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RESEARCH LETTER

16S rRNA profiling of the *Dermatophagoides farinae* core microbiome: *Enterococcus* and *Bartonella*

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To the Editor:

Characterization of the house dust mite (HDM; *Dermatophagoides farinae*) microbiome is important because it may exert immunomodulatory effects on the pathogenesis of allergic diseases, owing to its ability to generate microbe-associated molecules such as lipopolysaccharides and lipoteichoic acid.[1] In a recent study, whole-genome shotgun sequencing of *D. farinae* revealed that *Enterobacter* were the most abundant bacteria,[2] while another study using 16S rRNA cloning reported that *Bartonella* was the most abundant bacterial taxon.[3] In this study, a 16S rRNA amplicon analysis using high-throughput sequencing technology was performed; this is currently the most widely used and reliable method for microbiome community studies.[4] Bioinformatics analyses were performed using three different pipelines to determine the appropriate method for the HDM microbiome analysis. The genera *Bartonella* and *Enterococcus* constituted approximately 99% of the microbiome of *D. farinae*; this finding was supported by 16S rRNA cloning, culturing of mite homogenates and several staining methods. To eliminate the possibility of bacterial contamination, we used sterilized feed and a commercial cell culture flask. The microbiome of the mites in this study may represent the core bacteria or endosymbionts because the mites have been subcultured and have been commercially available for more than 10 years.

For *D. farinae* cultivation, mites were inoculated into a 75-cm^2 cell culture flask (SPL Life Sciences, Gyeonggi, Korea) and placed in a container maintained at 75% relative humidity and 25°C for 6 weeks. The feed was prepared by autoclaving a 1:1 mixture of inactivated dried yeast (Choheung Co., Gyeonggi, Korea) and fish feed (JeilFeed Co., Daejeon, Korea). To harvest mites, autoclaved saturated saline was used to separate mites from the debris. DNA was extracted by proteolytic degradation and mechanical disruption using the NucleoSpin® DNA Insect Kit (Macherey-Nagel, Duren, Germany). From the extracted DNA, the V3-V4 region of 16S rRNA was amplified through PCR using the bacterial universal primer pair of forward primer 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3′ and reverse primer 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC 3′. A limited-cycle amplification step was performed to add multiplexing indices and Illumina sequencing adapters. Libraries were normalized, pooled and sequenced on the MiSeq platform (Illumina MiSeq V3 cartridge [600 cycles]; Illumina, San Diego, CA, USA) in accordance with the manufacturer's instructions.

Three bioinformatics pipelines were used. The first was ChunLab. Raw reads were processed through a quality check and low-quality (<Q25) reads were filtered using Trimmomatic 0.32 (http://www.usadellab.org/cms/index.php?page=trimmomatic). Paired-end sequence data were then merged using PandaSeq (https://github.com/neufeld/pandaseq). Primers were then trimmed using the ChunLab in-house program applying a similarity cut-off of 0.8. Sequences were denoised using the mothur preclustering program (https://www.mothur.org), which merges sequences and extracts unique sequences, allowing up to 2 differences between sequences. The EzTaxon database (https://www.ezbiocloud.net/) was used for taxonomic assignment using BLAST 2.2.22 (https://blast.ncbi.nlm.nih.gov), and pairwise alignments were generated to calculate similarity. Uchime (https://www.drive5.com/uchime) and the non-chimeric 16S

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rRNA database from EzTaxon were used to detect chimeric sequences for reads with a best hit similarity rate of <97%. Sequence data were then clustered using CD-Hit (cd-hit.org) and UCLUST (https://www.drive5.com/usearch). The second pipeline was BaseSpace. The analysis was performed using Illumina BaseSpace® 16S Metagenomics v1.0.1 (May 2013 Greengenes (greengenes.secondgenome.com) as taxonomic database), provided by Illumina Inc. (http://basespace.illumina.com). The third pipeline used was mothur. The 16S rRNA sequence data were processed using mothur v1.39.5 as indicated in the MiSeq SOP (https://www.mothur.org/wiki/MiSeq_SOP). Sequences were aligned to the SILVA reference alignment (https://www.arb-silva.de).

