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Symbiotic bacterial communities in ants are modified by invasion pathway bottlenecks and alter host behavior

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Abstract. Biological invasions are a threat to global biodiversity and provide unique opportunities to study ecological processes. Population bottlenecks are a common feature of biological invasions and the severity of these bottlenecks is likely to be compounded as an invasive species spreads from initial invasion sites to additional locations. Despite extensive work on the genetic consequences of bottlenecks, we know little about how they influence microbial communities of the invaders themselves. Due to serial bottlenecks, invasive species may lose microbial symbionts including pathogenic taxa (the enemy release hypothesis) and/or may accumulate natural enemies with increasing time after invasion (the pathogen accumulation and invasive decline hypothesis). We tested these alternate hypotheses by surveying bacterial communities of Argentine ants (*Linepithema humile*). We found evidence for serial symbiont bottlenecks: the bacterial community richness declined over the invasion pathway from Argentina to New Zealand. The abundance of some genera, such as *Lactobacillus*, also significantly declined over the invasion pathway. Argentine ants from populations in the United States shared the most genera with ants from their native range in Argentina, while New Zealand shared the least (120 vs. 57, respectively). Nine genera were present in all sites around the globe possibly indicating a core group of obligate microbes. In accordance with the pathogen accumulation and invasive decline hypothesis, Argentine ants acquired genera unique to each specific invaded country. The United States had the most unique genera, though even within New Zealand these ants acquired symbionts. In addition to our biogeographic sampling, we administered antibiotics to Argentine ants to determine if changes in the micro-symbiont community could influence behavior and survival in interspecific interactions. Treatment with the antibiotics spectinomycin and kanamycin only slightly increased Argentine ant interspecific aggression, but this increase significantly decreased survival in interspecific interactions. The survival of the native ant species also decreased when the symbiotic microbial community within Argentine ants was modified by antibiotics. Our work offers support for both the enemy release hypothesis and that invasive species accumulate novel microbial taxa within their invaded range. These changes appear likely to influence invader behavior and survival.

Key words: bacteria; behavior; enemy release hypothesis; invasive species; pathogen accumulation; serial bottlenecks; survival.

Introduction

Biological invasions have become models to study a broad array of ecological processes including dispersal, community assembly, predator-prey and host-pathogen dynamics, and trophic theory (Elton 1958, Mack et al. 2000, Sakai et al. 2001). Invasive species are frequently subject to a bottleneck process where only a small number of individuals are introduced into a new environment. In a number of cases, successful invasions have occurred despite only a few individuals successfully surviving the migration process, establishing, and then spreading over entire countries or even continents (e.g., Lester et al. 2014, Tsuchida et al. 2014). Invading individuals may represent only a small proportion of phenotypic and genotypic diversity within the population of the native range (Sakai et al. 2001) and the "paradoxical" ecological success of invasions despite these limitations is the subject of intense study (Sax and Brown 2000, Allendorf and Lundquist 2003).

Several phenotypic characters that are not under genetic control can be influenced by invasion bottlenecks. While many micro-symbionts are inherited and show a strong phylogenetic signal, an organism's microbial community is also influenced by factors including its abiotic environment, diet, and social behavior (Russell et al. 2009, Muegge et al. 2011, Kwong and Moran 2016). For example, in England, considerable variation occurs in the spatial distribution of emerging infectious diseases within bumble bees and their likelihood of being infected by pathogens is associated with the geographic variation of

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disease in honey bees (Fürst et al. 2014). Thus, if a random propagule of bumble bees was inadvertently introduced to a new range from England, the propagules' microbial community would reflect that of honey bees from the population from which they originated, in addition to an array of other factors known to influence microbial communities in bees (Gherman et al. 2014). Invasive species may then undertake secondary and tertiary invasions as they spread further from their first introduced range. These invasions effectively represent additional, serial bottlenecks that likely influence the community ecology of symbiotic microbes.

Most animals probably have a microbial community that includes a core or obligate group of species, traditionally referred to as primary symbionts (Feldhaar 2011, Shapira 2016). In addition, secondary or facultative symbionts vary in presence and abundance between individuals. Obligate taxa are those that appear essential to digestive or other physiological processes. Bed bugs, for example, are reliant on the bacteria *Wolbachia* to provide essential vitamins and nutrients absent from blood meals (Hosokawa et al. 2010). Insects are known to carry core sets of bacteria (Engel et al. 2012, Russell et al. 2012, Kwong and Moran 2016) and we would expect introduced species to carry these primary bacterial symbionts wherever they successfully invade or they would not survive. Secondary or facultative symbionts include both beneficial and pathogenic bacteria. Beneficial taxa include *Lactobacillus* and *Bacillus* bacteria that inhibit infections via the production of antibiotic compounds for their hosts (Yoshiyama and Kimura 2009, Vásquez et al. 2012). The loss of such beneficial facultative symbionts during an invasion may inhibit the successful establishment in a new environment. Alternatively, the release or escape from pathogenic species may be extremely beneficial and forms the basis of the enemy release hypothesis (Keane and Crawley 2002, Torchin et al. 2003). For example, red imported fire ants (*Solenopsis invicta*) host less pathogenic infections in their introduced range than in their home range (Yang et al. 2010). An initial reduction in enemy abundance after invasion, however, may only be temporary. Increasing evidence shows that invasive species can accumulate pathogens or other natural enemies with increasing time after invasion, or that natural enemies might evolve an ability to attack a widespread invasive species (Flory and Clay 2013).

The Argentine ant (*Linepithema humile*) has been described as one of the most widespread, abundant and damaging invasive species (Holway et al. 2002). Native to South America, it has invaded all the world's continents except Antarctica, through serial jumps mediated by human activities (Suarez et al. 2001, Wetterer et al. 2009). The ants were introduced from Argentina to the Southeastern United States before spreading to California around 1905, then to Hawaii in 1916 (Wetterer et al. 2009, Vogel et al. 2010). They were first detected in Australia in 1931 (Wetterer et al. 2009) and were probably introduced from Europe during a secondary invasion event (Suhr et al. 2011). Around 1990, the supercolony in the East Coast of Australia provided the source of what was likely the single colony that successfully established around New Zealand (Corin et al. 2007*a, b*). The known colonization history makes this species ideal to examine patterns of bottlenecks or community assembly of microbes through space and time. Little is known about bacterial communities of Argentine ants with the exception of a documented loss of *Wolbachia* during the invasion process (Tsutsui et al. 2003, Reuter et al. 2005). Such results are suggestive that population bottlenecks in the invasion pathway may alter bacterial communities generally.

We examined the bacterial richness and diversity in Argentine ants from 14 populations representing their pathway of invasion from their native range to New Zealand. We use this data to test two alternate hypotheses: (1) That the bacterial diversity associated with Argentine ants would reflect serial bottlenecks, consequently decreasing in richness from Argentina, to the United States, then Australia, and finally to New Zealand. (2) Argentine ants would exhibit evidence of country-specific bacterial acquisition (as predicted by the Pathogen Accumulation and Invasive Decline [PAID] hypothesis; Flory and Clay 2013) particularly in New Zealand where many populations have experienced a collapse (Cooling et al. 2011). We also predicted that the ants would retain a core community of bacteria, perhaps in obligate relationships, throughout the invasion pathway. Finally, we used antibiotics to experimentally determine if changes in the bacterial community influence the behavior of Argentine ants.

