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ORIGINAL PAPER

Diversity of gut microbiota increases with aging and starvation in the desert locust

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Abstract Here we report the effects of starvation and insect age on the diversity of gut microbiota of adult desert locusts, *Schistocerca gregaria,* using denaturing gradient gel electrophoretic (DGGE) analysis of bacterial 16S rRNA genes. Sequencing of excised DGGE bands revealed the presence of only one potentially novel uncultured member of the *Gammaproteobacteria* in the guts of fed, starved, young or old locusts. Most of the 16S rRNA gene sequences were closely related to known cultured bacterial species. DGGE profiles suggested that bacterial diversity increased with insect age and did not provide evidence for a characteristic locust gut bacterial community. Starved insects are often more prone to disease, probably because they compromise on immune defence. However, the increased diversity of *Gammaproteobacteria* in starved locusts shown here may improve defence against enteric threats because of the role of gut bacteria in colonization resistance.

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Introduction

Studies on non-pathogenic relationships between insects and their microbiota have focused primarily on mutualistic mycetocyte-based associations and the roles of ecto- and endosymbionts in the digestion of refractory polymers (Dillon and Dillon 2004). The majority of apparently commensal relationships between insects and their gut microbial communities are much less understood, though recent studies suggest there are more to these associations than were first thought (e.g. Brummel et al. 2004; Behar et al. 2005; Broderick et al. 2006). Their adaptive significance is implied by the existence of mechanisms that promote the microbiota through suppression of the mucosal immune response (Ryu et al. 2008).

The "commensal" gut microbiota of the desert locust help to protect the host from invasion by pathogenic microorganisms by a process known as colonization resistance (CR) (Dillon and Charnley 2002). We have shown that bacterially derived phenolics play a key role in CR and provide components of the locust cohesion pheromone (Dillon et al. 2000; Dillon et al. 2002).

The use of cultural methods to study the gut microbiota of laboratory reared desert locusts revealed a variable bacterial community of limited diversity

that variously included *Escherichia coli, Enterobacter liquefasciens, Klebsiella pneumoniae, Enterobacter cloacae* and *Pantoea agglomerans*, as well as a number of Gram-positive cocci (Stevenson 1966; Hunt and Charnley 1981). The lack of complexity probably reflects the short through-put time and the simple gut structure. We have subsequently cultivated these bacterial species from wild caught Orthopteran species in countries as widely dispersed as Ethiopia, South Africa and Spain (Dillon et al. 2002).

It is generally believed that only a small proportion of the bacteria associated with insects may be amenable to cultivation (e.g. Reeson et al. 2003). Molecular techniques have shown a diversity of uncultured bacterial species in a variety of insects e.g. termites (Okhuma and Kudo 1996), and crickets (Domingo et al. 1998). One such technique, denaturing gradient gel electrophoresis (DGGE) analysis of bacterial 16S rRNA gene fragments generated by PCR (Muyzer et al. 1993), has been used to explore the gut microbiota of wood wasps (Reeson et al. 2003) and aphids (Haynes et al. 2003).

The gut bacterial community from four species of feral locusts and grasshoppers determined by DGGE revealed an effect of phase polymorphism on gut bacterial diversity in brown locusts (*Locusta pardalina*) from South Africa (Dillon et al. 2008). A single bacterial phylotype, closely related to *Citrobacter* sp. dominated the gut microbiota of two sympatric populations of Moroccan (*Dociostaurus moroccanus)* and Italian locusts (*Calliptamus italicus)* from Spain. However, sequence analysis of DGGE bands did not reveal evidence for a high proportion of unculturable bacteria and homologies suggested that bacterial species were principally *Gammaproteobacteria* from the family *Enterobacteriaceae* similar to those recorded previously in laboratory reared locusts (Dillon et al. 2008).

Here we report the use of PCR-DGGE analysis of bacterial 16S rRNA genes to investigate the effects of starvation and age on the gut microbiota of laboratory-reared desert locusts (*Schistocerca gregaria)*.