Mite 16S rRNA were cloned using PCR with full-length 16S rDNA primers, that is 27F (5′ AGAGTTTGATCMTGGCTCAG 3′) and 1492R (5′ TACGGYTACCTTGTTACGACTT 3′) and the TOPcloner™ TA Kit (Enzynomics, Daejeon, Korea). Cloned colonies were sequenced with the primers M13F and M13R. Homology was determined through NCBI BLAST searches.

For immunofluorescence staining, paraffin-embedded mites were cut into 7-μm serial sections. Rabbit anti-*Enterococcus* antibody diluted 1:1000 (ab19980; Abcam, Cambridge, UK) and a mouse anti-*Bartonella henselae* antibody diluted 1:50 (ab704; Abcam) were used as primary antibodies. Donkey anti-rabbit IgG-PE (sc-3745; Santa Cruz, Dallas, TX, USA) and goat anti-mouse IgG-Alexa Fluor 488 (A11001; Thermo Fisher, Waltham, MA, USA) were used as fluorescent secondary antibodies. Mite sections were silver stained using Warthin-Starry stain (ab150688; Abcam), which is specific for *Bartonella*, *Helicobacter pylori*, *Legionella pneumophila* and Spirochaetes.

The V3-V4 region of the 16S rRNA of bacteria colonizing *D. farinae* was amplified by PCR, and next-generation sequencing (NGS) was performed using the Illumina MiSeq platform. The microbiome was analysed using three different bioinformatics pipelines. Using the ChunLab pipeline, Illumina's BaseSpace pipeline, and mothur linked to the Silva database, 65 320 reads were assigned to 113 bacterial species, 67 601 reads were assigned to 163 genera and 49 408 reads were assigned to 33 genera, respectively. Using the ChunLab pipeline, *Enterococcus faecalis* accounted for 53.6% of reads, followed by *Bartonella* HQ806746 (39.0%); other species of *Bartonella* and *Enterococcus* were also detected (Figure 1). When the two genera were combined, over 99% of reads were assigned to *Bartonella* and *Enterococcus*, using this pipeline. Using BaseSpace, *Enterococcus* spp. represented 56.3% of reads, *Ochrobactrum* spp. accounted for 28.2% and *Bartonella* spp. accounted for 5.5%. In addition, *Devosia* spp. (3.8%), *Azorhizobium* spp. (2.8%), *Roseospira* spp. (0.93%), *Vagococcus* spp. (0.35%), *Agrobacterium* spp. (0.27%) and *Phaeobacter* spp. (0.13%) were detected. Using mothur, *Enterococcus* spp. was the most common (53.9%), followed by unclassified Alphaproteobacteria (40.5%) and *Bartonella* spp. (4.7%). For all three pipelines, *Enterococcus* was the most abundant bacterial taxon. The major difference among methods was the second most abundant bacteria, that is *Bartonella* spp. for ChunLab, *Ochrobactrum* spp. for BaseSpace and unclassified Alphaproteobacteria for mothur. These differences in results among bioinformatics pipelines might have resulted from the differences in algorithms and databases.

To determine whether the second most abundant bacterial genus was *Bartonella* or *Ochrobactrum*, we cultivated the mite's homogenate on bacterial culture plates. On blood agar, only one species of Gram-positive cocci was found; it was identified as *E. faecalis* through 16S rRNA sequencing. No growth was detected on MacConkey agar, where *Ochrobactrum* spp., but not *Bartonella* spp., is expected to grow.

