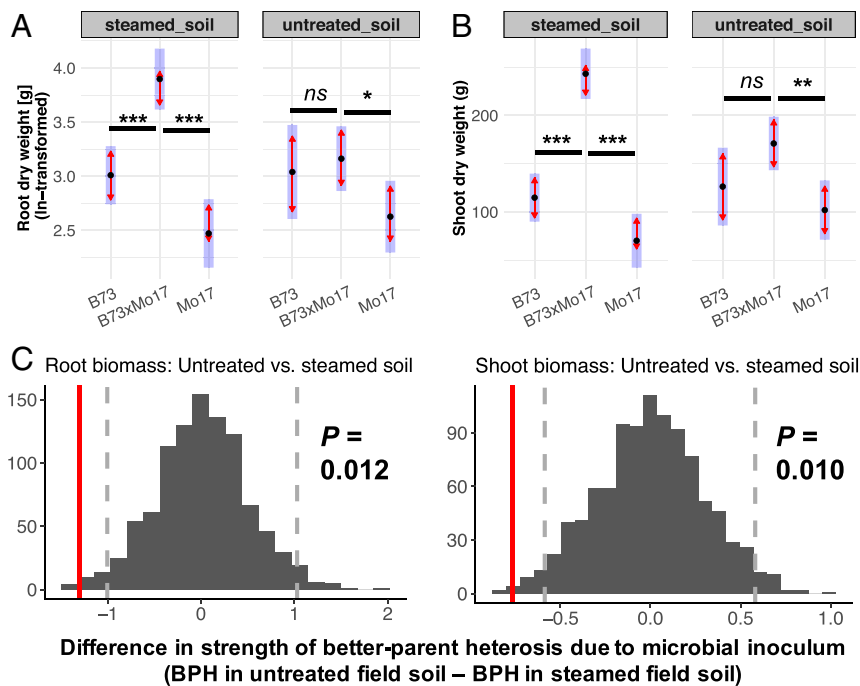


Fig. 4. In Experiment 4, we grew maize in a field in Kansas from seeds planted into untreated soil or steam-sterilized soil. After 8 wk of growth, heterosis of (A) root biomass and (B) shoot biomass was stronger in the steam-sterilized treatment. Black points show the estimated marginal mean (EMM) trait values for each genotype in each treatment; blue rectangles show the 95% CIs for the EMMs. The red arrows show the 95% CIs for pairwise tests between genotypes in each treatment after correction for the familywise error rate using Tukey's procedure; nonoverlapping arrows indicate statistically significant differences ($\alpha = 0.05$). Detailed statistical results are provided in *SI Appendix, Table 8*. Effects on emergence timing, plant height, developmental stage, and chlorophyll concentration are presented in *SI Appendix, Figs. S11, 12, 13, and 14*. $n = 45$ per genotype per treatment. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, $P > 0.1$ (Dunnett's test of contrasts between each inbred line and the hybrid). (C) The strength of BPH was calculated for each treatment using the EMM trait values. Observed Δ BPH is shown as a vertical red line. The histograms show the distributions of Δ BPH for 999 permutations of the data with respect to treatment (i.e., the distribution of Δ BPH if there were no effect of treatment). The gray dashed lines mark the 2.5% tails of the null distribution. Analogous results for MPH are provided in *SI Appendix, Table 9*.

to repel parasites, to avoid overreaction to harmless microbes, and to form beneficial symbioses is well illustrated by the co-evolution of rhizobia and their legume hosts to prevent immune reactions during colonization, which would be costly for both partners (30). If hybrids and inbreds differ in the balance between different strategies to modulate the defense response and to discern helpful from harmful microbes, then each of those strategies may work better in certain microbial communities than in others, with neither being optimal in all communities due to the enormous diversity of microbe-associated molecular patterns that induce varied reactions in the host (23). External factors, such as nutrient availability, likely complicate these strategies by affecting the potential benefit from symbiosis and the energetic cost of defense. For example, in phosphorous-limited conditions, mycorrhizal fungi increased shoot biomass of Mo17 and B73 by 106% and 800%, respectively, but when phosphorous was plentiful, the same microbes actually decreased biomass of both genotypes (31). Additionally, in the context of our work, the various sterilization treatments applied in the two field experiments likely did not uniformly impact all members of the soil communities, making interpretation more difficult. Clearly, much more work is needed to test the full range of natural soil microbiome diversity and the full range of plant genotypes.

