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# Bed bugs shape the indoor microbial community composition of infested homes

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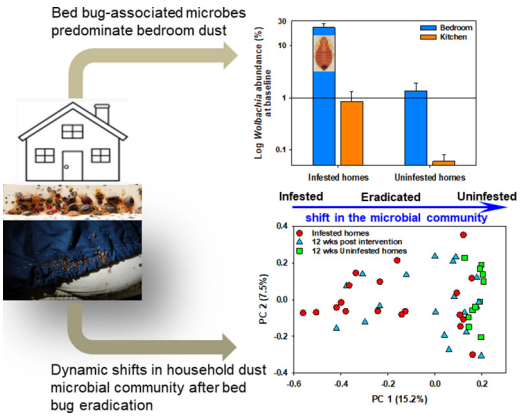
## HIGHLIGHTS

- Chronic bed bug infestations influence the microbial community associated with household dust.
- The microbiome of bed bug-infested homes is significantly different from uninfested homes, particularly in bedrooms.
- Eradication of bed bugs from infested homes shifted the microbial community towards the composition of uninfested homes.
- Further studies on the health risks associated with bed bugs and the microbial contaminants they deposit are warranted.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Indoor pests, and the allergens they produce, adversely affect human health. Surprisingly, however, their effects on indoor microbial communities have not been assessed. Bed bug (*Cimex lectularius*) infestations pose severe challenges in elderly and low-income housing. They void large amounts of liquid feces into the home environment, which might alter the indoor microbial community composition. In this study, using bed bug-infested and uninfested homes, we showed a strong impact of bed bug infestations on the indoor microbial diversity. Floor dust samples were collected from uninfested and bed bug-infested homes and their microbiomes were analyzed before and after heat interventions that eliminated bed bugs. The microbial communities of bed bug-infested homes were radically different from those of uninfested homes, and the bed bug endosymbiont *Wolbachia* was the major driver of this difference. After bed bugs were eliminated, the microbial community gradually shifted toward the community composition of uninfested homes, strongly implicating bed bugs in shaping the dust-associated environmental microbiome. Further studies are needed to understand the viability of these microbial communities and the potential risks that bed bug-associated microbes and their metabolites pose to human health.



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## 1. Introduction

Exposure to environmental microorganisms and their cellular components in the indoor environment has been linked to both positive and negative impacts on human health (Norbäck and Cai, 2020). Although exposure to certain environmental microbes in childhood was shown to have protective effects on children, exposure to potential pathogens, allergens and microbial metabolites adversely affects human health (Caillaud et al., 2018; Dannemiller, 2019; Mendell et al., 2011). It is essential to understand the factors that shape the indoor microbiota because (a) indoor microbes can affect indoor environmental quality, (b) people in developed countries spend >90% of their time indoors, and (c) air quality indoors plays critical roles in human health.

The microbial composition of built environments is influenced by a combination of biotic and abiotic factors, including the occupants (humans, pets, plants), temperature, humidity, and connectivity with the outdoor environment (Dunn et al., 2013; Hospodsky et al., 2012; Kettleson et al., 2015; Meadow et al., 2014). While indoor fungal communities appear to be influenced mainly by the outdoor environment and dampness, the bacterial communities are often associated with indoor biotic sources (Adams et al., 2013; Kettleson et al., 2015; Stephens, 2016; Tringe et al., 2008; Weikl et al., 2016). Along with the humans, pets, and plants, arthropods contribute to the biotic diversity of households (Bertone et al., 2016). However, most of the insect taxa examined to date have been scarce individuals of outdoors species that strayed indoors. The impacts of pests that establish chronic infestations indoors on the residential microbial composition have not been investigated. Two major obligate commensals of humans – cockroaches and bed bugs – can proliferate into enormous infestations and therefore potentially could shape the indoor microbiome. Kakumanu et al. (2018) showed that lab-reared and field-collected German cockroaches (*Blattella germanica* L.) had distinct microbiota, but their effects on the home microbiome have yet to be investigated.

The common bed bug (*Cimex lectularius* L.) is an obligate blood feeding insect that shelters and proliferates indoors in dark spaces such as on mattresses, linen, cracks and crevices of bed frames, furniture and walls (Delaunay et al., 2011). Bed bugs had almost disappeared in developed countries in the 1950s due to the extensive use of organochlorine insecticides indoors (Davies et al., 2012; Harlan, 2006), but they have resurged over the past two decades on a global scale, broadly protected by resistance to modern pyrethroid and neonicotinoid insecticides (Anderson and Leffler, 2008; Bernardeschi et al., 2013; Boase, 2001; Doggett et al., 2004; Krueger, 2000; Lee et al., 2008; Masetti and Bruschi, 2007). Bed bug infestations have become common in residences, hospitals, the hospitality industry and public transportation. Their rapid spread and establishment is particularly troubling in low-income communities, where apartments are inter-connected, re-infestations are frequent, and resources are limited for effective pest interventions (Wang et al., 2010).

Although bed bugs have not been established as vectors of any human pathogens, they are competent vectors of *Trypanosoma cruzi* and *Bartonella quintana*, the causative agents of Chagas disease and trench fever, respectively (Leulmi et al., 2015; Salazar et al., 2015). Bed bug infestations are associated with severe anemia in residents (Pritchard and Hwang, 2009), they produce copious amounts of environmental contaminants (e.g., histamine) (DeVries et al., 2018), and they adversely affect the quality of life through allergic responses to bites, secondary infections, sleeplessness, anxiety and ostracism (Goddard and de Shazo, 2012; Hwang et al., 2005; Reinhardt and Siva-Jothy, 2007; Romero et al., 2007; Susser et al., 2012).

An adult female bed bug can produce about 200–500 eggs in her lifetime, about 1–2 eggs each day as long as blood meals are readily available. The life cycle of *C. lectularius* consists of five nymphal stages before an adult emerges, and all mobile life stages obligately feed on the host. Each blood meal enables nymphs to grow and molt to the next stage every 3–7 days and enables females to lay eggs for about 10 days (Matos et al., 2017; Usinger, 1966). Short developmental stages, frequent blood feeding, high fecundity, cryptic habits, and high levels of resistance to modern insecticides result in exponential growth of bed bug populations (Delaunay et al., 2011; Harlan, 2010).

*Cimex lectularius* harbors several bacterial associates, including two transovarially transmitted endosymbionts, an obligate *Wolbachia* and an unclassified *Gamma-proteobacteria* (Chang and Musgrave, 1973; Meriweather et al., 2013), which are essential for host nutrition, reproduction, and survival (Hosokawa et al., 2010). Along with the endosymbionts, other bacteria were identified in bed bugs, including *Staphylococcus arlettae*, *Staphylococcus epidermidis*, *Micrococcus* and *Kocuria kristinae* (Cockburn et al., 2013). These bacteria are acquired either from the environment or sexually transmitted during a hemocoelic "traumatic" insemination (Bellinvia et al., 2020; Reinhardt et al., 2005). These microbes are propagated in the host insect and potentially could become an integral part of the indoor environment as bed bugs defecate, molt and die.

Bed bug infestations can range from just a few individuals to thousands of insects within a single residence. Large populations result in the recurrent deposition of moisture (from blood meals) and organic metabolites in the indoor environment in the form of feces, exuviae (shed cuticles) and dead bugs. This process not only dependably adds pest-associated microbes and microbial components and metabolites to the indoor environment but the excessive moisture and organic material is expected to support the proliferation of other microbes, with potentially detrimental effects on residents.

While the consequences of the indoor microbiome on human health are reasonably well established, the role of perennial pest populations in shaping the indoor microbial diversity and abundance and its health implications have not been investigated. As part of our long-term goal to understand the potential health risks associated with heavy indoor pest infestations and to develop and validate effective interventions, we aimed to characterize the microbiome of bed bug-infested homes. We set out to answer the following questions: 1) What microbes are associated with bed bugs? 2) Do bed bug infestations shape the bacterial communities in the indoor home environment? 3) Since bed bugs are more common in bedrooms and living rooms than in kitchens, do they differential affect the respective room microbiota? 4) How does the elimination of bed bug infestations affect the home microbiome?