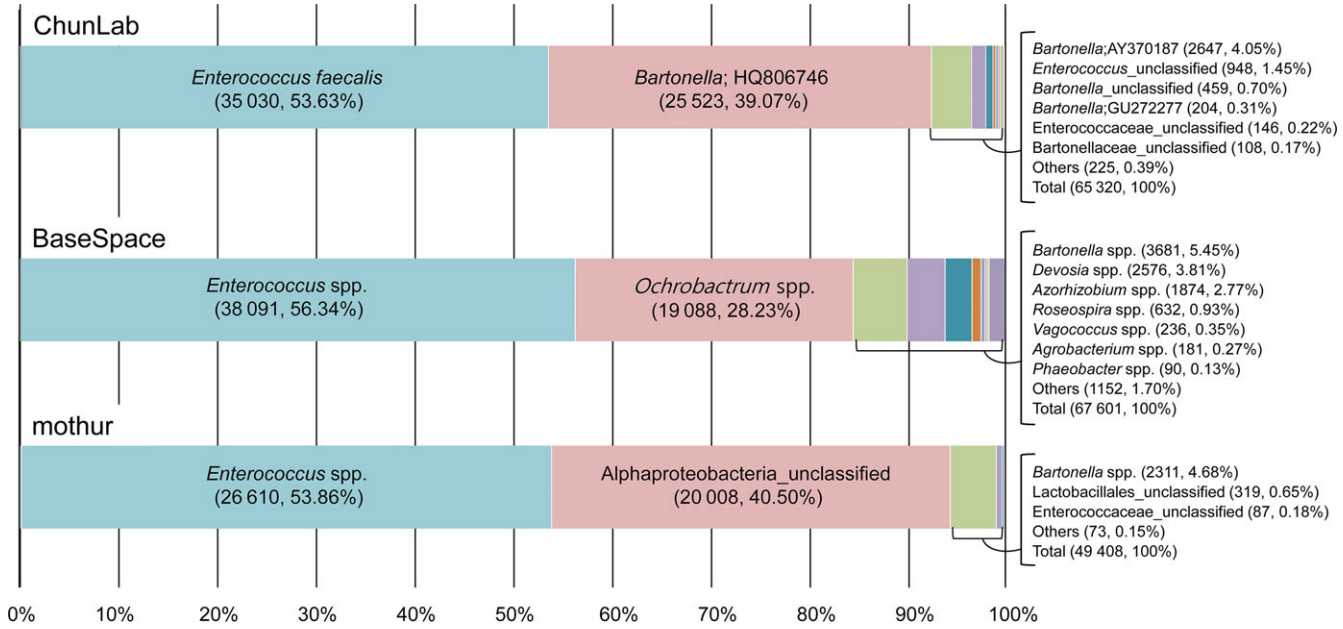


FIGURE 1 Results of a 16S rRNA amplicon analysis using high-throughput sequencing technology for the *Dermatophagoides farinae* microbiome by three different bioinformatics pipelines (read count, percentage). Bacteria occupying more than 0.1% of reads are shown

To confirm that *Bartonella* spp. are the second most abundant bacteria, we cloned and sequenced the full sequence of 16S rRNA (rather than the V3-V4 region) of the mite DNA and used NCBI BLAST for identification. Consequently, 21 of 31 colonies were identified as *E. faecalis* and 10 belonged to the genus *Bartonella*. Nine of the 10 *Bartonella* spp. were identified as the HQ806746 strain, which accounted for the majority of reads in the ChunLab pipeline results for the NGS analysis, and one was identified as the GU272277 strain. These results for *Bartonella* spp. were consistent with those obtained using the ChunLab pipeline, even at the strain level, and the proportion was also similar to that obtained through NGS. Other Alphaproteobacteria identified using the BaseSpace pipeline, such as *Ochrobactrum* spp., *Devosia* spp., *Azorhizobium* spp., *Roseospira* spp., and *Agrobacterium* spp., were not detected. Accordingly, the ChunLab pipeline was apparently the most suitable bioinformatics tool for analysis of the HDM microbiome.

