Microbial diversity determines the invasion of soil by a bacterial pathogen

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Natural ecosystems show variable resistance to invasion by alien species, and this resistance can relate to the species diversity in the system. In soil, microorganisms are key components that determine life support functions, but the functional redundancy in the microbiota of most soils has long been thought to overwhelm microbial diversity–function relationships. We here show an inverse relationship between soil microbial diversity and survival of the invading species *Escherichia coli* O157:H7, assessed by using the marked derivative strain T. The invader's fate in soil was determined in the presence of (*i*) differentially constructed culturable bacterial communities, and (*ii*) microbial communities established using a dilution-to-extinction approach. Both approaches revealed a negative correlation between the diversity of the soil microbiota and survival of the invader. The relationship could be explained by a decrease in the competitive ability of the invader in species-rich vs. species-poor bacterial communities, reflected in the amount of resources used and the rate of their consumption. Soil microbial diversity is a key factor that controls the extent to which bacterial invaders can establish.

community niche | invasiveness | resource utilization

Resistance to invasion by alien species represents a major life support function of terrestrial ecosystems (1). Theoretical (2–4) and experimental (5–8) studies have indicated that biologically diverse communities are often less prone to being invaded than simpler ones, but effects of microbial diversity on invading (micro)organisms have remained underexplored. The number of microbial, in particular bacterial, species in a single gram of soil can be enormous (9–11). Because several of the functions of the soil microbiota are key to soil functioning (12), the considerable functional redundancy has been thought to overwhelm any type of diversity–function relationship (13). However, microbial diversity was found to be inversely related to invasibility of the wheat rhizosphere by *Pseudomonas aeruginosa* (14) and also affected the ability of *Ralstonia solanacearum* to induce wilting disease in tomato (15), although this was not the case in potato (16). Soil bacterial diversity exerted a positive effect on the decline of this plant pathogen. However, this effect was dependent on soil type/management, occurring only in sandy soils under conventional agricultural management (17). Confounding factors, such as soil type and origin, may have led to conflicting results in these experiments, in which microbial diversity was strongly dependent on the soil used. Only by taking a "proactive" approach, manipulating bacterial diversity in a controlled experiment, can we clearly address the effects of microbial diversity on pathogen decline in soils.

The fate of the enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 in soil is of major concern (18–20). In this context, the microbial communities that established after soil fumigation were shown to determine the fate of the invading species, whereby reduction in microbial diversity due to progressively enhanced fumigation depths resulted in higher pathogen persistence in soil (21). Similarly, in 25 different manures, the decline rate of *E. coli* O157:H7 was negatively correlated with *Enterobacteriaceae* richness (22). Furthermore, the easily available carbon content of the manure explained this decline rate (22). A study in organic manure-amended soil showed a faster pathogen decline when rates of nutrient flow were reduced (23). Moreover, it has been shown that *E. coli* can survive at higher densities and for longer periods in sawdust than in sand livestock beddings (24). The lower survival observed in the sand was hypothesized to relate to the lower amount of organic matter and nutrients. In a follow-up experiment, evidence was provided for the contention that *E. coli* was suppressed in the sand as a result of the presence of several bacterial taxa (25). Thus, both microbial diversity and resource availability may play important roles in determining *E. coli* O157:H7 persistence in soil.

The underlying mechanisms of diversity–invasiveness relationships may lie in competition for the utilization of limiting resources [e.g., nitrate for plant communities (5)]. Theoretical tradeoff surfaces, as suggested by Tilman (4), might allow a prediction of the success of invasion. Moreover, systems harboring microbial communities with lower metabolic diversity might be more prone to invasion than those with communities capable of using a wider range of resources (26). Although competition for resources and components of diversity likely affect biological invasions, they are only pieces of the puzzle. Other mechanisms (e.g., predation and negative species interactions) might also determine the fate of invader species.

To better understand whether and how microbial diversity might hinder pathogen establishment in soil, we performed three experiments using a derivative of *E. coli* O157:H7 (strain T). Strain T is a genetically marked nontoxigenic *E. coli* O157:H7 (20), allowing survival and competition studies in soil. The aim of the first two experiments was to assess the effect of microbial diversity on invader establishment and survival. Along with assessing the effects of soil microbial diversity on invasibility of the system, we investigated whether protozoa exerted effects on the invader. The third experiment aimed to elucidate the mechanism behind the diversity–invasibility relationship that was found.

