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LETTER

Diversity of locust gut bacteria protects against pathogen invasion

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Abstract

Diversity–invasibility relationships were explored in the novel context of the colonization resistance provided by gut bacteria of the desert locust *Schistocerca gregaria* against pathogenic bacteria. Germ-free insects were associated with various combinations of one to three species of locust gut bacteria and then fed an inoculum of the pathogenic bacterium *Serratia marcescens*. There was a significant negative relationship between the resulting density of *Serratia marcescens* and the number of symbiotic gut bacterial species present. Likewise there was a significant inverse relationship between community diversity and the proportion of locusts that harboured *Serratia*. Host mortality was not negatively correlated with resistance to gut-invasion by *Serratia marcescens*, although there were significantly more deaths among pathogen fed germ-free insects than tri-associated gnotobiotes. The outcome is consistent with the predictions of community ecology theory that species-rich communities are more resistant to invasion than species-poor communities.

Keywords

Colonization resistance, diversity, germ free, locusts, *Schistocerca gregaria*, *Serratia marcescens*.

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INTRODUCTION

Theory suggests that species-rich communities are more resistant to invasion than species-poor communities (Robinson & Valentine 1979; Case 1990; Law & Morton 1996). Three, not mutually exclusive mechanisms have been invoked to explain this. Species-rich communities are more likely to contain a species that is particularly important to invasion resistance (dominance) (Huston 1997), occupy a wider range of niches (niche complementarity) (Tilman *et al.* 1997) and, contain species that facilitate each other's invasion resistance (positive interactions) (Bruno *et al.* 2003). Experimental tests of diversity–invasibility relationships have been ambiguous. However, where variation between communities in environmental heterogeneity, which may cause positive covariance between diversity and invasability (Jiang & Morin 2004), has been controlled for the predicted negative invasability–diversity relationships have been observed in a wide range of community types, including plants, marine invertebrates, protists and bacteria (McGrady-Steed *et al.* 1997; Hodgson *et al.* 2002). Here, we experimentally address diversity–invasibility relationships in an entirely novel system, where environmental heterogeneity should be comparable across replicate communities: the gut bacteria of desert locusts, *Schistocerca gregaria*. Many gut-colonizing organisms are pathogenic, hence invasion resistance of gut bacterial communities is likely to have important implications for host organisms.

It is well established for vertebrates that the indigenous gut microbiota can interfere with the establishment of newly ingested microbes. Furthermore, there is evidence from vertebrates that a more complex gut microbiota has a bigger protective effect than a monoassociation (Miller & Feeley 1975). The protective effect of gut bacteria that has variously been termed bacterial antagonism (Freter 1956), competitive exclusion (Lloyd *et al.* 1977), bacterial interference (Aly & Shinefield 1982) and colonization resistance (CR) (Van der Waaij 1989; Van der Waaij & Van der Waaij 1990; Rolfe 1997) is a significant component of host defence against pathogens. Resident flora may antagonize would-be gut colonizers by stimulating the host-immune response, increasing gut movement, competition for nutrients and binding sites, altering physiological conditions in the gut and the production of antimicrobials (Rolfe 1997; Reid *et al.* 2001). However, it is only in rare cases that it has been possible to identify the bacterial species responsible for CR and to elucidate the mechanisms involved (Hudault *et al.* 2001).

Colonization resistance has also been inferred in studies on insect gut microbiota from flies (Greenberg *et al.* 1970), caterpillars (Jarosz 1979) to termites (Veivers *et al.* 1982). However, the most complete evidence for a protective effect of the autochthonous gut microbiota in an insect comes from our extensive studies on the desert locust, *Schistocerca gregaria* (for review see Dillon & Charnley, 2002). We have shown that the locust gut microbiota play a major part in the suppression of enteric infections of a fungal pathogen, during periods of food deprivation when other components of the gut barrier are reduced, by the production of toxic phenols (hydroxyquinone, 3,4-dihydroxybenzoic and 3,5-dihydroxybenzoic acids) (Dillon & Charnley 1988). These phenolics, which are both antibacterial as well as antifungal, were absent from the gut fluid and faecal pellets of germ-free locusts. Monoassociation of germ frees with *Pantoea agglomerans*, a common inhabitant of locust guts, re-established production of 3,4-dihydroxybenzoic acid (Dillon & Charnley 1995). The absence of the other two phenols implies that a more diverse microbiota is required for the full cocktail of phenols.