Methods

Bacterial diversity and bottlenecks

Argentine ant workers were collected between 1999 and 2012 in four locations from their native range in Argentina. In the invaded range, they were sampled in the United States (four locations), Australia (one location), and New Zealand (five locations) (Fig. 1; Appendix S1). Ants were stored in 70–100% ethanol at either room temperature or −20°C. DNA was extracted from a pool of 30 ants from each sampling site using a PureLink® Genomic DNA Mini-Kit (Invitrogen). The extractions were further purified using a standard Phenol-Chloroform protocol, precipitated in ethanol and sodium acetate and dried and re-suspended in 100 μL TE buffer.

We used Roche 454 sequencing to amplify bacterial DNA with the 16S universal primers Gray28F 5′ GAGTTTGATCNTGGCTCAG 3′ and Gray519r 5′ GTNTTACNGCGGCKGCTG 3′ (Ishak et al. 2011, Kautz et al. 2013). These primers amplify a 490 bp sequence of the hypervariable V1-V3 region of the bacterial 16S ribosomal RNA gene. We barcoded the samples by tagging the Gray28F primer's 5′ end with 6-nucleotide barcodes generated by BARCLAW (Frank 2009). Each PCR reaction consisted of 15.0 μL master mix solution

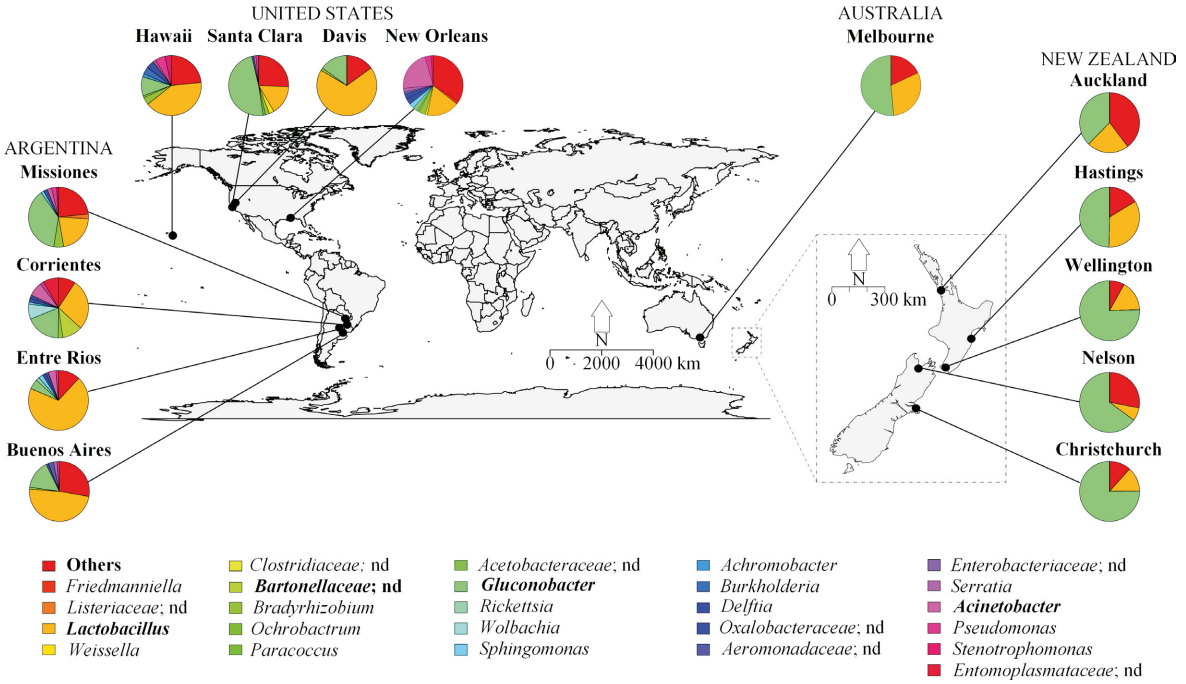

FIG. 1. Argentine ant sampling sites and bacterial diversity at the genus level. The pie charts show non-rare (>1%) bacteria at the genus level present in each sample. Bacterial genera present at >10% in at least one site are in bold. Bacteria names noted "nd" indicate that the genus has not yet been conventionally named.

containing approximately 20 ng DNA suspension, 1X BIOTAQ Buffer, 0.4 mg/mL BSA, 0.2 mM of each dNTP, 0.3 μM for each primer, 1.2 mM $MgCl_2$, 0.02 units Taq DNA polymerase (Fisher Biotec) and 9.1 μL molecular grade ddH_2O (Sigma Aldrich). The PCR thermal cycling consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. Amplifications of the 16S gene were confirmed using agarose gel (1.5%) electrophoresis and subsequent staining with ethidium bromide. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter) as per the manufacturer's instructions. The PCR product quality and quantity were assessed using a modified high range curve of a Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen). Final libraries consisted of 100 μL solutions each containing 500 ng tagged PCR products from samples preserved in TE Buffer. The libraries were sequenced by New Zealand Genomics Limited on GS Junior (Roche).

Sequence analysis was realized using QIIME 1.7.0 (Caporaso et al. 2010*b*). Sequences were assigned to their sample of origin, quality trimmed, and barcodes and primers removed using the QIIME default parameters. Our quality criteria followed standard practice (Kautz et al. 2013) and included sequences with a length between 200–1,000 bp, a minimum average quality score of 25, and a maximum of six homopolymer repeats. Sequences were clustered into Operational Taxonomic Units (OTUs) with 97% identity similarity using Usearch v5.2.236 (Edgar 2010). Sequences were denoised and chimeras filtered out with *de novo* chimera detection (Edgar 2010). Singleton OTUs (microbial sequences observed only once) were removed from the analysis. The most abundant sequence for an OTU was selected as a representative of its OTU cluster. Representative sequences of OTUs were aligned against the Greengenes Core reference alignment (De Santis et al. 2006) using PyNAST (Caporaso et al. 2010*a*). Representative sequence taxonomy was determined based on the Greengenes reference database v12.10 (McDonald et al. 2012) using RDP Classifier 2.2 (Wang et al. 2007). Due to the length of sequences used and the targeted 16S gene sequence, identification to species was not possible. Hence the lowest level of identification used was to genus. Samples were rarefied to the lowest number of reads observed in a sample set: 1,900 reads for the comparison between countries, and to 2,000 reads for the comparison between cities within New Zealand. Rarefaction curves were generated with the QIIME pipeline. Principal Coordinates Analysis (PCoA) and beta-diversity were calculated using UniFrac (Lozupone and Knight 2005). Australia was removed from the UniFrac analysis as only one sample was available. Differences between samples were tested based on the non-parametric method adonis implemented in QIIME 1.7.0. We used G-tests in QIIME 1.7.0 to analyze the difference in relative abundance of bacteria between countries. All tests were considered statistically significant when $P < 0.05$. Venn diagrams to compare bacterial diversity between samples were generated using the package VennDiagram in R v. i386 3.1.3.

