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Wolbachia infection alters the relative abundance of resident bacteria in adult *Aedes aegypti* mosquitoes, but not larvae.

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Abstract

Insect-symbiont interactions are known to play key roles in host functions and fitness. The common insect endosymbiont *Wolbachia* can reduce the ability of several human pathogens, including arboviruses and the malaria parasite, to replicate in insect hosts. *Wolbachia* does not naturally infect *Aedes aegypti*, the primary vector of dengue virus, but transinfected *Ae. aegypti* have anti-dengue virus properties and are currently being trialled as a dengue biocontrol strategy. Here, we assess the impact of *Wolbachia* infection of *Ae. aegypti* on the microbiome of wild mosquito populations (adults and larvae) collected from release sites in Cairns, Australia, by profiling the 16S rRNA gene using next generation sequencing. Our data indicate that *Wolbachia* reduces the relative abundance of a large proportion of bacterial taxa in *Ae. aegypti* adults, that is in accordance with the known pathogen-blocking effects of *Wolbachia* on a variety of bacteria and viruses. In adults, several of the most abundant bacterial genera were found to undergo significant shifts in relative abundance. However, the genera showing the greatest changes in relative abundance in *Wolbachia*-infected adults represented a low proportion of the total microbiome. In addition, there was little effect of *Wolbachia* infection on the relative abundance of bacterial taxa in larvae, or on species diversity (accounting for species richness and evenness together) detected in adults or larvae. These results offer insight into the effects of *Wolbachia* on the *Ae. aegypti* microbiome in a native setting, an important consideration for field releases of *Wolbachia* into the population.

Introduction

Interactions between insect hosts and their associated microbiome can have substantial impact on host physiology. Broad changes to the microbiome can affect functions including development, fecundity, metabolism and immunity/susceptibility to pathogens (Coon *et al.* 2014; Gaio Ade *et al.* 2011; Gall *et al.* 2016; Ridley *et al.* 2012). It is also well known that specific symbiotic bacteria of insects can play key roles in host functions (Douglas 2011). Two well-studied systems include the aphids where the

obligate symbiont *Buchnera aphidicola* synthesizes amino acids that are essential for aphid nutrition (Akman Gündüz & Douglas 2009) and the tsetse fly where *Wigglesworthia glossinidia* influences diverse host functions including reproduction, digestion and immunity (Pais *et al.* 2008). More broadly, the overall structure of the microbiome can also be important in the fitness of insects such as bees and mosquitoes, with changes to the composition of the microbiome shown to increase the mortality of bees following exposure to bacterial pathogens (Raymann *et al.* 2017) and to alter mosquito fecundity (Gaio Ade *et al.* 2011; Gendrin *et al.* 2015). It is evident in cases such as these where significant microbiome-host interactions occur, disturbing the insect bacterial population can adversely affect host fitness (Douglas 2011).

Factors influencing symbiotic relationships between microbiota and their invertebrate hosts are highly dynamic and have been well documented over the past decade (Osei-Poku *et al.* 2012; Valiente Moro *et al.* 2013; Wang *et al.* 2011). Most notably, host developmental stage, feeding status, and environmental factors including temperature have been shown to significantly influence symbiotic complexity (Lindh *et al.* 2005; Minard *et al.* 2013; Osei-Poku *et al.* 2012; Ramirez *et al.* 2012; Valiente Moro *et al.* 2013; Wang *et al.* 2011; Wernegreen 2012). Additionally, it is also becoming increasingly clear that resident or intrinsic species can shape the overall diversity of the microbiome. For instance, fleas infected with *Rickettsia felis* show a decrease in species richness in their associated microbiome (Pornwiroon *et al.* 2007) and the presence of *Asaia* species in *Anopheles stephensi* mosquitoes impedes vertical transmission of the endosymbiont *Wolbachia* (Hughes *et al.* 2014).

Wolbachia is a common, maternally inherited, facultative endosymbiont of a wide range of arthropod species. *Wolbachia* have been shown to alter insect reproductive behaviour and can reduce the ability of pathogens, such as the arbovirus dengue virus (DENV) and the malaria parasite, to replicate in hosts (Bian *et al.* 2013; Bian *et al.* 2010; McMeniman *et al.* 2009; Walker *et al.* 2011). *Aedes aegypti*, the primary vector for DENV, is naturally not infected by *Wolbachia*, but has been stably transinfected (McMeniman *et al.* 2009). Following large field trials conducted in Queensland, Australia, *Wolbachia* has invaded the *Ae. aegypti* population to near fixation (Hoffmann *et al.* 2011).

Similar releases are being carried out in a range of DENV endemic regions around the globe as part of a biocontrol initiative (Eliminate Dengue Program; http://www.eliminatedengue.com). Given the large-scale releases of *Wolbachia* into wild populations of *Ae. aegypti* globally, it is of interest to understand whether the presence of the endosymbiont has an impact on the mosquito microbiome. The mosquito microbiome has been reported to affect host development (Coon *et al.* 2014), fecundity (Gaio Ade *et al.* 2011) and vector competence (Bahia *et al.* 2014; Boissière *et al.* 2012; Hegde *et al.* 2015; Ramirez *et al.* 2012). As such, changes in the microbiome could negatively affect mosquito health or the anti-DENV effects of *Wolbachia*, and thereby impact the long-term efficacy of the biocontrol program.