Here, we explicitly test the prediction that diversity of gut bacteria confers resistance to invasion of a foreign, pathogenic bacterium in *Schistocerca gregaria*, and determine the impact of invasion resistance on host survival. We inoculated initially bacteria-free *Schistocerca gregaria* with either zero, one, two or three naturally associated bacteria, and then measured the ability of the bacterium *Serratia marcescens* to invade the locust gut, and determined survival rates of locusts. *Serratia marcescens* was chosen as the pathogen because it has been shown previously to cause disease in desert locusts (Stevenson 1959).

MATERIALS AND METHODS

Conventional locusts

Conventional (wild type) locusts that have an autochthonous gut microbiota were reared gregariously according to the method described by Hunter-Jones (1966) and fed on fresh wheat shoots, wheat bran (supplemented with dried brewer's yeast) and water. The colony was housed in a 28 °C constant temperature room with a 12-h light : 12-h dark cycle. Each cage was provided with a 60 W electric light bulb that allowed locusts to thermoregulate during the light period.

Bacterial isolates and their cultivation

We used three species of locust gut bacteria in our experiments: *P. agglomerans* (Sga40), *Klebsiella pneumoniae pneumoniae* (Sg16) and *Enteroccocus casseliflavus* (Sgs32) (Dillon *et al.* 2002). Although the gut microbiota of conventional locusts typically comprises three to 12 bacterial types, depending on nutritional status and age, these species were chosen because they are common members of the autochthonous gut microbiota of both laboratory cultured and wild locusts (Hunt & Charnley 1981; R.J. Dillon, G. Webster, A.J. Weightman, S. Blanford, M. Thomas and A.K. Charnley, unpublished observation). A strain of red pigmented isolate of *Serratia marcescens* (NCIB 1377), was selected for its pathogenicity to desert locusts: in a preliminary experiment, an oral dose of 1×10^6 CFU killed 10% of locusts in 5 days whereas an injection of 200 CFU was sufficient to kill 100% of conventional adult locusts in just 2 days. All insects that died from *Serratia* infection became red in colour. The bacterial strains were easily differentiated from each other by colony colour and shape, when growing in mixed cultures on solid media. Cultures of bacteria were grown overnight at 28 °C in Nutrient Broth (Oxoid Ltd, Basingstoke, UK) from a single colony of the bacteria grown on nutrient agar from frozen stocks. Suspensions of known concentration were prepared by reference to standard curve prepared in Nutrient Broth.

Production of gnotobiotic locusts

Bacteria-free (germ free) desert locusts, *Schistocerca gregaria* were reared initially from surface sterilized eggs inside flexible plastic isolators (Charnley *et al.* 1985). The bacteria-free status of the insects was checked by a combination of aerobic and anaerobic growth assays, and light and scanning electron microscopy. Locusts were fed a diet of freeze-dried grass and bran with vitamin supplement. First generation insects were bred to obtain further bacteria-free locusts.

Locusts were starved and deprived of water for 24 h prior to feeding with bacteria. Gnotobiotic locusts were generated by feeding teneral germ-free adults with a 50-μL aliquot of gut bacteria (unless otherwise stated, 6×10^7 CFUs) a week prior to the experiment; the same total quantity of bacteria were administered regardless of the number of bacterial strains being introduced. Germ free, gnotobiotic and conventional locusts were inoculated in the same way with 1.25×10^7 CFU of *Serratia marcescens*, placed in individual sterile plastic containers (11.3 cm in diameter, 8.9-cm height), fed sterile irradiated grass and put in a constant temperature room at 28 °C with a 12-h light : 12-h dark cycle. Forty Watt electric light bulbs were placed in front of the containers to produce a temperature gradient (36 °C at the front of the container and 29 °C at the back). This arrangement allowed the locusts to thermoregulate to their preferred temperature set point (36 °C for colony locusts, determined by an infrared thermal imager (NEC ThermoTracer TH5102, NEC San-ei Instruments Ltd, Tokyo, Japan) during the light period, but not to express behavioural fever (41–42.5 °C; Bundey *et al.* 2003). The

maximum temperature achieved by experimental locusts was 37 °C; determined with a copper constant thermocouple (Omega Engineering Ltd, Manchester, UK) inserted into the thorax and connected to a Hanna HI193551 thermometer (Hanna Instruments Ltd, Leighton Buzzard, UK).

