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Mutualistic ants as an indirect defence against leaf pathogens

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Summary

• Mutualistic ants are commonly considered as an efficient indirect defence against herbivores. Nevertheless, their indirect protective role against plant pathogens has been scarcely investigated.

• We compared the protective role against pathogens of two different ant partners, a mutualistic and a parasitic ant, on the host plant *Acacia hindsii* (Fabaceae). The epiphytic bacterial community on leaves was evaluated in the presence and absence of both ant partners by cultivation and by 454 pyrosequencing of the 16S rRNA gene.

• Pathogen-inflicted leaf damage, epiphytic bacterial abundance (colony-forming units) and number of operational taxonomic units (OTUs) were significantly higher in plants inhabited by parasitic ants than in plants inhabited by mutualistic ants. Unifrac unweighted and weighted principal component analyses showed that the bacterial community composition on leaves changed significantly when mutualistic ants were removed from plants or when plants were inhabited by parasitic ants. Direct mechanisms provided by ant-associated bacteria would contribute to the protective role against pathogens.

• The results suggest that the indirect defence of mutualistic ants also covers the protection from bacterial plant pathogens. Our findings highlight the importance of considering bacterial partners in ant–plant defensive mutualisms, which can contribute significantly to ant-mediated protection from plant pathogens.

Introduction

Plants have developed sophisticated strategies of resistance to deal with attacks from herbivores and pathogens. Direct defence is conferred by plant products and structures that deter or kill enemies, whereas indirect defence occurs when plants attract, nourish or house carnivorous organisms, the third trophic level, which can serve to reduce herbivore attack (Heil, 2008). Plant resistance to pathogens is mainly through direct mechanisms of defence, which include cell wall reinforcement (Schmelzer, 2002), production of reactive oxygen species (Lamb & Dixon, 1997), phytoalexin generation (Ahuja *et al.*, 2012) and pathogenesis-related protein accumulation (van Loon *et al.*, 2006). However, there is still very little information on whether the attraction of the third trophic level to plants, besides its defensive function against herbivores, can also confer protection against phytopathogens.

In defensive plant–ant mutualistic interactions, plants provide several different rewards such as food bodies, extrafloral nectar (EFN) and nesting space, while the ants attracted by these rewards in turn serve as an efficient indirect defence (Heil & McKey, 2003) and provide protection from herbivory (Davidson & McKey, 1993; Fonseca, 1994; Federle *et al.*, 1998), pruning of neighbouring plants (Davidson & McKey, 1993; Federle *et al.*, 1998) or microorganisms (Letourneau, 1998; Heil *et al.*, 2002; González-Teuber & Heil, 2010). Earlier studies found that certain ant plants produce PR proteins in insufficient amounts to protect themselves from pathogens (Heil *et al.*, 1999, 2000). Thus, these ant plants might depend on their ant colonies for this defensive function. Concordantly, previous studies showed that the presence of mutualistic ants reduced the abundance of fungal spores on host plant leaves (de la Fuente & Marquis, 1999; Heil *et al.*, 2002). *Macaranga* myrmecophyte plants could be infected with fungi when *Crematogaster* mutualistic ants were absent, but not in the presence of the ants (Heil *et al.*, 2002). The protection by ants against bacterial plant pathogens has not been tested before, and so far no molecular studies have surveyed the changes in the epiphytic bacterial community in the presence and absence of mutualistic ants. Epiphytic bacterial communities are diverse (Yang *et al.*, 2001) and probably shaped by several biotic and abiotic factors (Ercolani, 1991; Kadivar & Stapleton, 2003; Suda *et al.*, 2009). Some epiphytic bacterial taxa can act as pathogens, and their presence and abundance are correlated with the disease severity in plants (Rouse *et al.*, 1985; Stromberg *et al.*, 1999).

If ants indeed defend the plant against bacterial pathogens, is this protective service only provided by mutualistic ants? Plant–ant mutualisms are prone to exploitation (Bronstein, 1998) by nonmutualistic ant species that garner the rewards without providing any defensive service in return (Bronstein, 2001).

Furthermore, different ant species differ significantly in the defence efficiency provided to the host (Ness *et al.*, 2006; Miller, 2007; Chamberlain & Holland, 2009), and some studies have reported that certain ant species cannot effectively reduce herbivore damage or eggs and larvae of phytophagous insects (de la Fuente & Marquis, 1999; Raine *et al.*, 2004), causing negative effects on plant performance (Clement *et al.*, 2008).

For the present study, we used the Mesoamerican species *Acacia hindsii*. This obligate myrmecophyte plant species secretes EFN constitutively (Heil *et al.*, 2004) to nourish mutualistic ant colonies of *Pseudomyrmex ferrugineus*. Ants of *P. ferrugineus* permanently patrol the leaf surfaces and defend them aggressively against natural enemies. Nevertheless, several ant species have been observed to exploit this system, such as the nondefending parasitic ant species of *Pseudomyrmex nigropilosus* (Janzen, 1975) and *Pseudomyrmex gracilis* (Clement *et al.*, 2008; Kautz *et al.*, 2009). The different kinds of interactions that *Acacia* establishes with multiple ant partners render this genus highly suitable for a comparative study of the protective service by different ant partners.

The goal of this study was to compare the epiphytic bacterial community in the presence and absence of both ant partners by 454 pyrosequencing of the 16S rRNA gene, and to address the following questions. Do mutualistic ants of *P. ferrugineus* protect the host plant against microorganisms? If so, what is the mechanistic basis of this protective effect? Does the protection against pathogens differ when plants are inhabited by parasitic ants? In order to elucidate putative mechanisms associated with this protection effect from pathogens, we analysed 'indirect mechanisms' provided by ants (through a possible induction of the resistance state in the host plant) as well as 'direct mechanisms' (through a direct protection provided by ants).

Materials and Methods

Study system and field experiments

This study was conducted using the myrmecophyte plant *Acacia hindsii* (Fabaceae). This plant species was determined following Janzen (1974) and Seigler & Ebinger (1995), and by comparison with specimens held at the Herbario MEXU at the Universidad Autónoma de México in Mexico City. All plants used for the experiment grew at their natural sites in the coastal area of the state of Oaxaca, Mexico, 5 km northwest of Puerto Escondido (Pacific coast; *c.* 15°55′N and 97°09′W; elevation, 15 m). *A. hindsii* myrmecophyte plants are inhabited by specialised ants of the genus *Pseudomyrmex* during major parts of their life and the ants are entirely dependent on the food rewards and nesting space provided by their host. *Acacia* plants house their ant colonies with swollen thorns (domatia) and nourish them with EFN and food bodies. Mutualistic ants of the species *P. ferrugineus* effectively protect their host from herbivores and encroaching vegetation (Janzen, 1966, 1974), and they cannot be found nesting outside of the host plant. The parasite ant *P. gracilis* is considered a generalist, twig- nesting ant, but it has been reported to colonize thorns of myrmecophyte *Acacia* spp. (Skwarra, 1935; Ward, 1993; Clement *et al.*, 2008). A molecular phylogeny of the *Pseudomyrmex* genus showed that *P. gracilis* did not evolve from former mutualists, and no evidence for cheaters was found (Kautz *et al.*, 2009).