Mosquitoes have been the focus of several studies that elucidated the nature of their associated microbiome (Boissière *et al.* 2012; Dong *et al.* 2009; Gusmão *et al.* 2010; Minard *et al.* 2013; Muturi *et al.* 2017; Pidiyar *et al.* 2004; Rani *et al.* 2009; Valiente Moro *et al.* 2013; Wang *et al.* 2011). These include a recent study that has examined the microbiome of field-collected mosquito species *Culex pipiens* and *Ae. albopictus* that are naturally infected with *Wolbachia* (Muturi *et al.* 2017). However, comparisons across existing studies in an attempt to indirectly determine the potential effect of *Wolbachia* infection in *Ae. aegypti* are likely to only offer limited insight due to differences in geographical location, experimental design and mosquito species. To date, the most direct approach has been to characterise the microbiome of *Ae. aegypti* with and without *Wolbachia* infection using laboratory-reared *Ae. aegypti* stably infected with *Wolbachia* strain *w*Mel (Audsley *et al.* 2017) and laboratory-reared *An. stephensi* stably infected with *Wolbachia* strain *w*AlbB (Chen *et al.* 2016). Both of these studies indicated that *Wolbachia* had little impact on the microbiome, but as laboratory populations have considerably different microbiome compositions than wild mosquitoes (Boissière *et al.* 2012; Coon *et al.* 2014; Ramirez *et al.* 2012), laboratory based studies may not be fully informative. Thus, the potential of *Wolbachia* to affect the microbiome of wild populations of *Ae. aegypti* is still unknown.

In this study, we investigated the effect of *Wolbachia* infection on the microbiome of wild caught mosquitoes from Australia, collected from the 2013 release site of *Wolbachia w*Mel-infected mosquitoes from distinct locations in Cairns, Australia (Fig. S1). Using high throughput sequencing, we compared the microbiome structure of *Wolbachia*-negative and *Wolbachia*-positive *Ae. aegypti* larvae and adults from these sites. Our results show that *Wolbachia* infection significantly alters the relative abundance of resident bacteria in adult, but not larval, *Ae. aegypti*. Notably, however, *Wolbachia* did not affect overall species diversity of the microbiome at either life stage of *Ae. aegypti*.

Materials and methods

Sample collection

To capture both *Wolbachia*-infected and uninfected individuals simultaneously, fourth instar *Ae. aegypti* larval samples were collected from geographically distinct locations in Parramatta Park, Cairns, Australia (Fig. S1). Larvae were collected two weeks post the first release of *Wolbachia*-infected adult mosquitoes in February 2013, and as such, *Wolbachia*-positive larvae collected are first generation in the field. Buckets filled with water and some organic matter were placed in shaded areas and 14 days after placement 4th instar larvae were collected and identified to species by morphology. Adult *Ae. aegypti* mosquitoes were collected from the similar geographical locations as the 4th instar larvae (Fig. S1) using BG-Sentinel traps (Biogents). Adults were collected at 12 weeks post the first release of *Wolbachia* infected mosquitoes, which was 4 weeks post the final release to allow time for any remaining laboratory reared adult release material to have died and ensure that adults collected were at least first generation in the field. Individual larva and adults were placed in separate 3 ml collection tubes filled with 80 % (*v*/*v*) ethanol and stored at -20 °C until use.

DNA extraction & 16S ribosomal RNA gene amplification

DNA from each larval and adult sample was extracted using the DNeasy 96 blood and tissue kit (Qiagen) according to the manufacturer's specifications. Prior to extraction, each sample was washed twice, 5 min per wash in 5 ml 80% ethanol and subsequently rinsed in 5 ml sterile dH_2O. Extracted

DNA was resuspended in 50 μl sterile water, quantified using a spectrophotometer and the concentration adjusted to 5 ng/μl. We subsequently tested each sample for *Wolbachia* infection status and verified that each sample was *Ae. aegypti* using a multiplex TaqMan RT-PCR assay as described in (Caragata *et al.* 2013) using primers and probes specific to the WD513 gene of the *w*Mel *Wolbachia* strain and the *Ae. aegypti rps17* gene (Table S1).

Each distinct geographical site from which larval and adult samples were collected was treated as a biologically independent sample-set. In total we selected 9 sample-sets, each of which consisted of two pair-matched pools (with exception of one set of larvae that were collected from the same street but different sites; see Fig. S1). Each pool contained the DNA from 10 *Wolbachia*-positive larvae or female adult mosquitoes and the other containing the DNA from 10 *Wolbachia*-negative larvae or female adult mosquitoes. When preparing the *Wolbachia*-positive and *Wolbachia*-negative pooled DNA care was taken to ensure the pools contained equivalent amounts of DNA from each represented individual.

To prepare 16S amplicons for Illumina sequencing, we designed primers targeting the conserved 1100 bp – 1392 bp (for larvae) and 515 bp – 806 bp conserved regions (for adults) of bacterial 16S rRNA gene (Table S1). Different primer sets were used for the two developmental stages due to poor amplification from larval samples with primers designed against the 515 bp – 806 bp region. Illumina adaptor sequences that contained between zero - four random nucleotides at the 3' end were added to each primer to facilitate MiSeq sequencing, such that there were a total of four forward and four reverse primers for each region. Each set of four primers was combined in an equal ratio to a final concentration of 10 μM. The respective regions were then amplified using a standard PCR reaction using Phire Hotstart DNA polymerase (ThermoFisher) and 50 ng of DNA from each pool. The resulting amplification products were separated on a 1.2% agarose gel, the bands excised and subsequently recovered from the gel fragments using a QIAquick gel extraction kit (Qiagen) and the DNA dried under vacuum. Separate samples were multiplexed and sequenced using the MiSeq

Illumina platform at the Ramaciotti Centre for Genomics, University of New South Wales, Australia using a 2 x 250 bp sequencing run with a 500 cycle MiSeq reagent kit V2.