## 2. Methods

### 2.1. Ethics statement

The North Carolina State University Institutional Review Board (IRB) approved this study (#3840). Before participation, adults (>21 years old) provided informed consent.

### 2.2. Site selection, interventions and sampling

Detailed descriptions of the site, experimental design, sampling procedures and interventions were reported in DeVries et al. (2018). Briefly, a 140 unit multi-story apartment building in Raleigh, North Carolina, USA has been persistently infested with bed bugs for several years despite recurrent pest control interventions. Individual apartments in this building were initially surveyed for the presence of bed bugs by visual inspections and bed bugs were subsequently sampled with traps (ClimbUp Interceptor, Susan McKnight Inc., Memphis, TN). Based on the presence or absence of bed bugs, apartments were grouped into infested (bed bugs detected: 19 apartments) and uninfested (no bed bugs detected: 11 apartments) homes (Fig. 1). The 19 infested apartments were further divided into two treatment groups: infested-treated (intervention immediately after the baseline sampling; 7 apartments) and infested-controls (infested homes sampled for one month, then interventions implemented; 12 apartments). Two floor dust samples were obtained from each home immediately after recruitment

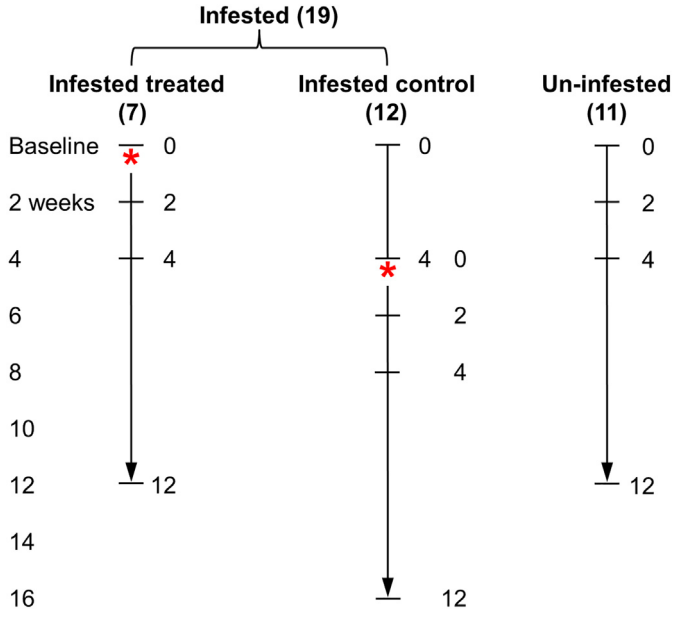


**Fig. 1.** Experimental design and the sampling plan of the study. The timeline is indicated on the left. Sampling times are indicated by horizontal lines. Interventions (spatial heat treatment) are indicated by *. Infested-control homes were sampled for 4 weeks, and then crossed-over to infested-treated homes and sampled for 12 weeks.

(considered the baseline sampling), one from the bedroom or living room and one from the kitchen.

*2.2.1. Heat-treatment of infested homes*

The 7 homes in the infested-treated group were heat-treated soon after the baseline sampling and dust was sampled recurrently at 2, 4, and 12 weeks after the intervention. The 12 infested-control homes were sampled 4 weeks after the baseline sampling and no interventions were done in these homes during this period. However, because all homes remained occupied throughout this study, ethical considerations limited the infested-control group to only 4 weeks. After the 4-week dust sampling, the 12 infested-control homes were crossed-over to the infested-treated arm of the project and heat-treated. Dust was sampled in these homes 2, 4, and 12 weeks after the intervention, as in other infested-treated homes. Thus, 19 infested apartments were included in the infested-treated group and received heat intervention – 7 infested homes treated at the start of the project and 12 infested homes treated 4 weeks later.

Heat treatments were conducted by a professional pest control company and consisted of raising the ambient temperature to ~50 °C for >4 h while fans circulated air throughout the apartment (Cooper, 2011). Residual insecticide sprays and dusts were also applied by the pest control technician to bed bug sheltering sites. Only apartments where bed bugs were not detected at all sampling times post-intervention were included in the infested-treated arm of the study.

*2.2.2. Uninfested homes*

The uninfested homes did not receive heat interventions (see Study limitations). However, bedroom dust samples were collected from these homes 2, 4 and 12 weeks after the baseline sampling, as in infested homes.

*2.3. Collection of dust samples and bed bugs*

Dust samples were collected from an area with the highest concentration of bed bugs in either the bedroom or living room. An area of the floor (3 m × 15 cm) near a wall and behind a bed or couch was sampled for 2 min using a Eureka Mighty-Mite 9.0-ampere vacuum cleaner (Eureka Company, Bloomington, IL) fitted with a Dustream® collector and filter (40 μm, Indoor Biotechnologies Inc., Charlottesville, VA). At baseline, a dust sample was also collected from the kitchen of each apartment. Samples were placed into glass vials and stored at −80 °C. Each dust sample was sieved (450 μm) to remove large particulates, and the fine dust was homogenized and used for DNA extraction.

Bed bugs were sampled with traps left in place for two weeks. ClimbUp Interceptor traps have been shown to be effective at detecting even low-level bed bug infestations (Wang et al., 2011). Traps were used to indicate the presence or absence of bed bugs, direct our dust sampling efforts, and assess the efficacy of the intervention in each home. Bed bugs collected in the traps were placed in ethanol in 2 ml collection tubes and stored at −30 °C until further use.

*2.4. DNA extraction from bed bugs*

DNA was extracted from bed bugs using the Puregene DNA extraction kit (Qiagen, Valencia, CA). Briefly, bed bugs were removed from collection tubes and ethanol was evaporated. Each individual bed bug was placed in a microcentrifuge tube, frozen in liquid nitrogen and ground thoroughly. The homogenates were incubated at 55 °C in cell lysis buffer and proteinase K for 5 h followed by ammonium acetate to precipitate proteins. DNA was pelleted out with isopropanol, washed with ethanol and resuspended in 50 μl TE buffer. DNA quality and concentration from each specimen were determined using the NanoDrop 1000 spectrophotometer (Thermo Fisher, Wilmington, DE). Equal volumes of DNA from 5 individual bed bugs from the same apartment were pooled and considered as one sample for sequencing and microbiome analysis.

Since DNA was extracted from ethanol-stored bed bugs without surface sterilization, we validated the procedure using additional individual bed bugs. Six bed bugs, two each from three different homes, were treated as follows: Three of the six bed bugs were surface sterilized with bleach, 70% ethanol and three washes of autoclave-sterilized water, whereas the other three bed bugs were directly taken from ethanol and used for DNA extraction without surface sterilization. Bed bugs were homogenized and DNA extracted using the Blood and Tissue DNA extraction kit (Qiagen, Cat: 69504). The homogenized bed bugs were initially incubated at 56 °C for ~5 h in lysis buffer containing proteinase K, and the rest of the procedure was as per manufacturer's protocol and eluted in 100 μl of nuclease-free sterile water.

*2.5. DNA extraction from dust samples*

The DNeasy Blood and Tissue DNA extraction kit was used for DNA extraction of dust samples. Approximately 10 mg of homogenized dust was incubated at 56 °C for ~2 h in lysis buffer 1 (proteinase K) and the rest of the procedure was as per manufacturer's protocol. The DNA was eluted in sterile nuclease-free water, quantified (Nanodrop 1000) and stored at −20 °C until further use. All the DNA samples were initially tested for PCR inhibitors by amplifying a fragment of the 16S rRNA gene using universal primers, 27F (5′-AGAGTT TGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). Any problematic samples or samples with low DNA were re-extracted.