Materials and methods

Insect production

A conventional colony of the desert locust *Schistocerca gregaria* viz one in which the insects have a full complement of normal microbes (Coates and Fuller 1977) was maintained on either hydroponically grown greenhouse wheat seedling and bran, or grass from local fields. Domestic crickets, *Acheta domestica*, were reared on wheat bran. A breeding colony of bacteria-free locusts, initiated from surface sterilized eggs, was maintained in flexible plastic isolators on irradiated freeze dried grass and bran with vitamin supplement (Charnley et al. 1985). The bacteria free status of the insects was checked by a combination of aerobic and anaerobic growth assays, direct microscopy and scanning electron microscopy (Charnley et al. 1985). Monobiotic locusts were obtained by inoculating newly hatched bacteria-free nymphs with a log phase broth culture of the bacterium *P. agglomerans* (Dillon and Charnley 1996). Adult male insects ca 21d old were used as fed or starved (food withheld for 5 days with access to water). All insects were maintained at 28°C under a 12:12 h day light/dark cycle.

Bacteria and growth media

Pantoea agglomerans strain Sga40 (16S rDNA accession number AY935243) was isolated from an adult *Schistocerca gregaria* from Addis Ababa, Ethiopia**.** Cultures of bacteria were grown overnight at 28°C in Nutrient Broth (Oxoid Ltd) from a single colony of the bacteria grown on nutrient agar from frozen stocks.

Extraction and purification of DNA

Whole insect guts were dissected rapidly in sterile saline (Gillespie et al. 2000) and suspended in homogenisation buffer with lysing matrix. Homogenisation was done using a bead beater (Fastprep instrument, MP Biomedicals) for 40 s. DNA extraction was carried out using the FastDNA Spin Kit for soil (MP Biomedicals) according to the manufacturer's instructions with the exception that material was centrifuged for 8 min at 14,000 g following cell lysis before transferring the supernatant to a clean tube with protein precipitation solution (Webster et al. 2003). DNA was stored in a 50 µl aliquot of DNase/pyrogen free water at −70°C. In addition, DNA template was also prepared from pure bacterial cultures as described previously (McCaig et al. 1994). Essentially, 1 ml of log phase culture was centrifuged for 4 min at 14,000×g. Supernatant was discarded and the cell

pellet was re-suspended in 100 µl of 5% (w/v) Chelex 100 (Sigma–Aldrich). The suspension was then heated at 100°C for 5 min prior to placing in ice for 5 min followed by a further heating and cooling step. The crude DNA lysate was then centrifuged as above and used directly for PCR amplification.

PCR amplification of 16S rRNA genes

Amplification of the 16S rRNA genes of *Bacteria* were performed using PCR-DGGE primers 357FGC-518R (Muyzer et al. 1993). Amplifications were carried out with 4 pmol ul^{-1} primers, 1 µl of DNA template, 1× reaction buffer (Promega), 1.5 mM $MgCl_2$, 1.5U Taq DNA polymerase (Promega), 0.25 mM each dNTP in a 50 µl PCR reaction mixture with molecular grade water. Positive (pure culture bacterial DNA) and negative controls (water) were routinely included. PCR conditions were 95°C for 5 min, 10 cycles of 94°C/30 s; 55°C/30 s; 72°C/60 s, 25 cycles of 92°C/30 s; 52°C/30 s; 72°C/60 s, followed by 10 min at 72°C. All PCR reactions were conducted with a PTC-100 thermocycler (M.J.Research Ltd).

DGGE analysis of PCR amplified products

DGGE was conducted according to previously described methods (Webster et al. 2002; Dillon et al. 2008). DGGE marker was prepared from a selection of bacterial 16S rRNA gene products to enable gel to gel comparison. PCR products were separated (ca 200 ng of each product) using a combined polyacrylamide and denaturant gradient between 6% acrylamide/30% denaturant and 12% acrylamide/60% denaturant. A 100% denaturing condition is equivalent to 7 M urea and 40% (v/v) formamide. Gels were poured with the aid of a 50 ml gradient mixer (Fisher Scientific,) and electrophoresis run at 200 V for 5 h at 60°C. Polyacrylamide gels were stained with SYBRGold nucleic acid gel stain (Molecular Probes) for 30 min and viewed under UV.