For these reasons and others, the implications of our findings for genetic crop improvement are not immediately clear. At this point, the microbial mechanism of heterosis that our results suggest cannot be directly incorporated into breeding programs. We are not suggesting that the differential effects of microbes on hybrids and inbreds are the only or dominant cause of heterosis nor that our data invalidate hypothesized genetic mechanisms for heterosis such as dominance and overdominance. Rather, we are demonstrating

that the strength of heterosis within a given environment depends, to some extent, on the local soil microbial community, in the same way that it has been shown to depend on abiotic factors such as nitrogen and water availability (8). To our knowledge, MDH has not been previously reported, nor are we aware of counter examples that carefully manipulated the microbial environment but observed no effect on heterosis. Some past studies used surface-sterilized kernels to study heterosis in maize seedlings (e.g., ref. 32), but these neither maintained sterile conditions after the initial removal of seed-associated biota nor intentionally exposed the seedlings to contrasting microbial treatments, which is necessary to test for MDH.

Altogether, our results shed unexpected light on the causes of heterosis, which have remained elusive despite over a century of investigation. They demonstrate the importance of the microbial environment for mapping genotype to phenotype and generate testable hypotheses about the mechanisms of this widespread and critically important phenomenon. Many questions remain, and future work will require careful experimentation to delve into the molecular and physiological mechanisms of MDH, to assess the evidence for or against the inbred immunodeficiency hypothesis and the inbred immune overreaction hypothesis, and to develop new hypotheses. Progress in this area will accelerate the integration of microbiome science into sustainable agricultural solutions, including prediction of cultivar responses to biologicals and microbiome optimization via genetic improvement of the host plant (33). In addition, it may reveal candidate genes that could be targeted in gene editing or conventional breeding programs to confer some benefits of heterosis to nonhybrid cultivars. These new avenues of research have high potential to advance our

understanding of heterosis in maize and many other crops and to lead to new innovations for agricultural sustainability and productivity.

Methods

The genotypes used for all experiments were B73, Mo17, and their F₁ hybrid B73xMo17. All data analysis was performed using R version 3.5.3, particularly the packages lme4, tidyverse, lmerTest, car, pbkrtest, and emmeans (34–39).

Experiment 1 (December 2018). In a laminar flow hood, we placed kernels of each genotype into a sterile 7.5" × 15" Whirl-Pak self-standing bag (Nasco) filled with 200 mL autoclaved calcined clay ("Pro's Choice Rapid Dry"; Oil-Dri Corporation). Immediately prior to planting, seeds were surface-sterilized using a 3-min soak in 70% ethanol (vol/vol) followed by a 3-min soak in 5% bleach (vol/vol) and three rinses with sterile deionized water; we plated extra seeds on malt extract agar to confirm that this protocol was effective (SI Appendix, Fig. 1C). To each growth bag, we added 120 mL either sterile 0.5× Murashige and Skoog basal salt solution (pH 6.0) or the same solution containing 10⁵ cells/mL (diluted from 10⁷ cells/mL in 1X phosphate-buffered saline [PBS]) of a SynCom of seven bacterial strains known to colonize maize roots (10). We planted 28 kernels of each inbred line and 14 of the hybrid, divided evenly between the SynCom and control treatments, 4.5 cm deep using sterile forceps. The growth bags were sealed with sterile AeraSeal breathable film (Excel Scientific, Inc.) to allow gas exchange and then placed in randomized positions in a growth chamber (Percival Scientific Inc.). No additional liquid was added after the growth bags were sealed. After 1 mo of growth (12-h days, 27 °C/23 °C, ambient humidity), we opened the growth bags, uprooted the plants, rinsed off adhering clay, and patted them dry before measuring fresh weight of shoots and roots. We applied two-way ANOVA to linear models of biomass with genotype, treatment, and their interaction as predictor variables. *F*-tests with Type III sums of squares were used for significance testing, and pairwise contrasts between the hybrid and each inbred were performed using Dunnett's post hoc procedure.

