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Microbiome and mycobiome interaction in house dust mites and impact on airway cells

Running head: Microbiome and mycobiome in mites

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Abstract

Background: Major allergen sources *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, and *Tyrophagus putrescentiae* have been reported to have distinct microbiomes. The purpose of this study was to investigate the effect of each mite on airway epithelial cells as a model of airway allergic disease.

Methods: Transcriptomic analysis (RNA-seq) of an airway epithelial cell line (BEAS-2B) was performed to compare gene expression patterns after treatment with extracts of three mite species (*D. farinae*, *D. pteronyssinus*, and *T. putrescentiae*). In addition, mycobiome deep sequencing of mites was employed to identify fungal species that interact with the microbiomes of the mites.

Results: Immune responses to bacteria were enriched only in the *D. farinae*-treated group as this species harbored larger numbers of bacteria than the other mites, and the high level of LPS in *D.*

farinae caused proinflammatory cytokine production in airway epithelial cells. In addition, antibiotic metabolism pathways were enriched in *D. pteronyssinus*-treated cells but not in *D. farinae*-treated cells. Subsequent experiments revealed that *D. pteronyssinus* had a high fungal load that inhibited bacterial survival in this mite species.

Conclusion: The large amount of bacteria in *D. farinae* causes airway epithelial cells to produce more allergy-related cytokines than *D. pteronyssinus*, since fungi present in *D. pteronyssinus* suppress the abundance of mite-associated bacteria.

Key Messages

- Comparative transcriptomic analysis of airway epithelial cells treated with three allergenic mites extracts was conducted.
- *Dermatophagoides farinae* harbors several bacteria with synergistic effects on allergic inflammation *in vitro*.
- *D. pteronyssinus* hosts abundant *Aspergillus penicillioides*, which suppresses bacterial microbiome growth in mites.

1. Introduction

Allergic diseases are caused by hypersensitivity of the host immune system of atopic individuals to environmental substances, usually not harmful to the non-atopic general population.¹ House dust mites (HDMs) and storage mites are well-known indoor or occupational causes of allergic diseases such as asthma, atopic dermatitis, and allergic rhinitis.²⁻⁴ *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* are major HDMs, while *Tyrophagus putrescentiae* is the predominant storage mite species in South Korea. Koreans store uncooked rice in rice bins or chests in their homes, and *T. putrescentiae* is often found in kitchens in urban areas,⁵ where asthma cases have been associated with the presence of *T. putrescentiae*.⁶ The predominant mite species in Korean homes were found to be *D. farinae* (65 to 77% of Korean homes), *D. pteronyssinus* (8 to 20%), and *T. putrescentiae* (6.5 to 8.5%).^{5, 7}

The major allergens in *D. farinae* and *D. pteronyssinus* belong to groups 1, 2, and 23, while those in *T. putrescentiae* belong to groups 2 and 3.^{3,8-11} The group 1 allergen is a cysteine protease, which destroys the tight junctions of epithelial cells and is located in the gut of mites.² The group 2 allergen is an MD-2-like lipid-binding protein, also located in the gut.² The group 3 allergen, which is the major one in *T. putrescentiae*, possesses serine protease activity.¹⁰

Previous studies on the microbiomes of *D. farinae*, *D. pteronyssinus*, and *T. putrescentiae* have reported different microbiota species compositions. The *D. farinae* microbiome is enriched in *Enterococcus faecalis* and *Bartonella*, whereas that of *T. putrescentiae* is enriched in *Bartonella* and Sphingobacteriales.¹²⁻¹⁵ Other studies have reported that the amount of lipopolysaccharide (LPS) is higher in *D. farinae* than in *D. pteronyssinus*.^{12,16} LPS is a critical stimulus of various immunological and allergic responses.¹⁷⁻¹⁹ In addition, *Aspergillus penicilloides* has been observed in the intestine of *D. pteronyssinus*.^{20,21} A change in fungal diet can affect the microbiome of *T. putrescentiae*, which is a fungivorous mite.²²⁻²⁴ This suggests that fungi may interact with bacteria in mites and thus modulate allergic disease.

Here, we performed a transcriptomic analysis (RNA-seq) of the airway epithelial cell line, BEAS-2B, to compare gene expression patterns after treatment with extracts from three species of mites (*D. farinae*, *D. pteronyssinus*, and *T. putrescentiae*). Pathway analysis and subsequent experiments revealed the interaction between microbiome and mycobiome in HDMs, which may help develop allergy immunotherapeutics.

2. Methods

A full description of the methods used in this study is provided in the Online Supplementary Materials.

Ethical aspects

An institutional review board (IRB) approval was not required. No humans or laboratory animals other than mites were used in this study.

Mite cultivation, DNA isolation, and protein extraction

Mites (*D. farinae* and *D. pteronyssinus*) were cultivated in the presence of an antifungal agent, amphotericin B powder, at a 10:1 ratio (Cayman Chemical, Ann Arbor, MI, USA) in autoclaved medium for four weeks. Detailed methods of the cultivation of mites (*D. farinae*, *D. pteronyssinus*, and *T. putrescentiae*), DNA isolation, and protein extraction are provided in Online Supplementary Materials.