Bioinformatics pipeline

A schematic representation of the bioinformatics pipeline we employed is shown in supplementary Fig. S2.

Operational Taxonomic Unit (OTU) picking with QIIME

Primers were removed from sequences, by calling the QIIME script split_libraries.py on the joined fastq files described above, using the following parameters: "-b 0" to disable demultiplexing in the script; "-z truncate_only" to remove reverse primers where found, without altering output where no primer was detected; and "-M 2" to allow two mismatches when matching primers because the primers used were degenerate. Quality control thresholds were set to remove any reads with an average quality score under 25 and any sequences under 200 nucleotides in length. We then used QIIME to pick OTUs (97 % identity threshold) using uclust (Edgar 2010), running the QIIME script pick_open_reference_otus.py with GreenGenes 13_8 as the reference OTU collection (Caporaso *et al.* 2010; DeSantis *et al.* 2006; McDonald *et al.* 2012; Wang *et al.* 2007; Werner *et al.* 2012). Any taxa/reads assigned to *Wolbachia* were subsequently filtered and removed using the filter_taxa_from_otu_table.py QIIME script, with parameter -n g_Wolbachia (Tables S2 and S3). On average *Wolbachia* comprised 8 % of reads in the *Wolbachia*-positive larvae (range 0.1 % - 22 %) and 68 % of reads in *Wolbachia*-positive adults (range 16 % - 85 %). Afterwards, our pipeline summarized the OTUs at the genus level for all *Wolbachia*-negative and all *Wolbachia*-positive samples (separately for larvae and adults) with the QIIME summarize_taxa.py script (Tables S4 and S5).

Calculation of differential relative abundance

For calculation of differential relative abundance, datasets of OTUs summarized at the genus level by QIIME (above) were imported into Phyloseq and filtered to remove genera with fewer than ten reads to avoid low-count taxa confounding the results. Since the historical method of rarefying counts has been shown to be inappropriate for differential abundance calculation (McMurdie & Holmes 2014), differential abundance of genera across *Wolbachi*a-positive and *Wolbachia*-negative samples (for both the adult dataset and larvae dataset) was calculated using the edgeR package (Robinson *et al.* 2010) in conjunction with the Phyloseq package (McMurdie & Holmes 2013, 2014). EdgeR performs robust differential expression analysis of count data from next generation sequencing (NGS) data, using statistical methods based on the negative binomial distribution. Differential abundance was calculated using two separate approaches: 1) with the datasets from the individual collection sites pooled so that all *Wolbachia*-negative larvae or adults were compared to all *Wolbachia*-positive larvae or adults using an exact test; and 2) with changes in relative abundance calculated where the collection sites were included as a factor in the statistical analysis. To include the collection site as a factor, we performed the edgeR differential abundance calculation using a linear model. First, we constructed an edgeR design matrix with collection site as a factor. We fitted this matrix to the data using the glmFit() function and performed the calculation for linear abundance using the glmLRT() function to execute a likelihood ratio test. In both cases, Benjamini-Hochberg correction was used to correct for multiple hypothesis testing (Benjamini & Hochberg 1995).

Correlation between the concentration (log counts-per-million; logCPM) and the differential relative abundance (log fold change; logFC) of genera undergoing significant changes in relative abundance in *Wolbachia*-positive adults used SPSS statistics software (version 22, SPSS Inc, IBM). logCPM and logFC values that were calculated using the linear model to include collection site as a factor of analysis (described above) were used to perform a Pearson's correlation using a two-tailed test.

Calculation of differential species richness/diversity

Phyloseq was also used to estimate species richness and diversity indices using unsummarized taxonomic assignments (raw OTU assignments, 97 % identity threshold) without filtering to remove low count OTUs, using the phyloseq estimate_richness function on the freshly-imported unsummarized QIIME OTU table. Alternate estimates of species diversity were also performed on the unsummarized taxonomic assignments following alpha rarefaction.

Results

Illumina sequencing output.

We examined differential abundance of taxa between *Wolbachia*-negative and *Wolbachia*-positive samples of *Ae. aegypti* larvae and adults by Illumina sequencing, assigning reads to OTUs, and using a statistical model to calculate fold changes in abundance. For the 18 larval samples a total of 16,673,721 pre-filtered reads were obtained. For the 18 adult samples, we obtained 14,226,463 pre-filtered reads. Two raw paired-end fastq files for each sample (read length 250 bp) were joined with fqjoin to produce a fastq file of overlapping reads. The mean read length of the joined larvae fastq files were 304.008, whilst the mean read length of the joined adult fastq files were 288.701.

The microbiome profiles of larval and adult Ae. aegypti collected from Cairns, Australia.