SynCom Effects on Germination. To test whether the SynCom affected germination, we conducted a 3 × 2 × 3 full factorial experiment manipulating plant genotype (B73, Mo17, and their F₁ hybrid), microbial inoculant (SynCom versus sterile control), and nutrient content (water, Hoagland's solution, or MS). Five surface-sterilized kernels were placed onto filter paper in five Petri dishes per genotype–inoculum–nutrient combination (*n* = 90 Petri dishes) and inoculated with 2 mL SynCom (diluted to 10⁵ cells · mL⁻¹ in nutrient solution) or a sterile nutrient solution control. Petri dishes were incubated in the dark at 30 °C and germination rate was recorded for each dish after 4 d. We used the Kruskal–Wallis test for main effects of genotype, inoculum, and nutrient treatment and for an interaction between genotype and inoculum. Mann–Whitney *U* tests were used for pairwise contrasts; *P* values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate (40).

Assessment of Root Colonization by SynCom Members in All Three Genotypes (January 2021). We sterilized and planted kernels of all three genotypes in Whirl-Paks with the SynCom (see Experiment 1 [December 2018]). In anticipation of genetic differences in germination success, we planted 40 kernels for Mo17, 25 for B73, and 15 for B73xMo17. Bags were randomized and placed on a shelf with light-emitting diode (LED) growth lights (16-h days, 23 °C, ambient humidity). Emergence of seedlings was documented daily, and roots were harvested for the colonization assay 10 d after emergence. Roots were gently rinsed in deionized water, and the primary root was cut and weighed for each plant. Roots were chopped, suspended in 1 mL sterile PBS with six 3-mm sterile glass beads (Sigma). Tubes were vortexed 3 × 1 min with a 10-s dwell time. The slurry was used to create a 10-fold dilution series with dilutions 10⁻¹ to 10⁻⁸ from which 10 μL was spotted on selective media for each of the seven strains as described in Niu and Kolter (10), with one modification. The medium for selective plates for *Enterobacter cloacae* was modified after tests by reducing the NaCl concentration from 9.3 to 4.65%. Plates were incubated at 30 °C for 16 to 60 h, and then colonies were counted. Colony counts were normalized to grams of root fresh weight.

Experiment 2 (January 2019). To determine whether natural soil microbial communities produced the same effect as the SynCom, we used the same gnotobiotic growth bags as in Experiment 1 to compare plant growth in the following: 1) a live soil slurry, 2) an autoclaved soil slurry, and 3) sterile buffer. We collected soil in November 2018 from field G4C at the Central Crops

Research Station and stored it at 4 °C until use. We mixed 200 g of this soil into 1 L PBS with 0.0001% Triton X-100 using a sterile spatula. The suspension was allowed to settle, filtered through Miracloth (22 to 25-μm pore size; Calbiochem), and centrifuged for 30 min at 3,000 × *g*. The resulting pellet was resuspended in 200 mL sterile PBS and immediately divided into two aliquots of 100 mL each. One aliquot was autoclaved for 30 min at 121 °C to produce a "killed" slurry concentrate. Live and killed soil slurry concentrates were diluted (10 mL slurry per liter 0.5× MS) to produce the final slurry treatments. An additional control consisted of diluted sterile PBS (10 mL PBS per liter 0.5× MS). Kernels were surface sterilized (see Experiment 1 [December 2018]), planted in 150 mL sterile calcined clay, and hydrated with 90 mL of one of these three treatments in the gnotobiotic growth bags described above (*n* = 20 per treatment for B73 and Mo17; *n* = 15 per treatment for B73xMo17). Prior to planting, the kernels were weighed and distributed evenly to ensure no systematic differences in seed size among the treatments. Bags were arranged into randomized, replicated blocks in a growth chamber in the Duke University Phytotron (12-h days, 27 °C/23 °C, ambient humidity) and uprooted after 1 mo of growth for measurement of shoot fresh weight and root fresh weight. Fisher's exact test was used to compare germination proportions between genotypes within each treatment. We applied two-way ANOVA to linear mixed-effects models of biomass with genotype, treatment, and their interaction as fixed predictor variables and block as a random-intercept term. *F*-tests with Type III sums of squares were used for significance testing of fixed effects, and pairwise contrasts between the hybrid and each inbred were performed using Dunnett's post hoc procedure. Likelihood ratio tests were used for significance testing of random effects.