Ant behavior after altering symbiont communities

Multiple nests of Argentine ants were collected in a vineyard near Hastings New Zealand (50.854° N, 0.574° E) in April 2014. In the laboratory we set up 20 colonies composed of five queens, 700 workers, and brood. Colonies were placed in plastic boxes (20.5 × 14.0 × 7.0 cm) coated with Fluon to inhibit ant escape (Polytetrafluoroethylene PTFE-30; BioQuip Products, Inc.). Each box contained three glass tubes (1.5 cm Ø; 15.0 cm long) filed 1/3 with water (blocked by a cotton ball) and covered in aluminum foil to create a favorable nesting environment. For the intraspecific interactions described below, one additional large colony of Argentine ants was created with one queen, ~5,000 workers and brood. The colonies were maintained at 20°C ± 1 under a 16/8 h light/dark cycle. They were fed twice a week with a cotton ball soaked in ~5 mL of a 20% honey solution (v/v) and ~1.5 g of freshly cut meal worms (*Tenebrio molitor* larvae). After 2 months of colony acclimation and prior to the experiments described below, we counted the number of workers and queens and all the brood were removed.

Argentine ant colonies were treated with antibiotics in order to modify the bacterial diversity present in the ants. We chose three different antibiotics as they may have different antimicrobial properties within their insect hosts (Matsuura 2001). Ampicillin, kanamycin and spectinomycin were selected. Argentine ant colonies underwent four treatments ($n = 5$ colonies each): no antibiotic (control), ampicillin, kanamycin or spectinomycin. Colonies were fed a 200 μL mix of 20% (w/v) sucrose and 5.00 mg/mL antibiotic (final concentrations). The control colonies were fed with a 20% (w/v) sucrose solution only. The antibiotic mix was fed to the colonies three times a week for three more weeks and ~1.5 g of freshly killed mealworms twice a week. Preliminary trials were conducted to ensure ants would consume the antibiotics at these concentrations and that these concentrations would not result in direct ant mortality. We also undertook a small experiment to demonstrate antibiotic treatment can alter symbiotic bacterial communities of ants (Appendix S2).

Intra- and interspecies interactions, and the survival of Argentine ants after the antibiotic treatments, were assessed 5 and 30 d after antibiotic treatment. Argentine ant aggression and survival were recorded during encounters with Argentine ants that were not treated with antibiotics. For interspecific interactions, we used the New Zealand native "bush ant" *Prolasius advenus*. Six colonies of bush ants were collected at Rivendell, in Kaitoke Regional Park in New Zealand (41.050°S, 175.179°E) in May 2014 and kept in the same conditions as the Argentine ants.

Each replicate consisted of 10 ants from each treatment or species, pitted against 10 from the other treatment or species. As a control group, untreated Argentine ants were marked with one small blue dot of paint on the gaster to differentiate them from the antibiotic-treated Argentine ant workers. Ants were randomly selected from the colonies for each replicate and were discarded after the experiment. The interaction arenas consisted of plastic boxes (13.5 × 9.0 × 8.0 cm) with walls coated in Fluon™. In each box, a plastic ring (5.0 cm Ø × 5.0 cm high) with both sides coated in Fluon™ was placed in the middle. Each ant group was randomly assigned to a side (outside or inside) of the ring. After placement, the ants were left to acclimatize for 20 min. The ring was then removed. During each interaction, interaction behavior was scored similarly to Suarez et al. (1999): score 0 – "ignore" (body contact with no reaction); score 1 – "touch" (one ant taps the other with its antennae); score 2 – "avoid" (after contact, one of the individual runs away from the other ant in the opposite direction); score 3 – "aggression" (one ant bites a body part of the other ant or raised its gaster); score 4 – "fighting" (prolonged fighting of at least 5 s). Behaviors for both Argentine and bush ants were scored for 25 s every 2.5 min, for a total period of 25 min. To assess survival, the number of dead ants of each species was recorded after 25, 30, 40, 50 and min, and 1, 2, 4, 8 and 16 h. Two groups of 10 ants from each colony participated in encounters with untreated marked Argentine ants and with bush ants. Ten replicates were used in total for each treatment. All analyses and figures were implemented in R version i386 3.3.0 (R Development Core Team 2016). Differences in aggression scores of treated Argentine ants were analyzed using a generalized linear model with treatment, time and opponent type (the scores of either untreated Argentine ants or bush ants encountering the treated ants) as response variables. Argentine ant survival was analyzed using the package *survival* (Therneau 2012). Comparison between survival probabilities of ants in different treatments was analyzed using Cox proportional hazard regression models. Statistical significance was assumed at $P < 0.05$.

Results

Bacterial diversity and bottlenecks

A total of 273,798 sequence reads were obtained from the 16S bacterial DNA amplified from Argentine ant samples. After quality trimming, a total of 89,076 reads were assigned to the samples from 14 locations. Following denoising, chimera and singleton exclusion steps using Usearch 7.0.1090, a total of 85,915 reads remained. These reads were assigned to the samples and were clustered into 763 Operational taxonomic units (OTUs) with 97% identity similarity between reads corresponding to 180 named genera. The number of OTU sequence reads identified are presented in Appendices S3–S5.

We hypothesized that the bacterial diversity associated with Argentine ants would reflect their invasion steps and bottlenecks, consequently decreasing in richness from Argentina, to the United States, then Australia, and finally to New Zealand. Rarefaction curves were used to estimate the number of bacterial operational taxonomic units (OTUs) observed for each country (Fig. 2a).

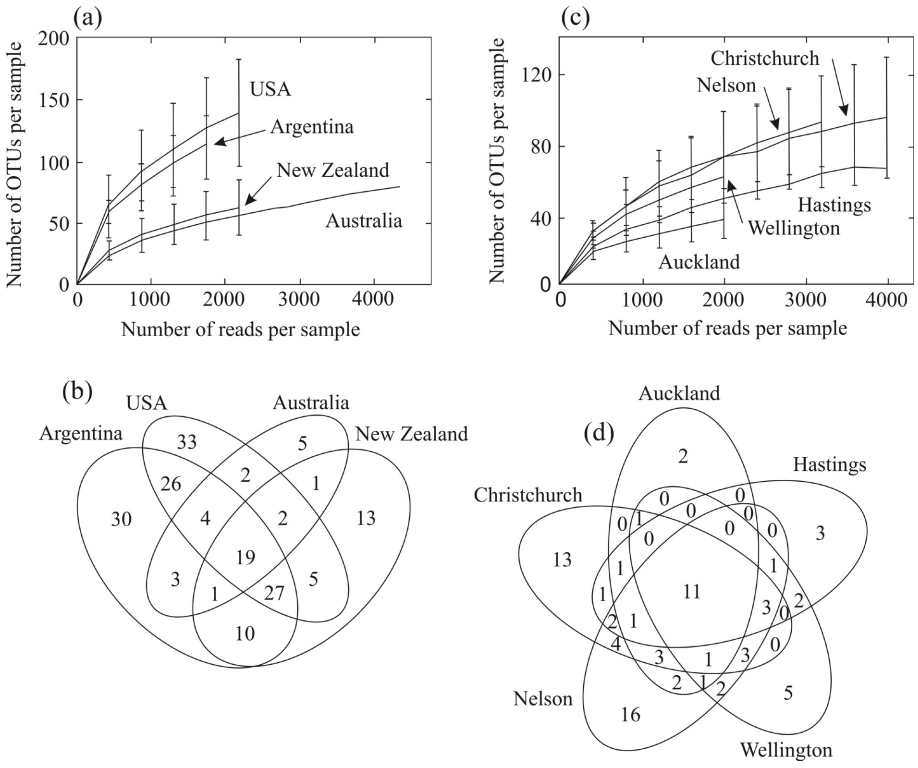

FIG. 2. Bacterial OTU richness (a, c) and distinctiveness based on genera identified from the OTUs (b, d). Data are from the four countries examined (a, b), and in the four sampling sites in New Zealand (c, d). (a, c) Error bars on the rarefaction curves represent the standard deviation. No error bars for the standard variation are presented for Australia as only one location was sampled. The length of the rarefaction curve for each country is limited by the lowest number of sequence reads in a sample from that country. (b, d) Venn diagrams of unique and shared bacteria identified to genus present in Argentine ants in the ant home range (Argentina) and invaded countries (b) or locations within New Zealand (d).