Mite extract treatment on cells and RNA-seq

Human bronchial epithelial cells (BEAS-2B, ATCC® CRL-9609™, Rockville, MD, USA) were sampled 24 hours after a single exposure to 100 µg/mL of extract from each mite. Three independent samples were examined following exposure to each mite extract or phosphate-buffered saline (PBS) as a control. Total RNA was extracted and sequenced by Macrogen (Seoul, South Korea) using a NovaSeq 6000 System (USA). Differentially expressed gene analysis was performed using DESeq2 six combinations were compared (*D. farinae*/PBS, *D. pteronyssinus*/PBS, *T. putrescentiae*/PBS, *D. farinae*/*D. pteronyssinus*, *D. pteronyssinus*/*T. putrescentiae*, and *D. farinae*/*T. putrescentiae*), and 912 genes exhibiting $\geq$ two-fold change and nbinomWaldTest raw p-values < 0.05 for at least one combination were selected for further study. Three samples for each group were analyzed as a single cluster to determine the level of similarity within the group. The numbers of clusters were independently assessed using hierarchical

clustering (Euclidean method, complete linkage) to construct a heatmap. Genes upregulated at least two-fold after treatment with each mite extract compared to PBS treatment and showing a false discovery rate (FDR) < 0.05 were used to construct a Venn diagram. Gene Ontology analysis was performed with G:Profiler (<https://biit.cs.ut.ee/gprofiler/gost>) using the term “GO biological process.” Terms with an FDR < 0.1 are shown in Table S1. See Online Supplementary Materials for additional details.

Fungus-specific TA cloning

Fungal ITS1 and ITS2 genes in mite DNA samples were amplified using polymerase chain reaction (PCR) with the following primer pair: forward primer, 5'-TCCGTAGGTGAACCTGCGG-3' and reverse primer, 5'-GCTGCGTTCTTCATCGATGC-3'.²⁵ TA cloning was performed and cloned colonies were sequenced, and homology was determined through NCBI BLAST searches.

Mycobiome deep sequencing and bioinformatics

From the mite DNA, the ITS region of fungal DNA was amplified by PCR using the following primer pair: forward primer (fITS9), 5'-GAACGCAGCAAAATGCGA-3' and reverse primer (ITS4), 5'-TCCTCCGC TTATTGATATGC-3'.²⁶ 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) following the manufacturer's instructions. Raw reads were processed using QIIME v1.9.1 and UNITE v18.11.2018, the fungal database, as indicated in previous literature.²⁷

*Inoculation of *E. faecalis* (green fluorescence protein) into mites*

E. faecalis OG1RF harboring pMV158GFP and pAM β 1 was grown on brain heart infusion (BHI) broth and agar medium with tetracycline (1 μ g/mL) and erythromycin (1 μ g/mL).²⁸

Mites (*D. farinae* and *D. pteronyssinus*, 1 g) were inoculated with 1 mL of *E. faecalis* solution (2×10^{11} CFU/mL) with or without amphotericin B (3 mg/mL). At 3, 6, and 10 days after inoculation, the mites (1 g) were homogenized and spread on BHI agar with antibiotics to count the remaining *E. faecalis*.

Culture of Aspergillus penicillioides and extract preparation

D. pteronyssinus homogenate was spread on BHI agar and cultured at 25°C for 7 days. Identified *A. penicillioides* colonies using fungal ITS1 and ITS2 gene sequencing were transferred to yeast extract sucrose agar medium and cultured at 25°C for 7 days.²⁹ To obtain an extract containing compounds that exhibit antibacterial activity, *A. penicillioides* extracts were prepared using a modified maceration method with CH₂Cl₂ and MeOH.³⁰ Control extract groups were prepared by applying the above method to *Pichia pastoris* (GS115) or PBS (vehicle control).

E. faecalis growth inhibition test with *A. penicillioides* extract

GFP-*E. faecalis* was cultured in BHI broth containing ampicillin overnight at 37°C with shaking at 200 rpm. Overnight culture broth was diluted to 1:1000 with BHI broth. Extracts (50 µL) of *A. penicillioides*, *P. pastoris*, or vehicle control were added to 500 µL of the diluted bacterial broth and incubated for 4 hours. After incubation, each bacterial broth was spread on BHI agar containing ampicillin and cultured overnight, following which colonies were counted.

Detailed the methods of LPS measurement, quantitative reverse transcription PCR, cytokine measurement, enzyme-linked immunosorbent assay (ELISA) against *Aspergillus*, and statistical analysis are described in Online Supplementary Materials.

3. Results

Comparative transcriptomic analysis of airway epithelial cells treated with three allergenic mites

To investigate the response of airway epithelial cells to different HDM species, BEAS-2B cells were treated with *D. farinae*, *D. pteronyssinus*, and *T. putrescentiae* extracts, and transcriptomic analysis (RNA-Seq) was performed to compare gene expression patterns. The similarity between gene expression profiles within each group was analyzed using one-way hierarchical clustering (Figure 1A). Overall, 912 genes were differentially expressed. The gene expression pattern observed in the PBS treatment group was different from that in each HDM group. In treatment groups, the expression patterns of *D. pteronyssinus*- and *T. putrescentiae*-treated cells were more closely clustered, indicative of more similar gene expression compared to the *D. farinae*-treated group. This observation was rather unexpected because *D. farinae* and *D. pteronyssinus* are closer in terms of phylogeny and allergen type. Thereafter, we identified genes that were upregulated in response to mite extract exposure when compared to PBS treatment.