Sequencing and analysis of excised DGGE bands

Bands were excised with sterile razor blades immediately after staining and visualisation of the gels. Gel bands were stored at −70°C, washed with 100 µl distilled water and DNA extracted with 10–20 µl of water depending on band intensity. DNA was re-amplified using the PCR-DGGE primers and products checked by agarose gel electrophoresis. The PCR products were purified using the Qia Quick 96 well PCR purification kit with the QIA Vac96 system (Qiagen Ltd). The products were directly sequenced with the 518R primer using an AB1377 Automated Sequencer (Applied Biosystems). Partial bacterial 16S rRNA gene sequences were subjected to a NCBI nucleotide blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify sequences of the highest similarity. Bacterial sequences obtained during this study were deposited at EMBL as accession numbers AM039786-AM039799 and AM050722-8.

Results

Establishing DGGE as a method for profiling the gut bacterial community of locusts

Preliminary experiments were undertaken to establish the validity and consistency of the DGGE technique when applied to the bacterial community of a locust gut. Firstly, germ-free locusts that had been mono-associated with *Pantoea agglomerans* strain Sga40 isolated from an Ethiopian desert locust (Fig. 1a) were investigated. DGGE analysis repeatedly revealed a single band with 100% sequence identity to DGGE band sequences derived from pure cultures of the same bacterial isolate and to *Pantoea agglomerans* 16S rRNA gene sequences in the database (accession number AY935243). Additionally, a band with a 100% sequence similarity to an insect 18S rRNA gene was also found, and in some instances PCR artefacts were identified (arrowed; Fig. 1a). Heteroduplex formation resulting in artefactual bands has been noted by others (Amann et al. 1995) and such spurious bands were readily identified by sequence analysis. Unless nucleotide sequence information indicated the presence of a "readable sequence", multiple and spurious bands at the bottom of gel lanes, irrespective of treatment were disregarded and removed from analysis. The second DGGE optimisation experiment was a comparison of the gut microbiota from a house cricket, *Acheta domesticus*, with that of a desert locust (*S. gregaria*). Previous work has shown a diverse microbiota in *Acheta* (Kaufman et al. 2000), and up to 20 bands were present on the corresponding DGGE gels in our

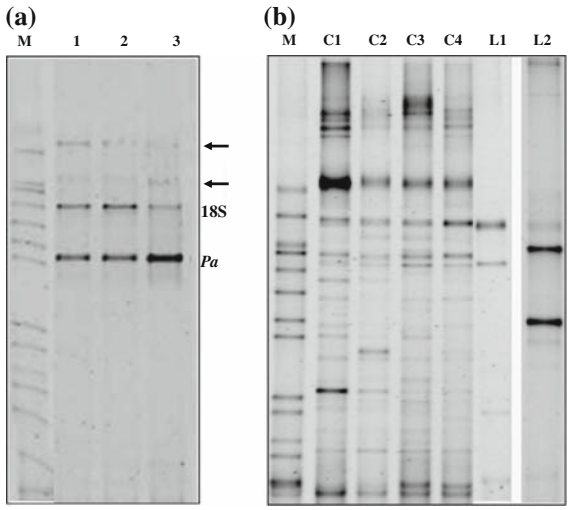

Fig. 1 **a** DGGE analysis of bacterial 16S rRNA genes from the guts of germ-free locust (*Schistocerca gregaria*) inoculated with *Pantoea agglomerans* strain Sga40. *Lanes* labelled 1–3, replicate locust guts; M, DGGE marker. *Bands* labelled Pa, *Pantoea agglomerans*; 18S, insect 18S rRNA genes; *arrows*, PCR artefacts as confirmed by sequencing. **b** DGGE analysis of bacterial 16S rRNA genes derived from the guts of house cricket (*Acheta domesticus*) and the desert locust (*Schistocerca gregaria*). *Lanes* labelled M, DGGE marker; C1–C4, replicate house crickets; L1, wheat- fed locust from Bath laboratory colony; L2, Grass fed locust from a laboratory colony in Addis Ababa, Ethiopia