*2.6. NGS library preparation and sequencing*

A total of 162 dust samples, 19 pooled bed bug samples and 6 individual bed bug samples were analyzed for microbial diversity. A 2 μl aliquot of genomic DNA isolated from individual dust samples or pooled bed bug samples was utilized to construct a sequencing library by the dual-index method (Kozich et al., 2013) targeting the variable V3 and V4 regions of the 16S rRNA gene. In brief, samples were amplified in duplicate according to the Illumina protocol, along with extraction and PCR controls. Once the negative controls were confirmed free of contamination, the two replicates of each

sample were combined and purified using AMPure beads (Cat. No: A63881, Agilent, Santa Clara, CA) per manufacturer's protocol. The samples were tested for the presence of the correct size bands on agarose gels followed by 8 cycle index PCR for multiplexing. The purified index-PCR product was quantified on QUBIT (Fisher) and the samples were normalized to 4 nM and pooled for sequencing. 16S libraries from 171 samples were sequenced at the Genomic Sciences Laboratory at North Carolina State University in three runs on the Illumina MiSeq platform (San Diego, CA) sequencing with v3 chemistry and 2 * 300 paired-end reads. Sequencing data from this study were deposited in the NCBI Sequence Read Archive (SRA) (BioProject ID: PRJNA612706). Weblink: https://dataview.ncbi.nlm.nih.gov/object/PRJNA612706?reviewer=d4hsummpijvl29p7m0kq1qdc8c.

### 2.7. Data analysis

Demultiplexed R1 and R2 sequencing reads files acquired from the sequencing facility were processed using the QIIME pipeline Version 1.9.1 (Caporaso et al., 2010). The demultiplexed reads were joined by sample and the sequences were taken through chimera checking. All the dust samples from three runs were merged and samples were clustered into operational taxonomic units (OTUs) at 97% similarity and taxonomic assignment of OTUs was done using the GreenGenes reference database v13.8 (DeSantis et al., 2006). OTUs that were observed <2 times, i.e., singletons, and OTUs that were identified as chloroplast were filtered from the dataset.

### 2.8. Bioinformatics of 16S sequences

Diversity analysis of dust samples was performed on a rarefied OTU table at a sampling depth of 21,000 reads/sample. Beta-diversity of the bacterial communities was calculated using Bray-Curtis, weighted and unweighted Unifrac methods (Lozupone and Knight, 2005). Principal coordinate analyses (PCoA) were conducted and diversity patterns were visualized using Emperor tools. Statistical comparisons between treatments were conducted using MRPP, adonis (Anderson, 2001) and Analysis of Similarity (ANOSIM) (Clarke, 1993). Rarefaction curves for OTUs, species richness, PD_Whole tree, Shannon diversity, and Chao1 were calculated for alpha-diversity (Hill et al., 2003) and number of observed species was used as metrics to plot alpha-rarefaction curves. Relative abundances of bacterial orders from different treatments (Infested vs Uninfested; Baseline vs 12 weeks after intervention) were compared using Mann-Whitney-*U* tests followed by Benjamini-Hochberg correction ($q$ value < 0.05) in the QIIME pipeline.

### 2.9. Quantitative PCR for determining bacterial loads

The absolute abundance of total bacteria in 162 dust samples from bed bug-infested and uninfested apartments were analyzed by quantitative PCR using 338F (ACTCCTACGGGAGGCAGCAG) and 518R (ATTACCGCGGCTGCTGG) primers (Fierer et al., 2005). Quantitative PCR assays were carried out on a CFX384 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and analysis was replicated twice. PCR reactions were conducted in 15 μl reaction mix containing 0.5 μM (each) gene-specific primers, 7.5 μl of SsoAdvanced SYBR Green supermix (Bio-Rad, USA), 1.5 μl of DNA template, and nuclease-free water to make up the 15-μl volume. The qPCR conditions were 3 min at 95 °C for initial activation, 40 cycles of 15 s at 95 °C for denaturation, 15 s at 55 °C for annealing, and 30 s at 72 °C, followed by a melt curve from 65 °C to 95 °C, with an increment of 0.5 °C. Positive (gDNA from *E. coli*) and negative (no DNA template) controls were included in all qPCR runs and the total copy numbers of 16S rRNA were calculated by using a standard curve generated by plasmid DNA with 16S rDNA insert (Kakumanu et al., 2018).

## 3. Results

### 3.1. Bed bug and dust samples

Bed bug DNA samples (5 bed bugs pooled per apartment) from 19 infested apartments were analyzed for the bed bug microbiome. Approximately 2.82 million total reads (156,710 ± 34,635 (SD) per sample, $n = 19$) remained after the quality check, which were analyzed for microbial community composition.

One hundred and sixty two dust samples from 30 apartments comprising 19 bed bug-infested and 11 uninfested homes were analyzed for microbiomes. Dust samples were collected from bedrooms or living rooms (primarily around the beds) at four time points from all 30 apartments and an additional sample was collected in infested controls ($n = 12$) one month after the baseline sampling (Fig. 1) before the heat intervention. Kitchen dust was sampled once during the initial sampling at baseline. A total of 15.65 million reads (96,609 ± 43,323 (SD) per sample, $n = 162$) were obtained from the analysis. The samples were processed, and rarefied to 21,000 reads per sample for performing the diversity analysis.

### 3.2. Bacterial taxa associated with Cimex lectularius

The bed bug microbiota is comprised predominantly of one phylum, Proteobacteria (~99%), and a very low percentage of Firmicutes and Actinobacteria. The two Proteobacteria genera *Wolbachia* (Family: Anaplasmataceae; Class: Alpha-Proteobacteria) and an unclassified member of Enterobacteriaceae (Class: Gamma-Proteobacteria) were the most abundant bacteria detected (Fig. 2), representing nearly 99% of the total bacterial abundance in all the bed bug samples, with *Wolbachia* ranging from 34% to 79% and the unclassified Enterobacteriaceae ranging from 20% to 65% across all samples. The bacterial genera *Acinetobacter*, *Enhydrobacter*, *Staphylococcus*, *Streptococcus*, *Pseudomonas* and *Corynebacterium* were detected in most of the samples, but on average they comprised <1% of the total microbial abundance. Other bacterial genera – *Bartonella*, *Sphingomonas* and *Enhydrobacter* – were present in bed bugs from some apartments, but at very low frequency (Fig. 2). The average Shannon diversity among all apartment-collected bed bugs from pooled samples was 1.7, ranging from 1.4 to 2.3 among different apartments.

The microbial composition of the six individual bed bugs from three apartments used for validation of our extraction procedures was similar to the pooled bed bug samples (data not shown). Overall, low diversity in the microbial communities was observed in all bed bugs.

### 3.3. Total bacterial load in bedroom dust samples

The bacterial abundance was analyzed for all the dust samples ($n = 162$) collected from bed bug-infested and uninfested homes at different time points. Total bacterial abundance in bedroom samples in general ranged from $10^6$ to $10^8$ 16S rDNA copies/mg dust. At baseline sampling, we did not find any significant differences between infested and uninfested homes in total bacterial abundance in bedroom dust ($t$-test, $p = 0.271$) (Fig. 3). However, we found a positive but non-significant correlation between the bacterial loads and bed bug counts in homes at baseline sampling (Fig. S1). A significant decline in the bacterial abundance was also detected after the heat intervention in the bedrooms of infested homes (paired $t$-test, $p < 0.001$), especially at 4 and 12 weeks after the treatment (Fig. 3). We did not observe any significant differences in the bacterial load in the uninfested homes across four time points ranging over 12 weeks.