The Venn diagram (Figure 1B) shows genes that were upregulated after exposure to each mite extract compared to the control (PBS treatment). Forty-three genes were upregulated in response to all three mite species treatments, and these were mainly involved in chemokine production. (Figure 1B, C). The expression of 63 genes increased only in response to *D. farinae*. Of note, these were related to the immune responses to bacteria. (Figure 1C). Furthermore, genes that were upregulated in the *D. pteronyssinus* and *T. putrescentiae* groups, but not the *D. farinae* group, were associated with antibiotic metabolism (Figure 1C). Detailed enriched pathways are listed in Table S1.

Amount of bacteria in *D. farinae* has a synergistic effect on allergic inflammation *in vitro*

The difference between *D. farinae* treatment and treatment with other mite extracts was related to airway epithelial cell responses to bacteria (Figure 1C). In particular, the expression of cytokines IL-8, CXCL-1, and CXCL-2, which are involved in allergic inflammatory responses and immune cell recruitment, was specifically upregulated in cells treated with *D. farinae* (Figure 2A).

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These results were confirmed by real-time PCR (Figure 2B). We hypothesized that these results would be due to the large amount of bacteria-derived substances, such as LPS, in *D. farinae*. Thus, bacterial DNA and LPS concentrations were measured in the three mite species' DNA and protein extract samples, respectively. The results revealed that *D. farinae* had 14,000-fold higher bacterial DNA concentration than *D. pteronyssinus* (Figure 3A). In addition, the LPS concentration was 25,270 EU/mg, 390 EU/mg, and 1123 EU/mg in the *D. farinae*, *D. pteronyssinus*, and *T. putrescentiae* extracts, respectively (Figure 3B). Furthermore, we decided to eliminate the effects of LPS in the mite extract. Our data revealed that treatment with an LPS inhibitor (polymyxin B) decreased the secretion of IL-6 and IL-8 in *D. farinae*-treated BEAS-2B cells but not in *D. pteronyssinus*- or *T. putrescentiae*-treated cells (Figure 3C). These data suggest that bacteria-derived substances in *D. farinae*, such as LPS and allergens, synergistically affect the secretion of allergy-related cytokines in airway epithelial cells.

D. pteronyssinus* hosts a large amount of *Aspergillus penicillioides

The antibiotic metabolic process pathway was not enriched in cells treated with *D. farinae*; however, it was enriched in those treated with *D. pteronyssinus* and *T. putrescentiae*, as revealed by the expression of CYP1A1 and ABCC2 genes (Figure 2A, B). In airway epithelial cells, including BEAS-2B cells, CYP1A1 expression can increase to support the metabolism and disposal of antibiotics.^{31, 32} In addition, the symbiotic relationship between mites and fungi in *D. pteronyssinus* has been previously described.^{23, 24, 33} Based on these results, we hypothesized that the difference in bacterial abundance among mite species depends on the presence of antibacterial substances produced by fungi in the mites.

The amount of fungal DNA in DNA extracted from the three mite species was measured. Interestingly, the relative amounts of total fungi and *Aspergillus* in *D. pteronyssinus* were over 1,000 and 100-fold higher, respectively, than those in *D. farinae* (Figure S1A, B). The opposite was observed for the relative amount of bacteria in these mites (Figure 3 A). Next, an antifungal agent, amphotericin B, was administered to mites for four weeks to reduce the amount of fungi. In *D. pteronyssinus*, the total amounts of fungi and *Aspergillus* decreased significantly (Figure 4A, B), whereas bacterial abundance did not change (Figure 4C). *D. pteronyssinus* may have originally hosted a limited number of bacteria; consequently, the abundance of bacteria did not increase in this short time period. In *D. farinae* and *T. putrescentiae*, no significant changes in fungi were observed, perhaps because the fungal load in these species was low to begin with.

PCR using fungus-specific primers (ITS1 and ITS2) was employed to analyze mycobiome changes in the three mite species following amphotericin B treatment. PCR of *D. pteronyssinus* revealed thick bands at approximately 300 bp (arrows), which became thinner following amphotericin B treatment (Figure 4D). These bands were excised, and TA cloning was performed. All sequences were identified as *A. penicillioides* (13 colonies from control *D. pteronyssinus*; 10 colonies from amphotericin B-treated *D. pteronyssinus*). Figure 4E shows the results of mycobiome analysis based on next-generation sequencing of fungus-specific ITS2 amplicons. *A. penicillioides* (red) was only found in *D. pteronyssinus*, but its abundance was greatly reduced following amphotericin B treatment. Another mold found only in *D. pteronyssinus* was *Penicillium cinerascens* (yellow). Some studies have previously reported that *A. penicillioides* and *P. cinerascens* produce antibacterial molecules.^{30,34} *Saccharomyces* (blue) was a component of

mite food used in this study and a typical yeast found in every mite sample. *T. putrescentiae* also hosted another yeast, *Meyerozyma* (green). These results revealed that only *D. pteronyssinus* harbored several mold-type fungi, which probably possess antibacterial activity. In addition, a high amount of *A. penicilliioides* protein was found in *D. pteronyssinus* as opposed to *D. farinae* extracts through ELISA using an anti-*Aspergillus* antibody (Figure S2).