To determine the possible protective effects of both mutualistic and parasitic ants against leaf surface bacteria, ant exclusion experiments were carried out in 10 plants of *A. hindsii* naturally inhabited by mutualistic ants and 10 plants of *A. hindsii* inhabited by the parasitic ant between March and May 2010. Ants of *P. ferrugineus* and *P. gracilis* were excluded for 6 wk from one branch of each of the individual plants by cutting off the inhabited thorns and mechanically removing ants. A second branch – which was the most similar to the ant exclusion branch with respect to its position within the plant and the number and quality of leaves – was selected as a control. Thorns were also removed from control branches in order to avoid potential damaging effects of cut thorns on plant leaves. Thus, the following four treatments were obtained: mutualistic ant presence (M+), mutualistic ant absence (M−), parasitic ant presence (P+), and parasitic ant absence (P−). After 6 wk, leaf samples coming from the different treatments were collected. For further enzyme activity and Chl analysis, leaf material was immediately frozen and stored at −80°C until laboratory analysis. In the laboratory, frozen leaf material was ground in an ice-chilled mortar with liquid nitrogen, and the resulting powder was kept frozen until further analysis.

Determination of leaf damage caused by pathogens

To determine natural leaf damage by pathogens in plants of *A. hindsii,* one representative leaf was collected per individual plant ($n = 6$) for the four different treatments (M+, M−, P+ and P−). Leaf damage was determined by counting leaflets with visible disease symptoms by pathogens in relation to the total number of leaflets per leaf. Damage by pathogens was visible as necrotic areas and leaf spots. The percentage of leaf damage by pathogens was arcsine-transformed before analysis.

Characterization of bacterial leaf abundance by culture-dependent techniques

Three leaflets coming from different plants of *A. hindsii* in the presence and absence of symbiotic and parasitic ants were placed in a 2 ml Eppendorf tube and filled with phosphate-buffered saline (PBS), pH 7. Samples were shaken for 24 h at room temperature to dislodge epiphytic microorganisms. For epiphytic microorganism determination, 30 µl of different dilutions (1 : 10, 1 : 100 and 1 : 1000) from each plant were cultivated in Luria-Bertani (LB) and potato dextrose agar media for further determination of bacteria and fungi, respectively. Plates were stored at 37°C for 48 h. Subsequently, colony-forming units (CFUs) cm^2 leaf $area^{-1}$ were counted in order to assess bacterial and fungal abundance. As we only found significant differences for the bacterial, but not fungal, abundance across the four treatments (see the Results section), further molecular analyses were performed only on epiphytic bacteria.

The rest of the PBS buffer was used for bacterial DNA extraction and 454 pyrosequencing analyses. Three replicates were analysed for each treatment. DNA extraction was done with QuickGene DNA tissue kit S (Fujifilm, Tokyo, Japan). DNA extractions started with 500 µl of PBS buffer. The partial 16S rDNA (1.5 kbp) was amplified by PCR using the primers 27F and 1492R. PCR analyses were carried out in 50 µl final volume containing 2 µl of DNA template, 0.5 µl of each primer (at a concentration of 100 µM for each primer), 2.5 µl deoxynucleoside triphosphate (at a concentration of 25 µM for each nucleotide; Bioline GmbH, Luckenwalde, Germany), 1.5 µl $MgCl_2$ (50 mM), 5 µl of 10× PCR buffer and 0.3 µl of Taq polymerase (5 U μl^{-1}). PCR was performed in a GeneAmp 9700 Thermal Cycler (Applied Biosystems Deutschland GmbH, Darmstadt, Germany) with the following programme: 4 min denaturation at 94°C, followed by 35 cycles at 94°C for 45 s, 55°C for 30 s and 72°C for 90 s. The final extension step was 10 min at 72°C. Amplified DNA was separated by electrophoresis in 1× TAE (50×: 242 g Tris, 57.1 ml acetic acid, 14.6 g EDTA, add water to 1 l) 0.8% w/v agarose gel.

Bacterial tag-encoded FLX amplicon pyroseqencing (bTEFAP) and data analysis

DNA samples were sent to an external service provider (Research & Testing Laboratories, Lubbock, TX, USA) for bTEFAP with 16S rRNA primers Gray28F and Gray519R (Ishak *et al.*, 2011; Sun *et al.*, 2011). A sequencing library was generated through one-step PCR with 30 cycles, using a mixture of HotStar and HotStar HiFidelity *Taq* polymerases (Qiagen). Sequencing extended from Gray28F, using a Roche 454 FLX instrument with Titanium reagents and procedures at the Research and Testing Laboratory (RTL, http://www.medicalbiofilm.org/). All low-quality reads (quality threshold = 25) and sequences < 200 bp were removed following sequencing, which left 263 462 sequences for analysis (mean ± SD = 20 266 ± 5735 per sample). The raw reads (sff files) were deposited in the sequence read archive (SRA) of the National Center for Biotechnology Information (NCBI; BioProject, SRP028563; BioSamples, SRS467661 + SRS467663-SRS467674; BioExperiments, SRX332215 + SRX332218-SRX332229). Analysis of the high-quality reads was conducted using QIIME (Caporaso *et al.*, 2010b). Cd-hit (Li & Godzik, 2006) and Uclust (Edgar, 2010) with 97% similarity thresholds were sequentially employed in a two-step operational taxonomic unit (OTU) picking strategy to cluster the sequences into OTUs. For each OTU, the most abundant sequence was chosen as representative sequence and aligned to the Greengenes core set (available from http://greengenes.lbl.gov/) using PyNast (Caporaso *et al.*, 2010a). RDP classifier was used for taxonomy assignment (Wang *et al.*, 2007), with a minimum confidence of 0.8 to record an assignment. An OTU table was generated describing the occurrence of bacterial phylotypes within the samples.