Results and Discussion

Assembly Experiment. In the first experiment, random bacterial isolates were obtained from a grassland soil in The Netherlands. Then, using batches of the same presterilized soil, bacterial communities consisting of 5, 20, or 100 random isolates were assembled in the soil, by adding isolate mixes in equal total cell numbers ($\approx 10^6$ g^{-1} dry soil) to the soil. In each treatment, 20% of the total added diversity encompassed actinobacterial morphs, which are known to produce antimicrobial compounds (27, 28). "Zero" control (no cells added; sterile soil) as well as natural soil

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microcosms were also included. The microcosms were kept for 30 d to allow the development of average soil population densities of 10^8 to 10^9 cells g^{-1} dry soil (20 °C, soil moisture content 18%, equaling 65% of water holding capacity). Then, strain T was added by pipetting a cell suspension into the soil followed by careful mixing, bringing the soil moisture content to 75% of water holding capacity. This treatment theoretically ensured the greatest contact between resident and invading populations. Richness and diversity of dominant bacterial communities were evaluated over time using PCR-denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes on the basis of soil-extracted DNA. Total and culturable bacterial numbers were determined by microscopic cell counting and dilution plating. The richness of dominant species (Sr) was approximated using the numbers of DGGE bands, whereas estimated diversity (*H'*; Shannon diversity index) was based on both the numbers and intensities of the bands.

The Sr and *H'* values differed significantly across treatments. The established communities revealed increasing richness [ANOVA $F_{(3,56)} = 572.94$; $P = 0.0001$] and diversity values [ANOVA $F_{(3,56)} = 89.84$; $P = 0.0001$] with increasing inoculum complexity. For each treatment, mean Sr and *H'* values were different from the values of the other treatments (Tukey's test, $P < 0.05$). Bacterial abundance after 60 d, as determined by microscopic counts, was similar across treatments, at approximately 10^9 cells g^{-1} dry soil. The numbers of culturable bacteria, determined via dilution plating on R2A agar, were dissimilar across treatments [ANOVA $F_{(3,8)} = 142.27$, $P = 0.0001$], although differences were small. The higher bacterial cfu numbers coincided with increasing community complexities as determined by bacterial PCR-DGGE data. Most bacterial cells were culturable, the ratio culturable/total bacteria being 0.85–0.95. This included the control treatment. The high culturability of soil bacteria observed in our experiment (which is often not the case for natural soils) could be explained by the use of relatively fast-growing bacteria when assembling the communities. This is confirmed by the positive correlation observed between the bacterial counts and community complexity.

Clear effects of diversity on strain T numbers (survival) were found. First, strain T survival was highest in sterile soil (Fig. 1), significantly exceeding that in the other treatments [Tukey's post hoc pairwise comparison $P < 0.05$; ANOVA $F_{(3,8)} = 102.24$, $P = 0.0001$]. In contrast, survival was lowest in the natural soil (Fig. 1, legend). Regarding the established communities, invader survival was higher in the presence of 5-strain communities than in those composed of 20 and 100 strains (ANOVA, $P < 0.05$). Moreover, species richness explained the differential survival in a progressively more robust manner over time, as evidenced from a richness–survival biplot (logarithmic model; Fig. 2). Thus, at 3, 30, and 60 d after invasion, the slopes of the curves became progressively steeper, revealing the fact that the effect of richness in decreasing survival of the invader was more pronounced toward the end of the experiment. This effect remained after correcting for the different amounts of culturable biomass [analysis of covariance $F_{(3,7)} = 16.05$; $P = 0.002$ at day 60]. The magnitude of the effect of species richness on invader survival in these communities likely reflected the relative simplicity of the respective communities (Fig. 1), and a comparison with survival in natural soil highlighted the impact of natural (high) diversity (Fig. 1, legend).

Dilution-to-Extinction Experiment. Thus, in an effort to compare survival of the invader in systems with more realistic species richness values, while still offering a litmus test as to the effect of diversity on invader survival, a second experiment was designed using the dilution-to-extinction method. In this setup, sterile soil microcosms are inoculated with a gradient of diversity present in serially diluted natural soil (14, 29–31), thus extending our observations with communities of fast-growing bacteria to those including oligotrophic and nonculturable cells. Natural soil served as the control. Three manipulative treatments of 10^1-, 10^3-, and 10^6-fold diluted cell suspensions from natural soil were used to establish the microcosms at similar final water contents as above. We were also interested in determining whether predation would play an important role in determining invasiveness by the pathogen, either by selective pathogen predation or by an overall reduction of microbial diversity. Therefore, in addition to the 10^1 treatment, a 10^1-filtered (10^1-F) treatment, in which the 10^1-fold diluted suspension was filtered over membranes with progressively smaller pore sizes (5, 3, 2, and 1 μm), was used to obtain communities free of protozoa. After incubating the microcosms at 20 °C for 30 d, strain T was added at 10^8 cells g^{-1} dry soil, as in the previous experiment. Survival of the invasive species, total and culturable bacterial biomass, as well as bacterial, fungal, and bacterial group-specific richness and diversity indices, were determined over 60 d.