A. penicilliioides* suppresses bacterial microbiome growth in *D. pteronyssinus

To determine whether *D. pteronyssinus* fungi have the ability to reduce bacteria in these mite species, highly concentrated *E. faecalis* was inoculated into mites. In our previous studies, *E. faecalis* was found to be a symbiont of *D. farinae*, but was not present in *D. pteronyssinus* and *T. putrescentiae*.^{35,36} By using *E. faecalis* harboring a green fluorescence protein gene plasmid, we were able to confirm the inoculation status of each mite under a fluorescence microscope and measure the amount of inoculated bacteria in each mite based on an antibiotic resistance gene. First, we confirmed that *E. faecalis* can be maintained in *D. farinae* but almost not in *D. pteronyssinus*. *E. faecalis*-concentrated solution was added to the harvested mites. At each time point, mite homogenates were spread on BHI agar with selection antibiotics to determine the number of colony-forming units (CFU). After inoculation, the amount of *E. faecalis* decreased exponentially in *D. pteronyssinus*, but the bacteria were maintained in *D. farinae* from 6 to 10 days (Figure 5A). After 10 days of inoculation, fluorescence microscopy revealed that *E. faecalis* settled in the gut of *D. farinae* (Figure S3).

Furthermore, to determine whether the reduction of fungi in HDM affects the bacterial populations, mites were inoculated with *E. faecalis* and treated with amphotericin B. On day 10 after *E. faecalis* inoculation, *D. farinae* had 7,950 CFU of *E. faecalis* per mite, whereas *D. pteronyssinus* had an average of only 6.7 CFUs per mite without amphotericin B treatment (Figure 5B). However, *D. pteronyssinus* treated with amphotericin B had an average of 261 CFUs of *E. faecalis* per mite, which was 40-fold higher than the number of CFU observed in *D. pteronyssinus* without amphotericin B treatment ($p = 0.0227$). In contrast, amphotericin B treatment did not promote the retention of *E. faecalis* in *D. farinae*. These results demonstrated that fungi in HDMs can inhibit bacterial growth within their hosts. In addition, *A. penicilliioides* was isolated from *D. pteronyssinus*, and its extract was prepared using the protocol to contain antibiotics. It was found

that the *A. penicillioides* extract can inhibit the growth of *E. faecalis in vitro* (Figure 5C, Figure S4).

4. Discussion

In this study, BEAS-2B cells were treated with *D. farinae*, *D. pteronyssinus*, or *T. putrescentiae* extract, and transcriptomic (RNA-seq) analysis was performed. Pathway analysis revealed that immune responses to bacteria were activated only in *D. farinae*-treated cells. This was attributed to the higher amount of bacteria and LPS in this species than *D. pteronyssinus* and *T. putrescentiae*. This observation is line with our previous studies reporting that *D. farinae* had *E. faecalis* and *Bartonella* as their core microbiome, and *T. putrescentiae* mostly hosted *Bartonella* and Sphingobacteriales.^{35,36} In contrast, *D. pteronyssinus* can be cultured almost aseptically in laboratory settings.³⁶ Other studies have also reported that *D. farinae* has higher bacterial counts or amounts of bacteria-derived substances, such as LPS.^{12,16}

In addition, LPS inhibitor (polymyxin B) treatment reduced the amount of IL-6 and IL-8 released from BEAS-2B cells in the *D. farinae* extract treatment group. This suggests that allergens and bacterial components, such as LPS in *D. farinae*, act synergistically on airway epithelial cells to stimulate an inflammatory response. HDMs induce a type 2 immune response with elevated expression levels of IL-6 and IL-8 in airway epithelial cells.^{37,38} Specifically, group 1 allergens and LPS from HDMs promote the expression of proinflammatory cytokines, such as IL-6 and IL-8, by stimulating the protease-activated receptor 2 (PAR-2) and TLR4, respectively.³⁹ Moreover, calcium levels in airway epithelial cells are elevated in response to the HDM extract, which, in turn, activates the NFAT/calcineurin signaling pathway to induce the transcription of the above-mentioned proinflammatory cytokines.⁴⁰ Furthermore, in a previous study, allergic airway inflammation was reduced in a mouse model when LPS in *D. farinae* extract was inhibited by polymyxin B.⁴¹ IL-8 is a major mediator of neutrophilia in acute severe asthma.⁴² Several studies have reported beneficial outcomes through therapies targeting IL-6 in patients with asthma and mixed neutrophilic/eosinophilic bronchitis.⁴³

