Multicellular organisms, including plants, are colonized by microorganisms, some of which are beneficial to growth and health. The assembly rules for establishing plant microbiota are not well understood, and neither is the extent to which their members interact. We conducted drop-out and late introduction experiments by inoculating Arabidopsis thaliana with synthetic communities from a resource of 62 native bacterial strains to test how arrival order shapes community structure. As a read-out we tracked the relative abundance of all strains in the phyllosphere of individual plants. Our results showed that community assembly is historically contingent and subject to priority effects. Missing strains could, to various degrees, invade an already established microbiota, which was itself resistant and remained largely unaffected by latecomers. Additionally, our results indicate that individual strains of Proteobacteria (Sphingomonas, Rhizobium) and Actinobacteria (Microbacterium, Rhodococcus) have the greatest potential to affect community structure as keystone species. Aside from geography, plant species, genotype and seasonal variation\(^\text{1,2-5}\), ecological forces such as assembly history—that is, the timing and order in which species arrive—may play a key role in community structure. Several groups have shown that priority effects, the imprint of arrival order on community structure\(^\text{6,7}\), play a role in shaping microbial communities of nectar yeasts\(^\text{8}\), fungi\(^\text{9-10}\) and cyanobacteria\(^\text{11}\). Additionally, arrival order can determine whether plant-protective bacteria or fungi are effective against pathogens\(^\text{12-14}\). For phyllosphere communities of greenhouse A. thaliana plants, a high variation in the relative abundance of various taxa was observed, which might indicate that stochastic colonization and historical contingency may also play a role in structuring microbial communities in plants\(^\text{15}\).

Here, we used a phylogenetically diverse, 62-leaf bacterial strain, gnotobiotic A. thaliana model system to conduct drop-out and late introduction experiments (Fig. 1) to shed light on the principles determining community structure in plants. Specifically, we aimed to understand the role of priority effects—that is, how the order in which strains are introduced to the plant affects the final outcome, and whether and to what extent single strains could drive community assembly.

**Results**

**Synthetic bacterial community structure.** We designed a 62-strain community that covers essentially all operational taxonomic units (OTUs) previously isolated from the A. thaliana phyllosphere\(^\text{16}\) and in which each single strain could be individually identified through 16S ribosomal RNA gene amplicon sequencing (Supplementary Table 1). This 62-strain ‘all’ input community comprised 32 Proteobacteria, 20 Actinobacteria, six Bacteroidetes and four Firmicutes (Supplementary Table 2). Strains were mixed and used to inoculate A. thaliana plants in a gnotobiotic system (for inoculum validation see Supplementary Fig. 1). The relative composition and intrinsic variability of the synthetic community that established in the phyllosphere was examined three and five weeks post-inoculation. As expected\(^\text{16}\), we confirmed that the community was stable between these time points (Supplementary Fig. 2; \(P \geq 0.01\)).
For initial overall community analysis, we thus combined leaf samples harvested at both time points resulting in a total of 48 replicates, each representing the community composition of one plant (Fig. 2).

The community was dominated by two strains, L68-Rhizobium and L203-Microbacterium, followed by L420-Devesia, L231-Sphingomonas, L177-Burkholderia and L233-Rhodococcus (Fig. 2 and Supplementary Dataset 1 and 2), all of which were detected in each of the 48 replicates. Notably, the relative abundance of a strain in the initial inoculum was not predictive of its colonization success (Fig. 2, Supplementary Figs. 1 and 3 and Supplementary Dataset 1). Of the 62 strains in the community, five remained undetected in all 48 samples, three of which were Bacillus and close relatives. At higher taxonomic levels, Proteobacteria was the most abundant phylum followed by Actinobacteria, Bacteroidetes and Firmicutes (Fig. 2). These data are in agreement with previous data on the Arabidopsis phyllosphere16,18,29,43,44.

Drop-out and late introduction effects. To investigate whether and to what extent priority effects influence community assembly, we combined leaf samples harvested at both time points resulting in a total of 48 replicates, each representing the community composition of one plant (Fig. 2).