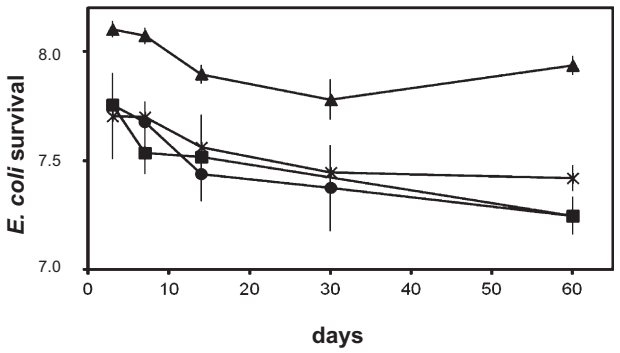

Fig. 1. *E. coli* strain T population dynamics (log cfu g^{-1} soil) over a 60-d period in soil with differently established diversities of culturable bacteria from the assembly experiment. ▲, control, no strains added; *, 5 strains; ■, 20 strains; ●,100 strains. Each symbol represents the mean value of three replicates. Bars represent SDs of the mean. *E. coli* strain T population dynamics in natural soil is not shown, because it would seriously mask the differences between the established communities; it was characterized by a near-linear decline of cfu numbers by approximately 6 log units, from approximately 10^8 cfu g^{-1} soil to 10^2 cfu g^{-1} dry soil, within 60 d after release.

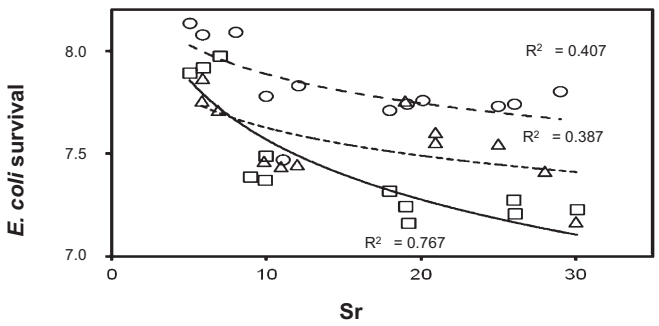

Fig. 2. Relationship between *E. coli* strain T population dynamics (log cfu g^{-1} soil) and the relative richness (Sr) of bacterial species from the assembly experiment, as estimated from bacterial PCR-DGGE assessments (number of bands) over a 60-d period, at days 3 ($R^2 = 0.407$; Survival = −0.21* ln (Sr) + 8.36), 30 ($R^2 = 0.387$; Survival = −0.20* ln (Sr) + 8.08), and 60 ($R^2 = 0.767$; Survival = −0.42* ln (Sr) + 8.54) after strain T introduction. At all time points, a logarithmic decay curve [Survival = −0.27* ln (Sr) + 8.35; ANOVA $F_{(1,58)} = 36.7$, $P = 0.0001$] fitted the data points best and better (higher R^2) than a linear one. The differences in the initial slopes were significant between d60 and d3/d30 ($P < 0.05$). Open circles, day 3; open triangles, day 30; open squares, day 60.

Total bacterial microscopic counts were similar between all constructed microcosms and over time, at approximately 4×10^9 cells g^{-1} dry soil, but slightly lower in natural soil, at 2×10^9 cells g^{-1} dry soil. As from day 10 after inoculation, the culturable bacterial cell numbers were stable over time in the constructed microcosms (range 5×10^8 to 10^9 cfu g^{-1} dry soil), being similar between treatments ($P > 0.05$). The cfu/total cell ratio ranged from 0.14 to 0.26. Significantly lower cfu counts of total bacteria (i.e., 2–5 $\times$ 10^7 g^{-1} dry soil) were obtained in the natural soil (Tukey's post hoc test, $P < 0.05$) at a cfu/total cell ratio of approximately 0.2%. These results are in line with the knowledge that the majority of bacterial cells in soil are unculturable.

