

those in health and under conditions of chronic immune stimulation.^{1,3} Further untangling of the functional properties of thymic B cells may lead to a better understanding of their role in central tolerance and autoimmune diseases.

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Comparative microbiome analysis of *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, and *Tyrophagus putrescentiae*

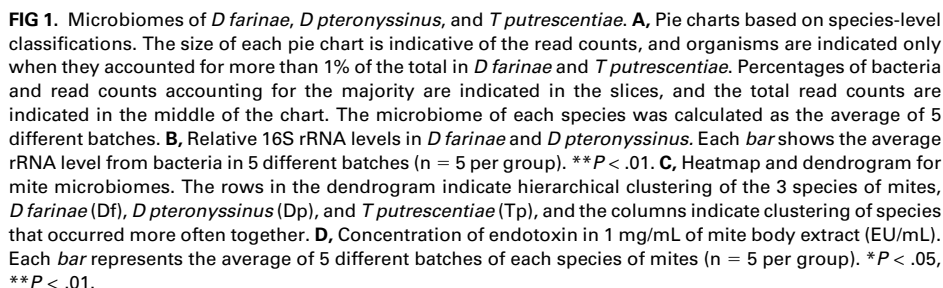


To the Editor:

Over the past few decades, there have been numerous studies on the effects of the human microbiome and environmental microbiome on allergic diseases.¹ However, little is known about the effects of the house dust mite (HDM) microbiome on allergic immune responses. HDMs are not only a major allergen source but also harbor various adjuvants triggering allergic responses, such as endotoxin and bacterial DNA from their microbiomes, which may exert immunomodulatory effects on allergic disease pathogenesis.² Therefore, we compared the microbiomes of 3 species of HDMs that are known to cause allergic diseases in humans and investigated the significance of these microbiomes to allergic immune responses. The microbiome compositions were determined from the 3 HDM species with 16S rRNA amplicon analysis using high-throughput sequencing technology. The detailed experimental procedures are described in this article's Online Repository available at www.jacionline.org.

We analyzed the microbiome compositions among 3 strains of mites, *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, and *Tyrophagus putrescentiae*, at the species level (Fig 1, A). *Bartonella* HQ806746 accounted for 97.94% and *Enterococcus faecalis* accounted for 1.57% of the microbiome in *D farinae*. *Bartonella* JX001274 accounted for 63.17% and JN236497, from the order Sphingobacteriales, accounted for 35.5% of the microbiome of *T putrescentiae*. In contrast, the microbiome of *D pteronyssinus* contained 64.58% *Klebsiella pneumoniae*, but the read count was very low with an average of 12.4 reads (see Table E1 in this article's Online Repository available at www.jacionline.org). Moreover, *D pteronyssinus* had approximately 600 times less bacteria than did *D farinae* (Fig 1, B). Notably, the read count of *K pneumoniae* was similar in the 3 mite species (16.4 in *D farinae*, 12.4 in *D pteronyssinus*, and 12.2 in *T putrescentiae*), which might reflect contamination during the experiment. We then performed heatmap and principal-components analyses based on the Bray-Curtis distance, which demonstrated significantly different microbiome patterns among the 3 mite species (Fig 1, C; see Fig E1 in this article's Online Repository available at www.jacionline.org).

Several studies have reported that *Bartonella* (or *Bartonella*-like bacteria) is the most dominant bacteria group in the microbiomes of both *D farinae* and *T putrescentiae*, which is in line with our results.²⁻⁵ In this study, *E faecalis* was also detected in *D farinae*, confirming our previous results.⁴ A previous study using a 16S rRNA cloning method demonstrated that *Enterococcus* species were the second most abundant bacteria in *D farinae*.⁶ Although the draft genome of *D farinae* indicated that *Enterobacter* species were predominant, we did not find any *Enterobacter* in our *D farinae* strain.³ Another study using 16S rRNA amplicon deep sequencing indicated that *Solitalea*-like bacteria belonging to Sphingobacteriaceae were present among various strains of *T putrescentiae* acquired from different sites,⁵ which is also in line with our results. *Bartonella* was also found to be abundant in *D pteronyssinus* based on 16S rRNA cloning.² However,