Multiple *t*-tests indicated a significant difference in both the average Chao1 richness and average number of species observed between Argentina and New Zealand (Bonferroni corrected $P < 0.05$), and between the United States and New Zealand (Bonferroni corrected $P < 0.05$ for both countries). No tests were performed with Australia as there was only one sample from this country. In total, 181 bacteria genera belonging to 16 different phyla were detected across all countries. Only 19 genera were shared by all countries (Fig. 2b). Populations from the United States shared the most bacterial OTUs with populations from the native range in Argentina, while New Zealand shared the least (118 genera vs. 78, respectively) (Fig. 2b). The bacterial community appeared to change through the invasion pathway from Argentina to New Zealand, with substantially more non-rare (>1%) bacteria genera present in Argentina than Australia or New Zealand (Fig. 1). Principal coordinates analysis further demonstrated the similarities between the Argentine and United States OTU communities (Fig. 3). The abundance of several bacterial genera also significantly declined over the invasion pathway, such as *Lactobacillus*, which was approximately half as abundant in New Zealand as in Argentina (Table 1). Other bacteria appeared to significantly increase in abundance, such as *Gluconobacter*, which was nearly 3-fold more abundant in New Zealand compared to Argentina (Table 1).

We predicted that the ants would carry a core community of bacteria, perhaps in an obligate relationship, throughout the invasion pathway. Core bacteria were defined as genera present in all sites and all cities sampled throughout the native and introduced range. Only nine genera were present in all sites (Table 2). They represented >50% (min 54.2% – max 92.1%) of the bacteria present in the ants in each location. The core genera were *Lactobacillus*, *Caulobacter, Bradyrhizobium, Gluconobacter, Achromobacter, Delftia, Acinetobacter, Pseudomonas,* and *Stenotrophomonas.*

In addition, we sought for evidence of country-specific bacterial acquisition. The vast majority of bacterial genera were classified as non-core taxa, with only nine core taxa out of the total of 181 genera identified throughout the study. The ants were assessed for evidence of country-specific bacterial acquisition. In the United States 33 unique genera were observed, and 13 were uniquely observed in New Zealand (Fig. 2b; Appendix S1). In the United States the most abundant unique genera were *Corynebacterium* and *Trebouxiophyceae* (respective relative abundance: 0.23% and 0.10%) and in New Zealand the most abundant unique genera were *Swaminathania* and *Rubrobacter* (respective relative abundance: 0.04% and

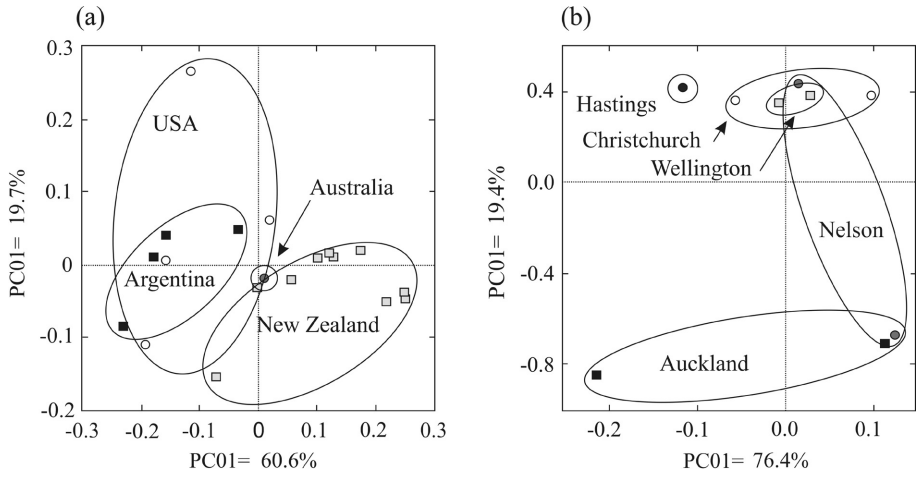

FIG. 3. Principal coordinate analysis based on weighted phylogenetic UniFrac distances generated in QIIME 1.7.0 for bacterial OTUs between Argentine ant samples from (a) the four countries examined, and (b) locations within New Zealand. Circles indicate the dispersion of samples from the same country/location.

TABLE 1. The mean relative abundance in percentage of bacterial genera in the Argentine ant home range and three introduced ranges, with the mean raw number of reads is in brackets. Only bacteria with conventional names are presented. To compare bacterial presence between countries, G-tests were undertaken on the relative abundance and on the number of reads of each genus after rarefaction to 1,900 reads. Significant *P*-values ($P < 0.05$) are indicated by a *. Australia was not included in the G-tests as it was represented by a single sample.

Phylum	Genus	Country				Bonferroni corrected *P*-value
		Argentina	USA	Australia	New Zealand	
Actinobacteria	*Tsukamurella*	0.03 (1.5)	0.03 (1.5)	0.01 (1.0)	0.07 (2.9)	NA
Bacteroidetes	*Chryseobacterium*	0.08 (4.5)	0.08 (3.5)	0.03 (2.0)	0.01 (0.5)	1
	Flavisolibacter	0.01 (0.8)	0.03 (1.3)	0.01 (1.0)	0.01 (0.6)	NA
	Segetibacter	0.02 (1.3)	0.16 (7.5)	0.04 (3.0)	0.01 (0.5)	NA
Firmicutes	*Lactobacillus*	33.79 (1856.5)	38.45 (1754.8)	30.57 (2246.0)	18.65 (794.5)	<0.001*
Proteobacteria	*Caulobacter*	0.19 (10.3)	0.21 (9.5)	0.01 (1.0)	0.04 (1.9)	1
	Phenylobacterium	0.00 (0.3)	0.01 (0.3)	0.01 (1.0)	0.00 (0.1)	NA
	Bradyrhizobium	3.16 (173.8)	2.32 (106.0)	0.31 (23.0)	0.30 (12.9)	<0.001*
	Methylobacterium	0.15 (8.0)	0.25 (11.5)	0.01 (1.0)	0.03 (1.5)	1
	Gluconobacter	22.04 (1211.0)	21.75 (992.8)	45.89 (3371.0)	60.72 (2586.6)	<0.001*
	Achromobacter	0.26 (14.3)	0.35 (15.8)	0.04 (3.0)	0.12 (5.0)	1
	Delftia	1.83 (100.8)	1.70 (77.8)	0.11 (8.0)	0.34 (14.3)	<0.001*
	Ralstonia	0.05 (2.5)	0.14 (6.3)	0.01 (1.0)	0.02 (0.8)	1
	Escherichia	0.13 (7.3)	0.19 (8.5)	0.01 (1.0)	0.02 (0.8)	NA
	Acinetobacter	3.63 (199.3)	4.21 (192.0)	0.65 (48.0)	0.27 (11.6)	<0.001*
	Pseudomonas	2.04 (112.3)	2.02 (92.0)	0.05 (4.0)	0.29 (12.6)	<0.001*
	Stenotrophomonas	0.87 (48.0)	1.09 (49.8)	0.10 (7.0)	0.19 (8.1)	<0.001*
Total OTU relative abundance OTUs (number of reads)		68.30 (3751.8)	72.97 (3330.8)	77.89 (5721.0)	81.10 (3454.3)	
Total OTU relative abundance (number of reads) not defined to the genus level		16.55 (909.3)	18.35 (837.8)	21.20 (1557)	18.02 (767.7)	