### 3.4. Bacterial diversity in bedroom dust samples at baseline

Irrespective of the infestation status, bedroom dust had a highly diverse microbial community, comprising bacteria in ~30 different

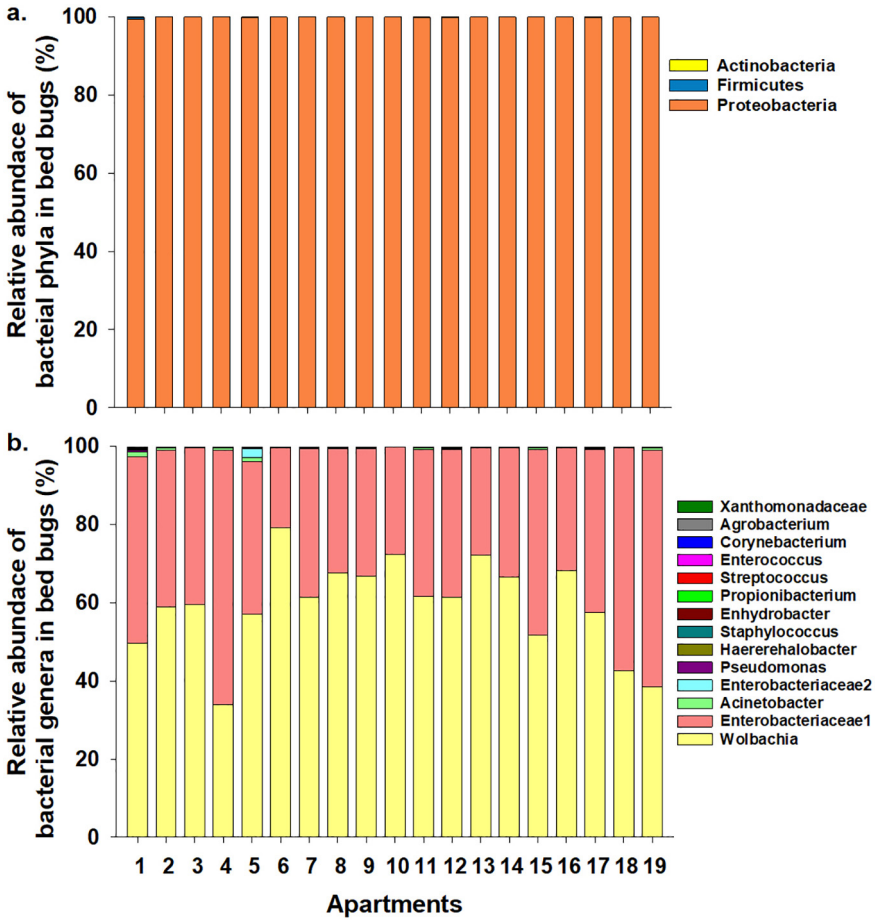


**Fig. 2.** Relative abundance of bacterial taxa (a. phylum; b. genus) in pooled bed bug (*Cimex lectularius*) samples collected from 19 bed bug-infested apartments. Each bar depicts the mean relative abundance value of independent replicates.

phyla. Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes were the most dominant phyla, comprising >95% of the bacterial communities in all the bedroom dust samples (Fig. 4a). However, overall microbial community composition in bedrooms varied significantly between infested and uninfested apartments. Gram-positive bacteria belonging to Firmicutes and Actinobacteria were more dominant in uninfested bedroom dust (53.1%), but they comprised only 34.3% of the dust-associated bacterial communities in infested bedrooms.

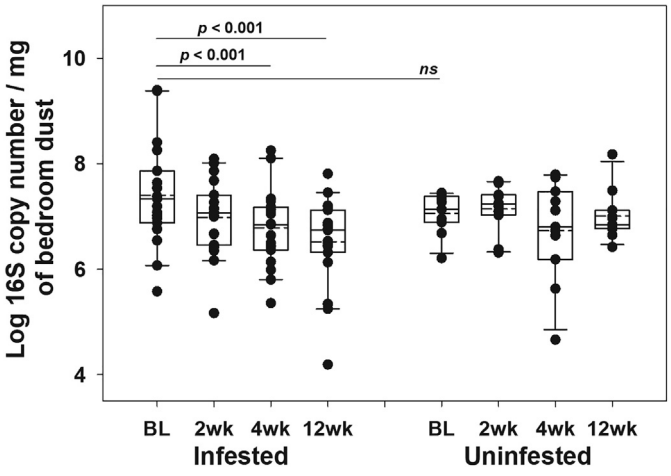


**Fig. 3.** Total bacterial abundance represented by 16S rDNA copy number per mg of bedroom dust. Dust samples were collected from bed bug (*Cimex lectularius*)-infested ($n = 19$) and uninfested ($n = 11$) apartments at baseline and 2, 4, and 12 weeks later. Infested apartments received a spatial heat intervention immediately after the baseline collection. Sample sizes are shown in Fig. 2. The horizontal solid line shows the median, dotted line defines the mean, the box represents the lower and upper quartiles, and the whiskers extend to the most extreme values (no more than 1.5 times the interquartile range from the box); all replicates are plotted as solid circles. NS indicates no significant difference between infested and uninfested apartments at baseline.

In general, the microbiome of bedroom dust comprised both bed bug-associated and human-associated bacteria. Members of the orders Rickettsiales, Actinomycetales, Bacillales, Clostridiales, Enterobacteriales and Pseudomonadales were the most dominant taxa found in the bedroom dust (Table S1 in Supplementary file1). *Wolbachia* was most abundant in infested homes (22.6% ± 17.63), followed by the unclassified member of Enterobacteriaceae (12.6 ± 10.24), although the proportion of these two taxa varied greatly among the homes, ranging from <5% (Apartments 1, 14 and 17) to >85% (Apartment 16) of total bacterial abundance (Fig. 4b). In contrast, however, the relative abundance of these two taxa was very low in bedrooms of uninfested homes, with the exception of one home (Apartment 25) where they constituted 31% of the total bacterial abundance. Thus, a significant difference in the abundance of *Wolbachia* was observed between infested and uninfested bedroom dust (Mann-Whitney, $p < 0.001$) (Fig. 5).

Other dominant bacterial genera detected in bedroom dust included *Corynebacterium*, *Staphylococcus* and *Streptococcus* (Figs. 4b, 5 and Table S2 in Supplementary file 1). While these bacteria were present in all the apartments, they formed a more dominant fraction of the dust microbiome in uninfested apartments. The genera *Propionibacterium*, *Pseudomonas*, *Acinetobacter*, *Bacteroides* and *Bartonella* were present in most of the apartments, but comprised 20–30% of the bacteria in some homes.