The community was dominated by two strains, L68-Rhizobium and L203-Microbacterium, followed by L420-Devesia, L231-Sphingomonas, L177-Burkholderia and L233-Rhodococcus (Fig. 2 and Supplementary Dataset 1 and 2), all of which were detected in each of the 48 replicates. Notably, the relative abundance of a strain in the initial inoculum was not predictive of its colonization success (Fig. 2, Supplementary Figs. 1 and 3 and Supplementary Dataset 1). Of the 62 strains in the community, five remained undetected in all 48 samples, three of which were Bacillus and close relatives. At higher taxonomic levels, Proteobacteria was the most abundant phylum followed by Actinobacteria, Bacteroidetes and Firmicutes (Fig. 2). These data are in agreement with previous data on the Arabidopsis phyllosphere16,18,29,43,44.

For each of the drop-out and late introduction experiments, we analysed the synthetic communities for potential effects on the remainder of the community (that is, all strains except the missing group) when a group was missing altogether (Fig. 3a, comparison I). Additionally, when the group was introduced late (Fig. 3a, comparison II). We also asked whether the remainder of the community established differently if a particular group was introduced early (that is, present in the initial inoculum) or late (Fig. 3a, comparison III). In addition, we investigated whether both the late-arriving group and the community as a whole established differently as a result of the arrival order (early versus late) of the group in question (Fig. 3a, comparison III). Because we introduced the missing strains in a MgCl₂ solution, we tested whether spraying the control community with MgCl₂ would affect its structure, but the effect was only minor (Supplementary Fig. 2).

We found that the drop-out and late introduction of entire groups resulted in significant ($P \leq 0.01$) effect sizes based on PERMANOVA analysis (Fig. 3b and Supplementary Fig. 4). The largest effect sizes were found for the impact of arrival time (early versus late) on the group that was introduced late (that is, the invading group; Figs. 3a (comparison III), 3b and Supplementary Fig. 4 (effect sizes 19–62%, $P \leq 0.002$)). Additionally, when considering all 62 strains, late introduction of various groups (Fig. 3a, comparison III) produced communities that were different from those in which all strains were present from the beginning (with large effect sizes of 12–28%, $P = 0.0001$), except in the case of the Gammaproteobacteria. When focusing only on the founding population (that is, excluding the late-arriving group) and its response to early versus late arrival of a drop-out group (Fig. 3a,b, comparison III), only small effect sizes (5–6%, $P \leq 0.01$) were found.
Removing any of the groups from the inoculation altered the community structure (that is, the relative abundance contribution of each strain) of the remainder of the community compared to the condition where all strains were present from the beginning (Fig. 3a, comparison I), with the removal of the Alphaproteobacteria (effect size $6.4\%$, $p=0.0002$) and the entire Proteobacteria (effect size $6.6\%$, $p=0.0001$) having a larger effect compared to the removal of the Beta- or Gammaproteobacteria (effect size $<3\%$, $p\leq 0.01$; Fig. 3b and Supplementary Fig. 4). Late introduction of the missing group (versus mock-spray with MgCl$_2$; Fig. 3a, comparison II) had no significant effect on the remainder of the community (Fig. 3b and Supplementary Fig. 4). Congruently, the late introduction of the missing group (versus mock-spray with MgCl$_2$; Fig. 3a, comparison II) had no significant effect on the remainder of the community (Fig. 3b and Supplementary Fig. 4).

We next assessed the contribution of individual strains to the observed community changes described above (Fig. 3c and Supplementary Fig. 5). This breakdown describes the directionality of the observed effects—that is, positive or negative consequences (Fig. 1b). Additionally it indicates whether strains within one phylogenetic group (and thus a longer shared evolutionary history) react similarly to changes in the community or following invasion at a later time point. The absence of Alphaproteobacteria in the early stages of colonization had exclusively beneficial effects for most Betaproteobacteria and several Actinobacteria (Fig. 3a,c, comparison I). Congruently, the late introduction of Alphaproteobacteria had significant negative effects on most of those strains when compared to the effect of adding back the Alphaproteobacteria versus mock-spray (comparison II). These reciprocal results confirm negative effects between members of the Alphaproteobacteria and individual strains of the Betaproteobacteria and Actinobacteria. Interestingly, most Alphaproteobacteria were largely able to invade a pre-existing microbiota and could establish similarly whether they were present at the early stages versus arriving late (comparison III).

The absence of Betaproteobacteria in the early stages of colonization (comparison I) had only a small effect on the remainder of the community, as it affected the relative abundance of only three strains ($P\leq 0.05$), and their late introduction was inconsequential with regard to the remainder of the community (comparison II). Notably, however, the Betaproteobacteria themselves were rather sensitive to the time of their introduction (comparison III), with an overall effect size of $62\%$ (Fig. 3b). Three out of eight strains (L160-Acidovorax, L220-Variorux and L416-Methylphilus) benefited significantly from late arrival while one strain (L177-Burkholderia) was negatively impacted ($P\leq 0.0001$). The absence or late introduction of Gammaproteobacteria (comparison I or III, respectively) had no significant consequences ($P\geq 0.01$), with the exception that the Betaproteobacterium L416-Methylphilus benefited when Gammaproteobacteria were introduced late.