0.03%). The relatively high abundance of *Corynebacterium* and *Trebouxiophyceae* in the United States and their complete absence from any other location suggests this result is not a sampling effect.

We examined the New Zealand samples to highlight the change in bacterial community composition after a single invasion event and following colonization process. Alpha diversity was analyzed through rarefaction curves

TABLE 2. The relative abundance in percentage of core bacterial genera present in Argentine ants in all locations. Core bacterial genera were defined as those genera present in all countries and locations. These percentage relative abundance estimates were generated from rarefaction analysis after standardizing the number of reads to 1900, which was the lowest number of reads in our sample of Argentina.

	Argentina				United States			Australia	New Zealand				
	Miss.	Corr.	Entr. Rios	Buen. Aires	HI	Sant. Clar.	Davis	Melb.	Auck.	Hast.	Well.	Nels.	Chri.
Firmicutes													
Lactobacillus	21.4	27. 6	69.7	48.4	41.0	15.8	68.5	30.6	22.7	34.1	16.5	7.2	13.2
Proteobacteria													
Caulobacter	0.1	0.4	0.1	0.2	0.2	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Bradyrhizobium	5.3	2.5	0.8	1.1	4.0	2.1	1.4	0.1	0.1	0.2	0.3	0.4	0.6
Gluconobacter	33.7	17.5	5.3	13.3	10.2	42.6	13.1	45. 9	37.5	44.1	75.8	65.1	75.8
Achromobacter	0.4	0.1	0.2	0.1	1.7	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.2
Delftia	2.1	2.1	1.8	1.2	4.4	1.1	0.8	0.1	0.2	0.2	0.2	0.4	0.7
Acinetobacter	2.4	7.8	4.3	0.9	1.3	1.1	1.0	0.7	0.1	0.2	0.1	0.4	0.5
Pseudomonas	2.7	2.0	1.3	1.3	5.6	1.5	0.8	0.1	0.2	0.1	0.1	0.4	0.7
Stenotrophomonas	1.0	0.8	0.6	0.8	3.5	0.9	0.4	0.1	0.1	0.1	0.1	0.2	0.4
Total relative abundance	69.1	60.8	84.0	67.4	71.8	65.4	86.1	77.7	60.9	79.1	93.3	74.1	92.1

Note: Location abbreviations: Miss. (Missiones), Corr. (Corrientes), Entr. Rios (Entre Rios), Buen. Aires (Buenos Aires), HI (Hawaii), Sant. Clar. (Santa Clara), Davis (California), Melb. (Melbourne), Auck. (Auckland), Hast. (Hastings), Well. (Wellington), Nels. (Nelson), Chri. (Christchurch).

based on the number of observed species for five cities in New Zealand (Fig. 2). The slopes of the curves for each sampling site are close to reaching the plateau, indicating that the sampling was sufficient to identify most of the bacteria present in the ants in each location. The observed number of bacteria was higher in the southern locations of Nelson and Christchurch than in the northern locations of Auckland, Hastings and Wellington. However, the variation in the number of observed bacteria in each location was high. Multiple *t*-tests indicated no significant differences for the number of observed OTUs between locations (Bonferroni corrected $P > 0.05$ for all pairs, not including Hastings from where we only had one sample). Similarly, no significant differences were observed for Chao1 richness indicator between locations (adonis, Bonferroni corrected $P > 0.05$). Of the 78

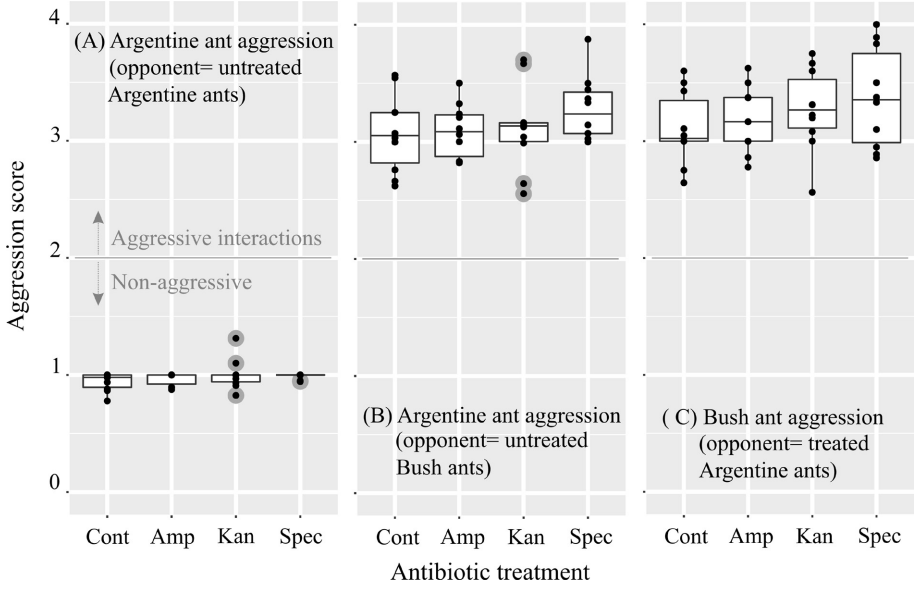

FIG. 4. Mean aggression scores of ants during interspecific encounters between (a) the control group or untreated Argentine ants; (b) Argentine ants given antibiotics and bush ants, and (c) bush ants interacting with treated Argentine ants. The antibiotics are ampicillin (Amp), kanamycin (Kan), and spectinomycin (Spec). Encounters are shown for Argentine ants treated with antibiotic treatment for 30. Lines are the means, boxes are lower and upper quartiles, error bars are the minimum and maximum values excluding outliers, and outliers are shown as large grey dots surrounding points.

bacterial genera identified in New Zealand, 11 were shared between all five locations (Fig. 4, Table 3). *Gluconobacter* (mean = 58.93 ± 8.35%) and *Lactobacillus* (mean = 19.67 ± 5.04%) were the only non-rare bacteria present in all locations (Table 3).