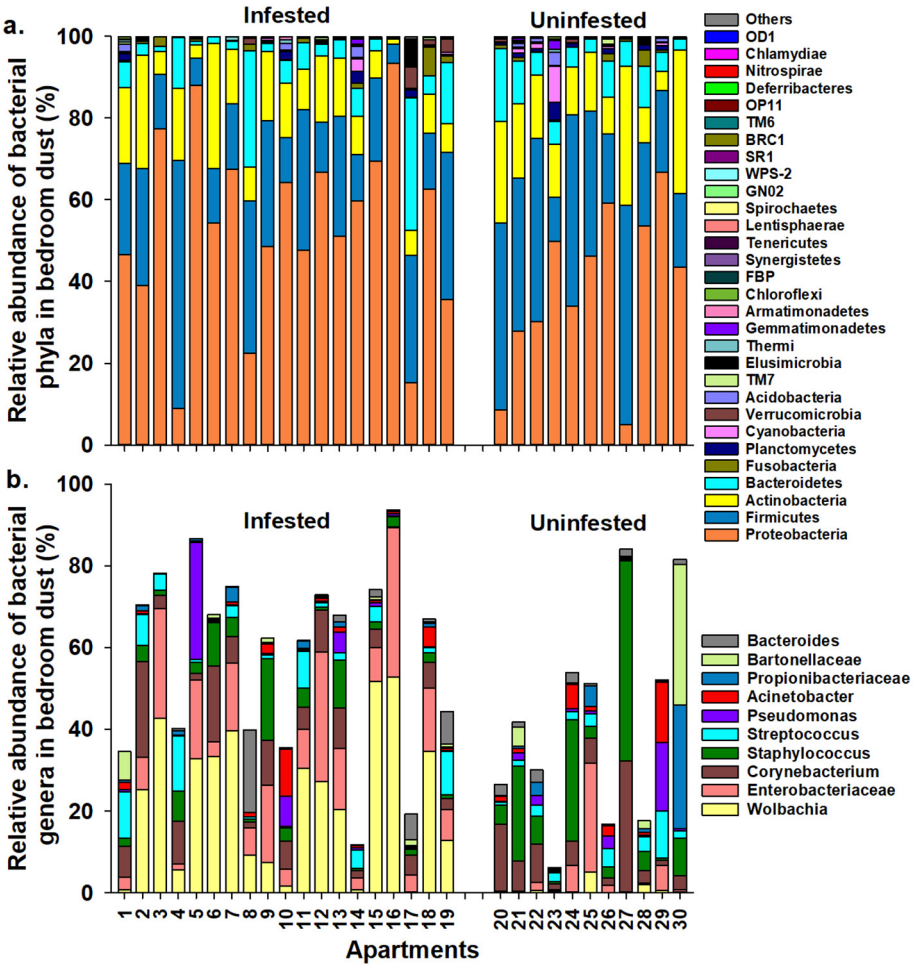


**Fig. 4.** Relative abundance of bacterial taxa in bedroom dust collected at baseline in bed bug (*Cimex lectularius*)-infested ($n = 19$) and uninfested ($n = 11$) apartments at the phylum level (a) and the 10 most dominant bacterial genera (b).

*3.4.1. Community analysis of bedroom dust samples*

Principal coordinate analysis of all baseline bedroom dust samples revealed a significant difference in the microbial communities between bed bug-infested and uninfested homes (MRPP, $p < 0.01$) (Figs. 6a and S2). PC1 (14.7% of the variance) separated infested from uninfested bedrooms. One bedroom dust sample from an uninfested apartment overlapped with dust samples from infested homes, suggesting that this apartment might have been infested in the recent past, or that our

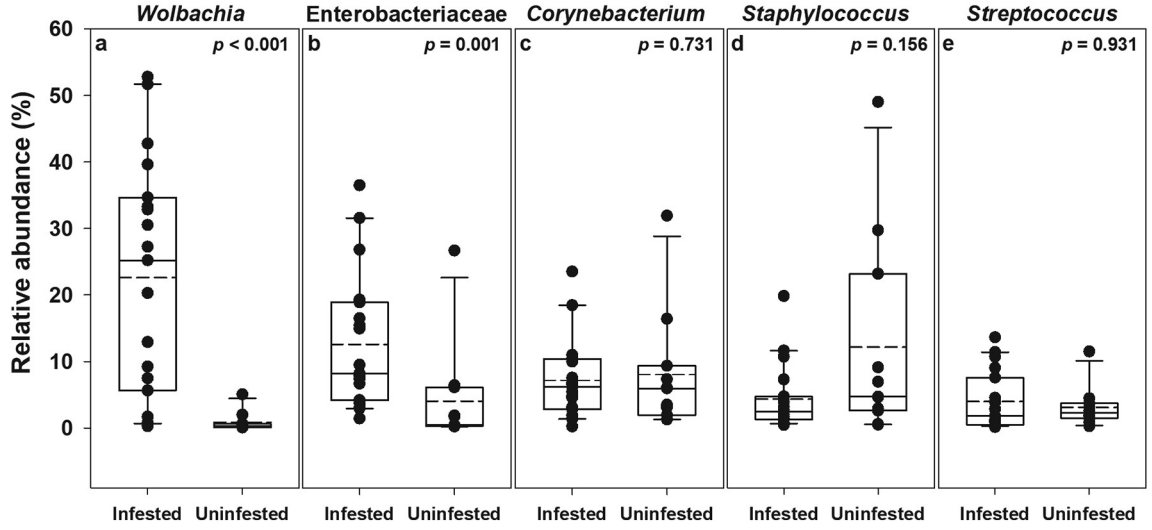


**Fig. 5.** Box plots showing the relative abundance of 5 dominant bacterial genera in bedroom dust of bed bug (*Cimex lectularius*)-infested ($n = 19$) and uninfested ($n = 11$) apartments at baseline sampling. The horizontal solid line shows the median, dotted line defines the mean, the box represents the lower and upper quartiles, and the whiskers extend to the most extreme values (no more than 1.5 times the interquartile range from the box); all replicates are plotted as solid circles.

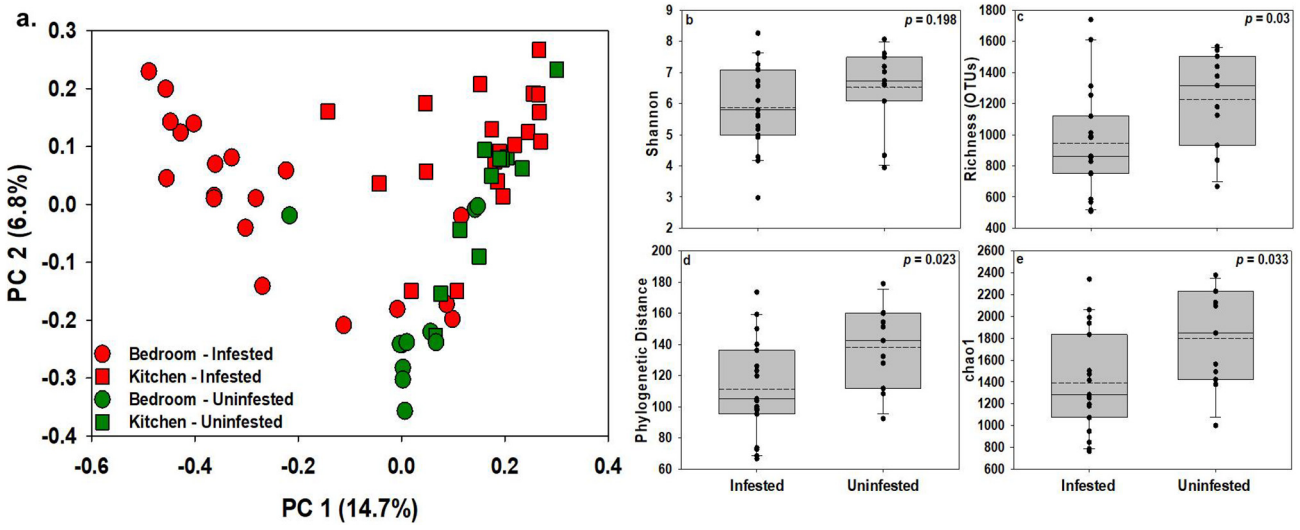


**Fig. 6.** Taxonomic composition and alpha-diversity of dust samples from bed bug (*Cimex lectularius*)-infested and uninfested homes. (a) Principal-coordinate analysis depicting differences in taxonomic compositions of bacterial communities among dust samples collected from bedrooms (circles) and kitchens (squares) of infested (red) and uninfested (green) homes at baseline sampling. Community composition dissimilarity is based on the Bray-Curtis dissimilarity metric. The percent variation explained by each component is indicated on the axis. (b–e) Box plots showing alpha-diversity indices of bedroom dust samples from infested ($n = 19$) and uninfested ($n = 11$) homes at baseline sampling. The horizontal solid line shows the median, dotted line defines the mean, the box represents the lower and upper quartiles, and the whiskers extend to the most extreme values (no more than 1.5 times the interquartile range from the box); all replicates are plotted as solid circles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inspection and traps missed bed bugs in this apartment (see Study limitations below). The alpha-diversity indices of bedroom dust at baseline showed significant differences between infested and uninfested apartments in species richness and phylogenetic diversity but not in Shannon diversity (Fig. 6b, c, d, e). Although the bed bug infestation rate did not appear to affect the microbial community composition, the alpha diversity indices showed a moderate negative correlation with bed bug counts at baseline (Fig. S3) which could be attributed to the predominance of bed bug-associated bacteria in infested homes.