The absence of the entire Proteobacteria phylum (comparison I) caused changes in various Actinobacteria and Bacteroidetes; however, the affected strains were not a direct sum of the strains impacted by the absence of Alpha-, Beta- and Gammaproteobacteria, respectively. On the other hand, following late introduction, the Proteobacteria subclasses seemed to react largely as they did in their individual class drop-outs (comparison III). None of the Firmicutes were affected by any of the drop-outs or late introductions; similarly, only two strains of the genus Flavobacterium in the phylum Bacteroidetes were responsive to any perturbations.

**Single-strain drop-out effects.** Next, we tested whether the removal of individual strains from the initial 62-strain inoculum could significantly alter community assembly by acting as keystone species. In total, 25 strains (Supplementary Table 2), spanning a median relative abundance of 0.01–27%, were individually dropped-out from the initial inoculum and community structure was examined five weeks post-inoculation. Of all strains tested, about one-third (that is, eight strains: L160-Acidovorax, L203-Microbacterium, L231-Sphingomonas, L233-Rhodococcus, L262-Rhizobium, L265-Pseudorhodoferax, L405-Chryseobacterium and L416-Methylphilus) had a significant impact on community structure (Fig. 4a and Supplementary Fig. 6; effect sizes $4$–$13\%$, $P\leq 0.001$). With regard to effect size, the absence of four of these strains, all of which had a median relative abundance $>1\%$, caused large, significant effect sizes—for example, L203-Microbacterium

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**Fig. 2** | Relative abundance in control community. Relative abundance of the 62-strain control community ($t_1$ and $t_2$, combined, $n=48$, one independent replicate). Violin plot with swarm plot overlay and pie chart are coloured according to strain phylogeny. For each strain, the horizontal line represents the median while points represent individual samples. Along the bottom, the number of replicates where a given strain was not detected are indicated by circle size and count.
Fig. 3 | Proteobacteria class drop-out and late introduction experiment. a. Schematic of drop-out and late introduction experiments showing the various comparisons analysed (I, II and III) among the control, the late arrival and the group-absent communities (n = 15–24 per condition, one independent replicate). b. Overall effects of class drop-outs and late introduction (PERMANOVA). Rows are labelled with the test groups under comparison according to a. Circle size and shading represent effect size, while asterisks denote significance. c. Strains affected by class drop-outs and late introductions (DESeq2). Column labels refer to the test groups under comparison according to a for each drop-out/late introduction condition. For mock-spray control, see Supplementary Fig. 2b. Strains are phylogenetically clustered and heatmap colours represent the log2 fold change of the test condition/control community. *P = 0.05, **P = 0.01, ***P = 0.001, ****P = 0.0001.

Generally, drop-outs of single strains affected the relative abundance of other strains (Fig. 4c and Supplementary Fig. 8 and 9). Interestingly, although the removal of a given strain impacted other strains positively or negatively, the affected strains themselves reacted to all tested drop-outs in only one direction (that is, positively or negatively, but not both). The only exception was...
L145-Arthrobacter, which responded positively to one drop-out but negatively to all others (Supplementary Fig. 8).

Next, we identified those strains that were affected by the various single-strain drop-outs (Supplementary Fig. 10). Consistent responses (that is, similar and significant changes in the same affected strains) were found following removal of those strains that had the largest, significant (P ≤ 0.01) effect sizes based on the PERMANOVA analysis (Fig. 4a,b; L203-Microbacterium, L231-Sphingomonas, L233-Rhodococcus and L262-Rhizobium generated from both independent replicates and the combined dataset. PC1, PC2, principal components 1 and 2, respectively; R1, R2, replicates 1 and 2, respectively. **P = 0.001, ****P = 0.0001. Exemplary principal component analysis plots of drop-outs L203-Microbacterium, L231-Sphingomonas, L233-Rhodococcus and L262-Rhizobium generated from both independent replicates and the combined dataset. PC1, PC2, principal components 1 and 2, respectively; R1, R2, replicates 1 and 2, respectively. Strains affected by single-strain drop-outs (P ≤ 0.01). Effector strains (left) and affected strains (right) are clustered and coloured by phylogeny. Blue and red lines represent negative and positive effects, respectively, inferred from the single-strain drop-out experiments. Line thickness correlates linearly with fold-changes.