Species richness was defined as the number of OTUs (number of OTUs was resampled to 12 302 sequences, the smallest dataset) present in each sample. α-Diversity estimators, the Shannon and Simpson indices, were calculated using QIIME (Caporaso *et al.*, 2010b). The relative abundance (percentage of total bacterial sequences) of the most representative bacterial families recorded on leaf surfaces (Lindow & Brandl, 2003) – Microbacteriaceae, Enterobacteriaceae, Pseudomonadaceae and Xanthomonadaceae – was also determined for each treatment group (M+, M−, P+ and P−). To assess differences in microbial community profiles across samples, principle coordinates were extracted based on the OTU table, using weighted and unweighted UniFrac metrics. Rarefaction curves (Supporting Information, Fig. S1) were obtained in QIIME by subsampling the OTU table with step increments of 1000 sequences and 100 iterations at each step.

Plant resistance traits

A possible mechanism by which mutualistic ants might achieve protection of the host plant against pathogens is to induce the resistance state in the host plant, that is, by an 'indirect mechanism'. The resistance state of host plants inhabited by either of the two ant partners was determined by the analysis of salicylic acid (SA) and PR-enzyme activities such as superoxide dismutases (SODs), peroxidases, chitinases and glucanases in leaf samples of the four treatment groups (M+, M−, P+, P−).

The analysis of SA was carried out according to Schulze *et al.* (2006) ($n = 5$ per treatment). SOD activity was determined spectrophotometrically at 450 nm using the commercially available SOD determination kit 19160 by Fluka (St Louis, MO, USA; $n = 5$ per treatment). For peroxidase quantification, a total volume of 197 µl of reaction solution contained 5 µl of protein extract, 0.83 µl H_2O_2 (30%), 1 µl guaiacol (99%) and 190 µl 50 mM Na-phosphate buffer at pH 6.0. The oxidation of the substrate was measured spectrophotometrically (Smax 190 PC) at 470 nm, as described previously (Hammerschmidt *et al.*, 1982; $n = 5$ per treatment). Chitinase and β-1,3-glucanase activities were determined following the procedure described by de Roman *et al.* (2011) ($n = 5$ and 6 per treatment, respectively).

Plant performance: Chl determination

Pathogen attacks result in a decreased rate of photosynthesis, and as a consequence yield loss (Berger *et al.*, 2007). To determine how photosynthesis was affected by differences in pathogen load in response to the presence/absence of mutualistic or parasitic ants, we measured Chl as a proxy to plant photosynthesis. Chl was measured by a modification of Arnon's method (Arnon, 1949). One hundred milligrams of powdered leaf material from each treatment group ($n = 7$ for plants inhabited with mutualistic ants, $n = 10$ for plants inhabited by parasitic ants) was extracted with 80% acetone. This procedure was performed under dark conditions to prevent Chl degradation. Readings were taken at wavelengths 664 and 647 using a spectrophotometer. The contents of Chl*a* and *b* were determined from the readings using the following equations derived from Porra *et al.* (1989): $Chla = 13.71 \times Abs664\ nm - 2.85 \times Abs647\ nm$; and $Chlb = 22.39 \times Abs647\ nm - 5.42 \times Abs664\ nm$. The concentration of pigments was expressed in nm ml^{-1}.

Statistical analysis

A mixed model analysis was used to determine the effects of the ant species and the presence/absence of ants on leaf damage by pathogens, bacteria abundance (CFU cm^2 leaf $area^{-1}$), number of OTUs, relative abundance of bacterial families, SA, enzyme activities and Chl content. Ant species and the absence of ants were considered as fixed effects, whereas the branches (presence/absence of ants) were nested on the individual plants. The relative abundance of the bacterial family's data (%) was arcsine-transformed before analysis. The model was fitted by restricted maximum likelihood (REML). All the analyses were done with SPSS Statistics 17.0 (Chicago, IL, USA).

Extracts of ant legs and inhibitory assays

As arboreal ants are permanently patrolling on the leaves of their host plants, legs are the structures that are permanently in contact with the surface of leaves and thus might be involved in the protection from pathogens. To elucidate a putative protection effect from pathogens provided by ant legs, we prepared methanol extracts of them in the laboratory in Mexico for further inhibitory assays. The legs of 30 ant workers from each ant species were cut with a scalpel under sterile conditions and extracted with absolute methanol (500 µl; $n = 3$ for mutualistic and for parasitic ants). After filtration, these crude methanol extracts were used for inhibitory assays against the plant pathogen *Pseudomonas syringae* var. *glycinea*. Inhibitory assays were performed following the agar diffusion method. *P. syringae* was grown in LB media broth for 16 h at 37°C and then inoculated onto LB agar plates (100 µl of culture bacteria for 20 ml of agar plate). Once solidified on the agar plates, 30 µl of each methanol extract (from mutualistic and parasitic ants) was pipetted onto sterile paper discs placed on the surface of the inoculated agar plates. Thirty microlitres of absolute methanol was used as a control. Agar plates were then incubated at 37°C for 24 h. Inhibitory effects of the methanol extracts were quantified as the diameter of clear zones of growth inhibition around each extract drop. The assay was done using three biological replicates for each ant species (each biological replicate represents a different ant colony).

Ant bacteria microbiota determination and inhibitory assays

To explore in more detail the putative protection effect from pathogens provided by ant legs, we isolated bacterial microorganisms associated with the legs of both ant species through the culture method. Legs coming from five ant workers of each ant species, *P. ferrugineus* and *P. gracilis*, were pooled, placed in 1–3 ml LB broth media and grown overnight at 37°C. LB broth-grown cells were then poured onto LB agar plates. Plates were incubated at 37°C until colonies were clearly visible. After that, single colonies were resuspended in 500 µl of sterile water and heated at 99°C for 15 min. The supernatant was used directly for PCR. Amplification of the 16S rRNA gene was carried out using the universal primer 27F and 1492R. A thermocycler with the following program was used: initial denaturation at 94°C for 1 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final extension step at 72°C for 8 min. PCR products were purified with the PCR purification kit Invisorb Fragment Clean Up (Invitek GmbH, Berlin, Germany) and then bidirectionally sequenced. Sequencing was carried out at the Max Planck Institute for Chemical Ecology, Jena, Germany. DNA sequences were cleaned and assembled using the DNASTAR Lasergene software package (DNASTAR Inc. Madison, WI, USA). The initial assembly of the sequences was performed with a 99% threshold. Consensus sequences were used for BLAST searches at the NCBI (http://www.ncbi.nlm.nih.gov). Bacterial 16S rRNA gene sequences have been deposited at the NCBI with accession numbers KF623095–KF623102 for *P. ferrugineus* OTUs and KF623103–KF623107 and KF623093 for *P. gracilis* OTUs.