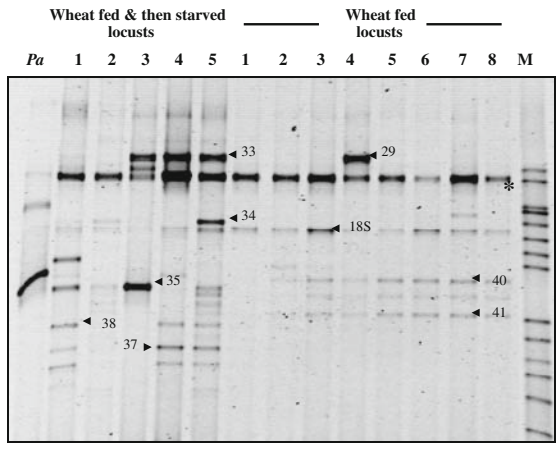

Fig. 2 Effect of nutritional status on the gut bacterial community of adult locusts fed on wheat, assessed by DGGE analysis of bacterial 16S rRNA genes in continuously fed and starved individuals. *Labelled bands* were excised and sequenced (see Table 1). *Bands* labelled *, similarity to *Photorhabdus sp.*, see bands 2, 22 and 79; 18S, high similarity to insect 18S rRNA genes, see bands 3, 26 and 81

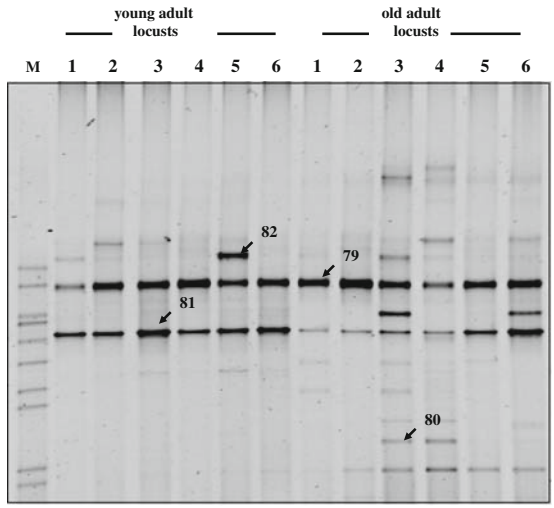

Fig. 3 Effect of age on the gut bacterial community of adult locusts. DGGE analysis of bacterial 16S rRNA genes in young and old adult locusts fed on barley. *Labelled bands* were excised and sequenced (see Table 1)

experiment. In contrast there were only 5–6 bands in our laboratory-reared desert locusts from Addis Ababa (Fig. 1b). It should be noted that for consistency in our experiments unless sequence information suggested otherwise we assumed that each band identified by DGGE corresponded to a different bacterial phylotype (Simpson et al. 2002).

The effect of feeding on locust gut bacterial community structure

Starved locusts (fed initially on wheat) had significantly more bacterial 16S rRNA gene bands (8.4 ± 2.3, $N = 5$) on DGGE gels than wheat fed controls (3.9 ± 1.2, $N = 8$) ($P < 0.001$, ANOVA) (Fig. 2), suggesting an increase in bacterial diversity.

The effect of locust age on gut bacterial community structure

Replicate DGGE experiments to compare the gut bacterial 16S rRNA gene profiles of young (7 d) and old (>28 d) adult locusts fed on barley (see Fig. 3 for example) were undertaken. DGGE profiles from older locusts had significantly more bands (6.4 ± 2.9, $N = 11$) than young insects (3.4 ± 1.5, $N = 9$) ($P < 0.014$, ANOVA), suggesting an increase in bacterial diversity with age.

Sequence analysis of excised DGGE bands

Representative bands excised from bacterial 16S rRNA gene DGGE profiles from locusts of different treatments (fed, starved, grass-fed, wheat-fed, barley-fed, young and old) were excised and sequenced (Figs. 2 and 3; Table 1). Additional bands were also taken from gels not displayed in this report.

Analysis of 16S rRNA gene sequences using the NCBI BLASTN search tool (http://www.ncbi.nlm.nih.gov/) revealed that most sequences derived from excised DGGE bands were similar to bacterial sequences previously isolated from *S. gregaria* (Hunt and Charnley 1981; Dillon et al. 2002). For example, bands 33 and 35 were similar (95–96% sequence similarity) to *Enterococcus* and *Klebsiella sp*ecies respectively and bands 4 and 5 had 98% sequence similarity to *Serratia* species (Table 1). However, since the sequence data from excised DGGE bands reported here is partial and the 150 bp sequence match should be treated with some caution and used as a guide. Although, it should be noted that the region of sequence used for analysis includes the variable V3 region of the bacterial 16S rRNA gene, which has previously been shown to be an excellent indicator of phylogeny e.g. Jensen et al. 2004; McCaig et al. 2001). In addition, since most sequences found in this study correspond to bacteria previously isolated by cultural methods this also gives confidence that the sequence identities are reliable (Hunt and Charnley 1981; Dillon et al. 2002). In contrast, bands 2, 22 and 79 had sequence similarity (88–90% sequence identity) to *Photorhabdus sp.* (Table 1). A number of other excised bands had a high sequence similarity to insect DNA sequences (18S rRNA gene) and plant chloroplast sequences.