**Fig. 4 | Single-strain drop-outs.** a. Overall effects of single-strain drop-outs (PERMANOVA). Data from two independent replicates (n = 11–12 per condition per replicate) were analysed separately and together. Strains are phylogenetically clustered and coloured. Circle size and shading represent effect size, while asterisks denote significance. P values (Benjamini–Hochberg corrected): *P = 0.05, **P = 0.01, ***P = 0.001, ****P = 0.0001. b. Exemplary principal component analysis plots of drop-outs L203-Microbacterium, L231-Sphingomonas, L233-Rhodococcus and L262-Rhizobium generated from both independent replicates and the combined dataset. PC1, PC2, principal components 1 and 2, respectively; R1, R2, replicates 1 and 2, respectively. c. Strains affected by single-strain drop-outs (P ≤ 0.01). Effector strains (left) and affected strains (right) are clustered and coloured by phylogeny. Blue and red lines represent negative and positive effects, respectively, inferred from the single-strain drop-out experiments. Line thickness correlates linearly with fold-changes.
Fig. 5 | Causal network (P ≤ 0.01) based on single-strain drop-outs. Nodes (strains) are coloured by phylogeny and labels refer to strain names. Rectangles represent effector strains that were individually removed, while ovals are strains that were affected by various drop-outs. Red and blue arrows (depicting positive and negative effects, respectively) represent edges (direct or indirect interactions). See Fig. 2 and Supplementary Table 2 for strain names and phylogeny.

L231-Sphingomonas, L233-Rhodococcus and L262-Rhizobium). Notably, these strains were found to cluster together when the experiments were combined for the DESeq2 analysis (Supplementary Fig. 8), as their absence produced similar changes in the community, predominantly by increasing the relative abundance of strains that were often undetected or had a very low median relative abundance (≤0.1%; for example, L70-Stenotrophomonas, L129-Pseudomonas, L182-Brevibacillus, L187-Exiguobacterium, L220-Variovorax, L222-Agromyces and L359-Flavobacterium). The most prominent sensitive strain across all drop-out conditions was L416-Methylphilus, with 15 of 25 drop-outs causing a significant (P ≤ 0.01) increase in its relative abundance. Because these sensitive strains had low relative abundance, we also tested whether there was a correlation between the relative abundance of a strain and the likelihood that it was impacted by a drop-out, but this was not the case (Supplementary Fig. 11; Pearson correlation = −0.180, P = 0.161).

The single-strain drop-out data provided a unique opportunity to create a causal network, because it was known which strain (that is, the dropped-out strain) affected other strains (Fig. 5). The network consists of effector and affected nodes (here, strains) connected by edges (that is, direct or indirect interactions), which are illustrated by directional arrows. The network based on the combined dataset was strictly hierarchical—that is, no bidirectional edges were observed for the strains tested (Fig. 5). As follows from the nature of the effects described above, the network was predominantly inhibitory and those keystone strains identified as having a reproducible, significant impact on the community based on the PERMANOVA analysis (Fig. 4a; strains L203-Microbacterium, L231-Sphingomonas, L233-Rhodococcus and L262-Rhizobium) affected many strains (more than ten strains, Fig. 5 and Supplementary Fig. 8; P ≤ 0.01). However, with the exception of the positive influences of L262-Rhizobium and L145-Arthrobacter on L203-Microbacterium, none of these keystone strains were themselves affected by any of the other tested dropped-out strains.

We then asked whether there was a correlation between outgoing node degree (the number of strains significantly affected by a node strain) and effect size following node removal, and found a strong positive correlation (Pearson correlation = 0.904, P = 5.8 × 10^{-10}; Fig. 6). Thus, we conclude that keystone strains, directly or indirectly, interact with more strains.

Discussion

The recent development of higher-complexity gnotobiotic systems\(^{26,46}\) has provided the opportunity to better simulate native-like conditions in the laboratory to understand the principles governing microbial community assembly and dynamics\(^{2,3}\). Though co-occurrence networks have emerged as a useful tool for the exploration of bacterial interactions through correlation analysis of relative abundance data\(^{4-10}\), this approach is insufficient for conclusively establishing causality or identifying keystone species\(^{8}\). Here, we moved towards this goal by conducting drop-out experiments and methodically removing groups or single strains in an effort to understand the nature and directionality of bacterial interactions (direct or indirect) in a complex community using a gnotobiotic Arabidopsis model system (Fig. 1b).