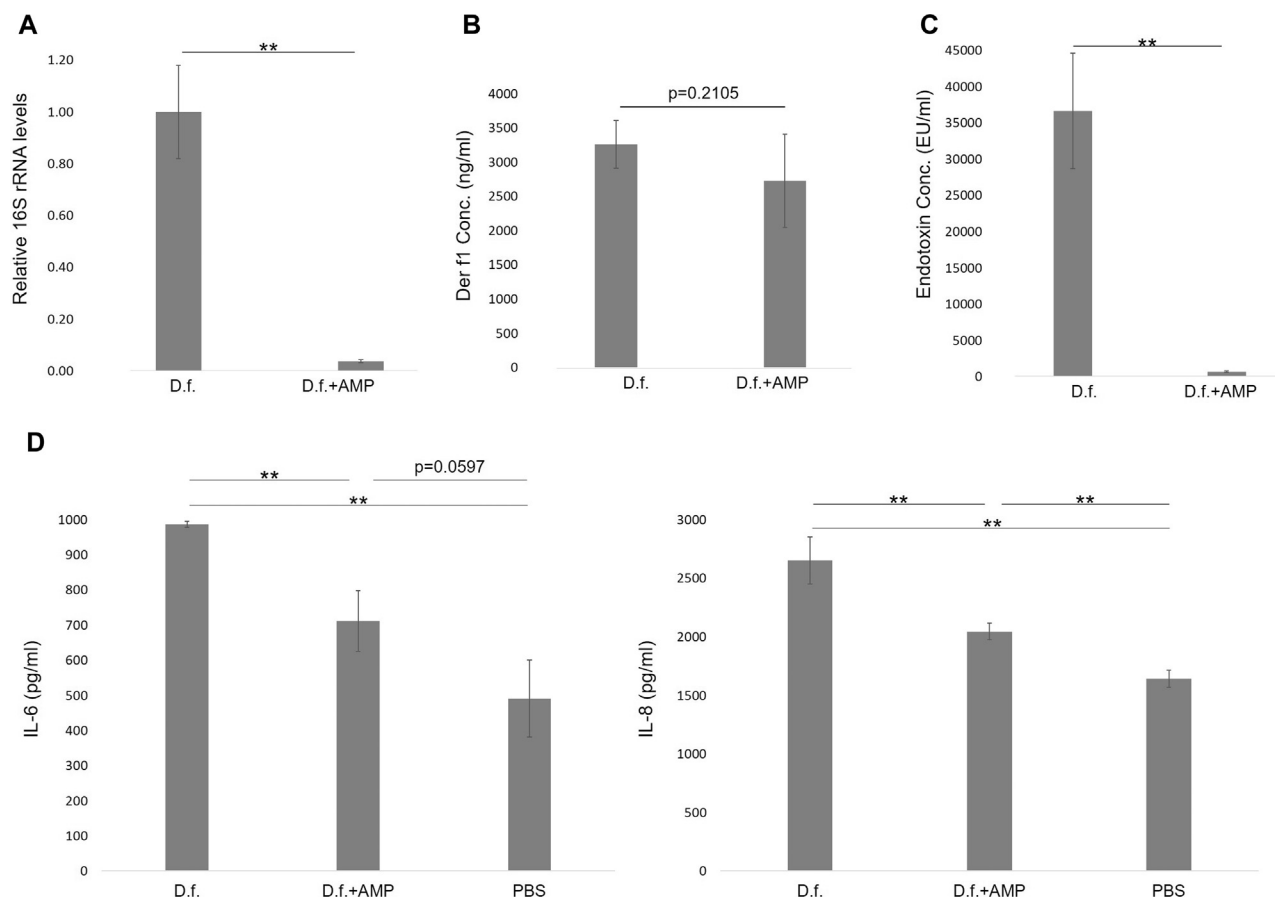


FIG 2. Change in the *D. farinae* (D.f.) microbiome following antibiotics treatment and its effects on the allergic inflammatory response. **A**, Relative 16S rRNA levels. **B**, Concentration of endotoxin in 1 mg/mL of *D. farinae* extract (EU/mL). **C**, Concentration of Der f 1 in 1 mg/mL of *D. farinae* extract (ng/mL). **D**, Concentrations of IL-6 and IL-8 of human bronchial epithelial cells (BEAS2-B) treated with the extract of *D. farinae* cultured under ampicillin (AMP) (pg/mL). Each bar shows the average of different batches (n = 4 per group). **P* < .05, ***P* < .01.

unlike the previous study, we used autoclaved media and analyzed the microbiome using a next-generation sequencing technique, and demonstrated that *D. pteronyssinus* can be cultured almost aseptically in the laboratory. Thus, application of such aseptic mites might be a useful strategy for developing more controlled allergy immunotherapeutic agents.