Bioassays were carried out to evaluate the putative inhibitory effects of ant leg-associated bacteria on *Pseudomonas* sp. (previously isolated from symptomatic *A. hindsii* leaves inhabited with parasitic ants; accession number KF623094), *P. syringae* var. *glycinea* and *Escherichia coli*. The inhibitory assays were performed following the agar diffusion method. Ant bacterial strains were grown in LB media broth for 16 h at 37°C. The pathogenic test bacteria were also grown in LB media broth for 16 h at 37°C and then inoculated onto LB media agar plates (100 µl of each culture bacteria with an OD = 0.6 was used for 20 ml of agar plate). Fifteen microlitres of each ant bacteria culture (OD = 0.6) was deposited on the agar plates. Agar plates were then incubated at 37°C for 24 h. Inhibitory effects of ant bacterial strains were quantified as the diameter of clear zones of growth inhibition around each ant bacterial drop. The diameter of the inhibition zone was measured with callipers (in mm). The experiment was repeated with three independent samples for every plant pathogen.

Results

Leaf damage by pathogens

Foliar damage by pathogens was significantly affected by the ant species ($F_{1,10} = 367.4$, $P = 0.000$, mixed model analysis) as well as by the presence of ants ($F_{1,10} = 46.1$, $P = 0.000$, mixed model analysis). It was significantly higher in plants inhabited by parasitic ants (presence of parasitic ants, 79.5 ± 3.3%; absence of parasitic ants, 83.7 ± 1.6%) than in plants inhabited by mutualistic ants (presence of mutualistic ants, 13.8 ± 1.6%; absence of mutualistic ants, 44.8 ± 3.2%). A significant 'ant species × ant presence' interaction was also found for foliar damage by pathogens ($F_{1,10} = 25.2$, $P = 0.001$, mixed model analysis).

Leaf bacterial abundance

The abundance of cultured bacterial colonies (CFU cm^2 leaf $area^{-1}$) was significantly affected by the ant species ($F_{1,14} = 118.0$, $P = 0.000$, mixed model analysis) as well as by the presence of ants ($F_{1,14} = 64.1$, $P = 0.000$, mixed model analysis). It was higher in plants inhabited by the parasitic ant than in plants inhabited by the mutualistic ant; nevertheless when both

ants were removed from the plants, the abundance of CFU increased significantly (Fig. 1). No significant ant species × ant presence interaction was found for bacteria abundance ($F_{1,14} = 4.11$, $P = 0.062$, mixed model analysis). In contrast to the bacterial abundance, fungal abundance was not significantly affected by the ant species ($F_{1,14} = 1.82$, $P = 0.198$, mixed model analysis), presence of ants ($F_{1,14} = 1.10$, $P = 0.312$, mixed model analysis) or ant species × ant presence interaction ($F_{1,14} = 0.07$, $P = 0.784$, mixed model analysis).

Diversity and comparison of epiphytic bacterial communities

The number of bacterial OTUs was only significantly affected by the ant species ($F_{1,4} = 9.97$, $P = 0.034$, mixed model analysis). It was significantly higher in plants inhabited by parasitic ants than in those inhabited by mutualistic ants (Table 1). No significant effects of the presence of ants ($F_{1,4} = 1.11$, $P = 0.329$, mixed model analysis) and ant species × ant presence interaction were found for number of OTUs ($F_{1,4} = 0.98$, $P = 0.377$, mixed model analysis). The Shannon and Simpson diversity indexes showed different results (Table 1). Whereas with the Shannon index there were significant differences in the epiphytic bacterial diversity between plants inhabited by mutualistic and by parasitic ants (ant species, $F_{1,4} = 22.98$, $P = 0.009$, mixed model analysis; presence of ants, $F_{1,4} = 3.45$, $P = 0.137$; interaction of factors, $F_{1,4} = 0.89$, $P = 0.398$, mixed model analysis), with the Simpson index there were not significant differences among the treatments (ant species, $F_{1,4} = 2.55$, $P = 0.185$; presence of ants, $F_{1,4} = 1.89$, $P = 0.241$; interaction of factors, $F_{1,4} = 0.09$, $P = 0.769$, mixed model analysis).

Table 1 Number of operational taxonomic units (OTUs; mean ± SE) and diversity indices calculated with the 16S rRNA gene libraries for the four groups: mutualistic ant presence (M+), mutualistic ant absence (M−), parasitic ant presence (P+) and parasitic ant absence (P−)

Samples	Number of OTUs	Diversity indices	
		Shannon	Simpson
M+	301 ± 36	4.48	0.89
M−	348 ± 32	4.65	0.86
P+	425 ± 33	5.08	0.91
P−	344 ± 46	4.79	0.89

The unweighted UniFrac principal component analysis (PCA) is a measure of community membership, whereas the weighted UniFrac PCA is a measure of community composition, as this analysis additionally considers bacterial abundances. In the unweighted UniFrac PCA (Fig. 2a), axis 1 and axis 2 contributed

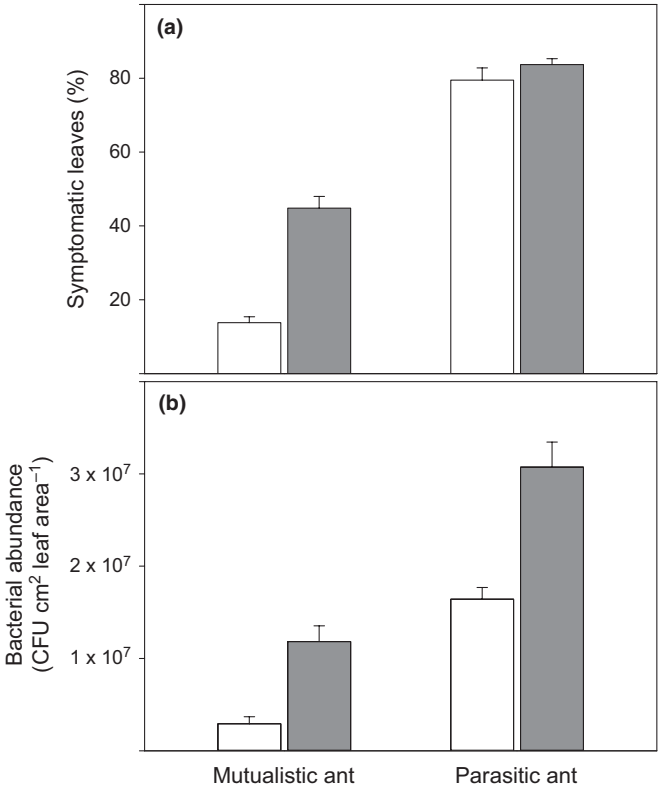

Fig. 1 Effects of the presence/absence of the mutualistic ant (*Pseudomyrmex ferrugineus*) and the parasitic ant (*Pseudomyrmex gracilis*) on the percentage of leaves (mean ± SE) affected by disease symptoms caused by pathogens (a) and on the bacterial abundance (colony-forming units (CFUs) on leaves of *Acacia hindsii* plants) (b) (mixed model analysis). Open bars, ants present; closed bars, ants absent.