The examination of variation among 48 control samples provided first insights on community composition and intrinsic variability. The overall community structure was broadly consistent with previous results in terms of phylum and class distribution\(^{16}\), despite considerable plant-to-plant variation (Fig. 2). As previously shown with the human microbiome, where even the most abundant OTU can vary nearly 5,000-fold across individual samples\(^{3}\), our finding suggests that stochastic variation has the potential to play a key role in microbiome structure at the level of individual plants. However, in contrast to the stochasticity seen in human microbiome samples, where variation is expected to arise due to the intrinsic heterogeneity of human lifestyles and genetics\(^{8,2,3}\), the compositional variation we observe in our experiments arises even when controlling for growth conditions and plant genotype. Though not investigated in detail, the stochastic variations may sometimes involve functional substitutions, for example by strains competing for similar substrates. For instance, in the rare cases where L90-Methyllobacterium or L86-Methyllobacterium was the most abundant strain (n = 2 and n = 6, respectively), the other Methyllobacterium and Methylphilus strains were below average relative abundance (Supplementary Dataset 2), with one exception. Since all of these strains presumably utilize methanol, they may compete for this carbon and energy source.

In both the single-strain and class drop-outs, there were significant changes in community structure as a result of strain removals, presumably due to changes in the interactions of the strains present. Overall, the frequency of effects suggests that interactions among strains in phyllosphere communities are common. A major conclusion is that, once established, an initial community is robust—that is, difficult to perturb (Fig. 3b). In each drop-out experiment, the initial community established without the drop-out group was not significantly altered following the introduction of the drop-out group (except perhaps Alphaproteobacteria, P = 0.03; Fig. 3a,b, comparison II). As previously reported in other studies\(^{2,3}\), this indicates that an initially established microbiome is likely to be
resistant to perturbations associated with the introduction of new species. Hence, founding taxa—that is, microbes that colonize a plant early—are likely to have a persistent, long-term influence on community structure even if the community is later exposed to other microbial populations. An interesting caveat is that, in many cases, new strains seem to be able to slip in without perturbing the pre-existing microbiota, suggesting that although the initial population is itself resistant to alteration (that is, the founding population remains stable when new strains are introduced), it is not resistant in the sense of impenetrable (that is, late-arriving strains can still colonize the host). Similar results were obtained when investigating invasion and exclusion patterns among co-existing *Pseudomonas syringae* strain pairs on leaves, where about half of the tested late-arriving strains could colonize the host after pre-inoculation with another strain. In our experiments, many strains that were introduced late established with a relative abundance similar to that obtained when present from the beginning (Fig. 3a,c, comparison III). However, as previously reported for certain *P. syringae* strains, though common, the ability to invade a pre-established microbiota is not universal, as some strains were significantly inhibited by late arrival. Combining these observations, our results point toward an initial competition for resources and a process of becoming established that has long-lasting effects; nevertheless, it appears that there are also unsaturated niches for newcomers to colonize even if they are not able to significantly change the pre-existing community. Analogously, late-arriving strains have also been shown to effectively colonize mouse caecal communities, where it was also hypothesized that many niches were unsaturated.

It is interesting to note that three of the Betaproteobacteria significantly benefited from late arrival (Fig. 3a,c, comparison III), suggesting they can grow best after other strains have had an opportunity to colonize the host. It is unclear whether late-arrivers benefit due to niche modification, either indirectly via changes induced in the plant or directly due to a dependency on metabolites, nutrients or other substances originating from other bacteria. However, benefiting from late arrival appears to be the exception, as most strains either showed no significant impact from late arrival or were inhibited by it. These findings are in line with previous research showing that inhibitory (rather than beneficial) priority effects are also predominant in other systems, and are of practical relevance regarding the use of biocontrol agents to prevent, for example, pathogen infection or frost damage. Overall, the class drop-out and late introduction experiments suggest that timing of bacterial arrival is an important determinant of phyllosphere community structure for incoming bacteria, and that minor changes in the order and timing of arrival probably play a role in shaping the microbiota.