Microbiome profiles of *Wolbachia*-negative and *Wolbachia*-positive adults and larvae were produced from datasets that had been corrected for *Wolbachia* and OTUs were summarized based on classical taxonomy (see materials and methods; Tables S4 and S5). For a broad overview of the microbiome variation across each of the collection sites profiles were constructed using relative abundance of phyla based on datasets summarized by QIIME at the level of phylum (Figs. 1A, 1B, 2A and 2B). Proteobacteria was the clearly dominant phylum, with OTUs belonging to this phylum comprising on average 89.9 % and 81.6 % of the bacteria detected in *Wolbachia*-negative larvae (Fig. 1A) and adults

(Fig. 2A), respectively. The proportion of Proteobacteria in the *Wolbachia*-corrected microbiome profiles of *Wolbachia*-positive larvae and adults were similarly high, comprising an average of 93.1 % (Fig. 1B) and 90.2 % (Fig. 2B), respectively. Thus, neither life-stage nor *Wolbachia*-infection status altered the strong dominance of this phylum.

Only the phyla Proteobacteria, Cyanobacteria and Actinobacteria were detected at a relative abundance of greater than 1 % in the larval samples, irrespective of *Wolbachia*-infection status. In total, OTUs belonging to 30 phyla were detected in the larval samples, but the majority of these were present at low relative abundances of less than 0.1 %. In *Wolbachia*-negative larvae, there were 5 bacterial phyla that were present at greater than 0.1 % mean relative abundance across the 9 collection sites, which were Proteobacteria, Cyanobacteria, Actinobacteria, Firmicutes, and Bacteriodetes (Fig. 1A). *Wolbachia*-positive larvae had a similar profile but with an additional phylum, Spirochaetes, detected at higher than 0.1 % average relative abundance, which was largely due to a higher relative abundance at a single collection site (site LA; Fig. 1B).

Similarly to the larvae, OTUs belonging to 30 phyla were also detected in adult *Ae. aegypti* and few of these phyla represented substantial proportions of the microbiome. We detected 7 classified bacterial phyla in *Wolbachia*-negative adults with a mean relative abundance over 0.1 % across the 9 collection sites, which were Proteobacteria, Cyanobacteria, Firmicutes, Actinobacteria, Bacteriodetes, Spirochaetes and Fusobacteria (Fig. 2A). Although the same 7 phyla also had the highest relative abundance in *Wolbachia*-positive adults (Fig. 2B), there were only 5 phyla with a mean relative abundance greater than 0.1 %, with the Spirochaetes and Fusobacteria reduced in relative abundance.

Wolbachia infection alters the relative abundance of bacterial taxa detected in adult Ae. aegypti.

To examine changes in relative abundance occurring at a higher level of taxonomic classification in *Wolbachia*-positive larvae and adults, we used the phyloseq package in conjunction with edgeR. Datasets that formed the basis of these analyses consisted of OTUs that had been assigned taxonomic

classifications (as described in the materials and methods) and summarized at the level of genus prior to statistical analysis of changes in relative abundance.

For the larval dataset, our analysis to examine relative abundance changes at the taxonomic level of genus compared 534 taxa between *Wolbachia*-positive and *Wolbachia*-negative samples. Since we could detect considerable variability in the relative abundance of some of the most abundant taxa between collection sites (see Supplementary Fig. S3 and S4), we first examined changes in relative abundance of taxa across all *Wolbachia*-negative larvae to all *Wolbachia*-positive larvae whereby the datasets from all collection sites had been pooled. Using this approach there were only 7 of the 534 taxa that were considered significantly altered between the two groups (Fig. 3, Table S6). When incorporating the individual collection sites as a factor in our analysis (see materials and methods for details), only unclassified *Brucellaceae* and unclassified *Chromatiales*, underwent statistically significant changes in relative abundance (Fig. 3, Table S7). Using either analysis approach, taxa found to undergo significant changes each comprised less than 0.01 % of the *Wolbachia*-negative microbiome, with the exception of *Lachnospiraceae* (Fig. 4). None of the 12 most abundant genera (Fig. S3) demonstrated significantly altered relative abundance.

For adult samples, our analysis of differential relative abundance compared 457 taxa. Comparison of all *Wolbachia*-negative adults to all *Wolbachia*-positive adults, where data from the collection sites were pooled, indicated that 277 of the taxa were significantly altered overall between the two groups (Table S8). However, when we included collection site as a factor in our analysis only 64 of these taxa were determined to undergo significant changes in relative abundance in *Wolbachia*-positive adults (Fig. 5, Table S9). Of these 64 genera, *Salinisphaera* was the only genus that had increased in relative abundance, and the only genus undergoing relative fold abundance change of > 5 log in either direction. The genera undergoing largest shifts in relative abundance (*Spironema*, *Campylobacter*, *Brevibacterium* and *Salinisphaera*) each comprise on average less than 1 % of the total microbiome in *Wolbachia*-negative adults (Fig. 6). However, a number of the taxa calculated to undergo smaller significant changes (decreases) in relative abundance are among the most abundant genera detected in

Wolbachia-negative adults, including unclassified *Comamonadaceae*, *Strephtophyta*, and *Streptococcus* (Fig. S4).

To investigate whether changes in relative abundance correlated with changes in absolute abundance we performed quantitative PCR (qPCR) for *Pseudomonas* as a representative taxon (Table S10). The 16S profiling indicated that *Pseudomonas* comprised on average 0.14 % and 0.09 % of the microbiome of *Wolbachia*-negative and *Wolbachia*-positive adults, respectively (Fig. 6). By qPCR *Pseudomonas* was detected above the lower threshold limit of the qPCR assay in 5 out of 7 *Wolbachia*-negative samples, but only 1 out of 7 *Wolbachia* positive samples. These data indicate a reduction in the abundance of *Pseudomonas* in *Wolbachia*-positive adults (Chi-square test, $p = 0.015$) that corresponds with our calculation of differential relative abundance by 16S rRNA gene sequencing and bioinformatics analysis using edgeR (Fig. 5).