Experiment 3 (September to November 2019). To determine whether MDH could be observed under field conditions, we conducted an on-farm soil sterilization experiment at the Central Crops Research Station, in a field that has been only in strawberries and cover crops for at least 5 to 6 y. Total bed width was 152 cm furrow to furrow, and beds were 20 cm high with a 76-cm width at the top. Five treatments were established in a complete block design: steam only (1 h, 5 bar); AITC (280 L/ha); AITC (280 L/ha) followed by steam (1 h, 5 bar); nontreated control; and chloropicrin (320 L/ha Pic-Clor 60). AITC and chloropicrin were applied on September 11, 2019 through shank application in raised beds. After fumigation, raised beds were covered with black totally impermeable film (TIF) plastic. Steam was applied on September 27th using a SIOUX SF-25 Natural Gas Steam Generator (SIOUX Inc.). The steam generator has a net heat input of 1.01 × 10⁶ BTU/hr and an average steam output of 383 kg/hr. The steam generator was mounted on a flatbed trailer and connected to natural gas tanks, a 1,300-L water tank, and a natural gas electrical generator (SI Appendix, Fig. 5F). Steam was applied consistently for 1 h at 5 bar, injecting steam at a 12-cm depth under TIF plastic using custom-made steam-graded spike hoses (SI Appendix, Fig. 5E). Temperature was monitored at different depths using HOBO U12 Outdoor/Industrial Data Logger (Onset Computer Corporation). The maximum temperatures reached in the steam-only treatment were 100 °C at the 12-cm depth; in the AITC + steam treatment, maximum temperatures of 66 °C were measured (SI Appendix, Fig. 4D). Kernels were hand planted 4 cm deep into slits in the plastic (6-inch spacing between slits with two seeds 3 inches apart on opposite ends of each slit) randomized within four blocks per treatment and seven subblocks per block. To reduce seed-borne microbial load while in the field, we soaked kernels in 3% hydrogen peroxide for 2 min and rinsed in sterile distilled water (dH₂O) immediately prior to planting. Plants were monitored for emergence three times (5, 8, and 12 d after planting), and height was measured twice (15 and 27 d after planting). After 27 d of growth, plants from three of the treatments (chloropicrin, AITC + steam, and control) were uprooted and oven dried for measurement of root and shoot biomass. For the biomass data, we applied two-way ANOVA to linear mixed-effects models with genotype, treatment, and their interaction as fixed predictor variables and block and subblock as random-intercept terms. For the height and leaf number data, we applied three-way repeated-measures ANOVA with genotype, treatment, date, and all interactions as fixed predictors and plant, block, and subblock as random-intercept terms. *F*-tests with Type III sums of squares were used for significance testing of fixed effects, and pairwise contrasts between the hybrid and each inbred were performed using Dunnett's post hoc procedure. Likelihood ratio tests were used for significance testing of random effects.