While the class drop-out experiments revealed that community structure was sensitive to the presence or absence of many strains, the single-strain drop-outs showed that (1) there are single key-stone taxa that play a major role in shaping phyllosphere community structure and (2) key-stone taxa seem to influence community structure mostly by affecting strains that have very low relative abundance. These results are in line with previous findings highlighting the importance of the ‘rare biosphere’ (that is, microbes occurring at low density) in microbial communities, where low-abundance microorganisms are thought to provide a robust reservoir of ecological function and resilience and can become more (or less) prominent in response to environmental changes.

About half of the strains (52%) were not affected by any of the single-strain drop-outs tested (Fig. 4c and Supplementary Fig. 8). This may reflect the diversity of the selected strains, with some bacteria engaging in competition or cooperation and others largely occupying niches that are relatively specialized and distinct, to largely avoid interaction with other taxa. Alternatively, this apparent robustness might reflect a high dynamic adaptability of these strains. To distinguish between these scenarios, dedicated follow-up experiments will be required. Interestingly, strains that were significantly affected by various drop-outs always responded either positively or negatively to all drop-outs, but not both (the exception being *L145-Arthrobacter*). This points towards particular strains having, for the most part, overlapping niches or requirements and more rarely relying on other strains for metabolites or nutrients. *L416-Methylphilus* reacted positively to the removal of most strains, which may suggest that this strain has a high niche overlap with most other strains. In either case, *L416-Methylphilus* itself is a key, albeit rather stochastic, component of the community, as mentioned above, as its presence/absence and relative abundance have a significant effect on community structure (Fig. 4a). Notably, the relative abundance of *Methylphilus* in natural *A. thaliana* populations has also been observed to be highly variable.

Several interesting observations about the nature of microbe–microbe interactions in the phyllosphere can be made based on the causal network (Fig. 5) produced through the single-strain drop-outs. For example, although incomplete, the network is predominantly inhibitory (~75%) with only two strains (*L262-Rhizobium* and *L145-Arthrobacter*) being responsible for half of the positive effects. Additionally, we note that the network is hierarchical and without observed bidirectional effects. Both of these observations support the hypothesis that competition (rather than cooperation) is prevalent in phyllosphere bacterial communities.

Despite our experimental system bearing similarity to a natural system, it is not clear to what degree priority effects dominate community assembly under environmental conditions. In our drop-out and late introduction experiments, we set up a strict inoculation timing cut-off where the initial population arrived at $t_0$ and the late population arrived at $t_1$ (three weeks later). This artificial timing cut-off allowed us to study and identify priority effects, but it is likely that historical contingency is much less pronounced in nature where the timing of strain arrival is more fluid. Nonetheless, the fact that we do see priority effects in the experimental system used here points towards historical contingency being relevant in nature.
Overall, both historical contingency and deterministic factors seem to play a role in shaping phyllosphere communities. In nature, however, removal of one strain would probably have a milder effect, as it is likely that there would be another very similar strain ready to fill the vacated niche—that is, they would be functionally redundant. Additional drop-out experiments utilizing many strains within the same OTU could address this question.

Future work analysing the role of single strains in this, and more complex, communities needs to be done to further elucidate microbe–microbe interactions in plants. However, it is clear that synthetic community studies are a powerful tool for understanding microbiota structure and dynamics in the phyllosphere and other host–microbe systems. The observed priority effects, and in some cases resistance to late arrival, suggest that practical manipulations of microbiomes could be more successful if applied early in a host’s life cycle when the microbiota is still developing14,19,20. Nevertheless, further work is needed to translate knowledge of microbiome interactions into a predictable science that could be harnessed. By gaining a better understanding of microbiota assembly and dynamics, it may be possible to selectively manipulate microbiomes in ways that provide advantages to the host—for example, by providing disease protection or enhancing crop yields.

**Methods**

**Plant growth conditions.** Plants were grown using calcined clay in round, gamma-sterilized microplates (model no. O118/80+OD118) fitted with an X+filler (Combines)19. Calcined clay (Diamond Pro Calcined Clay Drying Agent), used as an inert soil substitute, was washed with distilled water until runoff was almost clear (ten washes). Extra water was then poured off, and the clay was dried (open system were excluded (two opportunistic pathogens based on pre-test experiments in an agar-based growth system were dropped-out). Of these, 10 out of the 15 most abundant strains (based on the vacated niche—that is, they would be functionally redundant. For the single-strain drop-out, plants (1=4 d at 4 °C. To ensure that each box contained four plants at the time of inoculation, extra seeds were placed in each and removed before inoculation. Plants were watered weekly with 8 ml of 0.1x MS medium (Duchefa; pH 7.0; including vitamins, without sucrose) per microplate.