To assess whether there was a relationship between the log fold change and the relative concentration of genera showing differential relative abundance, we plotted log fold change against log counts-per-million from the datasets calculated using the linear model to include collection site as a factor (Fig. 7A, B). Genera undergoing significant changes in relative abundance between *Wolbachia*-positive compared with *Wolbachia*-negative samples are shown in red (FDR < 0.05). Of the taxa deemed to undergo significant differential relative abundance in the *Ae. aegypti* adults, there was no correlation of relative sample concentration to logFC (Pearson's coefficient = 0.242, $p = 0.054$). In larval samples a correlation was not calculated, since there were only two genera showing significant changes.

Wolbachia infection does not affect species diversity in Ae. aegypti larvae or adults.

To estimate species richness (total count of species) we used unsummarized taxonomic assignments of OTUs (97 % identity threshold, not summarized any further by classic taxonomy), corrected for *Wolbachia*, without filtering out low count OTUs. Species richness in larvae was not affected by the presence of *Wolbachia* (Table 1. Welch Two Sample t test: $p = 0.44$ for Observed *Wolbachia*-negative vs *Wolbachia*-positive larvae; $p = 0.56$ for Chao 1 *Wolbachia*-negative vs *Wolbachia*-

positive larvae). In contrast, we detected significantly higher species richness in *Wolbachia*-negative adults than *Wolbachia*-positive adults (Table 2. Welch Two Sample t tests: $p < 0.0001$ for Observed *Wolbachia*-negative vs *Wolbachia*-positive adults; $p = 0.00014$ for Chao 1 *Wolbachia*-negative vs *Wolbachia*-positive adults). This may relate to differences in sequencing coverage across the two life stages, with rarefaction analysis suggesting that we have achieved better coverage in larvae compared to adults (Fig. S5). However, Chao 1 estimates based on rarefied data also indicated higher species richness in *Wolbachia*-negative adults than *Wolbachia*-positive adults (Table S11).

To account for both species evenness and species richness, we used the Simpson's and Shannon diversity indices (Shannon 1948; Simpson 1949). In both adult and larval samples, we found no difference in Simpson's (Welch Two Sample t tests: $p = 0.23$ for adults; $p = 0.88$ for larvae) or Shannon (Welch Two Sample t tests: $p = 0.31$ for adults; $p = 0.48$ for larvae) diversity indices between *Wolbachia*-positive and *Wolbachia*-negative samples. Thus, despite an apparent reduction in species richness in the *Wolbachia*-positive adults, we did not detect a difference in the overall microbiome diversity due to the presence of *Wolbachia*.

Discussion

The composition of the mosquito microbiome has been of growing interest due to increasing evidence of its role in vector competence for viral and parasite transmission (Boissière *et al.* 2012; Cirimotich *et al.* 2011; Meister *et al.* 2005; Ramirez *et al.* 2012). *Wolbachia* infection of *Ae. aegypti* is known to decrease vector competence for viruses that significantly impact global health, including DENV, chikungunya virus, and Zika virus (Dutra *et al.* 2016; Moreira *et al.* 2009; van den Hurk *et al.* 2012). As such, the potential of this symbiotic relationship to be exploited as a method to reduce the transmission of arboviruses is currently being explored. Here, we used culture-independent methods to identify the impact of *Wolbachia* infection on the native microbiome of field-collected larvae and adult female *Ae. aegypti*. We found that *Wolbachia* lowers the relative abundance of approximately

14 % of bacterial taxa in adults when compared at the genus taxonomic level, but has no measurable effect on total species diversity at either life stage, when accounting for both species richness and evenness.

In *Anopheles* species it has been established that field-collected adult mosquitoes have limited diversity within their bacterial community, but that the composition can vary between individuals (Boissiere et al. 2012; Osei-Poku et al. 2012). This also appears to be the case for *Aedes* species (Minard *et al.* 2014; Valiente Moro *et al.* 2013). In field-collected *Ae. aegypti*, Ramirez and colleagues (2012) identified 34 cultivatable species belonging to four phyla in the midgut, with Proteobacteria the dominant phylum. Our study using culture-independent techniques identified genera belonging to 28 phyla detected above our lower threshold for low-count reads, but we similarly identified Proteobacteria as the dominant phylum. Despite variability in microbiome composition across species and individuals, numerous genera identified in our study have also been detected in other studies. These include by culture-dependent methods in field-collected mosquitoes (e.g. *Micrococcus*, *Staphylococcus*, *Comomonas*, *Acinetobacter*, *Enterobacter*, *Pseudomonas*), and by culture-independent methods in 'semi-field' conditions (e.g. *Novosphingobium*, *Elizabethkingia*, *Pseudomonas*, *Corynebacterium*) and laboratory-reared mosquitoes (e.g. *Elizabethkingia*, *Sphingobium*, *Chryseobacterium*, *Enterobacter*, *Comomonas*, *Acinetobacter*, *Fusobacterium*) (Audsley *et al.* 2017; Chen *et al.* 2016; Ramirez *et al.* 2012; Wang *et al.* 2011), adding positive support for our findings.