Sampling the gut microbiota

Experimental insects were fed every 2 days with approximately the same quantity of sterile grass (two blades) dipped in sterilized distilled water. Faecal pellets were collected 4 h after feeding from insects that had eaten all the provided food, and homogenized in 0.5 mL of 0.5% peptone water. Aliquots were plated out from a series of 10x dilutions onto nutrient agar and incubated for 24 h at 27 °C. The number of CFUs of each species on the agar plates was used to provide an estimate of the bacteria present in the pellets voided over a 4-h period. While not providing an estimate of absolute numbers, faecal bacterial densities have been found to correlate with gut bacterial communities across experimental treatments in simple gnotobiotic systems (e.g. Hudault *et al.* 2001). Therefore, faecal sampling is used widely in studies on vertebrate gut bacteria to track changes in intestinal microbiota (e.g. Filho-Lima *et al.* 2000).

Experimental design and analysis

Two experiments were carried out. The first compared the invasion resistance to *Serratia marcescens* of conventional and germ-free insects in five blocks carried out at different times (total $n = 65$), with *Serratia marcescens* densities measured every day over a 4-day period. For each locust, the average density through time was calculated, and $\log_{10}$ transformed to normalize residuals and homogenize variance between treatments. Treatment, block and their interaction were fitted as factors in a general linear model.

The second experiment measured the invasion resistance of germ-free and gnotobiotic insects, with all possible combinations of the three chosen gut bacteria species, in two blocks carried out at different times ($n = 71$). Here, density of both gut bacteria and *Serratia marcescens* were measured every 2 days, over a 9-day period. Because high rates of mortality greatly reduced the sample sizes of some of the specific treatments, locusts were grouped into diversity treatments of 0, 1, 2 or 3 gut bacteria for most analyses. Average density through time of both gut bacteria and *Serratia marcescens* were calculated for each locust. *Serratia marcescens* density could not be normalized, and other error distributions were not appropriate, so we used a non-parametric Kruskal–Wallis test to compare density across diversity treatments. To allow for our *a priori* expectation that density of *Serratia marcescens* would decrease with bacterial diversity, we used the ordered heterogeneity test (a combination of the Kruskal–Wallis test and a Spearman rank correlation) (Rice & Gaines 1994) to generate our final test statistic. We also compared the proportion of locusts across treatments that had undetectable densities of *Serratia marcescens* by logistic regression (Crawley 1993), with block fitted as an additional factor. We used a Kruskal–Wallis test to compare the density of resident gut bacteria across diversity treatments. Finally, we compared locust survivorship across treatments using survival analysis, with census data (whether alive or not at the end of the experiment) fitted as the response variable, ln(time to death) as an offset, and diversity and block as factors. All analyses were carried out using Genstat 6.2 (Lawes Agricultural Trust, Rothamsted, UK).

RESULTS

Our data suggest that the autochthonous gut biota of *Schistocerca gregaria* afforded protection against establishment by *Serratia marcescens* in the gut: the density of *Serratia marcescens* recovered from germ-free animals was *c.* five times higher than from conventional insects (Fig. 1; $F_{1,59} = 16.52$, $P < 0.001$). However, mortality was very low in both treatments, and clearly not significantly different (two and one locusts respectively).

To test the hypothesis that the diversity of gut bacteria contributes to their protective effect, germ-free locusts were inoculated with various combinations of one to three species of locust gut bacteria: *P. agglomerans*, *K. pneumoniae pneumoniae* and *E. casseliflavus*. After the mono-, di- and tri-associations had become established (1 week), the gnotobiotic locusts were fed an inoculum of *Serratia marcescens*. The dynamics of *Serratia marcescens* in the different diversity treatments are shown in Fig. 2. In all cases there was a significant decline in pathogen population with time.