Ant behavior and survival after altering symbiont communities

We first examined intraspecific aggression and survival after the Argentine ants were given antibiotics. Only non-aggressive interactions were observed in intraspecific aggression tests examining antibiotic treated Argentine ants and marked non-treated Argentine ants (Fig. 4). No significant change in aggression was observed between Argentine ant groups between treatments ($F_{3,72} = 0.288$; $P = 0.834$), over time ($F_{1,72} = 0.679$; $P = 0.413$), or in the time × treatment interaction ($F_{3,72} = 0.076$; $P = 0.973$). The Cox regression analysis showed no change in survival in intraspecific interactions between the main effects of the antibiotic treatments, but did show a significant effect of time ($P = 0.028$) and a significant ampicillin × time interaction ($P = 0.009$; Table 4). This result reflected an occasional ant dying in some of the replicates of the Ampicillin treatments over the 16-h trial, which was not related to intraspecific aggression (Figs. 4 and 5). Otherwise the survival probability during intraspecific encounters was close to 100% for all the other intraspecific treatments.

In interspecific interactions, the mean aggression scores between Argentine ants and bush ants were all above 2, which indicated a prevalence of highly aggressive interactions. No significant change in interspecific aggression was observed between antibiotic treatments ($F_{3,72} = 1.582$; $P = 0.201$) or over time ($F_{1,72} = 3.083$; $P = 0.083$), or in the time × treatment interaction ($F_{3,72} = 0.452$; $P = 0.717$). However, the highest aggression levels, as well as the highest mean levels of aggression, were observed in the kanamycin and the spectinomycin treatments (Fig. 4). These slightly (but not significantly) higher levels of aggression appeared to influence Argentine ant survival. Compared to the control treatment, significantly lower rates of survival were observed in the kanamycin and the spectinomycin treatments ($P < 0.001$; Table 4). We attribute these results to some Argentine ants in these treatments engaging in aggressive interactions more intensely, for longer periods, resulting in higher mortality rates.

The reciprocal aggression displayed by bush ants resembled patterns observed with Argentine ants. The highest levels of aggression displayed by bush ants were towards Argentine ants treated with kanamycin and the spectinomycin (Fig. 4). No significant change in interspecific aggression displayed by bush ants were observed between antibiotic treatments ($F_{3,72} = 2.094$; $P = 0.109$) or over time ($F_{1,72} = 1.622$; $P = 0.207$), or in the time × treatment interaction ($F_{3,72} = 1.139$; $P = 0.339$). The survival of bush ants was significantly lowered by the treatment of Argentine ants with the antibiotic ampicillin ($P = 0.026$; Table 4). Similarly to the results for intraspecific trials, there was a significant negative effect of time ($P < 0.001$) and a significant time × Ampicillin interaction ($P = 0.024$; Table 4).

Table 3. The mean relative abundance in percentage of core bacterial genera in cities of the introduced range of New Zealand, with the mean raw number of reads is in brackets. For Hastings, no means were calculated as only one sample represented the location. In order to compare bacteria presence between locations, G-tests were conducted in adonis using QIIME on the number of reads of different genera after rarefaction to 2,000 reads. Bonferroni corrected *P*-values are presented for each genus. Non-classified OTUs and reads ("unclassified" and "other phyla") were marginal (<0.05%) in all locations. *Lactobacillus* and *Gluconobacter* were the only two genera present >1% in all locations. Note that these estimates differ slightly from those presented in Table 2, which are based on a minimum number of 1,900 reads. Significant *P*-values ($P < 0.05$) are indicated by a *.

Phylum	Genus	Location					Bonferroni corrected *P*-value
		Auckland	Hastings	Wellington	Nelson	Christchurch	
Firmicutes	*Lactobacillus*	28.5 (1376)	34.1 (2820)	15.0 (1272)	6.7 (558)	14.0 (1124)	<0.001*
Proteobacteria	*Caulobacter*	0.1 (1)	0.1 (2)	0.1 (5)	0.1 (3)	0.1 (6)	NA
	Bradyrhizobium	0.1 (7)	0.2 (12)	0.4 (22)	0.4 (28)	0.5 (47)	1
	Ochrobactrum	0.1 (1)	0.1 (2)	0.1 (3)	0.1 (3)	0.1 (13)	NA
	Gluconobacter	34.9 (2272)	44.1 (3647)	76.4 (5849)	64.1 (5076)	75.1 (6435)	<0.001*
	Sphingomonas	0.1 (1)	0.2 (14)	0.1 (3)	0.2 (17)	0.3 (23)	NA
	Achromobacter	0.1 (2)	0.1 (4)	0.4 (18)	0.1 (6)	0.2 (15)	1
	Delftia	0.1 (10)	0.2 (17)	0.3 (15)	0.4 (30)	0.6 (57)	1
	Acinetobacter	0.1 (8)	0.2 (19)	0.1 (8)	0.4 (30)	0.4 (39)	1
	Pseudomonas	0.1 (9)	0.1 (8)	0.2 (9)	0.2 (28)	0.7 (59)	0.32
	Stenotrophomonas	0.1 (8)	0.1 (10)	0.1 (6)	0.2 (15)	0.4 (34)	1
	Total named OTUs	64.3 (3715)	79.6 (6583)	93.7 (7251)	74.2 (5938)	93.4 (7939)	NA
	Total OTUs	NA (6061)	NA (8269)	NA (7720)	NA (7797)	NA (8490)	NA
	Total number present in all cities	64.0 (3695)	79.3 (6555)	93.2 (7210)	72.9 (5794)	92.6 (7852)	NA

TABLE 4. Effects of antibiotic treatment on the ant survival in interspecific interactions, using a Cox regression analysis. Argentine ants were given antibiotics for 5 and 30 d. Bush ants were not treated with antibiotics. Groups of ten Argentine ants and 10 bush ants were then placed in an arena and allowed to interact (fight). Each antibiotic treatment is compared to the control treatment, which was given the same diet but no antibiotics.

	Argentine ants survival (control)			Argentine ant survival (bush ants)			Bush ant survival (Argentine ants)		
Factor	Coef (± SE)	*z*	*P*-value	Coef (± SE)	*z*	*P*-value	Coef (± SE)	*z*	*P*-value
Ampicillin	−0.355 (0.493)	−0.721	0.471	0.068 (0.077)	0.883	0.377	−0.203 (0.092)	−2.219	0.026*
Kanamycin	0.658 (0.391)	1.683	0.092	0.371 (0.073)	5.095	<0.001**	0.081 (0.085)	0.948	0.343
Spectinomycin	0.643 (0.391)	1.647	0.100	0.344 (0.073)	4.713	<0.001**	0.036 (0.086)	0.419	0.675
Time (day 5 vs. day 30)	−2.311 (1.049)	−2.203	0.028*	−0.063 (0.080)	−0.789	0.430	−0.315 (0.094)	−3.356	<0.001**
Ampicillin × time	3.011 (1.146)	2.626	0.009**	0.192 (0.109)	1.771	0.077	0.301 (0.134)	2.25	0.024*
Kanamycin × time	0.442 (1.219)	0.363	0.717	−0.183 (0.106)	−1.723	0.085	−0.190 (0.133)	−1.425	0.154
Spectinomycin × time	−15.77 (1927)	−0.008	0.993	−0.065 (0.105)	−0.617	0.537	−0.156 (0.134)	−1.165	0.244

*Statistical significance at $0.050 > P > 0.010$.
**Statistical significance at $P < 0.001$.