We also found a high endotoxin concentration in both *D. farinae* and *T. putrescentiae*, whose microbiomes generally comprised gram-negative strains such as *Bartonella* HQ806746, *Bartonella* JX001274, and *Sphingobacteriales* JN236497 (Fig 1, D). *D. pteronyssinus*, which harbored relatively few bacteria, showed an approximately 11-times lower endotoxin concentration than did *D. farinae*. This finding is in line with previous studies showing that *D. farinae* extracts have more endotoxin than *D. pteronyssinus* extracts, and that *D. farinae* harbors a greater bacterial abundance than does *D. pteronyssinus*.^{2,7}

To determine whether there is a difference in the microbiome according to the site of origin of the mite strain, we further analyzed the microbiome of a *D. farinae* strain collected from a house in Japan (see Fig E2 in this article's Online Repository available at www.jacionline.org). This Japanese strain has been maintained in our laboratory for more than 15 years using the same methods applied for cultivating the Korean strain of *D. farinae*. The results showed that *Bartonella* HQ806746 was the dominant taxon among Japanese mites, exhibiting an identical microbiome pattern to that

of Korean mites. Thus, when bred in the same environment, mites of the same species showed almost identical microbiome patterns regardless of their country of origin. This also suggests that the difference in the microbiomes of the 3 species of mites examined in this study was not related to the collection site, but rather truly reflects unique symbionts for each mite species.

Furthermore, to determine whether the change in the microbiome in HDM affects allergy development, the mites were cultured in medium containing the antibiotic ampicillin. In our pilot study, ampicillin treatment did not affect the growth of mites at a medium:ampicillin ratio of 10:1 (data not shown). Therefore, the mites were cultivated in medium with a 100:1 ampicillin ratio, which reduced the amount of bacteria in mites by 25-fold, and resulted in a decrease in the proportion of *Bartonella* (Fig 2, A; see Fig E3 in this article's Online Repository available at www.jacionline.org). Furthermore, the endotoxin concentration was reduced by 100-fold by antibiotics treatment (Fig 2, B). However, the allergen (Der f 1) concentration of the mite extract did not differ between the ampicillin treatment group and the control group (Fig 2, C).

Furthermore, treatment of a human bronchial epithelial cell line (BEAS-2B) with the extract of antibiotic-treated mites significantly reduced the secretion levels of the proinflammatory cytokines IL-6 and IL-8, which are related to allergic airway inflammation (Fig 2, D). This suggests that allergens and bacterial

components such as endotoxin synergistically act on airway epithelial cells to stimulate the inflammatory response. Group 1 allergens and endotoxins have been reported to be recognized by protease-activated receptor 2 and Toll-like receptor 4, respectively, to stimulate IL-6 and IL-8 secretion in airway epithelial cells.⁸ In line with this finding, allergic airway inflammation was reduced in a mouse model when the endotoxin of our *D farinae* extract was depleted.⁹

Although we focused only on the bacterial composition of the mite microbiome, further study on fungal composition in mites could help to provide more integrated perspectives on bacterial and fungal endosymbiosis in different mite species, and their associations with the development of allergic diseases.⁵

In conclusion, we found that 3 species of mites, *D farinae*, *D pteronyssinus*, and *T putrescentiae*, showed distinct microbiomes, and that a change in the microbiome could affect the ability of the mite to induce allergic diseases.