As expected, bacterial PCR-DGGE analyses revealed the presence of communities with different richness values in different treatments (Fig. S1) [ANOVA $F_{(4,40)} = 69.56$; $P = 0.0001$] over the entire experiment. Natural soil showed the highest mean species richness value and the 10^6 treatment the lowest. Species richness did not differ between the 10^1 and 10^1-F treatments, indicating that predation by protozoa did not affect the bacterial richness. Fungal communities also showed progressively reduced richness [ANOVA $F_{(4,40)} = 15.67$; $P = 0.0001$] with increasing dilution. The 10^1-F treatment showed lower fungal richness than the 10^1 treatment, probably resulting from filter-retention of fungal mycelia (Fig. S1). The diversity indices revealed patterns similar to those observed for the richness values. Furthermore, species richness data were supported by phospholipid fatty acid (PLFA) analyses performed at day 60. Of a total of 25 PLFAs, 21 were detected in the 10^1, 19 in the 10^1-F, 17 in the 10^3, and 12 in the 10^6 treatments. Because the chemical composition of the PLFA biomarkers depends on the type of microorganism (32), this provides an additional indication that the dilution treatment had resulted in progressively decreasing species richness. Filtration partially removed the effect of dilution treatment on survival. Total soil PLFA revealed the occurrence of the protozoan markers (21, 32) 18:3ω6, 20:2ω6, and 20:4ω6 only in the 10^1 and natural soil treatments, at 0.63 ± 0.17 and 0.53 ± 0.08 nmol g^{-1} dry soil (detection limit 0.05 nmol g^{-1} dry soil). The fact that only the natural soil and 10^1 treatments showed the presence of protozoa (33) might explain the differences between 10^1 and 10^1-F and indicated the role of protozoans in reducing pathogen survival. Nevertheless, the mechanisms behind the protozoan effect remain speculative. Protozoa can affect bacterial communities by influencing conditions for growth (34) as well as through selective or nonselective feeding, and members of the *Enterobacteriaceae* may constitute food sources that stimulate protozoan growth (35). The fact that bacterial richness was not affected by filtration suggested that nonselective feeding of dominant bacterial species took place. On the other hand, filtration might have affected other microbial members (e.g., fungi) as well. In this context, the effects of predation cannot be completely isolated from the microbial diversity effect, and only a full description of the microbial communities in terms of species composition and abundance would provide support for these hypotheses.

In all treatments, invader survival could be characterized as a progressive decline of strain T abundance (as determined by cfu numbers over time; Fig. 3). Differences between treatments became evident after day 6, and they increased toward the end of the experiment. Results of univariate repeated-measures ANOVA, in which the effects of treatment diversity on survival over the experiment were tested, are shown in Table 1. Survival of the invader was affected by time, treatment, and their interaction (Table 1). Thus, soil microbial diversity, established by dilution-to-extinction, affected invader survival. The decline rate was also impacted by filtration (Table 1, comparing treatments 10^1 and 10^1-F). One-way ANOVA performed for the day-60 values confirmed the analyses, showing significant differences between mean invader abundance between treatments [$F_{(4,10)} = 112.27$; $P = 0.0001$]. Pairwise comparisons significantly separated all mean values ($P < 0.05$; Tukey's test), except for the natural soil and 10^1 treatments (Fig. 3), which clustered together.

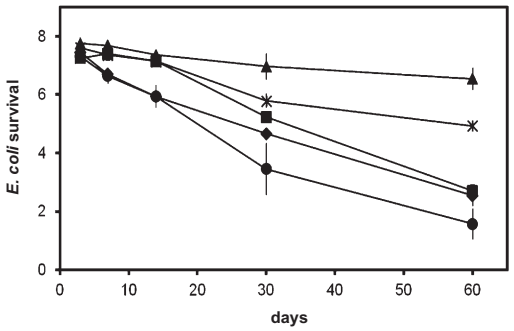

Fig. 3. *E. coli* strain T population dynamics (log cfu g^{-1} soil) over a 60-d period in soil with differently established microbial diversities using a dilution-to-extinction approach. ▲, 10^6 treatment (number indicates the dilution factor of the sample microbial community; *, 10^3; ●, 10^1; ■, 10^1-F (F indicates sequential filtering over membrane filters to remove higher organisms, including protozoa); ◆, natural soil. Each symbol represents the mean value of three replicates. Bars represent SDs of the mean.