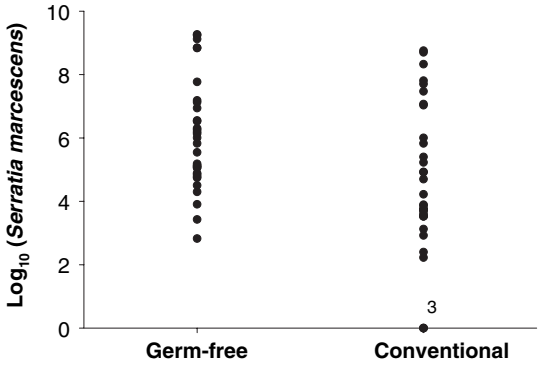

Figure 1 Density of *Serratia marcescens* in faeces from conventional and germ-free locusts. Each point represents a single locust, and numbers show multiple locusts with undectable densities.

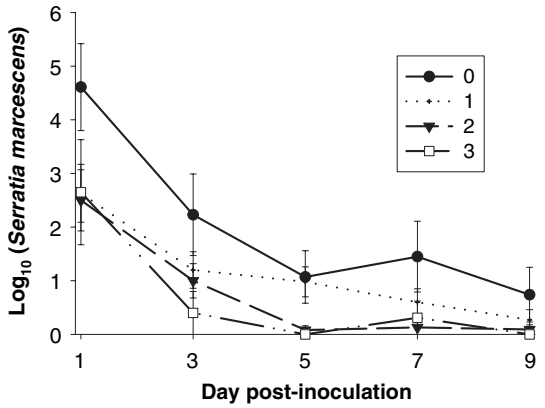

Figure 2 Mean (± 1 SEM) density of *Serratia marcescens* in faeces through time in the different diversity treatments. 0, 1, 2, 3 represents number of bacterial species fed prior to inoculation with the pathogen.

However, unlike a fed inoculum of *Flavobacter indologenes* (representative of the phylloplane bacterial flora ingested with food by locusts), which was quickly removed from the gut, and could not be isolated from faeces ≥ 4 h (data not shown), *Serratia marcescens* clearly replicated and became established in the guts of a proportion of the inoculated locusts.

There was a significant negative relationship between the density of *Serratia marcescens* and the number of gut bacterial species present: pathogen density was lowest in insects with the most diverse gut bacterial communities (Fig. 3a; $r_s P_c = 0.999$; $P < 0.0001$). Likewise there was also a significant inverse relationship between community diversity and the proportion of locusts that were infected (Fig. 3b; $\chi^2_1 = 13.08$, $P < 0.001$). These results could not be explained by high pathogen densities in the germ-free treatment: when these insects were excluded from the analysis, the relationships remained ($r_s P_c = 0.83$, $P = 0.03$; $\chi^2_1 = 4.87$, $P < 0.01$). Furthermore, our data suggest that the increased probability of diverse communities containing any one particular gut bacterial species was not responsible for this relationship between density and diversity (dominance effect): there was no difference in *Serratia marcescens* density between mono-associated locusts infected with the different bacterial species Kruskal–Wallis ($n = 31$, $H = 1.82$, $P = 0.4$).

Each locust was inoculated with same overall number of symbiotic bacteria, whether one, two or three species were included in the cocktail. However, there was a significant positive relationship between the size of the resulting gut microbiota and the number of species inoculated (Fig. 4; $H = 11.54$, $P = 0.003$). However, there was no significant correlation between the density of *Serratia* and density of symbionts ($r_s = -0.11$, $P = 0.41$; excluding germ-free insects).