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Late thymic deficiency after HLA-haploidentical hematopoietic stem cell transplantation for severe combined immunodeficiency



To the Editor:

In patients with severe combined immunodeficiency (SCID), hematopoietic stem cell transplantation (HSCT) is highly successful without conditioning when donors are HLA-identical siblings. In contrast, in HSCT from alternative donors, such as haploidentical parents, results are more variable.¹

We analyzed long-term outcome of HSCT in 129 patients with SCID transplanted from HLA-haploidentical parental donors at

TABLE I. Details of patients and transplantation outcomes

Characteristic	No conditioning (n = 41)	Conditioning (n = 88)	P value
Baseline characteristics			
Female sex	9 (22)	29 (33)	.221
B+ SCID variant	31 (82)	42 (54)	.004
Omenn syndrome	1 (2)	7 (8)	.434
Maternofetal transfusion	19 (49)	39 (44)	.701
Pulmonary infection at diagnosis	19 (48)	40 (46)	1
Abnormal liver function	8 (20)	11 (12)	.299
Transplant characteristics			
Transplantation after 1995	12 (29)	56 (64)	<.001
Donor			
Mother	23 (56)	60 (68)	.236
Father	18 (44)	27 (31)	.167
Peripheral blood stem cells*	13 (32)	56 (64)	.001
Immune reconstitution			
B cells of donor origin	5 of 25 (20%)	52 of 59 (88%)	<.001
IVIG independence†	6 of 25 (24%)	49 of 56 (88%)	<.001
Any myeloid engraftment‡	1 of 25 (4%)	47 of 59 (80%)	<.001
Full myeloid engraftment	1 of 25 (4%)	38 of 59 (64%)	<.001
Early posttransplant events			
Acute GvHD ≥ grade 2	6 (16)	28 (34)	.051
GvHD de novo‡	0 (0)	9 (10)	.057
Chronic GvHD	3 (9)	17 (22)	.115
Veno-occlusive disease or capillary leak	0 (0)	15 (17)	.003
Early fatalities	12 (29)	20 (23)	.512
Late posttransplant events			
Late fatalities	3 of 21	1 of 59	
Pulmonary complications	8 of 21	3 of 59	.008
Recurrent infections	5 of 21	2 of 59	
Chronic lung disease	3 of 21	1 of 59	
CD8 ⁺ T-cell proliferative disorders	2 of 21	—	
Autoimmunity	—	4/59	
Tumors	—	(3§+1)/59	
Growth retardation (<5th percentile)	2§/21	(7§+1)/59	

Values are n (%).

IVIG, Intravenous immunoglobulin.

*All other grafts were bone marrow.

†IVIG independence was defined as stable, normal immunoglobulin serum levels and absent clinical complications secondary to humoral deficiency.

‡de novo GvHD was defined by no evidence of materno-fetal transfusion.

§Patients affected by *DCLRE1C* deficiency.

||Myeloid engraftment was defined as donor myeloid chimerism >10% measured either in granulocytes or in CD34⁺ bone marrow precursors.⁹

Online Repository Material

Comparative microbiome analysis of *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, and *Tyrophagus putrescentiae*

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11 **Methods**

12 *Mite cultivation*

13 Inactive dry yeast (Ottogi Co., Korea) was mixed with feed for fish (KKW1Q0040; JeilFeed
14 Co., Korea) at a ratio of 1:1, and the mixture was autoclaved twice. *D. farinae* and *D.*
15 *pteronysinus* were collected from beds in a Korean household (1998,1999), Japanese *D.*
16 *farinae* was collected from a bed in a Japanese household (2002), and *T. putrescentiae*, a
17 storage mite, was collected from a rice barrel in a Korean house (2005); all mites have been
18 continuously cultured and maintained in our laboratory for more than 15 years. The mites
19 were inoculated into the autoclaved medium in each of five 75T cell culture flasks (70075,
20 SPL Life Sciences, Korea) and cultivated at 25°C for 6 weeks; subculture was performed
21 every 6 weeks for 18 weeks. *D. farinae* with antibiotics was cultivated in the autoclaved
22 medium at a 100:1 ampicillin ratio.