To evaluate the effects of the diversities of all microbial groups monitored via PCR-DGGE, a global richness indicator was established (36). Repeated-measures ANOVA performed at 3, 30, and 60 d revealed an effect of dilution treatment on global richness [$F_{(4,10)} = 36.62$, $P = 0.0001$], but no effects of time or time $\times$ treatment. Thus, global richness was different between treatments and remained so over time. Natural and 10^1-treated soils exhibited high global richness values, whereas the 10^6-treated soil showed the lowest value (Tukey's post hoc test, $P < 0.05$). Global richness values of treatments 10^1-F and 10^3 were statistically similar, the former slightly exceeding the latter. Interestingly, global richness values correlated with, and thus predicted, invader survival: 3, 30, and 60 d after introduction, significant negative relationships were observed between strain T survival rate and global richness (Fig. 4). Thus, global richness was able to explain the decline of the invading species in the microcosms—as global richness increased, *E. coli* survival decreased. Moreover, the effect was magnified over time. Bacterial counts (cfu) evaluated at the same time points did not show any relationship with invader survival ($P > 0.05$). These results

Table 1. Univariate repeated-measures ANOVA, separately testing for effects of dilution treatment and filtration on *E. coli* strain T survival at five time points along the experimental period

Test	Effects	df	*F*	*P*
Dilution treatment				
Between-subject effects	Dilution	4	193.70	<0.0001
	Error	10		
Within-subject effects	Time	4	424.79	<0.0001
	Time × treatment	16	21.36	<0.0001
	Error (time)	40		
Filtration				
Between-subject effects	Filtration	1	114.51	0.0004
	Error	4		
Within-subject effects	Time	4	180.64	<0.0001
	Time × filtration	4	4.16	0.0170
	Error (time)	16		

For dilution treatment, all treatments were considered. For filtration, the 10^1 and 10^1-F data were used to evaluate the filtration effects on the same sample community.

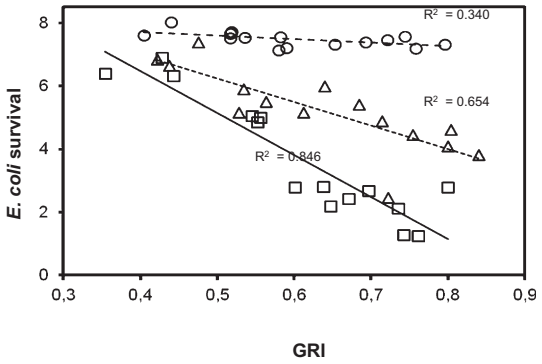

Fig. 4. Relationship between *E. coli* strain T population dynamics (log cfu g^{-1} soil) and the global richness indicator (GRI) at different times after introduction of the invader. The GRI (34) was derived from the sum of five richness values (number of bands on DGGE gels) obtained from the following microbial groups: total bacteria, total fungi, pseudomonads, actinobacteria, and bacilli, each normalized per group, and divided by 5. Linear correlations between *E. coli* survival and GRI increased over time (R^2 = 0.34, R^2 = 0.654, and R^2 = 0.846 for 3, 30, and 60 d after pathogen inoculation, respectively). Open circles, day 3; open triangles, day 30; open squares, day 60.

confirm the previous findings obtained with simpler communities (assembly approach), clearly indicating that the reduction in pathogen density is not observed only in fast-growing bacterial communities. In fact, by creating more realistic soil microbial communities, containing oligotrophic and nonculturable organisms, a steeper decline over time in pathogen population size was observed.

Inverse Relationships Between Invader Survival and Richness of Different Groups. Significant inverse linear relationships between survival of the invader and richness at day 60 were found for most microbial groups under study (i.e., bacteria, fungi, pseudomonads, and Actinobacteria; Fig. S2), survival being particularly affected by bacterial and actinobacterial richness. To a lower extent, it was also negatively correlated with the *Pseudomonas* and fungal richness values, whereas it did not show a clear relationship with *Bacillus* richness. The negative correlations were corroborated by similar regression performed with the Shannon diversity indices. Multiple regressions, in which richness for all five microbial groups at day 60 was related to invader survival, were also run. The correlation between invader survival and actinobacterial richness was highly significant (R^2 = 0.893; P = 0.0001), indicating that Actinobacteria are major contributors to this model. Actinobacteria have the capacity to produce ample secondary metabolites involved in soil microbiostasis (27, 28), indicating that negative interspecies interactions play an important role in determining the fate of invading species. The notion that specific groups of bacteria play a specific role in *E. coli* suppression is not new (25). It was not clear, however, whether the microbial suppression of *E. coli* was due to competition for vital resources or to direct antagonism. To confirm the presence of suppressors of strain T, future experiments should harness the assemblage approach, selecting either antagonists or competitors for the same resources.