At the level of phylum, the microbiome profiles of adult *Ae. aegypti* were similar between collection sites, but at higher taxonomic classifications there was high variability between *Wolbachia*-negative samples and when determining the effect of *Wolbachia*. This was the case for even the most abundant taxa, where it could be seen that the magnitude and direction of *Wolbachia's* effect on some genera were variable. Since individual mosquitoes/larvae were pooled in our study to create one sequencing library per collection site for each *Wolbachia*-positive and *Wolbachia*-negative samples, within-site replication would be important for understanding whether *Wolbachia* could have different effects

across sites. Such differences may be accounted for by factors including host genetic background, mosquito age or environmental factors that are not controlled for within this study. The effect of *Wolbachia* on the abundance of 64 genera, however, was consistent regardless of collection site. This group includes some of the most abundant genera, belonging to a variety of phyla: Proteobacteria, Cyanobacteria, Firmicutes, Actinobacteria, and Bacteriodetes.

One consideration when assessing changes to the native microbiota in response to *Wolbachia*, is their potential involvement in the expression of 'pathogen-blocking'. When collection site was included as a factor, 63 genera were significantly decreased in relative abundance and only a single genus was increased in relative abundance in *Wolbachia*-positive adults. However, a greater number of genera were identified that were increased in relative abundance when all collection sites were pooled, including *Vagococcus, Salinisphaera* and *Achromobacter* that underwent a fold change of greater than 5 log (Table S8). To our knowledge, only a few of the taxa that were altered in either direction in *Wolbachia*-positive adults have been examined in the literature with respect to viral interactions in mosquitoes. *Pseudomonas* and *Comamonas,* which were reduced in relative abundance, have been shown previously to have no effect on DENV titres in mosquitoes in artificial feeding experiments (Ramirez *et al.* 2012). *Vagococcus,* which was increased in the pooled analysis, has been previously identified in *Wolbachia*-positive insects (Palavesam *et al.* 2012) and *Vagococcus salmoninarium* isolated from *Ae. albopictus* has been shown to directly reduce titres of La Crosse virus by 40 % *in vitro,* indicating it may have antiviral activity (Joyce *et al.* 2011). Although our study did not quantify changes in absolute abundance of taxa, it is it is conceivable that *Vagococcus* could contribute to 'pathogen-blocking' in *Wolbachia*-positive *Ae. aegypti* in the field. The antiviral effect reported in *Wolbachia*-positive *Ae. aegypti* appears stronger than that of *Vagococcus in vitro* (Dutra *et al.* 2016; Joyce *et al.* 2011; Walker *et al.* 2011) but future studies may consider testing for the abundance of *Vagococcus* species in *Wolbachia*-positive adults.

Despite the fact that *Wolbachia* are vertically transmitted, our results indicate that *Wolbachia* infection has minimal effect on the larval microbiome, which is thought to largely be acquired from their surrounding environment (Coon *et al.* 2014). Recent work has revealed that while *Ae. aegypti* require live bacteria for development, they do not rely on particular species (Coon *et al.* 2016). The authors suggest that the variable nature of aquatic environments would prevent mosquitoes from reliably encountering particular species. In field-collected *Ae. aegypti* larvae and water from their collection sites, Coon *et. al.* (2016) detected 28 taxonomic orders present at greater than 2 % relative abundance. In our field-collected larval samples from geographically distinct locations in Cairns, Australia, we detected species from 22 phyla that had sequence counts above our lower limit for abundance comparisons (when OTUs were summarized at genus taxonomic level), and 26 orders identified from these phyla overlap with those identified by Coon *et al.* (2016). However, in our study only taxa from one phylum were considered significantly altered relative abundance between the two groups when we include collection site as a factor of our analysis.

One possible explanation for that the lesser impact of *Wolbachia* on the larval microbiome in comparison to the adult microbiome is the lower relative abundance of *Wolbachia* in larvae, such that competition for resources may be a greater factor in *Wolbachia*-positive adults than *Wolbachia*-positive larvae. However, it is notable that the reduced relative abundance of a large proportion of taxa in *Wolbachia*-positive adults is in keeping with the broad bacterial protection effects of *Wolbachia* (Ye *et al.* 2013), and thus may be related to upregulation or 'priming' of immune pathways such as Toll and IMD (Rancès *et al.* 2012). While there is evidence to suggest that *Wolbachia* upregulates expression of immune gene in *Drosophila* larvae (Zheng *et al.* 2011), it is unclear if *Wolbachia* plays a role in pathogen protection in *Ae. aegypti* during their larval stage. Therefore, it is also possible that life stage-specific differences in immune responses and/or priming, may contribute to the reduced impact of *Wolbachia* on the relative abundance of other taxa in larvae compared with adults.

A previous study of *An. gambiae* reared in "semi-natural" habitats found larvae and pupae to have higher diversity than adults (Wang *et al.* 2011). Comparison of Chao1 and ACE species richness estimates from our study comparing all adults with all larvae (without accounting for *Wolbachia* infection status) indicate that field-collected *Ae. aegypti* larvae had greater species richness than adults, while the reverse was true for diversity estimates (Simpon's and Shannon), which account for both richness and evenness. However, there are several caveats to our study that may influence our estimates of species richness and evenness differentially across the two life stages. *First*, it is possible that the artificial breeding sites used to sample the larvae may have influenced/limited their microbiome diversity. *Second*, the use of different primer sets for amplifying the 16S rRNA gene for adults compared to larvae could potentially introduce different bias into the microbiota detected, making direct comparisons between the life stages problematic. *Third*, species richness estimates in *Wolbachia*-positive adults may be affected by the greater dominance of *Wolbachia*, potentially limiting the lower abundance taxa sampled by NGS. Nevertheless, our data indicate that *Wolbachia* infection status had no significant effect on species diversity in either adults or larvae, when comparing between *Wolbachia*-positive and *Wolbachia*-negative samples from a single life stage.