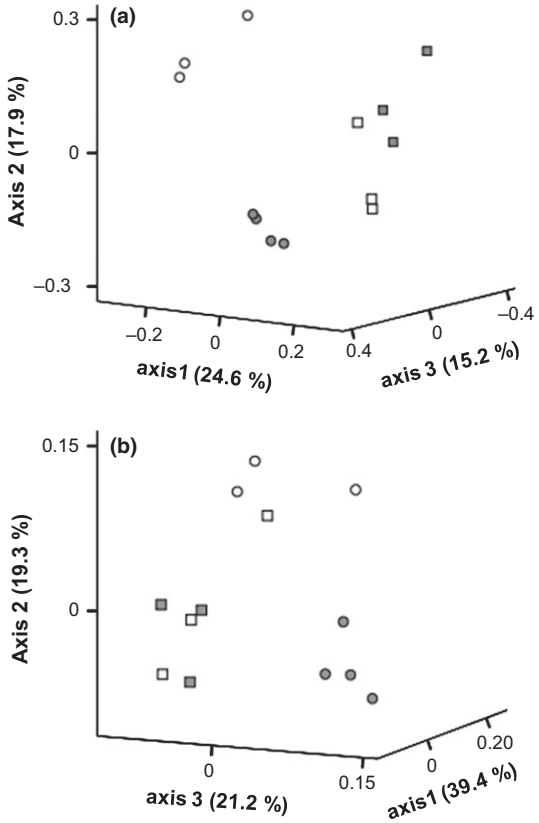

Fig. 2 Principal component analyses based on unweighted (a) and weighted UniFrac distances (b) across the four treatments. White circles, mutualistic ants present; grey circles, mutualistic ants absent; white squares, parasitic ants present; grey squares, parasitic ants absent.

significantly to the variation among the four groups (axis 1, $F_{3,9} = 101.65$, $P = 0.000$; axis 2, $F_{3,9} = 19.64$, $P = 0.000$; axis 3, $F_{3,9} = 0.50$, $P = 0.687$, univariate ANOVA), allowing a grouping among M+ and M− from P+ and P−. While for axis 1, M+ and M− were significantly different from the other groups, P+ and P− did not differ significantly between them (Tukey *post hoc* test). For the weighted UniFrac (Fig. 2b), PCA axes 2 and 3 contributed to the variation among the groups (axis 1, $F_{3,9} = 0.80$, $P = 0.522$; axis 2, $F_{3,9} = 11.94$, $P = 0.001$; axis 3, $F_{3,9} = 4.97$, $P = 0.026$, univariate ANOVA). For axis 2, M+ was significantly different from the other three groups, and for axis 3, M− differed significantly from P+ and P−, thus allowing a clear separation in the bacterial community composition between plants inhabited by mutualistic and those inhabited by parasitic ants.

Table S1 shows the BLAST results for the four treatments (M+, M−, P+, P−) according to the closest bacterial genus designation. Regarding the most representative bacterial families for epiphytic surfaces, the relative abundance of the bacterial families Pseudomonadaceae and Enterobacteriaceae was not significantly affected by the ant species (Pseudomonadaceae, $F_{1,4} = 1.20$, $P = 0.746$; Enterobacteriaceae, $F_{1,4} = 1.05$, $P = 0.362$, mixed model analysis), but it was significantly affected by the presence of ants (Pseudomonadaceae, $F_{1,4} = 9.27$, $P = 0.038$; Enterobacteriaceae, $F_{1,4} = 40.63$, $P = 0.003$, mixed model analysis). Whereas the relative abundance of Pseudomonadaceae increased when ants were removed from the plant, the abundance of Enterobacteriaceae decreased in their absence (Fig. 3). No significant ant species × ant presence interaction was found for the relative abundance of Pseudomonadaceae ($F_{1,4} = 1.90$, $P = 0.240$) and Enterobacteriaceae ($F_{1,4} = 7.39$, $P = 0.053$). For the families Xanthomonadaceae and Microbacteriaceae, neither the ant species nor the presence of ants significantly affected their relative abundances (Xanthomonadaceae: ant species, $F_{1,4} = 3.95$, $P = 0.117$; presence of ants, $F_{1,4} = 0.07$, $P = 0.802$; ant species × ant presence interaction, $F_{1,4} = 12.04$, $P = 0.026$, mixed model analysis; Microbacteriaceae: ant species, $F_{1,4} = 3.14$, $P = 0.151$; presence of ants, $F_{1,4} = 0.99$, $P = 0.376$; ant species × ant presence interaction, $F_{1,4} = 0.17$, $P = 0.698$, mixed model analysis).

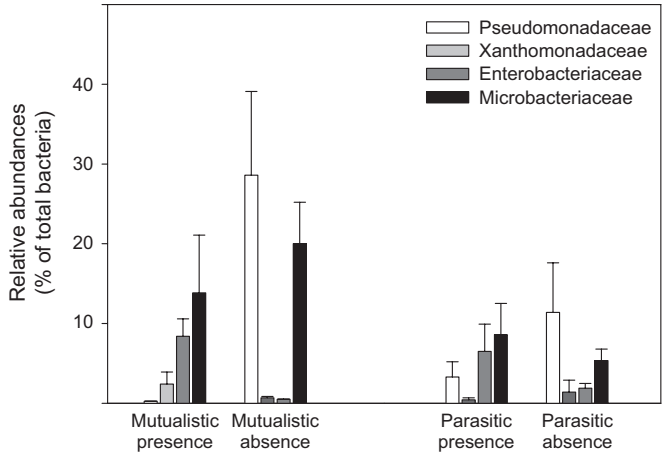

Fig. 3 Effects of the presence/absence of the mutualistic ant (*Pseudomyrmex ferrugineus*) and the parasitic ant (*Pseudomyrmex gracilis*) on the relative abundance (% of total bacterial sequences, mean + SE) of the four most representative bacterial families for epiphytic surfaces in plants of *Acacia hindsii* (mixed model analysis).

Plant resistance traits

The SA concentration was significantly affected by the ant species and by the ant presence (Table 2, Fig. 4). A higher concentration of SA was observed in plants inhabited by parasitic ants than in those inhabited by mutualistic ants and on those branches where ants were removed from the plants. Nevertheless, this ant presence effect was only significant for plants inhabited by mutualistic ants (mutualistic ant, $Z = 2.02$, $P = 0.043$; parasitic ant, $Z = 1.21$, $P = 0.221$, Wilcoxon test for matched pairs; Fig. 4). No significant ant species × ant presence interaction was found for SA concentration (Table 2).