Competition Between Invader and Resident Community. Competition for nutrients is an important mechanism that may limit invasions in highly diverse communities (5). In resource-based niche theories, the establishment of invading species is dependent on the amount of (limiting) resources that are left unconsumed by native species, as well as by the rate at which native and invader species consume the existing resources (4, 37). We hypothesized that the uptake of diverse resources plays important roles in the invasibility of microbial communities by strain T. Thus, to explain the effect of microbial diversity on strain T, we evaluated the competitive ability of the latter in the presence of bacterial communities of increasing richness, focusing on single niche factors (i.e., the carbon source). Thus, we compared the resource utilization patterns of communities varying in richness (1, 5, 10, and 20 species), with or without strain T, on 31 individual carbon sources typical for soil (38). The dissimilarity in resource utilization patterns of bacterial communities in the presence and absence of strain T was used as a proxy for the competitive ability of strain T, with higher dissimilarities indicating higher dominance of strain T in the community. The competitive ability of strain T indeed decreased with increasing species richness (Fig. 5). The significant negative correlation observed between strain T's competitive ability and species richness, for both the amount of each resource (R^2 = 0.456, P = 0.0001) and the rates of utilization (R^2 = 0.334, P = 0.0001), indicated that the more diverse communities were, the better they were able to acquire resources, and at a higher rate, than strain T. As expected, in the absence of the invader, the communities with higher species richness were able to consume more of the individual resources (Fig. S3; R^2 = 0.39, $P < 0.0001$).

Rate of Resource Utilization. Considering that species with higher consumption rates will be more efficient in extracting resources, and thus more competitive, we could make a parallel to the resource requirement parameter R* proposed by Tilman (4, 37). In his work, it was proposed that species with lowest R* would outcompete those that require higher resource levels. Briefly, considering a fixed amount of resource, species with higher growth rates will have a lower R* (assuming that loss rate due to

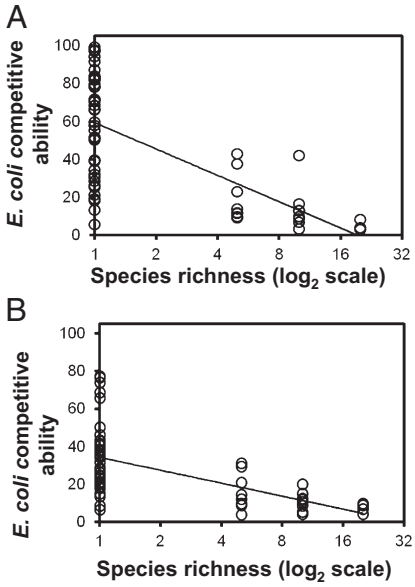

Fig. 5. Relationship between manipulated species richness and the competitive ability of *E. coli* strain T. The competitive abilities are expressed both in relation to the amount of resources consumed (*A*) and their rate of consumption (*B*) and represent the dissimilarity in resource utilization patterns, obtained by pairwise comparisons of bacterial communities of increasing richness (1, 5, 10, and 20 species), in the presence and absence of strain T. Each circle represents a pairwise comparison. Resource utilization patterns correspond to the average in the amount (*A*, R^2 = 0.46, P = 0.0001) or in the rate of consumption (*B*, R^2 = 0.33, P = 0.0001) of 31 carbon sources present in the Biolog Ecoplate. Similar data were obtained when only the 10 carbon sources used by *E. coli* strain T were analyzed (R^2 = 0.47, $P < 0.0001$ and R^2 = 0.43, $P < 0.0001$, for amount and rate of consumption, respectively).

predation is similar in the experiment). Thus, species with higher consumption rates would use the resources faster and, therefore, reduce the probability that less-competitive strains would thrive. In this context, we calculated, for each individual strain and on each carbon source, the rate at which the resources were consumed. The growth rates of strain T were, indeed, significantly lower compared with those of the set of 40 bacterial species used in the experiments. Considering the 10 resources used by *E. coli*, its average growth rate was 4.17 ± 0.77 omnilog units h^{-1} (average ± SE), whereas the average rate of resource utilization of the other 40 strains on the same set of resources was 14.51 ± 0.63 omnilog units h^{-1} (average ± SE). These results support the view that efficiency of resource utilization by the invader vs. that by the established community was at the heart of the effect of microbial diversity seen in the soil microcosms, at least when considering species interactions by one niche factor at a time. It is important to note that the relative rates of resource utilization observed do not necessarily reflect the actual in situ rates in soil, where complex interactions between substrates and resident microbial communities are likely to be found. Nevertheless, they provide an indication of the potential activities of representatives of the soil microflora. See *SI Discussion*.