Discussion

The present molecular biological (16S rRNA genes) analysis of the gut bacterial community of locusts shows similarities with previous culture-based studies (Hunt and Charnley 1981; Dillon et al. 2002). In addition, this study also supports the previous findings (Stevenson 1966; Hunt and Charnley 1981) that the locust gut bacterial community comprises of only a few bacterial species. The bacterial phylotypes found in this study from desert locusts fed on grass, barley or wheat, starved, young or old mostly belonged to the *Enterobacteraceae* and *Enterococcaceae*. A comparable analysis of the gut bacterial communities of 4 species of feral locusts and grasshoppers using DGGE with bacterial 16S rRNA gene fragments also suggest a simple microbiota dominated by *Gammaproteobacteria* from the family *Enterobacteriaceae* (Dillon et al. 2008).

Gut microbial communities containing relatively few species have been reported for honeybees (Babendreier et al. 2007), ground beetles (Lehman et al. 2009), aphids (Haynes et al. 2003) and gypsy moths (Broderick et al. 2004). This contrasts with the situation in termites (Okhuma and Kudo 1996), and scarabid beetles (Egert et al. 2003).

A potentially novel bacterial phylotype identified by DGGE profiles from several experimental locusts was found to give 88–90% similarity to 16S rRNA gene sequences from the *Photorhabdus* genus of bacteria that are associated with insect pathogenic nematodes (Munch et al. 2008). These sequences may be derived from bacteria that belong to a *Photorhabdus*-related member of the *Gammaproteobacteria* that is peculiar to the locust gut.

Amongst the Enterococci identified, *E. casseliflavus* (Broderick et al. 2006; Aarestrup et al. 2002; Müller et al. 2001) and *E. sulfureus* (Miller and Miller 1996; Müller et al. 2001) have been isolated from both plants and insect guts. *Acinetobacter* sp has also been found previous among insect gut microbiota (Indiragandhi et al. 2007; Murrell et al. 2003). *Serratia* spp are commonly associated with insects (e.g. Indiragandhi et al. 2007; Broderick et al. 2006).

It is well documented that PCR based methods of 16S rRNA gene analyses may not reveal the full extent of diversity within an environment (Amann et al. 1995) and this is probably true for the locust gut. It is possible that locusts have additional bacterial taxa in their gut that yield poor or no 16S rRNA gene products with the PCR primers used and/or have too low a template abundance (Von Wintzergerode et al. 1997). It is also evident from extensive sequencing of the DGGE bands that some of the sequences identified by DGGE can be attributed to non-bacterial sources (e.g. insect and plant DNA), probably due to the low concentration of bacterial template compared to the large concentration of non-target DNA. Similar findings were reported by Normander and Prosser (2000) using