Several stains were used to determine the distribution of bacteria. In Gram staining, Gram-positive cocci considered as *Enterococcus* spp. were distributed throughout the intestine of *D. farinae* and were densely gathered in the stool at the hindgut region (Figure 2A,B). Immunofluorescence staining showed the same results (Figure 2C). Gram-negative intracellular *Bartonella* spp. were difficult to distinguish from surrounding tissues by Gram staining, but they were detected in the haemocoel (probably in hemocytes) through silver staining and immunofluorescence staining (Figure 2D-F). No bacteria were found in the eggs. Reports indicate that the hemotropic bacteria, *Bartonella*, could be present in tick or louse haemocoel, and our mites seem to be healthy because they have breed normally.[5,6]

In this study, 16S rRNA amplicon sequencing and subsequent analyses revealed that *E. faecalis* and *Bartonella* HQ806746, both of which are detected in numerous mammals and arthropods, constitute the core microbiome of *D. farinae*. These results were consistent with several previous findings. In a microbiome analysis of *D. farinae* using 16S rRNA cloning, *Bartonella* spp. was predominant.[3] In another study using similar methods, *Enterococcus* spp. was the second most abundant bacteria in *D. farinae*.[7] Some previous findings differ from the current findings. Study of the draft genome of *D. farinae* reported *Enterobacter* spp. was the most predominant (63.5%) and *Bartonella* spp. accounted for only 1.7%.[2] Another study also stressed that *Bartonella* was not detected in *D. farinae*.[7] These differences might be explained by differences in sequencing methods, feeding, breeding facilities and mite strains.

Bartonella spp. and *E. faecalis* might have immunomodulatory roles in allergic disease development. Lipopolysaccharides of Gram-negative bacteria bind to Toll-like receptor 4, which is involved in HDM-induced asthma induction.[8] In a mouse model using *D. farinae* extract that we provided, allergic asthma features were reduced when lipopolysaccharide was depleted in HDM extract.[8] Besides, some studies have reported that *Bartonella* lipopolysaccharides serve as an antagonist of Toll-like receptor 4.[9] Additionally, lipoteichoic acid of *E. faecalis* may affect the development of allergies via Toll-like receptor 2.[10] Meanwhile, recent reports confirmed that *D. farinae* allergens thus far reported are not derived from its microbiome, so the bacteria identified in this study are unlikely to be the allergen sources.[2]

Thus, in this study, *E. faecalis* and *Bartonella* spp. were identified as core microbiome of *D. farinae*, and are expected to have immunomodulatory roles in allergic disease development. It is important to characterize and control the HDM microbiome and their