Materials and Methods

Soil and Microcosms. Fresh samples of a species-rich loamy sand soil denoted Wildekamp (W; pH-KCl 5.5; approximately 2% organic matter, 16–18% moisture upon sampling), obtained from the upper 10 cm of the Wildekamp field (Wageningen, The Netherlands) in autumn (September 2006), were used throughout. The field was covered by permanent grass, and regular mowing in summertime was the only treatment applied. Part of the soil was sterilized by γ irradiation (50 kGy), and another part was kept for <7 d at room temperature in closed plastic bags that were regularly aerated. The first was used as the matrix of soil microcosms, whereas the latter was used, in the dilution-to-extinction experiment, as an inoculum.

Experimental Setup. Varying treatments were established in soil microcosms. One type of manipulation consisted of the addition, to the γ-sterilized W soil, of consortia consisting of mixtures of random bacterial isolates, of increasing complexity, whereas a second one consisted of the addition of microbial communities obtained via a dilution-to-extinction approach. Microcosms were constructed in 200-mL bottles that contained 50 g of W soil at final moisture content of 65% of water-holding capacity (WHC). Control microcosms contained natural or sterilized soil without added cells. Three replicates were used per treatment per time. Flasks were distributed in plastic trays following a randomized design. The soil microcosms were incubated at 20 °C in the dark and at constant moisture. Microcosms were destructively sampled at each time point during the experimental period (3, 7, 14, 30, and 60 d) and monitored with respect to invader population density and microbial diversity (details below).

In a first experiment, the sterilized W soil was inoculated with 0, 5, 20, or 100 bacterial strains isolated from the same soil, before sterilization. For each diversity level, 20% of the bacterial strains encompassed typical actinobacterial morphs. Bacterial isolation was performed by shaking 10 g of soil in 90 mL of sterile 0.1% sodium pyrophosphate plus 10 g of gravel (2–4 mm diameter) for 30 min. Serial 10-fold dilutions of this mother suspension were spread onto R2A medium (Oxoid), and colonies appearing at up to 12 d were randomly picked, purified by streaking, and stored until their utilization in the bacterial mixtures. For introduction, cells obtained from individual pure fresh colonies were suspended in sterile water to obtain an absorbance at 600 nm (A_{600}) of 1. Assuming that an A_{600} of 1 corresponds to 10^9 cells mL^{-1}, we set up inocula for each treatment by adding different amounts of each bacterial suspension to obtain mixtures with the same amount of cells but different strain richness (5, 20, or 100 strains). As expected, approximately 5×10^8 to 10^9 bacterial cfu g^{-1} soil were established before the introduction of strain T in each microcosm (checked by plating on R2A).

In the second experiment, a dilution-to-extinction technique was adopted (30, 31). Specifically, microcosms containing 50 g of γ-sterilized W soil were inoculated with 5-mL aliquots of different suspensions obtained by serially diluting (1:10) natural W soil suspensions in sterile water. The systems were carefully mixed avoiding contamination, and covered to prevent water evaporation. As inocula, the 10^1, 10^3, and 10^6 dilutions were used. The 10^1 dilution was also used after vacuum-filtration through membranes with progressively smaller pore sizes (5, 3, 2, and 1 μm), thus yielding treatment "10^1-filtered" (10-F).The aim of this successive filtration was to exclude protozoa and other soil mesofauna, but it is likely that larger (>1 μm) bacterial and fungal cells have been left out as well. Natural soil did not receive inoculant cells, but soil humidity was maintained at 65% of WHC.

Pathogen Introduction. In both microcosm experiments, after allowing 30 d for the establishment and stabilization of the microbial community at a comparable level across all of the treatments, an invading bacterial pathogen [i.e., the genetically marked, nonpathogenic *E. coli* O157:H7 derivative strain Tn5 *luxCDAEB* (20)] was introduced into the soil microcosms at approximately 10^8 cfu g^{-1} dry soil. This established a soil moisture content of 75% of WHC.