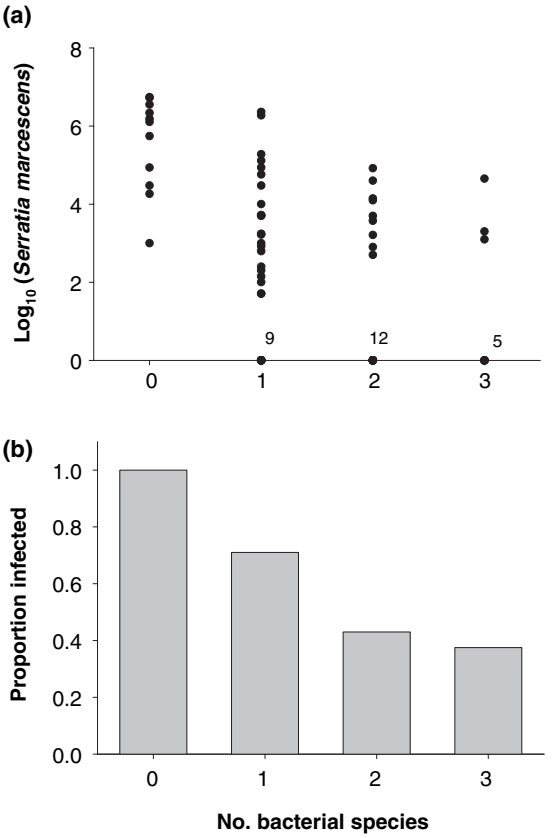

Figure 3 The impact of gut bacterial diversity on the colonization of *Serratia marcescens*. (a) Density of *Serratia marcescens* in faecal pellets through time. Each point represents a single locust. Numbers are locusts with undetectable *Serratia marcescens*. (b) Proportion of locusts with detectable *Serratia marcescens* at any time point.

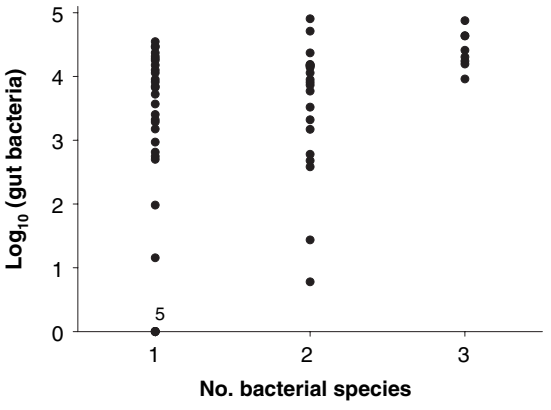

Figure 4 Density of gut bacteria in faeces from conventional and germ-free locusts. CFUs isolated from faeces averaged over the 9 days of the experiment. Each point represents a single locust.

Resistance to invasion by *Serratia marcescens* did not show a comparable negative correlation with host mortality, although there were significantly more deaths among pathogen fed

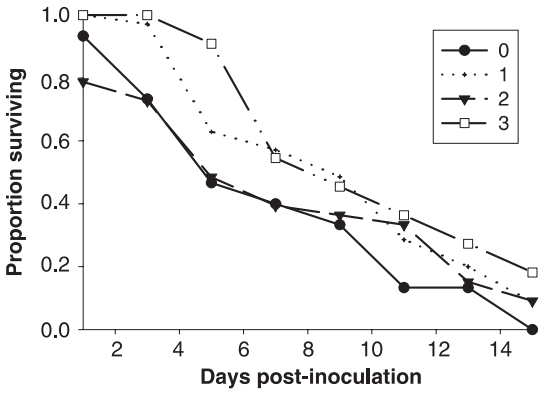

Figure 5 Proportion of insects with different numbers of gut bacteria surviving through time. 0, 1, 2, 3 represents number of bacterial species fed prior to inoculation with the pathogen.

germ-free insects than tri-associated gnotobiotes (Fig. 5; survival analysis: $F_{4,89} = 2.86$, $P = 0.03$, differences between mortality rate of germ-free and tri-associated gnotobiotes, $t = 1.98$, $P = 0.05$).