Superoxide dismutase activity was only significantly affected by the ant species (Table 2, Fig. S2a). Its activity was higher in plants inhabited by parasitic ants than in those inhabited by mutualistic ants. Neither the presence of ants nor the interaction 'ant species × ant presence' significantly affected SOD activity.

Peroxidase activity was significantly affected by the ant species and by the presence of ants, as well as by the interaction of both factors (Table 2, Fig. S2b). In plants inhabited by parasitic ants, peroxidase activity was barely detectable, whereas in plants inhabited by the mutualistic ants, there was an induction of its activity on those branches where ants were removed.

Glucanase activity was significantly affected by the ant species, but it was not affected by ant presence (Table 2, Fig. S3a). Glucanases were higher in plants inhabited by parasitic ants than in those inhabited by mutualistic ants. No significant ant species × ant presence interaction was found for glucanase activity.

Chitinase activity was not significantly affected by the ant species, but it was significantly affected by ant presence (Table 2, Fig. S3b). No significant ant species × ant presence interaction was found for chitinase activity.

Table 2 Effects of ant species and the presence of ants on plant defence traits of *Acacia hindsii*

	Salicylic acid	Superoxide dismutase	Peroxidase	Glucanases	Chitinases
Ant species (A)	11.26*	53.41***	8.35*	7.08*	0.59 ns
Ant presence (P)	5.46*	0.04 ns	5.79*	0.13 ns	5.47*
A × P	0.19 ns	0.21 ns	5.78*	0.32 ns	3.00 ns

F- values are shown (df = 1.8 for all variables). Significant differences: *, $P < 0.05$; ***, $P < 0.001$. ns, not significant (mixed model analysis).

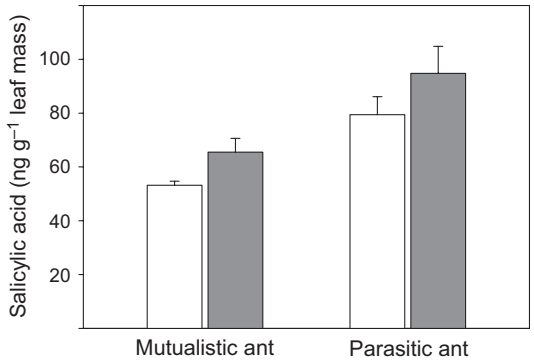

Fig. 4 Effects of the presence/absence of the mutualistic ant (*Pseudomyrmex ferrugineus*) and the parasitic ant (*Pseudomyrmex gracilis*) on the salicylic acid concentration (mean + SE) in plants of *Acacia hindsii* (mixed model analysis). Open bars, ants present; closed bars, ants absent.

Chlorophyll determination

The content of Chl*a* and *b* in plants of *A. hindsii* was significantly affected by the ant species (Chl*a*, $F_{1,15} = 12.92$, $P = 0.003$; Chl*b*, $F_{1,15} = 16.67$, $P = 0.001$, mixed model analysis) and by the presence of ants (Chl*a*, $F_{1,15} = 10.46$, $P = 0.006$; Chl*b*, $F_{1,15} = 4.59$, $P = 0.048$, mixed model analysis). The Chl content decreased significantly in those plants inhabited by parasitic ants and when mutualistic ants were removed from the plant (Fig. 5a,b). No significant ant species × ant presence interaction was found for Chl*a* ($F_{1,15} = 4.47$, $P = 0.052$, mixed model analysis) but a significant interaction was found for Chl*b* ($F_{1,15} = 6.05$, $P = 0.026$, mixed model analysis).

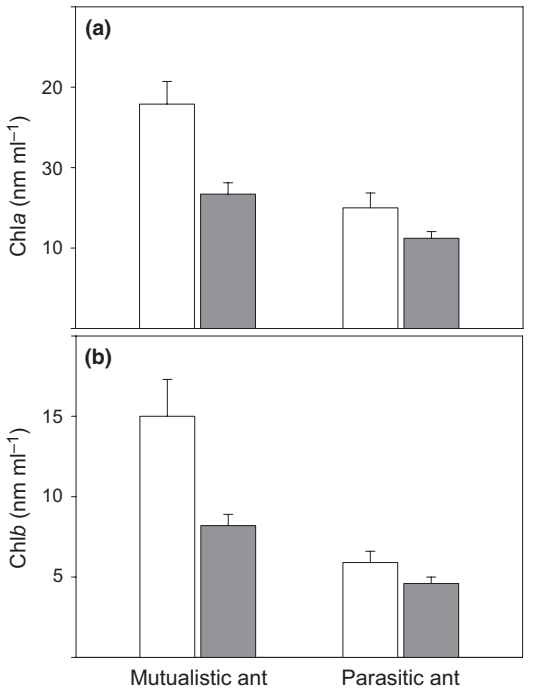

Fig. 5 Effects of the presence/absence of the mutualistic ant (*Pseudomyrmex ferrugineus*) and the parasitic ant (*Pseudomyrmex gracilis*) on Chl*a* content (a) and Chl*b* content (b) (mean + SE) in the host plant *Acacia hindsii* (mixed model analysis). Open bars, ants present; closed bars, ants absent.

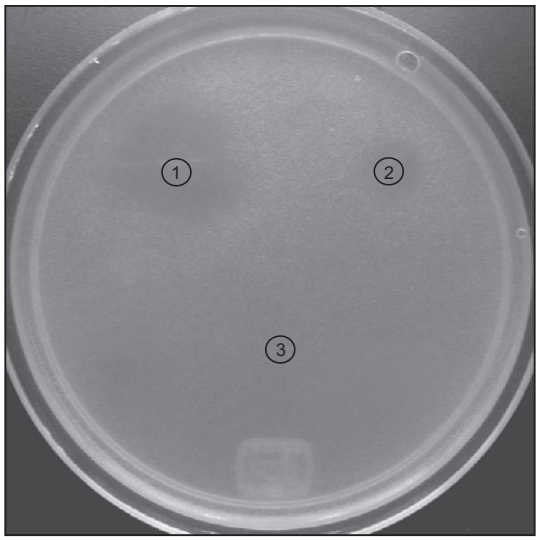

Fig. 6 Antimicrobial effects of methanol extracts of legs from mutualistic ants (*Pseudomyrmex ferrugineus*) (1) and parasitic ants (*Pseudomyrmex gracilis*) (2) tested on the bacterial pathogen *Pseudomonas syringae*. Pure methanol was used as a control (3).

Ant leg bacterial microbiota determination and inhibitory assays

We found that the crude methanol extracts of legs from both ants, mutualistic and parasitic, effectively inhibited the growth of the plant pathogen *P. syringae* (mutualistic ant extract, inhibition zone = 3.6 ± 0.8 mm; parasitic ant extract, inhibition zone = 2.3 ± 0.3 mm; Fig. 6).