Several bacteria have been found previously to negatively affect *Wolbachia* density or transmission in naturally infected insect hosts. These include *Spiroplasma* (*Drosophila melanogaster*) (Goto *et al.* 2006), *Asaia* (*Anopheles stephensi*) (Hughes *et al.* 2014; Rossi *et al.* 2015), and co-infection with alternate species of *Wolbachia* (*Callosobruchus chinensis*) (Kondo *et al.* 2005). *Ae. aegypti* are not naturally infected with *Wolbachia* species and we did not detect any sequences that could be assigned at the genus or family taxonomic level to *Spiroplasma* or *Asaia* in our abundance OTU comparisons for adults or larvae. Thus it is plausible that these known "*Wolbachia*-incompatible" microbiota are not an impediment to *Wolbachia* infection/transmission of *Ae. aegypti* in the geographical locations tested in our study.

Taken together, our results indicate that *Wolbachia* does not substantially alter the diversity of the microbiota in mosquitoes and has the largest effects on the relative abundance of taxa that comprise a small proportion of the adult *Ae. aegypti* microbiome. These data therefore do not offer obvious microbial partners that may be assisting with the expression of pathogen blocking. Regardless, the potential involvement of the low frequency genera such as *Vagococcus* should be further examined experimentally. Perhaps more importantly, however, the limited effects on native microbiome diversity suggest a low potential for *Wolbachia* to impact host fitness in this capacity. This is especially pertinent in the context of using *Wolbachia*-infected *Ae. aegypti* as a biocontrol strategy against DENV, which relies on vector health for success.

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Data accessibility

Raw Illumina sequencing reads are available at MG-RAST under project ID mgp4380 (http://metagenomics.anl.gov/mgmain.html?mgpage=project&project=mgp4380). QIIME and phyloseq/edgeR analyses are available from the Dryad Digital Repository doi:10.5061/dryad.37m5c (https://doi.org/10.5061/dryad.37m5c).

Author contributions

DAJ, SLO and EAM designed the study. MDA and DAJ carried out the work. AS, MDA, MW and DAJ analysed the data. All authors contributed to the writing of the manuscript.

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Legends

Table 1. Species richness and diversity in *Ae. aegypti* larvae

Table 2. Species richness and diversity in adult female *Ae. aegypti*

Fig. 1 Bacterial 16S rRNA gene profiles for *Wolbachia*-negative and *Wolbachia*-positive *Ae. aegypti* larvae collected from Cairns, Australia. Microbiome profiles for (A) *Wolbachia*-negative larvae and (B) *Wolbachia*-positive larvae. The 10 bacterial phyla with the highest mean relative abundance are displayed, with all remaining bacterial phyla shown as a single group (sum of low abundance phyla). Legends are ordered from lowest to highest mean relative abundance for each group. Each column represents an individual collection site with letter codes corresponding to the collection sites indicated on the map in Fig. S1.

Fig. 2 Bacterial 16S rRNA gene profiles for *Wolbachia*-negative and *Wolbachia*-positive *Ae. aegypti* adult females collected from Cairns, Australia. Microbiome profiles for (A) *Wolbachia*-negative adults and (B) *Wolbachia*-positive adults. Details are as described in the legend for Fig. 1.

Fig. 3 Bacterial taxa undergoing significant changes in relative abundance in *Wolbachia*-positive *Ae. aegypti* larvae. *Wolbachia*-corrected OTU datasets summarized at the genus level were used for comparisons of relative abundance, calculated as log fold change. A negative value denotes greater abundance in the *Wolbachia*-negative dataset as compared to the *Wolbachia*-positive dataset. Taxa that demonstrated significant differential abundance in *Wolbachia*-positive larvae calculated by comparing all *Wolbachia*-negative samples to all *Wolbachia*-positive samples (data from collection

sites pooled prior to analysis) are shown coloured by phylum. Open circles indicate that the taxon also demonstrated significant differential abundance in *Wolbachia*-positive larvae calculated using a linear model to include collection site as a factor. A p value < 0.05 after applying Benjamini-Hochberg correction for multiple hypothesis testing (false discovery rate; FDR) was considered significant. Left Y-axis indicates the genus undergoing differential relative abundance. *Where no genus could be assigned the highest classification available is listed; "other" indicates that the taxonomic level following that listed was an ambiguous assignment.

Fig. 4 Relative abundance of bacterial taxa undergoing significant changes in *Wolbachia*-positive larvae. Taxa that were shown to undergo significant changes in relative abundance in *Wolbachia*-positive larvae (Fig. 3) are plotted to show relative abundance as a fraction of the total microbiome. Data points show the relative abundance of the taxa for each collection site, with columns indicating the mean relative abundance across all sites ± standard error of the mean (SEM).

Fig. 5 *Wolbachia* infection is associated with altered relative abundance of bacterial taxa detected in *Ae. aegypti* adults. *Wolbachia*-corrected OTU datasets summarized at the genus level were used for comparisons of relative abundance, calculated as log fold change. A negative value denotes greater abundance in the *Wolbachia*-negative dataset as compared to the *Wolbachia*-positive dataset. Taxa that demonstrated significant differential abundance in *Wolbachia*-positive adults calculated using a linear model to include collection site as a factor are shown coloured by phylum. Other details are as described in the legend for Fig. 3; blank categories on the right Y-axis indicate that no order was assigned.