62-strain synthetic community selection. The V5–V7 region of the 16S rDNA gene was amplified16, and 320 to 340 bp of the forward primers and 796 to 817 bp of the reverse primers were sequenced to ensure that the strains were not related. To ensure that no one strain was dominant, one strain, each of the remaining 47 strains. Plants and boxes were prepared as described above. Twenty-six mixtures were prepared—an ‘all’ control and 25 mixtures each lacking one of the following strains: L33, L61, L68, L83, L68, L88, L90, L145, L160, L203, L231, L262, L265, L288, L269, L335, L334, L339, L371, L390, L405, L416 and L420 (see Supplementary Table 2 for strain details and phylogeny). A core mix of 37 strains that was common to all mixtures was pre-mixed (1 ml/v, 750 µl/mix) and then added to the medium. The phyllosphere of each plant was harvested using sterilized tweezers and stored until further processing. Additionally, a Detonococcus strain, which was the only representative in its phylum, was also excluded. See Supplementary Table 2 for detailed information on the 62 strains selected.

Proteobacteria class drop-out and late introduction. All 62 strains were streaked onto R-2A agar (Sigma-Aldrich) supplemented with 0.5% v/v methanol and allowed to grow for 6 d at 22 °C. A sterile, 1-µl plastic loop was used to scratch off a ‘loopful’ of each strain and re-suspend it in 1 ml of 10 mM MgCl2. Tubes containing the re-suspended strains were then vortexed for 10 min, and strains that formed aggregates were filtered through a 10 µm filter (CellTrics, Symex Suisse AG) before mixing. Strains were mixed into their respective phylogenetic groups (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and ‘others’) in equal ratio (v/v, 750 µl/mix). Each drop-out inocula were then prepared by mixing these groups according to the experimental design and adjusting the total volume so that all mixtures contained the same total (6.2 ml). For example, for the ‘No Alphaproteobacteria’ inoculum, 0.8 ml of Betaproteobacteria (eight strains), 0.3 ml Gammaproteobacteria (three strains), 3 ml others (30 strains) and 2.1 ml of 10 mM MgCl2 (to account for the volume of the 21 Alphaproteobacteria strains) were added. Each mixture was adjusted to an OD550 of 0.007, and one-week-old seedlings were inoculated via pipetting (1 µl per seedling). Because of the way in which these mixtures were prepared, the inoculum density of individual strains in the class drop-outs was higher (maximum by a factor of two) than that in the ‘all’ community control. This change was considered tolerable (not expected to cause observable effects linked to a difference in bacterial density), as it has previously been shown that the synthetic community established with the full A. thaliana collection was robust against an even stronger imbalance in the inoculums (factor of four)19. Axenic control plants were mock-inoculated with 10 mM MgCl2.

Phyllosphere samples were harvested (n=15–18 per condition) three weeks post-inoculation, to examine microbial community composition (see harvest procedure below). Half of these harvested plants were harvested as-is, while the other half were sprayed with 10 mM MgCl2 immediately post-inoculation to assess the effect of the spray on the community (mock-spray control). Other plants (n=15–16 per condition) were either sprayed with the missing group of strains (for example, adding back Alphaproteobacteria to the condition where they were originally lacking) or mock-sprayed with 10 mM MgCl2 (n=15–24 per condition) and allowed to grow for another two weeks.

For the late introduction of missing strains, all 62 strains were grown on R-2A plates as described above. Each strain in the Proteobacteria phylum and the others’ (not Proteobacteria) was mixed equally (v/v, 750 µl/mix) into its respective group (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and ‘others’). For the single-strain drop-out experiment, a proportionate volume of a single number of strains (10 ml of 10 mM of MgCl2 to dilute each strain to the same concentration as in the ‘all’ community). For the single-strain drop-out, plants (1=4 d at 4 °C. To ensure that each box contained four plants at the time of inoculation, extra seeds were placed in each and removed before inoculation. Plants were watered weekly with 8 ml of 0.1x MS medium (Duchefa; pH 7.0; including vitamins, without sucrose) per microplate.