We isolated eight and six bacterial strains from legs of mutualistic and parasitic ants, respectively (Table 3), with representatives of the genera *Bacillus*, *Lactococcus*, *Enterococcus*, *Staphylococcus*,

Table 3 Identification of bacterial strains isolated from legs of the mutualistic ant *Pseudomyrmex ferrugineus* and the parasitic ant *Pseudomyrmex gracilis*

Ant species	Identification	Accession number	Identity
P. ferrugineus – 1	*Bacillus* sp.	JF937058.1	98
P. ferrugineus – 2	*Enterobacter* sp.	EU260129.1	99
P. ferrugineus – 3	*Escherichia* sp.	DQ013851.1	99
P. ferrugineus – 4	*Pantoea* sp.	HM222646.1	99
P. ferrugineus – 5	*Burkholderia* sp.	FJ392830.1	99
P. ferrugineus – 6	*Lactococcus lactis*	HM638416.1	99
P. ferrugineus – 7	*Klebsiella* sp. 1	HQ616650.1	99
P. ferrugineus – 8	*Klebsiella* sp. 2	GU301269.1	99
P. gracilis – 1	*Ralstonia* sp.	AY509958.1	99
P. gracilis – 2	*Burkholderia* sp.	JQ791544.1	99
P. gracilis – 3	*Bacillus* sp. 1	JN613469.1	99
P. gracilis – 4	*Bacillus* sp. 2	JX566650.1	99
P. gracilis – 5	*Staphylococcus* sp.	JX291543.1	98
P. gracilis – 6	*Enterococcus mundtii*	AB680486.1	99

Ralstonia, *Burkholderia*, *Enterobacter*, *Escherichia*, *Klebsiella* and *Pantoea*. Several bacteria isolated from the mutualistic ant *(Bacillus* sp., *Pantoea* sp., *Lactococcus lactis* and *Klebsiella)* as well as from the parasitic ant (*Burkholderia* sp., *Bacillus* sp. and *Enterococcus mundtii)* were able to inhibit both *Pseudomonas* strains as well as *E. coli* (Table 4).

Discussion

The aim of this study was to investigate with molecular and cultivation-dependent approaches whether the defensive service by ants to myrmecophyte plants also covers the protection from bacterial pathogens. Here we provide information about the difference in the effectiveness of protection from pathogen bacteria between mutualistic and parasitic ants, as well as the putative mechanisms involved in this ant protection service from pathogens.

Plants of *A. hindsii* inhabited by the parasitic ant of *P. gracilis* showed a higher percentage of pathogen-inflicted leaf damage than plants inhabited by mutualistic ants (Fig. 1a). The latter provides evidence of a direct beneficial effect provided by the mutualistic ants against microbial pathogens. Moreover, leaf bacterial abundance (CFUs; Fig. 1b) as well as the number of bacterial OTUs (bacterial richness) also increased when plants were inhabited by parasitic ants (Table 1). In comparison to plants inhabited by mutualistic ants, plants inhabited by parasitic ants suffered from the attack of 45 new bacterial OTUs. The removal of both mutualistic and parasitic ants increased leaf bacterial abundance in plants; however, this occurs to a different extent depending on the ant species removed (Fig. 1b). Thus, parasitic ants would also be able to reduce epiphytic bacteria, but less effectively than mutualistic ants. The Unifrac unweighted and weighted PCAs showed that the bacterial community composition on plant leaves, considering the community membership as well as their bacterial abundances, changed significantly and predictably when mutualistic ants were removed from the plant or when plants were inhabited by parasitic ants (Fig. 2a,b). The latter strongly suggests that the absence of the right partner from the host plant, the mutualistic ant, led to a significant change in the community composition of bacteria OTUs on leaf surfaces in plants of *A. hindsii*.

Pseudomonas, *Pantoea*, *Brevundimonas* and *Achromobacter* were the dominant bacterial genera detected on the leaf surface of *A. hindsii* plants. Some of these genera, such as *Pantoea* (Enterobacteriaceae) and *Pseudomonas* (Pseudomonadaceae), have previously been found as the main bacterial inhabitants on other leaf surfaces (Lindow & Brandl, 2003; Hunter *et al.*, 2010; Vorholt, 2012). Putative benefits provided by epiphytic microorganisms to the plant are not yet well established (Vorholt, 2012), and although there are some leaf surface bacteria, such as *Sphingomonas*, that can be involved in plant protection against bacterial pathogens (Innerebner *et al.*, 2011), the majority of microorganisms on leaf surfaces appear to be commensal without known detrimental or beneficial roles to the plant (Hirano & Upper, 2000; Lindow & Brandl, 2003). On the other hand, the establishment of certain antagonistic bacteria, such as *Pseudomonas* strains, can strongly influence the composition of epiphytic bacterial communities (Lindow & Brandl, 2003), acting as plant pathogens with the potential of causing disease (Hirano & Upper, 2000). In *A. hindsii* plants, the bacterial composition changed significantly in the absence of mutualistic ants. Whereas members of the Pseudomonadaceae family (represented mainly by the genus *Pseudomonas*) increased significantly in the absence of mutualistic ants, members of the Enterobacteriaceae family decreased (Fig. 3, Table S1). To our knowledge, this is the first study that demonstrates with a molecular approach that the absence of mutualistic ants from the host plant can lead to changes not only in the overall composition of the bacterial community but also in the abundance of potentially pathogenic bacteria on plant leaves. Ant-mediated protection against fungi have been assessed before (de la Fuente & Marquis, 1999; Heil *et al.*, 1999). Our findings complement previous research on ant-mediated defence, showing that defence by ants also covers the protection from pathogenic bacteria.