### 3.5. Bacterial diversity in kitchen dust samples at baseline

Kitchen floors were sampled in all the apartments only at baseline to assess differences between bed bug-infested and uninfested apartments. In general, the microbial communities of kitchen dust and bedroom dust were significantly different (MRPP, $p < 0.001$) (Fig. 6a) but this was more prominent in bed bug-infested homes. All the statistics are shown in Table 1. Kitchen samples from bed bug-infested homes were separated from kitchen samples from uninfested homes along PC1, as were bedroom samples (Fig. 6a). The kitchens were dominated by taxa from bacterial orders *Pseudomonadales*, *Enterobacteriales*, *Actinomycetales* and *Lactobacillales*. The genera *Acinetobacter*, an unknown genus of *Enterobacteriaceae*, *Lactococcus*, *Pseudomonas*, *Corynebacterium*, *Staphylococcus*, *Erwinia*, *Sphingomonas* and several others were detected in all the kitchen samples (Table S3 in Supplementary file 1). However, unlike the bedroom dust, none of the genera comprised >6% of the total bacterial abundance. *Wolbachia* was detected in the kitchens of infested homes, but it comprised only 2–8% of the total bacterial abundance in some homes, and was negligible in others. Beta-diversity analysis of kitchen dust samples from infested and uninfested homes showed no significant difference (MRPP, $p = 0.166$) in the microbial community composition, consistent with the observation that bed bugs forage and rest mainly in close proximity to where their hosts sleep.

On the other hand, we found a significant difference between bedroom and kitchen dust samples in both infested and uninfested apartments (largely related to PC2 in Fig. 6a), suggesting a clear distinction in the environmental microbiomes of these two rooms. The differences were much more pronounced, however, in bed bug-infested apartments.

### 3.6. Stable microbial communities in uninfested and infested homes

#### 3.6.1. Uninfested homes

Dust samples were collected from the bedrooms of uninfested homes at baseline and 2, 4 and 12 weeks later to document the effects

**Table 1**
Statistical analysis of dust samples based on infestation status, area of sampling and time of sampling.

| | Infestation | Area | Time of sampling | Bray Curtis | Weighted Unifrac | | Unweighted Unifrac | |
|---|---|---|---|---|---|---|---|---|
| | | | | MRPP | Adonis | ANOSIM | Adonis | ANOSIM |
| Infestation status | Infested | Bedroom × kitchen (38)[a] | Baseline | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |
| | Uninfested | Bedroom × kitchen (22) | Baseline | 0.001 | 0.009 | 0.011 | 0.001 | 0.001 |
| Area of sampling | Infested × Uninfested | Bedroom (30) | Baseline | 0.002 | 0.004 | 0.005 | 0.003 | 0.196 |
| | Infested × Uninfested | Kitchen (30) | Baseline | 0.166 | 0.559 | 0.096 | 0.863 | 0.612 |
| Time of sampling | Infested-controls | Bedroom (24) - 2 time points | Baseline vs 4 weeks after baseline | 0.987 | 0.95 | 0.927 | 0.995 | 0.965 |
| | Uninfested | Bedroom (44) - 4 time points | Baseline, 2, 4 and 12 weeks after baseline | 1.000 | 0.999 | 0.992 | 0.998 | 0.948 |
| | Infested | Bedroom (76) - 4 time points | Baseline and 2, 4 and 12 weeks post-intervention | 0.971 | 0.963 | 0.87 | 0.900 | 0.981 |
| | Infested | Bedroom (38) | Baseline vs 12 weeks post-intervention | 0.207 | | | | |
| | Infested × Uninfested | Bedroom (60) | Baseline vs 12 weeks post-intervention | 0.001 | | | | |

[a] Number in the parenthesis indicates the sample number used in the analysis.

of abiotic factors (e.g., seasonal) and biotic factors (e.g., occupants' behavior) on the home microbiota. However, beta-diversity analysis of these time points showed no significant differences in the microbial community composition over time (MRPP, $p < 1.000$) and hence robust stability of the bacterial communities in uninfested homes.

*3.6.2. Infested-control (untreated) homes*

Of 19 bed bug-infested apartments 12 were randomized as infested-controls for 4 weeks before they were crossed-over to the infested-treated arm and received heat intervention. Dust samples were collected from the bedrooms of these homes at baseline and 4 weeks later. As in the uninfested homes, the microbial communities were stable in these infested homes as no significant changes in the microbial community composition were evident between the baseline and one-month samples (MRPP, $p = 0.987$). At both sampling times, the dust microbiomes were similar both phylogenetically and in bacterial composition.

*3.7. Elimination of bed bugs changes the dust-associated microbiomes*

Dust samples collected from the bedrooms of 19 bed bug-infested homes at baseline and 2, 4 and 12 weeks after heat intervention were analyzed to understand the effect of bed bug elimination on the indoor microbial community structure. We observed a gradual shift in the community composition from the pattern represented by the baseline (infested) samples toward the pattern of uninfested homes (Fig. S4). While this shift was detected in the 2-week samples, the difference was most prominent between the baseline and 12-week samples (Fig. 7). Principal coordinate analysis of the baseline and all the post-intervention bedroom dust samples from infested homes showed a shift in the microbial community toward the uninfested dust samples, but no significant differences were observed over time (MRPP, $p = 0.997$). Similarly, the beta-diversity of dust samples from infested homes 12 weeks post-intervention was not significantly different from the baseline samples (MRPP, $p = 0.203$) (Fig. 7), likely because of small sample sizes and the inherent variation among homes. However, when the beta-diversity of all 60 infested and uninfested homes was compared between baseline (30 homes) and 12 weeks (30 homes), a significant difference in the community composition was observed (MRPP, $p = 0.01$; Interaction: Time of sampling x Infestation was significant).

The post-intervention shift in the microbial communities in infested homes was primarily associated with a decrease in the relative abundance of *Wolbachia* and Enterobacteriaceae. The *Wolbachia* levels in the infested homes showed a gradual significant decline after the heat treatment, whereas in the uninfested apartments *Wolbachia* and Enterobacteriaceae remained unchanged during the experimental period (Fig. 8). Of the top twenty orders comprising >0.05% of the reads, we observed declines in the relative abundance of taxa belonging to Rickettsiales, Enterobacteriales, Niesierrales and Xanthomodales, and increases in the relative abundance of members of Psuedomonadales, Burkholderiales, Rhizobiales, Sphingomonadales, Fusobacteriales and Flavobacteriales (Table S4 in Supplementary file 1). No difference was evident in the abundance of Clostridiales and Bacillales between the baseline samples in the infested homes and dust samples collected 12 weeks post-intervention.