In this study, pathways related to antibiotic metabolism were enriched in *D. pteronyssinus*- and *T. putrescentiae*- but not in *D. farinae*-treated cells, as indicated by the increase in CYP1A1 and ABCC2 expression. In bronchial epithelial cells, including BEAS-2B, CYP1A1 expression has been shown to increase in response to antibiotics, such as penicillin, to facilitate their metabolism.^{31, 32} We observed that fungal loads were higher in *D. pteronyssinus* than

in *D. farinae* and that *A. penicilliioides* was the predominant fungal species in *D. pteronyssinus*. *A. penicilliioides* is a xerophilic fungus living on low-moisture substrates such as indoor air, house dust, and stored dried food contaminants.^{44,45} The presence of *A. penicilliioides* has been suggested to play a role in the reproduction of *D. pteronyssinus*.⁴⁶ Notably, *A. penicilliioides* produces aurantiamide acetate, which displays antibacterial and antifungal activity.^{30,47} *Penicillium cinerascens*, the second most common fungus observed in *D. pteronyssinus* in this study, is known to have antibacterial activity due to the presence of gliotoxin and spinulosin.³⁴ We speculated that these antibacterial substances might act on BEAS-2B to upregulate antibiotic metabolism-related genes.

A symbiotic relationship between mites and fungi, such as *A. penicilliioides*, was observed in *D. pteronyssinus* and *T. putrescentiae*. In addition, fungi have been previously reported to shape the microbiomes of mites.^{23,24,33} Moreover, in the current study, we observed that *D. pteronyssinus* could not harbor inoculated *E. faecalis*, whereas *D. farinae* could. *E. faecalis* has been identified as a core part of the microbiome of *D. farinae* in our previous study, and it may affect the development of allergies.^{14,35,48} An antifungal agent was used to confirm whether this phenomenon was related to the presence of fungi. In *D. farinae*, amphotericin B treatment did not affect inoculated *E. faecalis*. However, in *D. pteronyssinus*, amphotericin B treatment increased the abundance of inoculated *E. faecalis*. The antifungal agent allowed *E. faecalis* to survive in *D. pteronyssinus*, which suggests that the relatively poorer microbiome of *D. pteronyssinus* is related to the presence of specific species, such as *A. penicilliioides*, in the mite mycobiome.

Previous studies have shown that fungi in insects affect the growth of bacteria.⁴⁹⁻⁵¹ Little *et al.* found that symbiotic black yeasts acquire nutrients from the bacterial mutualists of ants and suppress bacterial growth.⁴⁹ *Beauveria bassiana* (a fungus) oosporein can act as an antimicrobial compound to limit microbial competition in *B. bassiana*-killed hosts, allowing the fungus to maximally utilize host nutrients for growth and sporulate on infected insect cadavers.⁵⁰ In *Megachile rotundata* (a bee), antifungals lowered bacterial abundance but increased the diversity of surviving fungi, suggesting that bacteria inhibit the growth of some fungi in the gut through chemical or competitive interactions.⁵¹ In this study, amphotericin B treatment allowed *D. pteronyssinus* to retain bacteria. In this process, fungal compounds such as aurantiamide acetate produced by *A. penicilliioides* might act as antibiotic agents.

This study showed that the LPS derived from bacteria in HDMs has a synergistic effect on allergic inflammation, and fungi in HDMs suppress the growth of bacteria in HDMs. In addition, it has been reported that in HDMs, fungi affect the production of allergens including proteases, lipid-binding proteins, and chitin-related proteins, which may be associated with allergic sensitization and symptoms.⁵² Therefore, when cultivating HDMs for clinical use, such as for allergic diagnosis and immunotherapy, strict control and monitoring of the bacteria and fungi in mites are required.

IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) are common inflammatory cytokines in the airway epithelium; they are associated with airway hyperresponsiveness.⁵³ In our RNA-Seq analysis of BEAS-2B cells, transcripts of IL-25, IL-33, and TSLP were not detected in any of the samples, except for the case of one sample treated with *D. pteronyssinus* extract, in which three transcripts of TSLP were detected. This result was obtained likely because the expression levels of these cytokines were too low to be detected and analyzed by RNA-Seq. Further analysis is required to compare the gene expression and protein production of these cytokines in airway epithelial cells treated with different mite species.

5. Conclusion

In conclusion, it was shown that the large amount of bacteria in *D. farinae* causes airway epithelial cells to produce allergy-related cytokines, whereas fungi in *D. pteronyssinus* suppress bacteria within the mite. The concept that fungi in HDM inhibit the bacterial microbiome, which is associated with allergic responses, may open new avenues for the development of allergy immunotherapeutics. Therefore, standardization of mite cultivation and the production of mite extracts with respect to the microbiome and mycobiome is essential for the development and improvement of diagnostic and immunotherapeutic agents for allergic diseases.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

JYK, MHY, and TSY conceived and designed experiments. In-Yong Lee cultured mites. SL and DY collected samples and associated metadata. JYK and MHY analyzed the experimental data. JYK and SSY performed cloning experiment. All authors contributed to writing the manuscript. The authors read and approved the final manuscript.

Ethical Statement

Not applicable.

Availability of data and material

Raw sequence data are available in NCBI GenBank under Bioproject PRJNA729580.