DISCUSSION

We have established that the gut microbiota of the desert locust significantly reduce colonization of the gut by the pathogen *Serratia marcescens*. This complements our previous observation that enteric invasion by the entomopathogenic fungus, *Metarhizium anisopliae*, is suppressed by the microbiota (Dillon & Charnley 1986). More importantly, we have shown that diverse communities of gut bacteria (two and three species) provide greater protection than a single resident species. These data are consistent with data from numerous community ecology studies including grassland (reviewed in Levine & D'Antonio 1999), marine invertebrate (Stachowicz *et al.* 1999) and *in vitro* microbial communities (McGrady-Steed *et al.* 1997; Hodgson *et al.* 2002) that suggest that species-rich communities are more resistant to invasion by foreign species, than are species-poor communities.

The model experiments described here have ecological relevance because the normal autochthonous gut microbiota of locust is simple (three to 12 bacterial types). This is true of locusts of different age, diet and nutritional status (fed or starved) from laboratory desert locust to wild Moroccan locusts (*Dociostaurus maroccanus*) and Italian locust (*Calliptamus italicus*) (from Spain) and brown locusts (*Locusta pardalina*) (from South Africa) (R.J. Dillon, G. Webster, A.J. Weightman, A.K. Charnley, unpublished observation). All have bacteria of the same taxa with many common types; indeed the guts both species of the sympatric Spanish locusts were dominated by a single bacterial phylotype.

Locust thermal ecology has been taken into consideration here. Experimental locusts were allowed to thermoregulate to their preferred set point temperature (36–37 °C). We have shown previously that an injected dose of the strain of *Serratia marcescens* used here caused behavioural fever; the 41 °C fever temperature achieved prevented septicaemia (Bundey *et al.* 2003). As we did not want to prevent infection here the experimental conditions did not allow fever temperature to be reached. This is ecologically relevant as behavioural fever is only possible on sunny days and bacteria in the haemocoel invoke behavioural fever but not those in the gut (unpublished data).

It is unclear exactly why diverse communities of gut bacteria should be more resistant to invasion than depauperate communities, but the data suggest that it is not simply because more diverse communities have a higher probability of containing a gut bacterial species that confers particularly high invasion resistance. Locusts monoassociated with *P. agglomerans* produced 3,4-dihydroxybenzoic acid, one of three antimicrobial phenols that contribute to CR against *M. anisopliae* (Dillon & Charnley 1995). If these phenols play a part in the antagonism of *Serratia marcescens* [and this is by no means certain as a different isolate of *Serratia marcescens* to the one used in the present work showed resistance to the phenols (Dillon & Charnley 1995)], then species of bacteria that can provide the other two compounds may be needed. A number of the Enterobacteriaceae that colonize locust guts including *P. agglomerans* and *K. pneumoniae pneumoniae* can produce the related phenolic guaiacol, probably by decarboxylation of vanillin (Dillon *et al.* 2002). *Enterococcus casseliflavus* produces little guaiacol *in vitro* (Dillon *et al.* 2002) and there is no information on the ability of either *K. pneumoniae pneumoniae* or *E. casseliflavus* to produce hydroxyquinone or 3,5-dihydroxybenzoic acids. In terms of the wider applicability of this work it is important to note that bacterially derived antimicrobial phenolics are widely present in locusts and grasshoppers of different stage and nutritional state (Dillon & Charnley 1995).

Despite the presence of (diverse) gut bacterial species affording greater invasion resistance against *Serratia marcescens*, the protective effects of gut bacteria in terms of insect mortality was much less pronounced. In the first study, mortality did not differ between germ-free and conventional locusts, and in the second study, although mortality was considerably higher, mortality rates only differed significantly between insects with no and three gut bacterial species. It is not clear why the difference in pathogen establishment in the gut between different treatments was not reflected in insect mortality. All dead insects were red in colour indicating that mortality was caused by *Serratia marcescens*. Control mortality among unioculated conventional, germ-free and gnotobiotic locusts of comparable age was negligible over the time scale of the experiment (data

not shown). The experimental conditions prevented the fever temperature (41 °C) required for elimination of the bacterium from the haemocoel (Bundey *et al.* 2003). However, 37 °C (maximum body temperature of the experimental locusts) may have been sufficient to reduce bacterial growth in the haemocoel following invasion from the gut (optimum 34 °C, unpublished data) and improve the immune response of the locust to such an extent that infection was minimized. Increased body temperature is known to improve the efficiency of the immune response in some other invertebrates (Kozak *et al.* 2000). The higher mortality in the second experiment compared with the first may just reflect the variability inherent in the system and the thin dividing line between success and failure in host–pathogen interactions when an intrahaemocoelic dose of as few as 200 CFU is lethal.