Table 1 Closest nucleotide sequence matches to excised DGGE bands

DGGE band number	Age, nutritional status of locusts	Closest sequence match (accession number)***	% Sequence similarity (length (bp))
20	Old, grass-fed	*Enterococcus sulfureus*	99
		(AJ301841)	(175)
21		*Zea mays*, chloroplast	97
		(X86563)	(94)
22		*Photorhabdus luminescens*	89
		(EU930333)	(105)
23		*Klebsiella pneumoniae*	98
		(AP006725)	(154)
24		*Klebsiella pneumoniae*	98
		(AP006725)	(154)
28		*Enterococcus faecalis*	96
		(GQ337884)	(145)
39		*Klebsiella* sp.	99
		(FJ999767)	(142)
14	Young, grass-fed	*Zea mays* chloroplast	95
		(X86563)	(128)
26	Young, grass-starved*	Insect 18S rRNA	100
		(ABU65116)	(95)
2		*Photorhabdus luminescens*	88
		(EU930333)	(105)
3		Insect 18S rRNA	100
		(ABU65116)	(95)
4		*Serratia marcescens*	98
		(FM213393)	(172)
5		*Serratia marcescens*	98
		(GQ351502)	(170)
12		*Klebsiella pneumoniae*	98
		(GQ214541)	(176)
29	Young, wheat-fed	*Enterococcus casseliflavus*	98
		(GQ337020)	(164)
40		*Serratia marcescens*	96
		(EU660208)	(171)
41		*Serratia marcescens*	98
		(AY616169)	(166)
33	Young, wheat-starved**	*Enterococcus casseliflavus*	95
		(FJ357239)	(155)
34		*Acinetobacter soli*	98
		(FJ976568)	(120)
35		*Klebsiella pneumoniae*	97
		(EU913821)	(133)
37		*Klebsiella pneumoniae*	97
		(AP006725)	(177)
38		*Klebsiella* sp.	95
		(FJ711774)	(148)

Table 1 continued

DGGE band number	Age, nutritional status of locusts	Closest sequence match (accession number)***	% Sequence similarity (length (bp))
82	Young, barley-fed	*Enterococcus casseliflavus*	99
		(GQ337020)	(175)
81		Insect 18S rRNA	100
		(AF423803)	(123)
79	Old, barley-fed	*Photorhabdus luminescens*	90
		(EU930333)	(105)
80		*Klebsiella pneumoniae*	96
		(AP006725)	(171)

Band number corresponds to bands labelled on Figs. 2 and 3 and from additional gels not shown

* Fed grass prior to starvation

** Fed wheat prior to starvation, Young = 7d, Old = >28d

*** Closest sequence match determined by NCBI nucleotide blast search (blastn; http://blast.ncbi.nlm.nih.gov/Blast.cgi)

the same PCR-DGGE methodology to look at the bacteria community in the barley phytosphere. This may highlight a potential pitfall in the interpretation of DGGE profiles, however, as these sequences were sufficiently different from bacterial 16S rRNA genes they were easily identified and eliminated by sequencing.

The results of the present work suggest that there is no characteristic gut bacterial community associated with the desert locust though diversity increased with age. Similarly the DGGE profile of larvae of the wasp *Vespula germanica* were not consistent between individuals of the same or different nests (Reeson et al. 2003). In contrast the majority of isolates from gypsy moths were found in >90% of the larvae examined and all were found in >50% of the larvae in any given treatment (Broderick et al. 2004). Two 16S rRNA gene sequences, *Enterococcus faecalis* and an uncultured *Enterobacter* sp were found in all larvae, regardless of treatment.

At present there is no evidence that the desert locust gut microbiota make a significant contribution to host nutrition, at least under optimal conditions (Charnley et al. 1985). A similar situation pertains in *A. domesticus*, but in this insect it is known that the gut microbiota make an impact during periods of nutritional deficiency (Domingo et al. 1988). An increase in microbial diversity in starved locusts could improve the likelihood of a bacterial contribution to host nutrition. Starved insects are potentially more prone to disease but the increased diversity of *Gammaproteobacteria* in starved locusts shown here would improve insect host defence against enteric threats because of the role of gut bacteria in colonization resistance (Dillon et al. 2005). Increased bacterial diversity in starved locusts may be due to reduced gut peristalsis; in fed insects food through put may reduce bacterial colonization.

The gut of newly fledged adult locust has a limited microbiota but within 4–7 days it acquires an extensive microbiota (authors, unpublished results). Purging of the gut and shedding of the cuticular lining of the fore- and hind gut at the last larval moult presumably accounts for the sparse biota in young adults. A similar situation occurs also in house flies (Greenberg 1959). In both cases recolonization of the gut is *via* the food. DGGE analysis revealed an increase in bacterial diversity with locust age (7d–28d). Our recent study (Dillon et al. 2005) suggests this may improve colonization resistance critically during the onset of reproduction. We associated germ-free locusts 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 gut bacterial species present. Likewise there was a significant inverse relationship between community diversity and the proportion of locusts that harboured *Serratia* (Dillon et al. 2005). The results presented in this study provide additional data indicating that the changes in the diversity

profile of the gut microbiota in locusts may benefit the host in terms of colonization resistance against pathogens.

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