Fumigation Effects on *Pythium* Survival (Experiment 3). To evaluate the efficacy of the steam, steam + AITC, and chloropicrin treatments on *Pythium ultimum* and *Fusarium* wilt, mixed soil samples (6 inches deep) were taken after treatment. Five samples were combined into one mixed sample taken

from a 5 × 5 ft sample area. Four mixed soil samples were taken per treatment. The survival of *P. ultimum* was assessed using the wet-plating method on semiselective medium (41). Corn meal agar (Sigma-Aldrich) was prepared (17 g · L⁻¹) and sterilized. After sterilization, a nonionic detergent (Tween 20, Thermo Fisher Scientific) (1 mL · L⁻¹) was added. Rose bengal (Thermo Fisher Scientific) (25 µg · mL⁻¹), rifampicin (Thermo Fisher Scientific) (10 µg · mL⁻¹), ampicillin (Sigma-Aldrich) (25 µg · mL⁻¹), pimarcidin (Sigma-Aldrich) (5 µg · mL⁻¹), and benomyl (Sigma-Aldrich) (40 µg · mL⁻¹) were added to the agar at 50 °C. After cooling, 0.5 to 1 g wet inoculum was spread (same dilution) on a total of five plates in three replicates. Plates were incubated at room temperature in the dark. *P. ultimum* colonies were counted during the first 2 d after plating. The mean propagules per gram soil were calculated for all three replicates (41).

Fumigation Effects on Soil Microbial Community Viability (Experiment 3). To assess the effects of the fumigation treatments on the field soil, we collected soil samples weekly, beginning immediately before planting and ending 4 wk (56 time points) after planting, when plants were harvested. Four soil cores per week were collected from each treatment to a depth of 25 cm; each core was divided into subsamples taken from depths 3 to 5 cm and 17 to 20 cm, kept on ice for 4 to 5 h, and then stored at 4 °C overnight and used for bacterial counts the following day. Soil suspensions were prepared by mixing 1 g fresh soil in 9 mL 0.95% NaCl. The suspension was then homogenized with a micro homogenizer (OMNI International, Inc.) at 12,000 rpm for one 60-s cycle. After homogenization, serial dilutions were prepared up to 10⁻⁵. Samples were plated on Reasoner's 2A (R2A) 1/10 and VxylG media (42) using the 6 × 6 drop plate method (43) for dilutions 10⁻² to 10⁻⁵. Plates were incubated in the dark at 25 °C for 3 wk. CFUs were counted at 3, 7, 14, and 21 d; we determined that 14 d was the best time to count colonies, and thus we used only that time point to calculate CFU per gram soil.

Fumigation Effects on Bacterial and Fungal Microbiomes (Experiment 3). We used high-throughput amplicon sequencing to assess how the on-farm fumigation methods affected the bacterial and fungal communities in the soil at large. Bulk soil samples were collected from the treated blocks at three time points: 1, 4, and 6 wk after the treatments were applied. After the final measurements of plant phenotype, the roots of a representative subsample of plants in the control, chloropicrin, and AITC + steam treatments were harvested for microbiome quantification. DNA was extracted from soil and root samples using the DNeasy PowerSoil kit (Qiagen, Inc.) and used as a template for PCR amplification of the V4 region of the bacterial 16S rRNA gene and the fungal ITS1, following established protocols (9). The resulting 16S-V4 and ITS1 amplicons were then sequenced in parallel on the Illumina MiSeq platform (V2 chemistry, 250-bp paired-end reads) to census the bacterial and fungal components of the microbiome, respectively.

Established bioinformatic pipelines were used to quality filter, denoise, and assign taxonomy to the raw sequence reads (9). Sequences that were derived from plants or that could not be identified at the kingdom level were discarded; samples with insufficient data (<500 bacterial reads or <500 fungal reads) were removed from the dataset. Finally, amplicon sequence variants (ASVs) that were not detected at least 10 times (for fungi) or 25 times (for bacteria) in at least three samples were removed from the dataset. The final bacterial dataset included 75 samples with a median of 30,109 reads per sample, comprising 1,307 ASVs. Sequencing depth was lower on average for fungi; as a result, the fungal dataset included 57 samples with a median of 6,466 reads per sample, comprising 122 ASVs. To reduce stochastic variation due to differences in sequencing depth, we applied the variance-stabilizing transformation (44) to the resulting ASV counts; additionally, we calculated the standardized, log-transformed sequencing depth for each sample to use as a nuisance variable.