DISCUSSION

Our study found remarkably similar within-country microbial communities of an invasive ant species, but these communities showed considerable between-country variation along their invasion pathway. Bacterial diversity was highest in the native range and lowest in the most recently invaded populations in Australia and New Zealand. Only nine bacterial genera were consistently observed throughout the native range. We found evidence for both the apparent loss and acquisition of new bacterial genera specific to each invaded country. Changes in the ant's microbiota following the application of antibiotics may influence the survival of both the host and of native ant species in interspecific interactions.

The enemy release hypothesis proposes that invasive species become abundant because of the absence of natural enemies such as pathogens and parasites in an introduced range (Keane and Crawley 2002, Torchin et al. 2003). A loss of microbial pathogens during invasion has been reported in a variety of invasive species, including shrimp (*Dikerogammarus villosus*), sparrows (*Passer domesticus*) and fish (*Silurus glanis*, *Lepomis gibbosus*) (Marzal et al. 2011, Arundell et al. 2015, Sheath et al. 2015). Red

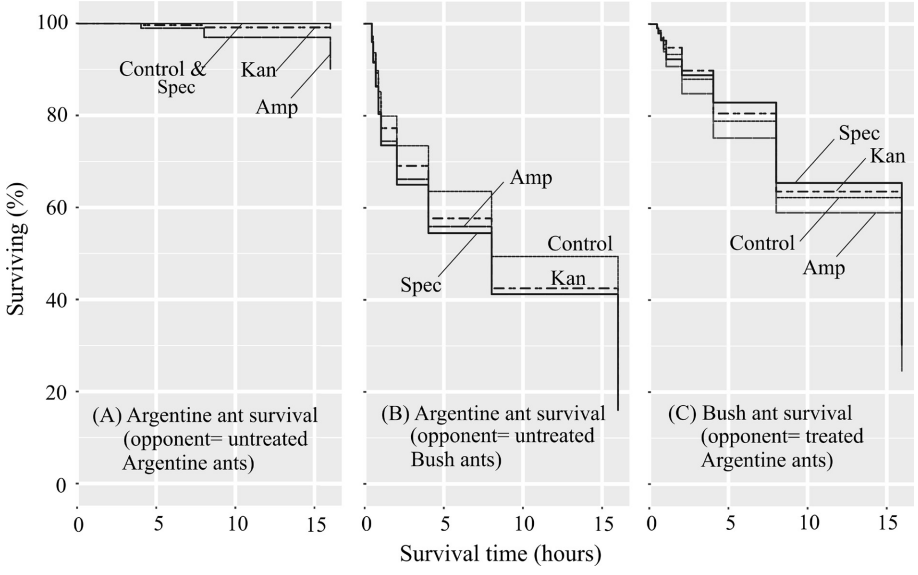

FIG. 5. Effects of antibiotic treatment on ant survival in interspecific interactions, shown as Kaplan-Meier survival curves. Survival probability of encounters between antibiotic treated Argentine ants and bush ants (*Prolasius advenus*) after 30 d of antibiotic treatment. Survival plots are shown for: (a) antibiotic treated Argentine ants against untreated Argentine ants, (b) treated Argentine ants against bush ants, and (c) the survival of bush ants against treated Argentine ants. Argentine ants given the antibiotics ampicillin (Amp), kanamycin (Kan) and spectinomycin (Spec).

imported fire ants (*Solenopsis invicta*) also harbor a lower number of pathogens in their invaded range than in their home range in South America (Yang et al. 2010). Our results supported our hypothesis that bacterial diversity declines through the invasion pathway. Some of these bacteria are likely to be pathogenic. Examples of genera observed only in Argentina or the United States include *Brevibacterium* and *Erwinia*. Genera such as *Brevibacterium* have been observed in other invasive ants (Powell et al. 2014) and are known to have pathogen effects in insects (Selvakumar et al. 2011). The specific role of such bacteria in the life history and fitness for most insects including Argentine ants is unknown and requires further assessment. Nevertheless, the loss of bacterial genera along the invasion pathway offers support for the enemy release hypothesis.

From our Argentine ant samples, the two bacterial genera *Wolbachia* and *Rickettsia* were amongst the bacteria lost during the invasion process. This result is in agreement with previous studies recording *Wolbachia* infections in Argentine ants throughout their native range but only in one introduced population in Hawaii (Tsutsui et al. 2003, Reuter et al. 2005). Our samples from Hawaii did not harbor *Wolbachia*, which may be due to the marginal presence of the endosymbiont in the ants or limited population level sampling. Additional sampling in each country would help confirm the loss of *Wolbachia* and the other bacteria we assume to have been lost along the invasion pathway. *Rickettsia* was found in both Argentina and the United States, but not in Australia or New Zealand. *Wolbachia* and *Rickettsia* are facultative endosymbionts that are transmitted both vertically and horizontally (Viljakainen et al. 2008, Weinert et al. 2009). These taxa can have both beneficial and detrimental effects on their hosts (Zug and Hammerstein 2015). Previous researchers have suggested that the mechanisms for the loss of *Wolbachia* in ants may due to random founder effects, selection against *Wolbachia* co-infection in the native range, or even environmental effects such as heat exposure (Shoemaker et al. 2000, Reuter et al. 2005, Rey et al. 2013). *Wolbachia* has been considered deleterious in ant populations, altering ant fitness (Wenseleers et al. 2002). Hence, it seems most likely that the loss of bacteria such as *Wolbachia* in Argentine ants is due to random founder effects as proposed in the enemy release hypothesis.

The pathogen accumulation and invasive decline hypothesis posits that with increasing time in a new range, an invasive species will accumulate an increasing diversity of pathogens that will eventually lead to a decline in its density and distribution (Flory and Clay 2013). This hypothesis has found support in several studies of invasive plants (Orrock et al. 2012, Stricker et al. 2016). Our results provide evidence that supports the hypothesis that invasive species can acquire microbial taxa specific to their introduced range. Of the invaded range sites, Argentine ant populations in the United States (the invaded country where they have been longest) had a total of 33 genera that were observed nowhere else. There have been multiple introductions into the United States (Brandt et al. 2009), however, and the taxa observed in the United States may also be representative of microbial diversity in Argentine ants un-sampled in the native range. Perhaps stronger evidence for the acquisition of microbial taxa comes from the New Zealand samples. New Zealand is at the end of this introduction pathway and the invasion likely consisted of a single nest that subsequently spread throughout the country (Corin et al. 2007*a, b*). Despite this common ancestry the microbial flora of the New Zealand samples still had 13 distinct genera, and individual cities within the country all had uniquely represented genera. Our work in viral pathogens of these ants similarly suggests that they both bring in viral taxa from their home range and acquire local strains of viruses with high pathogenicity in bees (Sébastien et al. 2015). Together, these results offer support for the loss and accumulation of microbial taxa along the invasion pathway. Whether or not this change in microbial community has influenced observed declines in Argentine ant density and distribution (Lester and Gruber 2016) remains unknown.