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Figure legends

Figure 1. Analysis of the transcriptional differences between airway epithelial cells (BEAS-2B)

exposed to *D. farinae* (D.f), *D. pteronyssinus* (D.p), and *T. putrescentiae* (T.p) extracts, or control (PBS). (A) Hierarchical clustering of the 912 differentially expressed genes after 24 h of exposure to each house dust mite and PBS. Genes satisfying the criteria of $\geq$ two-fold change and p value < 0.05 are shown. (B) Venn diagram representing the number of upregulated genes after treatment with each house dust mite extract compared to PBS treatment. Genes satisfying the criteria of $\geq$ two-fold change and false discovery rate (FDR) < 0.05 were counted. (C) Pathway analysis performed with genes from (B), using G:profiler with “GO biological process.”

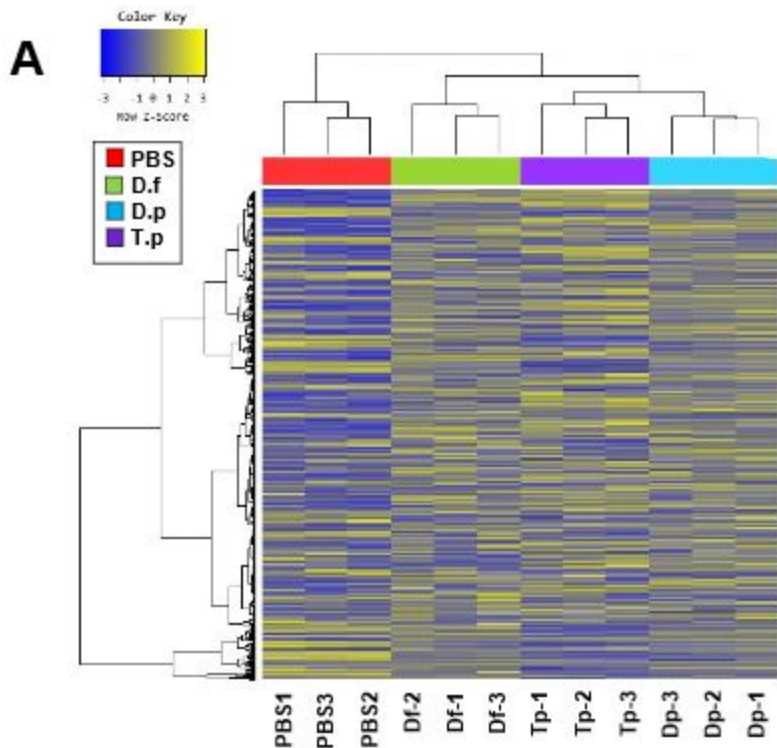
Figure 2. Transcript expression of genes upregulated in airway epithelial cells (BEAS-2B) treated with *D. farinae* (D.f), *D. pteronyssinus* (D.p), and *T. putrescentiae* (T.p) extracts (A) Fragments per kilobase million (FPKM) of IL-8, CXCL1, CXCL2, CYP1A1, and ABCC2 in BEAS-2B cells 24 h after stimulation. (B) Quantitative PCR data for IL-8, CXCL1, CXCL2, CYP1A1, and ABCC2 in BEAS-2B cells 24 h after stimulation. Data are presented as the mean $\pm$ SD of at least three independent experiments.

Figure 3. LPS in *D. farinae*, *D. pteronyssinus*, and *T. putrescentiae* and its effects on mite extract-induced inflammatory cytokine production in airway epithelial cells (BEAS-2B). (A) Relative 16S rRNA gene levels in *D. farinae* (D.f), *D. pteronyssinus* (D.p), and *T. putrescentiae* (T.p). (B) Concentration of endotoxin (LPS) in 1 mg/mL of mite extract (EU/mL). Effects of LPS on mite extract-induced (C) IL-6 and (D) IL-8 production in BEAS-2B cells. Each extract was pretreated with the LPS inhibitor polymyxin B. Extracts with and without pretreatment were then applied to cells. Data are presented as the mean $\pm$ SD of at least three independent experiments.

Figure 4. Measurement and identification of fungi in *D. farinae*, *D. pteronyssinus*, and *T. putrescentiae*. Relative amount of (A) fungal, (B) *Aspergillus*, and (C) bacterial genes (16 rRNA gene) in DNA extracted from *D. farinae*, *D. pteronyssinus*, and *T. putrescentiae* following treatment with antifungal agent amphotericin B. (D) Results of PCR using primers specific for fungi (ITS1 and ITS2). The visibly thinned bands following amphotericin B treatment of *D. pteronyssinus* (red arrows) were excised, sequenced, and identified as *Aspergillus penicillioides*.

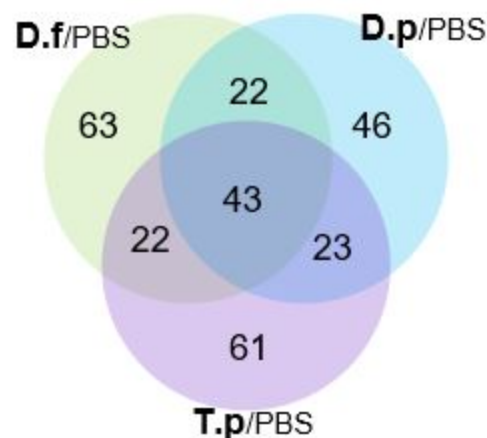
(E) Mycobiome composition of *D. farinae*, *D. pteronyssinus*, and *T. putrescentiae*. Fungus-specific ITS2 amplicon sequencing based on next-generation sequencing was performed.