Monitoring of Survival of the Invader as Well as Total Bacterial Communities. After *E. coli* O157:H7 derivative strain T addition, we monitored the microcosms at different time points over a 60-d period, during which all cultivation-dependent and -independent analyses were performed. The survival of strain T, expressed as the number of cfu g^{-1} dry soil, was then determined by selective dilution plating on tryptic soy agar (TSA) plus respective antibiotics, in accordance with van Elsas et al. (21). *E. coli* counts were determined after 24 h incubation at 37 °C. Survival of strain T was thus based on the cfu numbers observed on selective medium at each sampling time, during a 60-d period. The number of total culturable bacteria, expressed as cfu g^{-1} dry soil, was determined by dilution plating onto unselective R2A medium followed by colony counting after prolonged incubation (up to 12 d) at 28 °C. Total bacterial biomass was determined using dichlorotriazinylaminofluorescein-assisted microscopy, and total protozoa were estimated using the most-probable-number method of Darbyshire et al. (33). We further determined the prevalence of different indicator PLFAs by using MIDI-FAME analysis (21).

The modified microbial diversities established in the two types of microcosms were assessed using soil-DNA-based PCR-DGGE. The richness (number of different amplicon types) and diversity (Shannon index) of total fungi and bacteria, as well as of specific bacterial groups (only for the dilution-to-extinction experiment), were evaluated on the basis of PCR-DGGE analyses performed at day 3, 30, and 60 after invasion by *E. coli* strain T. In the group-specific PCR-DGGE approach, we assayed those microbial groups of which members were likely responsible for antagonism toward invading microorganisms (i.e., *Pseudomonas* spp., *Bacillus* spp., and *Actinobacteria*). In all cases, DNA was extracted from soil using standard soil DNA extraction (MoBio Ultraclean Extraction Kit). PCR amplifications and DGGE separation procedures were applied as indicated for total bacteria, fungi, and *Actinobacteria*, *Pseudomonas*, and *Bacillus* (27). The patterns obtained were compared across treatments by using GelCompar version 4.0 (Applied Biosystems) and diversity measures, such as band (taken as indicative for species) richness and the Shannon index of diversity (including a richness and evenness component), were calculated. On the basis of the observed richness values, a global richness indicator (GRI) was calculated (36), by adding the normalized richness values obtained for five microbial groups (total bacteria, total fungi, actinobacteria, pseudomonads, and bacilli) and dividing it by 5. A range of statistical analyses was performed to assess the relationship between the survival of strain T and the diversity parameters established.

Competition Experiment. Bacterial strains were isolated from soil (as described previously), and a set of 40 different strains, distinguished according to colony morphology and BOX-PCR (39), was selected. Briefly, the strains were used to create communities of increasing species richness, following a broken stick model (39, 40) to create communities varying in species richness and composition. Bacterial species were randomly ordered, creating one stick, which was then subsequently "broken", giving two assemblages of 20 species, four of 10 species, and eight of 5 species. Extra assemblages were constructed by creating a third and fourth stick of 20 species, containing the species located at the center or ends of the first 20-species sticks. Further division of these sticks provided an additional four assemblages containing 10 species. Thus, four, eight, and eight assemblages containing respectively 20, 10, and 5 species were created, in addition to the 40 monocultures. Microcosms consisted of Ecoplates (Biolog) containing 31 carbon sources commonly found in soil, replicated three times (38). Microcosms were inoculated with the assemblages at similar final cell densities (OD_{600} = 0.1). Thus, each microcosm received the species at a cell density of $OD_{600} = 0.1/s$, where s is the number of species in the community inoculum (1–20). For microcosms containing strain T, the latter was introduced at OD_{600} = 0.02, regardless of the established species richness. The data based on monocultures in the absence of the pathogen were used to calculate the growth rates of individual strains (using slopes, see below) on the respective C sources. *E. coli* strain T was also used as a single strain at initial densities corresponding to OD_{600} = 0.02 and OD_{600} = 0.1, for comparisons in the com-

petition and growth rate experiments, respectively. Incubation was in the omnilog apparatus (Biolog) for 48 h at 25 °C, and reading was every 15 min. The quantity of substrate used and the rates of consumption were calculated according to the area under the curve and the slopes of increase, respectively, for each of the 31 individual C sources, using the software provided. Pairwise comparisons of the bacterial assemblages, in the presence and absence of strain T, were performed by using Bray Curtis dissimilarity implemented in the software PRIMER 5 (PRIMER-E). Similar relationships were obtained by using the distance measures Gower and Euclidean Distance.

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