Nine bacterial genera from two phyla appear to represent the core bacteria present in Argentine ants. *Lactobacillus* and *Gluconobacter* dominate these core bacteria. *Lactobacillus* is a known genus of favorable facultative symbionts, especially studied in honey bees and bumblebees (Corby-Harris et al. 2014, Praet et al. 2015). In these bees the bacteria are thought to produce antimicrobial compounds and promote host health (Forsgren et al. 2009, Yoshiyama and Kimura 2009). Similarly, *Gluconobacter* can be largely found in the guts of bees as a facultative symbiont (Anderson et al. 2011, Martinson et al. 2011). It is possible that these bacterial genera may be essential to the survival and spread of Argentine ants. Additional experimental work is needed to demonstrate that Argentine ants have such a core bacterial group or primary symbionts, as has been observed in other animals including social insects (Feldhaar 2011, Shapira 2016).

Hu et al. (*in press*) recently compared the bacterial communities of Argentine ants in relation to variation in the trophic position the ants occupy among native and populations in the USA. Their results parallel some of our finding including the identification of a core community of symbionts that include *Lactobacillus* and *Acinetobacter*. Their study also found an apparent acquisition of new bacterial taxa and a substantial change in the abundance of other genera such as *Rickettsia* in introduced populations. The consistency of our results with those of Hu et al. (*in press*) provides support for our conclusions despite possible methodological concerns related to sample age. For example, the samples from Argentina were the oldest and could have degraded leading to inflated diversity estimates. Hu et al. (*in press*), however, used samples from a similar range of collection dates and found no significant evidence for degradation over time. In addition, our samples passed quality assessment criteria at the sequencing facility. We also acknowledge that the use of a sample blank or control in our metagenomics analysis would have been ideal. Salter

et al. (2014) found sample blanks with up to 90 different bacterial genera, due to contamination of extraction kits, particularly when the starting template is of low concentration. Our samples exceeded the quality criteria for template concentration and we used the same extraction kits for all samples. We found 49 of the genera identified by Salter et al. (2014) as present in sample blanks, however, these genera were not present in all samples as would be expected if there was contamination. Many of these taxa are also known or hypothesized symbionts of ants including *Burkholderia* and *Acinetobacter* (e.g., Russell et al. 2009, Hu et al. *in press*). Further, the majority of these taxa (35 of the 49 genera that we observed) were also found by Hu et al. (*in press*) after controlling for false positives owing to degradation. Nevertheless, we note that our diversity estimates could still have been influenced by sample age and false-positive results. Future research using similarly aged samples, blank controls and multiple samples from each sampling location will help avoid some of these concerns.

In addition to playing vital roles in nutrition and physiology, symbiotic bacteria can also directly alter the behavior of their animal hosts. Accumulating evidence suggests that gut microbiota communicate with their hosts' nervous system, altering brain function and behavior (Cryan and Dinan 2012). A variety of direct and indirect mechanisms could enable bacteria to alter their hosts' behavior. For example, bacteria are known to produce a range of neurotransmitters and neuromodulators. *Lactobacillus* spp. and *Bifidobacterium* spp. produce GABA (gamma-amino butyric acid), *Saccharomyces* spp. produce noradrenalin, and *Bacillus* spp. produce dopamine (Lyte 2011, Forsythe and Kunze 2012). Although this type of work is undertaken primarily in vertebrates, it has been hypothesized that symbiont-mediated alterations in host behavior may be common in insects (Feldhaar 2011). Neurotransmitter-producing bacteria such as *Lactobacillus* and *Bifidobacterium* spp. are common in insects such as bees and ants (e.g., Ishak et al. 2011, Engel et al. 2012). Bottlenecks in invasive species pathways that alter symbiont composition communities may consequently alter insect behavior, as well as their physiology.

We suspected that a change in the ant's symbiotic microbiota might alter their host's behavior due to the increasing evidence that bacteria can alter brain function and behavior (Cryan and Dinan 2012). Our experimental manipulation of the Argentine ant microbial fauna using antibiotic treatments indicated that the microbial flora of the ants does seem to influence their interspecific interactions. We examined three different antibiotics that each likely had different effects on the microbial population within the ants. The alteration of the microbial fauna via two of the three antibiotics (kanamycin and spectinomycin) had a significant influence on Argentine ant survival in interspecific interactions. Studies on vertebrates have shown spectinomycin to act on the nervous system and even modulate pain (Mercado et al. 2015), resulting in a range of responses including reduced rates of autotomic behavior (~gnawing off your limbs) (Krsljak and Stajcic 2004). Within social insects, both inter- and intraspecific aggression of termites have been observed to be altered after treatment with spectinomycin (Wei et al. 2007). Matsuura (2001) similarly observed a change in termite aggression after antibiotic treatment. He concluded that behavior was likely altered due to the antibiotics changing the termites' bacterial communities and odor in feces, which appear to be a key nest-mate recognition cue. We observed no such change in intraspecific aggression in Argentine ants after antibiotic treatment, indicating microbial symbionts play a limited role in intraspecific recognition. Other research has observed no intraspecific aggression in interactions between Argentine ant colonies collected from widely separated countries including New Zealand and the United States (van Wilgenburg et al. 2010), despite our results demonstrating that the populations from these countries have very different bacterial communities.

While we found no evidence that intraspecific recognition cues are altered by symbiotic bacteria, there may be consequences for the ants via microbial taxa interactions with the nervous system as seen in vertebrates (Cryan and Dinan 2012, Mercado et al. 2015). In other work, we gave the antibiotic ampicillin to Argentine ants and examined the changing microbial community using 454 sequencing (Sébastien 2016). We observed ampicillin to increase the abundance of *Lactobacillus* and *Sphingomonas,* but decrease the abundance of *Achromobacter*. The ampicillin treated ants had decreased long-term survival in the absence of intra- or interspecific interactions (Sébastien 2016). It is clear from these results that a modified symbiotic bacterial community can influence the population dynamics of this invasive ant. The mechanism for bacteria's influence on their host's behavior or survivorship remains to be determined. Nevertheless, as the evidence grows that symbiotic bacteria can substantially influence their host's behavior and longevity (Cryan and Dinan 2012), we expect that these microbial communities will be observed as important partners in the outcome of biological invasions.

Examining bacterial communities of invasive ants can be a powerful mechanism for identifying pathways of invasion, possible biocontrol agents, and the structure and function of their microbiota (Shoemaker et al. 2000, Tsutsui et al. 2003, Reuter et al. 2005, Yang et al. 2010, Ishak et al. 2011, Sébastien et al. 2012, Hu et al. *in press*). Our results contribute to a growing body of literature on the ecology and evolution of the microbiota of ants. Most work in this area has concentrated on gut microbes that are correlated with diet and show a strong phylogenetic pattern (Russell et al. 2009, 2012, Anderson et al. 2012). Bacterial communities are most divergent among ants that have predatory or herbivorous diets and an ants' gut microbiota can facilitate dietary specialization by providing essential, limiting nutrients such as Nitrogen (Russell et al. 2009, Anderson et al. 2012, Hu et al. 2014). Notably, Argentine ants are dietary generalists and have a relatively depauperate gut microbiota compared to

other ants (Russell et al. 2009, Anderson et al. 2012). However, the intraspecific variation in bacterial diversity we found among colonies in this study may be a common feature in ants (Hu et al. 2014) mandating a need for more correlational and experimental studies to determine assembly rules for community structure in ant microbial communities.

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