## 4. Discussion

The emergence of culture-independent sequencing technology to efficiently characterize microbiomes has propelled investigations into factors that shape microbial communities of the built environment and their associated health implications. The microbiomes in a wide array of building types have been investigated, including in residences (homes, dormitories, hotels), health-care facilities, offices, classrooms, restaurants and other food handling facilities, transportation (vehicles and stations), libraries, and many other urban, rural and farm buildings (Adams et al., 2015; Kirjavainen et al., 2019; Lax and Gilbert, 2015; Leung et al., 2014). Several prominent factors play major roles in contributing to and shaping indoor microbiomes, including the residents or occupants themselves, pets, plants, HVAC and ventilation, dampness, building design and the local outdoor environment (Dannemiller et al., 2016; Prussin and Marr, 2015). Surprisingly however, indoor pests are rarely mentioned in surveys of microbial communities in the built environment. Further, homes in disadvantaged low-income communities are rarely included and therefore the impacts of large established arthropod infestations on the indoor microbiome remain uninvestigated. Large pest populations can generate significant moisture and organic media for microorganisms, especially in high-occupancy homes that

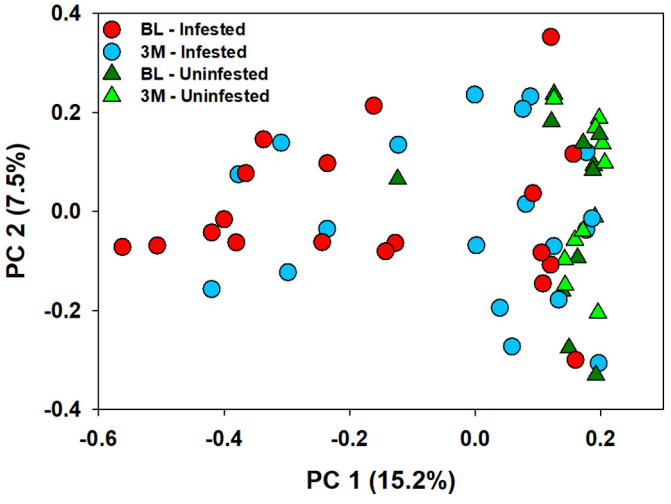


**Fig. 7.** Principal-coordinate analysis depicting differences in taxonomic compositions of bacterial communities among dust samples collected at baseline and 12 weeks post-intervention from bedrooms of bed bug (*Cimex lectularius*)-infested (circle, red and blue respectively) and baseline and 12 weeks samples from uninfested (triangle, green and light green respectively) homes. Community composition dissimilarity is based on the Bray-Curtis dissimilarity metric. The percent variation explained by each component is indicated on the axis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

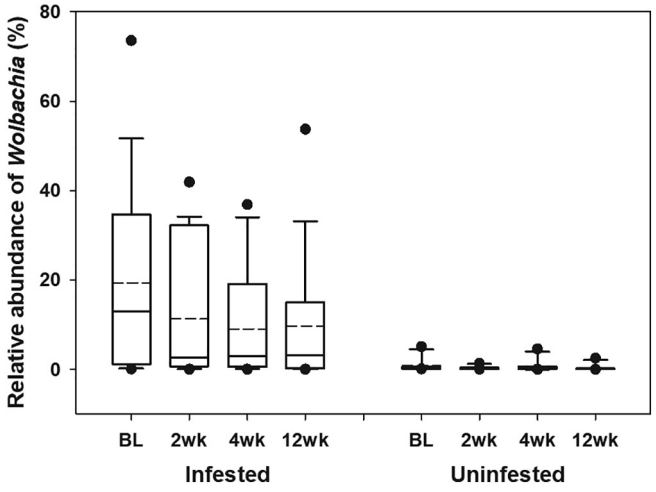


**Fig. 8.** Box plots showing the relative abundance of *Wolbachia* in bedroom dust samples at baseline (BL) and three subsequent time points from bed bug (*Cimex lectularius*)-infested ($n = 19$) and uninfested ($n = 11$) homes. In infested homes, spatial heat interventions were implemented immediately after the baseline sampling, followed by dust collection at 2, 4 and 12 weeks post intervention. The horizontal solid line shows the median, dotted line defines the mean, the box represents the lower and upper quartiles, and the whiskers extend to the most extreme values (no more than 1.5 times the interquartile range from the box); all replicates are plotted as solid circles.

are common in low-income communities. To date, the relationship between persistent synanthropic pest populations and the indoor microbiota has not been examined. Therefore, the primary aims of this study were to (a) determine if bed bug infestations influence the microbial diversity of household dust and if so, (b) assess whether the elimination of bed bugs also affects the indoor microbiome.

### 4.1. Bed bug microbiome

Although the focus of this investigation was the household dust-associated microbiome, it was essential to characterize the bed bug-associated microbial communities to infer an effect of bed bugs on household microbiomes. We found that the bed bug microbiome was unusually sparse compared with other insects, including the German cockroach, another indoor pest (Kakumanu et al., 2018). Two Proteobacteria genera – *Wolbachia* (Order: Rickettsiales) and an unclassified gamma-proteobacterium – comprised 98–99% of the total bacterial abundance in bed bugs, consistent with previous reports (Fisher, 2017; Hosokawa et al., 2010; Hypša and Aksoy, 1997; Meriweather et al., 2013; Sakamoto and Rasgon, 2006). These two endosymbionts are vertically transmitted from mother to offspring (Hypša and Aksoy, 1997) and they play a role in development and reproduction in bed bugs (Chang, 1974; Hosokawa et al., 2010). *Wolbachia* resides in specific tissues called bacteriomes (Chang and Musgrave, 1973) associated with the gonads, and is a nutritional mutualist in bed bugs, primarily involved in provisioning B-vitamins to its host (Hosokawa et al., 2010). The unclassified gamma-proteobacterium, which is closely related to BEV-like symbionts in plant hoppers and *Pectobacterium* (Enterobacteriaceae) (Degnan et al., 2011; Hypša and Aksoy, 1997), is found in other tissues but little is known about its function in bed bug biology.

We detected *Staphylococcus*, mainly *S. epidermidis*, in all the bed bug samples, albeit at low abundance, which likely relate to the bed bug's association with human skin. We have isolated *S. epidermidis* from the fecal and gut samples of bed bugs in our laboratory. Other bacteria included Gram-positive *Micrococcus* sp., *Kocuria kristinae*, *S. arlettae*, *Bacillus* and *Arthrobacter*, and Gram-negative *Enterobacter*, which were also previously found in bed bugs, including on their exterior surface (Cockburn et al., 2013; Delaunay et al., 2011; Reinhardt et al., 2005). Most of the bacteria detected are environmental bacteria and bed bugs might have acquired them either on their surface or during their unusual traumatic mating that might involve sexual transmission of microbes (Bellinvia et al., 2020; Otti et al., 2017; Reinhardt et al., 2005). Nevertheless, because we validated that surface bacteria were excluded from our analysis, it appears that all these bacteria were extracted from within the bed bugs.

### 4.2. Influence of bed bug infestations on the indoor microbiome

Dust-associated microbiomes vary significantly among homes, particularly in floor samples, and due to sharing of taxa with the occupants' microbiome (Lax et al., 2014). In general however, residential microbiomes are dominated by members of Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes (Adams et al., 2015; Dunn et al., 2013; Kembel et al., 2012; Lax et al., 2014; Rintala et al., 2008). These phyla also dominated the microbial communities in dust samples in uninfested homes in the current study, which had high relative representation of the orders Actinomycetales, Bacillales, Lactobacillales, Enterobacteriales and Rhizobiales. Bacteria belonging to the genera *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Propionibacterium*, *Pseudomonas* and *Acinetobacter* were detected in all the uninfested apartments. These genera are commonly found in indoor environments as either human-associated microbes or microbes that are shared with the outdoor environment (Adams et al., 2015; Jeon et al., 2013). We also found substantial amounts of chloroplast DNA in the dust, likely plant and pollen originated; these reads were removed from the analysis.

Within uninfested homes, the bacterial diversities in kitchen and bedroom dust samples were significantly different. In general, residents spend more time in bedrooms and their microbiome might be shared and be an important component of the bedroom dust microbiome. In kitchens, on the other hand, diverse microbial communities are influenced not only by humans, but also by microbes associated with food, refuse, more lighting and aerosolized faucet water, among other factors (Flores et al., 2013; Kelley and Gilbert, 2013; Lax et al., 2014).