Fig. 6 Relative abundance of bacterial genera of interest undergoing significant changes in *Wolbachia*-positive adults. Genera of interest that were shown to undergo significant changes in relative abundance in *Wolbachia*-positive adults (Fig. 5) are plotted to show relative abundance as a fraction of the total microbiome. Data points show the relative abundance of the genera for each collection site, with columns indicating the mean relative abundance across all sites ± SEM.

Fig. 7 Correlation of relative concentration of taxa with differential relative abundance between *Wolbachia*-negative and *Wolbachia*-positive samples. Change in taxa relative abundance in *Wolbachia*-infected *Ae. aegypti* (log fold change) is shown against relative concentration (log counts per million) for larval (A) and adult (B) samples. Data points indicated in red are those calculated to undergo significant changes (FDR < 0.05) in relative abundance using a linear model to include collection site as a factor.

Table 1. Species richness and diversity in *Ae. aegypti* larvae

Wolbachia status	Observed	Chao1	se.chao1	ACE	se.ACE	Shannon	Simpson	InvSimpson	Fisher
Negative (site LH)	3108	4768.262	124.85	4903.274	40.295	3.644	0.915	11.72	484.86
Negative (site LC)	2527	2839.557	32.503	3036.075	26.245	1.645	0.63	2.706	342.577
Negative (site LJ)	2038	3068.605	98.421	3151.122	31.471	2.889	0.818	5.49	281.413
Negative (site LK)	2428	3229.366	73.989	3380.3	31.245	2.098	0.711	3.462	314.335
Negative (site LG)	2721	3790.639	87.754	4002.416	35.135	3.309	0.898	9.845	400.443
Negative (site LF)	1458	2340.291	101.861	2321.815	27.228	1.796	0.524	2.102	189.447
Negative (site LE)	1555	2428.918	98.126	2331.69	25.969	2.125	0.555	2.25	235.808
Negative (site LA)	2637	4931	177.933	5064.228	45.337	3.446	0.834	6.031	454.935
Negative (site LB)	3147	4303.637	87.237	4565.92	37.817	2.12	0.661	2.949	441.897
Positive (site LG)	3955	5956.534	131.047	6308.151	46.07	3.468	0.905	10.533	568.633
Positive (site LA)	8422	9383.046	57.204	9944.87	47.152	4.492	0.935	15.429	1336.425
Positive (site LJ)	2199	3492.36	114.433	3604.891	35.078	3.1	0.773	4.4	357.849
Positive (site LI)	1674	2782.095	115.195	2759.085	30.06	1.917	0.559	2.27	223.198
Positive (site LB)	2932	4345.85	106.404	4628.758	40.411	2.204	0.639	2.767	440.144
Positive (site LH)	2379	4257.367	152.042	4500.417	41.6	3.394	0.887	8.822	391.251
Positive (site LK)	2693	3842.187	96.05	4088.093	36.573	2.169	0.664	2.98	352.182
Positive (site LC)	2552	4202.602	131.837	4494.628	40.263	2.916	0.783	4.612	382.035
Positive (site LF)	1061	1834.52	98.975	1798.541	24.424	1.918	0.545	2.197	163.684

Table 2. Species richness and diversity in adult female *Ae. aegypti*

Wolbachia status	Observed	Chao1	se.chao1	ACE	se.ACE	Shannon	Simpson	InvSimpson	Fisher
Negative (site AA)	2082	2881.053	75.128	3107.891	31.937	2.157	0.716	3.52	271.724
Negative (site AB)	2408	910.923	54.543	922.951	17.073	3.501	0.909	10.975	117.776
Negative (site AC)	3764	3713.038	110.642	3974.964	37.621	2.479	0.762	4.207	334.32
Negative (site AG)	1568	5591.78	123.721	6091.43	46.88	3.548	0.879	8.265	549.5
Negative (site AH)	3073	3126.642	164.973	3201.289	35.958	3.208	0.861	7.179	237.115
Negative (site AF)	2844	4772.653	122.276	5341.325	46.342	3.08	0.852	6.775	453.085
Negative (site AJ)	4664	1712.944	70.114	1751.413	22.798	3.754	0.931	14.528	205.824
Negative (site AE)	1820	1017.296	71.129	996.185	17.874	2.936	0.854	6.851	105.711
Negative (site AI)	2697	1311	85.452	1353.817	22.209	2.217	0.735	3.767	117.903
Positive (site AA)	601	954.75	84.051	911.31	18.156	3.006	0.862	7.224	93.931
Positive (site AJ)	1166	4621.188	136.344	4884.205	41.905	3.054	0.855	6.892	386.873
Positive (site AC)	608	6271.04	99.946	6919.329	47.986	3.703	0.902	10.219	675.524
Positive (site AG)	739	934.452	73.64	937.639	17.931	3.272	0.868	7.598	105.61
Positive (site AF)	512	1136.028	25.762	1198.263	17.159	0.903	0.259	1.35	122.347
Positive (site AI)	525	3227.441	139.219	3405.328	35.773	3.342	0.88	8.312	279.34
Positive (site AB)	977	853.548	74.442	844.925	17.574	2.811	0.812	5.308	87.279
Positive (site AE)	465	1062.221	48.945	1138.463	19.225	1.74	0.55	2.222	112.475
Positive (site AH)	744	4714.533	151.867	5321.212	47.833	2.823	0.798	4.953	390.202