The protective effect of locust gut bacteria against *Serratia* within individual insects is not on the scale observed for CR in some vertebrate models (e.g. Filho-Lima *et al.* (2000). However, CR could also have a population level impact among members of a locust swarm. Communal living of gregarious locusts facilitates microbial transmission. Therefore CR, by decreasing population levels of the bacterium, could reduce also the likelihood of the acquisition of a disease-causing inoculum by susceptible individuals. In other words CR, by lowering *Serratia* populations in locust guts, could reduce carrier status of the individuals concerned.

The locust symbiotic gut microbiota contribute to host defence against enteric disease. However, they are only conditionally mutualistic and there are life history trade-offs involved; analogous to trade-offs between resistance to parasitoids and intraspecific competitive ability (e.g. Kraaijeveld & Godfray (1997). Germ-free adult male locusts are significantly heavier than conventionals (Charnley *et al.* 1985) and germ-free locusts of both sexes live longer (Charnley *et al.* 1985; unpublished data). Similar results have been reported in vertebrates (Lochmiller & Deerenberg 2000), which has led to the prophylactic feeding of antibiotics to boost livestock production (Doluschitz & Zeddies 1991).

If locust gut bacteria are a mixed blessing, namely a protection against pathogens but either themselves opportunistically pathogenic (Lysenko 1985) or a drain on the innate immune response (Lochmiller & Deerenberg 2000), then they should be tolerated only if the risk of infection by virulent forms is high (van Baalen & Jansen 2001). A testable prediction from this hypothesis is that gregarious locusts, which are most at risk from communally acquired infections, should have a more diverse gut microbiota than solitaries. Interestingly, Wilson *et al.* (2002) have shown recently that the former invest more in their intrinsic immune defences than the latter.

In addition to potential costs to the host of gut microbiota *per se*, there may be costs to the hosts of harbouring diverse relative to single species communities. Much theory suggests that competition for host resources imposes selection for greater rates of host exploitation, potentially increasing the amount of damage caused to hosts (Bremermann & Pickering 1983; van Baalen & Sabelis 1995; Frank 1996). More recently, it has been suggested that other types of competitive strategies may evolve as a result of competition between bacterial lineages (Chao *et al.* 2000; Brown *et al.* 2002; West & Buckling 2003). First, selection may favour bacteria that exploit extracellular molecules, such as iron-scavenging siderophores, produced by other bacteria (West & Buckling 2003; Griffin *et al.* 2004). Such parasitic strategies will reduce the growth rate of the total bacterial community and potentially the damage directly caused to the host by the bacterial community (West & Buckling 2003) but also potentially reduce community invasion resistance. Second, competition may favour the evolution of costly anti-competitor toxins, which again will reduce total community density as above (Gardner *et al.* 2004; Massey *et al.* 2004) but may also increase invasion resistance if the toxins also act against the invader. Precisely how diverse gut bacterial will affect host fitness relative to single species communities depends on the extent of niche separation between coexisting species (and hence the intensity of competition), the type of competitive interactions that have evolved, and whether or not bacteria can respond facultatively to the presence of competitors.

Here, we have shown that species-rich communities of locust gut bacteria are more resistant to invasion by a bacterial pathogen than species-poor communities. This has a number of important implications. First, it is a demonstration of community ecology theory in an entirely novel setting. Second, it supports the probiotic principle that diverse communities of gut bacteria are likely to result in reduced host morbidity and mortality following infection by gut pathogens (Tannock 1999). Third, a treatment that combines an inoculum of a gut pathogen with a suppressor of the commensal flora may provide a novel method for biocontrol of insect agricultural pests. Consistent with this (Broderick *et al.* 2000) showed that the antibiotic zwittermicin A acts synergistically with *Bacillus thuringiensis* against the gypsy moth *Lymantria dispar*.

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