Table 4 Inhibitory effects of bacterial strains isolated from legs of mutualistic (*Pseudomyrmex ferrugineus*) and parasitic (*Pseudomyrmex gracilis*) ants on three tested bacteria (*Pseudomonas* sp., isolated from symptomatic *Acacia hindsii* plants, *Pseudomonas syringae* pv. *glycinea* and *Escherichia coli*)

Ant species	Ant bacteria isolates	*Pseudomonas* sp. (*A. hindsii* isolate)	*Pseudomonas syringae* pv. *glycinea*	*Escherichia coli*
P. ferrugineus	*Bacillus* sp.	2 ± 0.1	2.3 ± 0.3	3.6 ± 0.3
P. ferrugineus	*Enterobacter* sp.	–	–	–
P. ferrugineus	*Escherichia* sp.	–	–	–
P. ferrugineus	*Pantoea* sp.	–	2 ± 0.3	3 ± 0.0
P. ferrugineus	*Burkholderia* sp.	–	–	–
P. ferrugineus	*Lactococcus lactis*	2 ± 0.2	1.8 ± 0.2	2.3 ± 1.1
P. ferrugineus	*Klebsiella* sp. 1	–	–	–
P. ferrugineus	*Klebsiella* sp. 2	–	2.5 ± 0.1	2.8 ± 0.2
P. gracilis	*Ralstonia* sp.	–	–	–
P. gracilis	*Burkholderia* sp.	2.8 ± 0.4	–	2.6 ± 1.4
P. gracilis	*Bacillus* sp. 1		3 ± 0.0	–
P. gracilis	*Bacillus* sp. 2	–	–	–
P. gracilis	*Staphylococcus* sp.	–	–	–
P. gracilis	*Enterococcus mundtii*	–	2.5 ± 0.16	3.1 ± 1.0

The inhibition zone (mm) of ant bacterial strains was quantified as the diameter of clear zones of growth inhibition around each ant bacterial drop. Values are means ± SE of three biological replicates. The dash (–) indicates no inhibition.

We observed that plants inhabited by parasitic ants and/or plants from which mutualistic ants were removed displayed a classical response to pathogen attack: an increase in SA concentration (Fig. 4), that is, synthesized in response to infection and whose concentration positively correlates with the plant's degree of resistance against pathogen infection (Loake & Grant, 2007); a high activity of SOD (Fig. S2a), which constitutes the first line of defence against reactive oxygen species (Bolwell & Daudi, 2009); an activation of chitinases (van Loon & van Strien, 1999; Fig. S3b); and a decrease in the Chl content (Fig. 5). These induced defence responses in both plant groups (parasitic presence and/or mutualistic absence) indicate that changes in the microbial community observed in the absence of the mutualistic ant partner indeed had an effect on the physiological state of the plant. Thus, the higher the pathogen-inflicted leaf damage in plants of *A. hindsii*, the higher the resistance state of the plant. Furthermore, a decrease in photosynthesis or Chl measurement in pathogen-infected tissue has been proposed as a plant strategy to switch off photosynthesis and other assimilatory metabolisms in favour of respiration and other processes required for defence (Berger *et al.*, 2007; Bolton, 2009). Consequently, a decrease in the Chl content may result in poor plant performance.

There can be different mechanisms involved in ant-mediated protection from leaf pathogens. For example, some putative mechanisms associated with this phenomenon can be related to ant-associated microorganisms with a potential function of antibiotic secretions (Currie *et al.*, 1999), as well as to chemical secretions produced by exocrine glands of the ants themselves (Morgan, 2008) and/or to leaf plant endosymbionts with potential inhibitory effects against pathogens (Arnold *et al.*, 2003). In the present study, we focused whether ant-associated microorganisms might be involved in this ant-mediated protection from leaf pathogens. Fig. 6 indicates that methanol extracts of ant legs of both mutualistic and parasitic ants were able to inhibit the growth of the plant pathogenic bacterium *P. syringae*, suggesting that the legs of both ants have the ability to inhibit the growth of pathogenic bacteria. To explore in more detail the origin of this protective effect provided by ant legs, bacterial strains from legs of both ants were isolated and identified (Table 3). For *P. ferrugineus*, many of these identified bacteria have been described before as members of the bacterial community associated with this ant species (Eilmus & Heil, 2009). The genera *Bacillus*, *Lactococcus*, *Pantoea* and *Burkholderia* could effectively inhibit the growth of two *Pseudomonas* strains (Table 3), one of them directly isolated from *A. hindsii* symptomatic leaves inhabited by parasitic ants. Some of these bacterial genera are known for their ability to produce antibiotic compounds. For example, *Burkholderia*, which has been isolated from fungus gardens of the leaf cutting ant *Atta sexdens*, can produce antibiotics (Santos *et al.*, 2004), or several species of the genus *Bacillus* also produce plenty of secondary metabolites, many of which have specific antibacterial properties (Mannanov & Sattarova, 2001). Several members of lactic acid bacteria (LAB), such as *Lactococcus*, are known to produce antibacterial substances. For example, LAB microbiota associated with the honeybee *A. mellifera* could effectively inhibit the honeybee bacteria pathogen *Paenibacillus* (Forsgren *et al.*, 2010). Moreover, LAB can be effective in the control of plant pathogens (Visser *et al.*, 1986; Visser & Holzapfel, 1992). These results suggest that ant leg-associated bacteria from both ants might effectively contribute, through metabolites with antimicrobial activity, to the protection provided by ants from phytopathogens. Different factors, however, might simultaneously affect a higher foliar pathogen load. For example, higher amounts of herbivory as a result of the absence of defending ants might also act as vectors for foliar pathogens. Thus, ant-mediated protection from pathogens might be the result of a combination of several mechanisms, which still remain to be elucidated in detail.

Protection mediated by mutualistic bacteria in insect hosts seems to be more common than is currently appreciated (Oliver *et al.*, 2003; Kaltenpoth *et al.*, 2005; Brownlie & Johnson, 2009; Kaltenpoth, 2009). Among ants, for example, fungus-growing ants use symbiotic bacteria living in specific regions on their cuticle to inhibit growth of pathogenic fungi on their crops (Currie *et al.*, 1999). In our system, the specific location and prevalence of the leg-associated bacteria in both species of *Pseudomyrmex*, as well as differences in behaviour between mutualistic and parasitic ants, might be relevant factors determining differences in protective efficacy against bacterial pathogens between both ant species.

Our results show that the indirect defence of mutualistic ants also covers the protection from bacterial leaf pathogens, and that bacteria associated with ants' legs can potentially contribute to this protective effect by ants. Thus, the study of ant–plant defensive mutualisms would benefit from widening the current concept (Heil & McKey, 2003) and considering bacterial partners, which can contribute significantly to the ability of arboreal ants to protect the host plant from pathogens. The results of the present study raise the question as to whether only arboreal ants can fulfil this function of protection from pathogens or whether it is a potentially widespread function among ants. Further studies, however, are necessary to understand the mechanistic basis of the ant-provided protection from pathogens in ant–plant mutualisms in more detail.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Rarefaction curves for the microbial community analyses by bTEFAP based on the raw OTU table.

Fig. S2 Activity of superoxide dismutases (a) and of peroxidases (b) in leaves of *Acacia hindsii* in the presence and absence of mutualistic and parasitic ants.

Fig. S3 Activity of glucananses (a) and chitinases (b) in leaves of *Acacia hindsii* in the presence and absence of mutualistic and parasitic ants.

Table S1 Relative abundances of epiphytic bacterial genera in plants of *Acacia hindsii* in the presence and absence of mutualistic and parasitic ants

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