23 *Isolation of DNA and preparation of mite body extracts*

24 Autoclaved saturated salt water was used to harvest mites separated from the culture medium
25 and debris. DNA was then extracted from the mites using NucleoSpin DNA Insect Kit
26 (MACHEREY-NAGEL, Germany). The rest of the harvested mites were used to prepare a
27 homogenate by sonication with the Qsonica sonicator (Q500) (Pronextech, Korea) and
28 centrifuged at 10,000 *g* for 30 min using Mega 17R (Hanil, Korea). The extract was obtained
29 by removing foreign substances from the homogenate, including bacteria, with a sterile
30 Millex-GP syringe filter with a 0.22-μm pore-size hydrophilic polyethersulfone membrane
31 (EMD Millipore, USA).

32 *Amplification of 16S rRNA by polymerase chain reaction (PCR)*

33 The V3–V4 region of 16S rRNA was amplified by PCR using the following primer pair:

34 forward primer, 5'-
35 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3';
36 reverse primer, 5'-
37 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-
38 3' (Illumina MiSeq V3 cartridge (600 cycles); Illumina, USA).

39 *Next-generation sequencing*

40 A limited-cycle amplification step was performed to add multiplexing indices and Illumina
41 sequencing adapters. Libraries were normalized, pooled, and sequenced on the MiSeq
42 platform (Illumina MiSeq V3 cartridge (600 cycles); Illumina) in accordance with the
43 manufacturer's instructions.

44 *Endotoxin measurement*

45 The mite extracts were diluted 1:2,000 in Limulus Amebocyte Lysate (LAL) water, and the
46 concentration of endotoxin was measured using an LAL QCL-1000 Kit (LONZA,
47 Switzerland).

48 *Real-time PCR*

49 The genes for house dust mites were amplified using the forward primer 5'-
50 ACCCGTGAACATGCTTTGCT-3' and reverse primer 5'-CACCATTCTCTCAAGCTCGT-3',
51 and the genes for bacteria were amplified using the forward primer (BACT1369) 5'-
52 CGGTGAATACGTTTCYCGG-3' and reverse primer (PROK1492R) 5'-
53 GGWTACCTTGTTACGACTT-3' from XenoTech with AMPIGENE qPCR Mixes (ENZO,
54 USA) for the all bacterial 16S rRNA.

55 *Enzyme-linked immunosorbent assay (ELISA)*

Der f 1, the group 1 allergen of *D. farinae*, was quantified using the Der f 1 ELISA kit (Indoor Biotechnologies, UK) with a 1:1000 diluted extract of *D. farinae* cultivated in medium with antibiotics.

Cytokine measurement

The human bronchial epithelial cell line BEAS-2B (American Type Culture Collection, USA) was cultured in a six-well cell culture plate (SPL, Korea) in Dulbecco's modified Eagle medium/F12 (1:1) with 10% fetal bovine serum and 1% penicillin (Gibco, USA) for 24 h at 37°C in a 5% CO₂ incubator. Each extract (100 µg/mL) was applied to each well, and the supernatant was collected after 24 h of culture. The concentrations of cytokines secreted from BEAS-2B cells were measured from the supernatants with the DuoSet ELISA human IL-6 kit (R&D Systems, USA).

Bioinformatics and statistical analysis

Sequencing data obtained from the Illumina MiSeq system were processed using Mothur v1.39.5. Contigs between paired-end reads were assembled and aligned to EzBioCloud's 16S database from ChunLab (ChunLab Inc., Korea). Chimeras were removed using UCHIME, and remaining sequences were classified using the EzBioCloud reference [1]. Heatmap analysis and principal components analysis (PCA) were performed using R 2.15.1 with the Vegan package 2.0-10 v2.15.3.

ELISA results are presented as the mean ± standard deviation, and statistical significance was determined using Student's t-test or analysis of variance, with Bonferroni correction as a post-hoc analysis. Differences with p values of 0.05 or less were considered statistically significant.

78 **Reference**

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81 7.