Figure 5. Suppression of bacterial growth by *A. penicilliioides* in *D. pteronyssinus*. (A) Settlement of inoculated *E. faecalis* in *D. farinae* and *D. pteronyssinus* over time. *E. faecalis* containing a GFP plasmid was inoculated into the mite gut, and the number of remaining bacteria in mites (CFU per mite culture flask) was counted at each post-inoculation time point. Brain heart infusion (BHI) agar containing antibiotics was used to selectively culture *E. faecalis* containing the GFP plasmid. (B) Remaining inoculated *E. faecalis* in mites treated with amphotericin B 10 days after bacterial inoculation. Ten days after inoculation, homogenates of 25 mites for each group (n=6) were spread on BHI agar containing antibiotics to determine CFU per mite. (C) *E. faecalis* CFU after 4 hours of treatment with *A. penicilliioides* extract. *E. faecalis* was cultured in BHI broth medium with *A. penicilliioides* extract, and the broth was spread on BHI agar after 4 hours of treatment. Yeast extract (GS115) was used as a positive control.

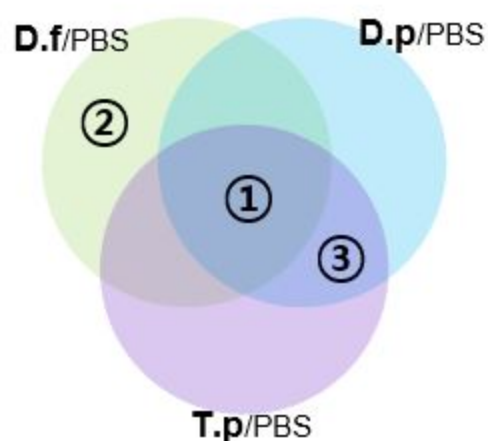


B

The number of upregulated genes



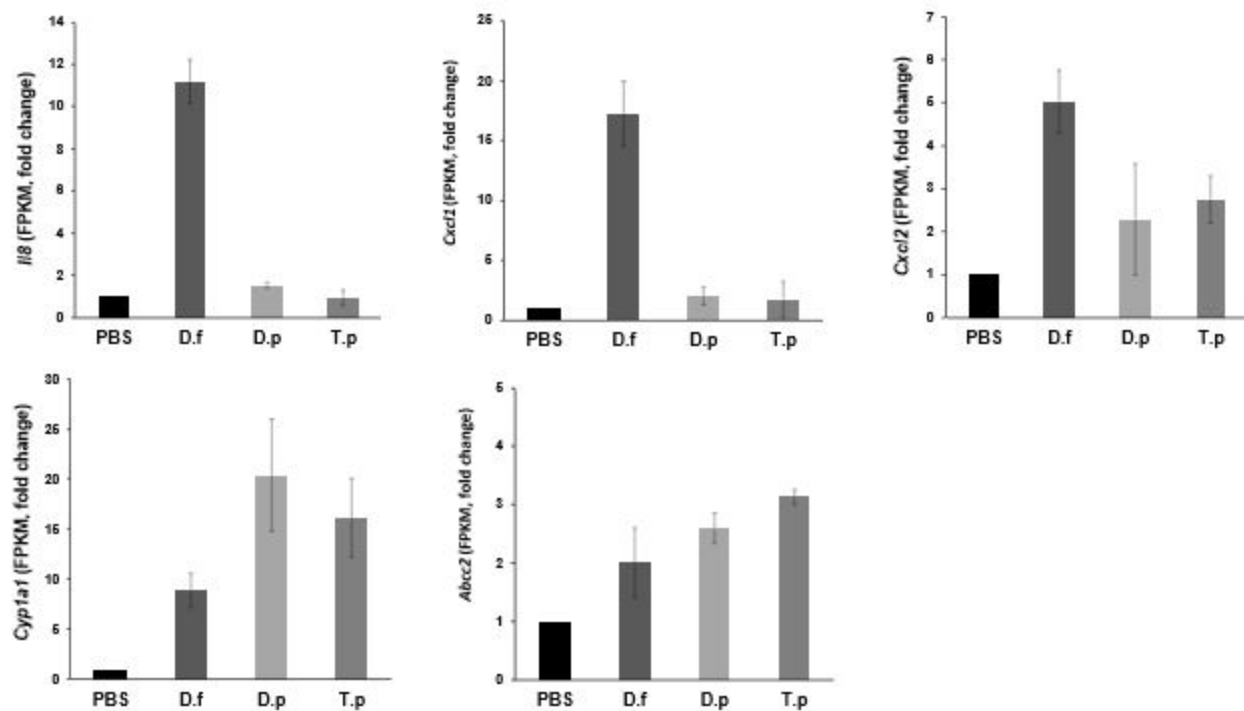
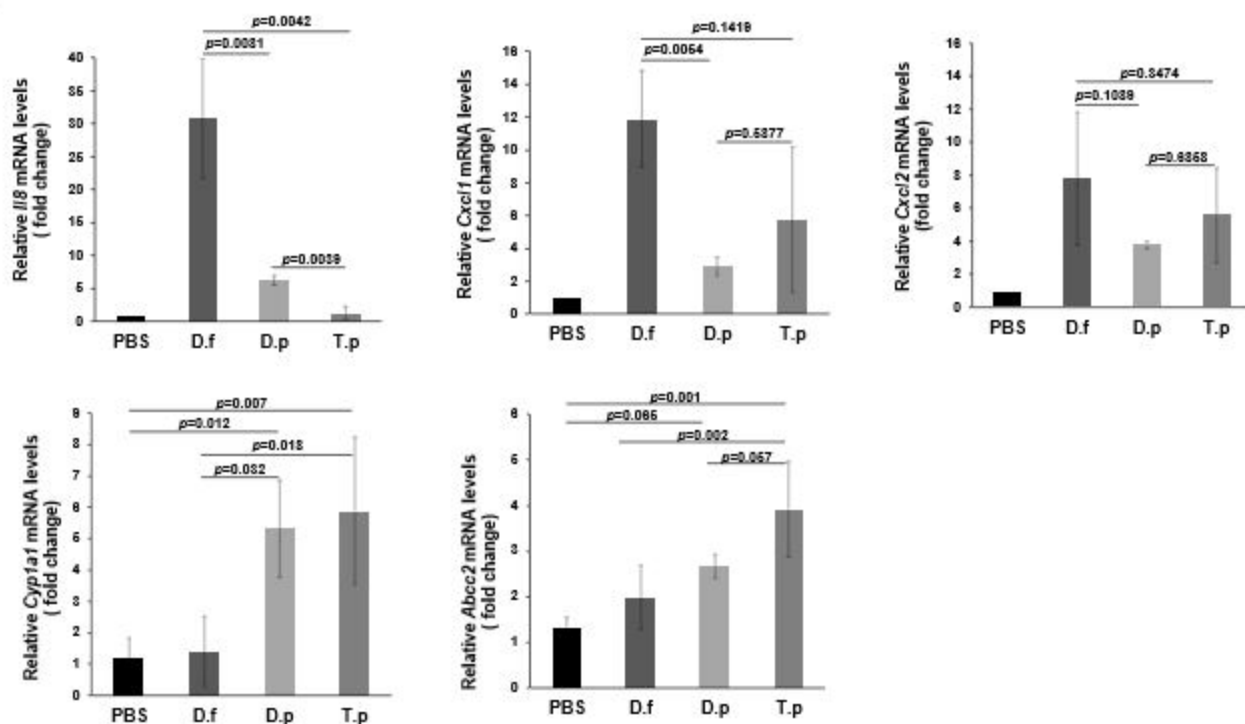
C