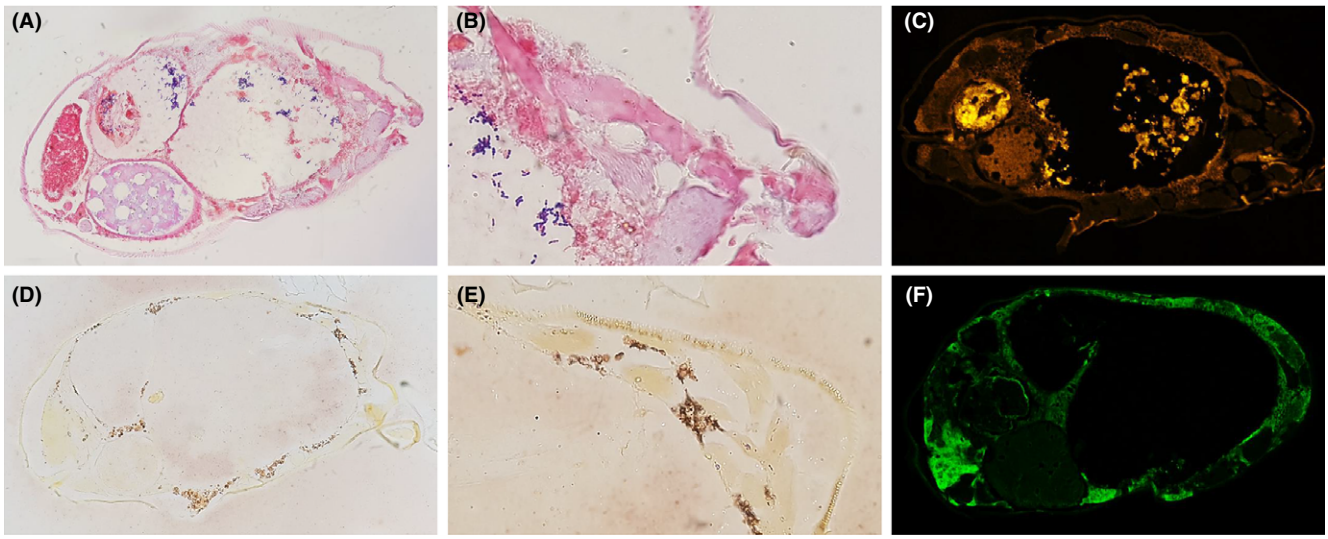


FIGURE 2 Staining methods for *Dermatophagoides farinae* showed that *Enterococcus faecalis* was distributed throughout the intestine and *Bartonella* was detected in the haemocoel. A, Gram staining (×400). B, Gram staining (head region, ×1000). Gram-positive cocci, considered as *Enterococcus* (blue-coloured cocci in clusters and short chains), were distributed throughout the intestine and the stool. C, Immunofluorescence staining using an anti-*Enterococcus* antibody (orange). D, Warthin-Starry silver staining (×400). E, Warthin-Starry silver staining (head region, ×1000). Gram-negative intracellular rods, considered as *Bartonella*, were detected in the haemocoel, but not in the intestine or egg. F, Immunofluorescence staining using an anti-*Bartonella* antibody (green)

immunomodulatory molecules for allergy prevention, treatment and immunotherapy development.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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REFERENCES

1. Stewart GA. Studies of house dust mites can now fully embrace the "-omics" era. *J Allergy Clin Immunol*. 2015;135:549-550.
2. Chan TF, Ji KM, Yim AK, et al. The draft genome, transcriptome, and microbiome of *Dermatophagoides farinae* reveal a broad spectrum of dust mite allergens. *J Allergy Clin Immunol*. 2015;135:539-548.
3. Valerio CR, Murray P, Arlian LG, Slater JE. Bacterial 16S ribosomal DNA in house dust mite cultuers. *J Allergy Clin Immunol*. 2005;116:1296-1300.
4. Poretsky R, Rodriguez-R LM, Luo C, Tsementzi D, Konstantinidis KT. Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. *PLoS ONE*. 2014;9:e93827.
5. Reis C, Cote M, Le Rhun D, et al. Vector competence of the tick ixodes ricinus for transmission of *Bartonella birtlesii*. *PLoS Negl Trop Dis*. 2011;5:e1186.
6. Previte D, Olds BP, Yoon K, et al. Differential gene expression in laboratory strains of human head and body lice when challenged with *Bartonella quintana*, a pathogenic bacterium. *Insect Mol Biol*. 2014;23:244-254.
7. Hubert J, Kopecký J, Perotti MA, et al. Detection and identification of species-specific bacteria associated with synanthropic mites. *Microb Ecol*. 2012;63:919-928.
8. Ryu JH, Yoo JY, Kim MJ, et al. Distinct TLR-mediated pathways regulate house dust mite-induced allergic disease in the upper and lower airways. *J Allergy Clin Immunol*. 2013;131:549-561.
9. Malgorzata-Miller G, Heinbockel L, Brandenburg K, van der Meer JW, Netea MG, Joosten LA. *Bartonella quintana* lipopolysaccharide (LPS): structure and characteristics of a potent TLR4 antagonist for in-vitro and in-vivo applications. *Sci Rep*. 2016;6:34221.
10. Kaesler S, Volz T, Skabytska Y, et al. Toll-like receptor 2 ligands promote chronic atopic dermatitis through IL-4-mediated suppression of IL-10. *J Allergy Clin Immunol*. 2014;134:92-99.

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