Online Repository Tables

Comparative microbiome analysis of *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, and *Tyrophagus putrescentiae*

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11 **Table E1. Read counts at the species and genus levels**

12 (A) Species level

Taxon	D.f	D.p	T.p
Actinomyces_odontolyticus	0.6	0.2	0
Corynebacterium_glutamicum	1.4	0	0.2
Microbacterium_kyungheense	0.2	0	0
Asaccharobacter_celatus	0.6	0	0
Bacteroides_vulgatus	5	0	0
Pedobacter_panaciterrae	0.4	0	0
Anaerobacillus_macyae	1	0	0.4
Bacillus_bingmayongensis	0.8	0	0
Bacillus_pseudofirmus	1.2	0	0
Enterococcus_faecalis	180	0	0
Lactobacillus_apodemi	7.2	0	1.4
Streptococcus_mutans	0.8	0	0
EU509291_s	0.4	0	0
EU006478_s	0.4	0	0
HM124173_s	0.6	0	0
FJ678358_s	0.2	0	0
HQ806746_s	11211.6	0	0
APJD_s	0.2	0	0
Devosia_chinhatensis	0.2	0	0
Phyllobacterium_brassicacearum	0.2	0	0
Rhizobium_laguerreae	1.4	0.2	0.4

Rhizobium_multihospitium	0.6	1.8	0.4
Rhizobium_nepotum	0.4	0	0
Rhizobium_tropici	0.8	0	0
Sphingomonas_leidy	0.6	0	0
Delftia_acidovorans	2.6	0.4	0.6
Variovorax_gossypii	0.4	0	0
Enterobacter_cloacae	6.6	0.6	1.6
Escherichia_coli	0.4	0	0
Klebsiella_granulomatis	2.6	1.6	0.4
Klebsiella_pneumoniae	16.4	12.4	12.2
Leclercia_adecarboxylata	1.2	0.8	0.2
Propionibacterium_acnes	0	0.2	0
AY642552_s	0	0.2	0.2
AVBN_s	0	0.4	0
Burkholderia_contaminans	0	0.4	0
JN236497_s	0	0	522.2
Bacillus_nanhaisediminis	0	0	0.2
Lysinibacillus_sphaericus	0	0	0.2
Lactobacillus_salivarius	0	0	0.4
AB606382_s	0	0	0.2
JX001274_s	0	0	929.4
Delftia_lacustris	0	0	0.6

13 (B) Genus level

Taxon	D.f	D.p	T.p
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Actinomyces	0.6	0.2	0.2
Corynebacterium	1.4	0	0
Microbacterium	0.2	0	0
Adlercreutzia	0.6	0	0
Bacteroides	5	0	0
Pedobacter	0.4	0	0
Anaerobacillus	1	0.2	0.8
Bacillus	0.8	0	0
Bacillus_g6	1.2	0	0
Enterococcus	180	0	0
Lactobacillus	7.2	0	1.8
Streptococcus	0.8	0	0
AB626912_g	0.4	0	0
Acetatifactor	0.4	0	0
Clostridium_g24	0.6	0	0
Oscillibacter	0.2	0	0
Bartonella	11211.6	0	929.4
Bradyrhizobium	0.2	0	0
Devosia	0.2	0	0
Phyllobacterium	0.2	0	0
Rhizobium	3.2	2	0.8
Sphingomonas	0.6	0	0
Delftia	2.6	0.4	1.2
Variovorax	0.4	0	0

Enterobacter	6.6	0.6	1.6
Escherichia	0.4	0	0
Klebsiella	19	14	12.6
Leclercia	1.2	0.8	0.2
Burkholderia	0	0.4	0
Mesorhizobium	0	0.4	0
Propionibacterium	0	0.2	0
JN236497_g	0	0	522.2
Lysinibacillus	0	0	0.2
JN713389_g	0	0	0.2

14 Results represent the average total reads for the three species of mites as determined by next-
15 generation sequencing of 16S rRNA amplicons.

16

Online Repository Figures

Comparative microbiome analysis of *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, and *Tyrophagus putrescentiae*

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Fig. E1. Analysis of the variance of microbial communities in three species of house dust mites using principal components analysis (PCA). PCA score plots for *D. farinae* (red), *D. pteronyssinus* (green), and *T. putrescentiae* (purple) are shown. Each point represents one batch (n = 5).

17 **Fig. E2.** Pie chart of the microbiome of *D. farinae* from Japan cultured under the same
18 condition as Korean *D. farinae* based on species-level classification.

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Fig. E3. Microbiome of *D. farinae* cultured with antibiotics.