To test whether fumigation treatments altered soil microbiome composition, we used permutational multivariate analysis of variance (MANOVA) to partition variance in the bacterial and fungal communities among several sources: sequencing depth, time point, treatment, and the interaction between time point and treatment. To test whether fumigation treatments were also detectable in plant-associated communities at the end of the experiment, we conducted another permutational MANOVA to partition root microbiome variation among sequencing depth, genotype, treatment, and the interaction between genotype and treatment.

Experiment 4 (June to August 2020). To investigate whether microbe-dependent heterosis could be observed in a very different field site and whether it could persist throughout the lifetime of the plant, we conducted another field experiment at the University of Kansas Field Station in Lawrence, KS. Since the 1940s, this site has been used to grow corn in rotation with soy.

In 2016, the site was left fallow and remained fallow with annual mowing up until this experiment. Bottomless pots were constructed from 6-inch segments of advanced drainage systems pipe (12-inch diameter) and filled with either steam-sterilized or nonsteamed soil that was dug adjacent to the site. Soil was steam sterilized for 4 h at 150 °C twice over 48 h using the SG15 Steam Generator (Siebring Manufacturing). Each genotype (B73, Mo17, and B73xMo17) was represented by 45 replicates per treatment (*N* = 270), and replicates were randomly arranged into six blocks. Seedling emergence was monitored daily for 4 wk. Chlorophyll concentration in the youngest fully expanded leaf was measured weekly from 4 to 8 wk after planting using a MC-100 Chlorophyll Concentration Meter (Apogee Instruments); three measurements were taken from a location two-thirds of the distance from stalk to leaf tip and then averaged for each plant. Developmental stage and height were recorded weekly until 8 wk after planting, when plants were uprooted for biomass measurements. Root systems and shoots were oven dried for 7 d at 60 °C and then weighed. For quantitative analysis, the developmental stage was coded as an integer, so that emergence = 1, first leaf collar = 2, second leaf collar = 3, ..., tasseling = 18, and silking = 19. Proportion emergence was compared between the hybrid and each inbred using Fisher's exact test. For the emergence-time data, we applied two-way ANOVA to linear mixed-effects models with genotype, treatment, and their interaction as fixed predictor variables and block as a random-intercept term. Root weight was natural log-transformed, and height was square-root-transformed to improve heteroscedasticity; the resulting estimated marginal means were back transformed prior to calculations of heterosis. For height, developmental stage, and chlorophyll concentration, we applied three-way repeated-measures ANOVA with genotype, treatment, date, and all interactions as fixed predictors and plant and block as random-intercept terms. *F*-tests with Type III sums of squares were used for significance testing of fixed effects, and pairwise contrasts between the hybrid and each inbred were performed using Dunnett's post hoc test. Likelihood ratio tests were used for significance testing of random effects.

Statistical Tests for Changes in Strength of Heterosis. For all experiments, we performed permutation tests to assess whether the change in strength of heterosis between sterile and nonsterile treatments was statistically significant. First, we used the estimated marginal means from fitted linear mixed models to calculate the BPH and MPH for each trait in each treatment:

$$BPH = \frac{B73xMo17 - \max(B73, Mo17)}{\max(B73, Mo17)}$$

$$MPH = \frac{B73xMo17 - \frac{B73 + Mo17}{2}}{\frac{B73 + Mo17}{2}}$$

Second, we calculated " Δ_{BPH} " and " Δ_{MPH} " as the difference in BPH or MPH between nonsterile and sterile treatments. Positive values of Δ_{BPH} or Δ_{MPH} indicate that heterosis was stronger in nonsterile conditions than in sterile conditions. Third, we recalculated Δ_{BPH} for 999 datasets that had been permuted with respect to microbial treatment, creating a distribution of Δ_{BPH} values that would be expected if treatment had no effect on heterosis. Finally, we compared the observed Δ_{BPH} to this distribution to examine the null hypothesis that heterosis is equally strong in perturbed versus unperturbed microbiomes.

Data Availability. All raw data and original R code that support the findings of this study are freely available in a public repository (<https://doi.org/10.5281/zenodo.4107065>) (45). Raw sequence reads are available in the National Center for Biotechnology Information Sequence Read Archive under BioProject accession no. PRJNA669388.

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