Bed bugs are commonly found close to where their hosts rest or sleep (Eddy and Jones, 2011) and therefore, they are much more abundant in bedrooms and living rooms than in kitchens. Indeed, as we hypothesized, the dust-associated microbial communities in the bedrooms of infested homes were significantly different from kitchen microbiomes. Also, the diversity of dust-associated microbiomes of bed bug-infested and uninfested homes was significantly different. The bed bug-associated bacteria were highly abundant in the bedroom dust of infested homes, whereas human-associated and environmental bacteria predominated the bedroom dust of uninfested homes and in the kitchens of both infested and uninfested homes. In particular, the relative abundances of *Wolbachia* and the unclassified gamma-proteobacterium were high in the bed bug-infested bedrooms. These bacteria, and their cellular components, are deposited by bed bugs into the home environment either in their feces, exuviae or as components of dead bed bugs. Thus, bed bug-associated microbes and their metabolites become an integral part of the indoor dust microbiota, which in turn can be readily linked with bed bugs by their presence. Because of their relatively short lifespan, rapid proliferation and the abundance of dead bed bugs (due to natural death and pest control efforts), bed bugs have a high potential to impact the microbial community structure of infested structures. In contrast, *Wolbachia* was undetected, or at very low abundance, in the uninfested homes, indicating that bed bugs were likely the sole source of this bacterium. It is important to note in this context that some of the uninfested homes with low abundance of *Wolbachia* might have been previously infested or possibly were infested but below our capability to detect live bed bugs.

Bed bugs feed on blood every few days and defecate copious amounts of feces (Darrington, 2015). Thus, bed bugs add organic matter and moisture to the indoor environment that could sustain greater populations of microbes. Contrary to our expectations, however, we did not find higher abundance of bacteria in infested than uninfested homes. It remains to be determined whether the abundance of fungi is affected by bed bugs.

### 4.3. Elimination of bed bugs alters the indoor microbiome

Our longitudinal sampling showed that eliminating bed bugs resulted in a shift in the bedroom microbial community structure toward the structure of uninfested bedrooms. Although the post-interventions samples were not significantly different from the baseline samples, a clear shift toward the bedroom community composition of uninfested homes suggests that bed bugs contributed to the bedroom microbiome. This shift was affected by a reduction in the bed bug-associated bacterial taxa in the intervention homes, resulting in a more diverse and even microbial community. The results suggest that the changes in the indoor microbiome are gradual and require more time than was available in our three-month sampling. We predict that intensive cleaning of homes after bed bug elimination might accelerate the shift toward the microbiome structure of uninfested homes (Dannemiller et al., 2016; Kettleson et al., 2015).

### 4.4. Health risks and implications of bed bug infestations

While bed bugs are not known as vectors of any human pathogens, their presence in the residential environment poses a number of health risks. Their bites can lead to severe allergies and even secondary infections, frequent blood feeding may cause anemia, and their presence

can adversely impact the quality of life, sleep patterns, anxiety and stress. Moreover, bed bugs defecate large amounts of histamine (DeVries et al., 2018) and exposure to dust-associated histamine might exacerbate allergic disease and asthma. Bed bugs were also shown to carry several human pathogens such as *Borrelia recurrentis* and *Rickettsia* (El Hamzaoui et al., 2019; Potts et al., 2020). Finally, residual insecticides are commonly used to control bed bug infestations, also potentially exposing residents to environmental contaminants.

Two additional health risks related to the bed bug-associated microbiome include (a) proliferation of potentially pathogenic microbes associated with bed bugs and household dust, and (b) microbes and microbial metabolites that bed bugs deposit in the indoor environment. Among the diverse bacterial taxa we detected in home dust were some potential pathogens similar to those detected in other studies (Dunn et al., 2013). *Bartonella quintana*, the causative agent of trench fever, was previously detected in *C. lectularius* (Angelakis et al., 2013), and we found members of Bartonellaceae in dust samples from 28 of 30 homes (93%) in this study, but at very low abundance. In a previous survey of 99 bed bugs from 10 apartments in the same building we did not detect any *Bartonella*, but found *Burkholderia multivorans*, an important pathogen in nosocomial infections, in four apartments (40%), based on 16S-23S rRNA intergenic transcribed spacer sequences (Saenz et al., 2013). Interestingly, we again detected the genus *Burkholderia* in 25 of 30 apartments (83.3%) in the current study; however, species-specific screening is warranted to determine whether *Burkholderia multivorans* has become more common in these homes. Overall, our detection rates of potential human pathogens were low and no association was evident between pathogen occurrence and bed bug infestation.

An additional health risk may be related to the non-pathogenic microbes shed by bed bugs and the microbial communities supported by their fecal excretions. While we found a significant difference in the bacterial diversity, no significant difference was detected in bacterial loads in infested and uninfested homes. However, it remains to be determined whether bed bug excretions and dead bed bugs support bacterial communities in specific microhabitats that were either not sampled by our limited vacuum sampling, or sieved out during sample processing; fungal communities are yet to be determined (see Study limitations below).

Notably, the bed bug endosymbionts are Gram-negative bacteria and presumably release large amounts of endotoxins into the environment. A recent survey of 831 homes throughout the U.S. found high endotoxin concentrations in bedrooms, family rooms and kitchen floors, and significant positive correlations between endotoxin levels and asthma (Thorne et al., 2005); these relationships were strongest for bedroom floors. As household endotoxin exposure is a significant risk factor for increased asthma prevalence, the potential contribution of bed bugs to endotoxin levels in the bedroom cannot be ignored.

Overall, the human microbiome and health can be affected by intimate contact with the home and pet microbiomes (Trinh et al., 2018), and bedroom allergens associated with house dust mites and cockroaches adversely affect children's health (Salo et al., 2018). We suspect that intimate contact with large populations of bed bugs and their microbiome and metabolites in the bedroom environment will be found to also pose similar health risks.

### *4.5. Study limitations*

We acknowledge several constraints and challenges in the design and implementation of this study. First, the relatively small sample size and inherently large variation among homes constrained our ability to detect small differences among treatments. For example, we found no significant difference in the abundance of non-bed bug-associated bacteria in bed bug-infested and uninfested homes, counter to our expectation. It is possible that the inclusion of more homes might unveil the impact of bed bug infestation on other microbes. Second, contributing to the variation among infested homes was the inclusion of homes in this group independent of the infestation level, which ranged from 8 to 227 bed bugs trapped per home during our baseline sampling. The impacts of bed bugs are expected to vary greatly with their abundance, confounding this arm of the design. Third, ethical considerations and our IRB limited the use of an infested-control (untreated) group to only one month before it was crossed-over to an intervention arm. We detected no differences in longitudinal sampling over a month in these homes and the strong effect of bed bugs would likely remain beyond one month. Finally, we were unable to include a heat intervention in uninfested homes to control for the effects of spatial heat on the home microbiome. These interventions were conducted by professional pest management services independently of our study, and because they are costly (>$1000 per small apartment) and guided by the presence of bed bugs, they were only implemented in infested homes. The effects of heat on the home microbiome, independent of the elimination of bed bug, are unknown. However, the gradual shift in the dust-associated microbiome toward the community structure of uninfested homes suggests that the elimination of bed bugs had a greater effect than exposure to heat.

## 5. Conclusions

Global demographic shifts in the form of urbanization pose new challenges, especially related to over-crowded communities and homes (Sarkar and Webster, 2017) which create congenial conditions for pest infestations, including bed bugs and cockroaches. Infestations can reach very high densities and are especially problematic in low-income and elderly homes. The results from this study provided strong evidence that bed bugs can shape the indoor microbiome of infested homes. The shift in the microbial community structure toward the community composition of uninfested homes after elimination of bed bugs strongly suggests that bed bugs influence the indoor microbiome. Along with ecology-based analysis of indoor microbiota, it is imperative to investigate pest-associated factors like mycotoxins, microbial volatile organic compounds and potential emerging contaminants (e.g., histamine) to understand how microbes associated with insect infestations affect human health.

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## CRediT authorship contribution statement

**Madhavi L. Kakumanu:** Methodology, Investigation, Formal analysis, Data curation, Writing - original draft. **Zachary C. DeVries:** Methodology, Investigation, Writing - review & editing. **Alexis M. Barbarin:** Methodology, Investigation, Writing - review & editing. **Richard G. Santangelo:** Methodology, Investigation, Writing - review & editing. **Coby Schal:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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