**Growth chambers** (CU-414A, Percival) were fitted with a mixture of full-spectrum lights (Philips Master TL-D 18W/950 Graphica) and lights emitting some UVA and UVB light (Sylvania ReptiStar F18W/6500K) to simulate natural conditions. Combined light intensity was set to 190–200 µmol·m−2·s−1 for wavelengths 400–700 nm (photosynthetically active radiation), and 4–5 µmol·m−2·s−1 for wavelengths 280–400 nm (UV). Growth chambers were set to 22 °C, 54% humidity and 11-h photoperiod.
Generation of 16S rDNA-based community compositional data. Reference sequences were extracted from draft genomes using USEARCH v.8.0.1623_win32 (ref. 44) by performing an in silico polymerase chain reaction (-search_pcr) with primers 799F (ref. 61) and 1193R (refs. 44,62) and allowing for a maximum of two mismatches, a minimum/maximum amplicon length of 100/600 bp, respectively, and searching on both DNA strands. Sequences with ambiguous base pairs were then corrected using the available Sanger sequences16.

Paired-end sequencing reads were merged using the USEARCH v.10.0.240_i86linux64 (ref. 44) command, fastq_mergepairs, with a minimum overlap of 16 bp and a minimum identity of 90%. Merged reads were quality filtered using -fastq_filter, with a maximum expected error of 0.1 and a minimum length of 100 bp. The reads were then classified and counted using -otutab with a minimum identity of 97% to the 16S reference sequences, producing an initial OTU table with a count for each reference in each sample. Additional bash code totalled the number of unclassified sequences in each sample to add to the end of the OTU table.

If an experimental sample was contaminated by a bacterium outside of the 62-strain community, we would expect its 16S sequence also to have been amplified and sequenced. To check for such a case and correctly quantify the relative abundance of the references, the unclassified reads were de-replicated (-fastx_uniques) and clustered using the UPARSE algorithm (-cluster_otus65, with a minimum cluster size of 1 and a fixed identity threshold of 97%). The representative sequences from these de novo OTUs were then added to the 16S reference sequences before all quality-filtered reads were re-classified and counted using -otutab, with a minimum identity of 97%. This final OTU table was checked against the initial OTU table and, since there was neither substantial change in the counts of the reference sequences nor the presence of high-abundance de novo OTUs, we concluded that there had been no significant contamination.

Phylogenetics. The phylogeny of the strains was constructed based on their full-length 16S rDNA gene sequences (see Supplementary Table 2 for accession numbers), which were aligned using SINA v.1.3.3 (ref. 52) and the SILVA SSU Ref NR database release 132, December 2017. PhyML v.3.3.20180214 (ref. 53) was then used, with default parameters, to build a maximum likelihood phylogeny from the alignment.

Data analysis. For each experiment, the final OTU table was imported and processed in R v.3.3.3. The data were organized into compatible datasets, each consisting of samples from a single condition and the control samples. The OTU table was then log-normalized for sequence depth and variance-stabilized by DESeq2 v.1.14.1 (ref. 54). For the class drop-out experiment, samples were compared by treatment (Effect of Mock Spray in Controls, Late Arrival versus Mock (II)), time point (Effect of Time in Controls) or initial condition (Group Absent versus Control (I), Late Arrival versus Control (III)). For the single drop-out experiment, DESeq2 was designed for analysis of differential expression and assumes that the distribution of expression of a single gene can be fitted to a negative binomial distribution, and that most genes are not differentially expressed. For our data, these assumptions imply that the distribution of relative abundance of a single strain can be fitted to a negative binomial distribution, and that most strains do not show differential abundance, which we consider reasonable.

For visualization of the overall effects on communities, principle component analysis was applied to the transformed OTU table using the prcomp command. The effect size, which is the variance explained by the compared factor, and the Pvalue of the comparison across a specific factor, were calculated by PERMANOVA using the adonis function of the package vegan v.2.5–4 (https://cran.r-project.org/web/packages/vegan/) with Euclidean distance. For the single drop-out experiment, PERMANOVA was modified to account for the batch effect between replicates with the strata argument.

Data visualization. For the figures that summarize the relative abundance of each strain, we chose to represent the relative abundance as a comparison between samples, relative abundance values were calculated by proportional normalization of each sample by its sequencing depth so that they would be more accessible to the reader. For the figures that correlated relative abundance against other factors, the median value of the DESeq2-transformed OTU table was used instead. For the pie chart of relative abundance, counts were summed across control samples.

The following R packages were also used during data analysis and visualization: corrplot, plotrix, circlize, ape, calibrate, igraph, and beeswarm. Cytoscape (v.3.7.0) was used to illustrate the causal network.

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