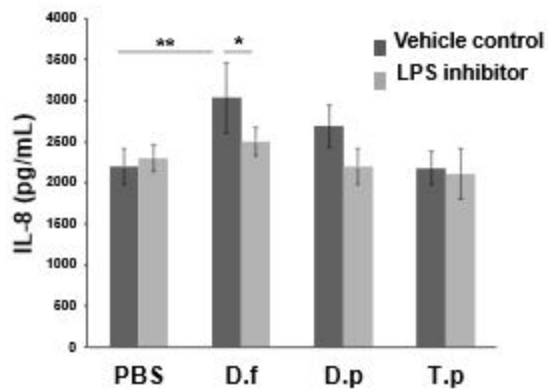
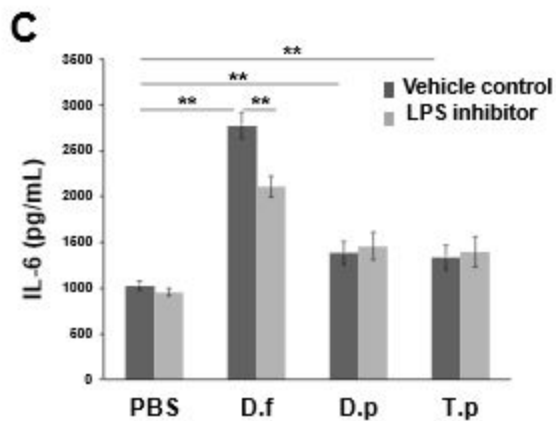
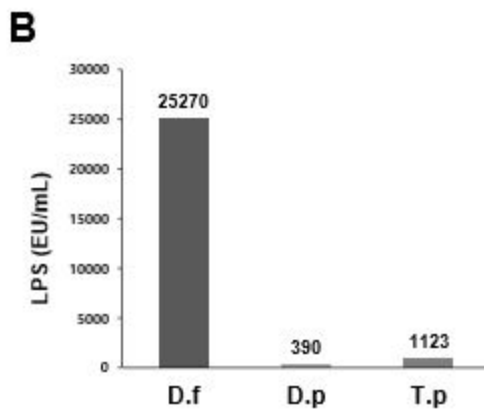
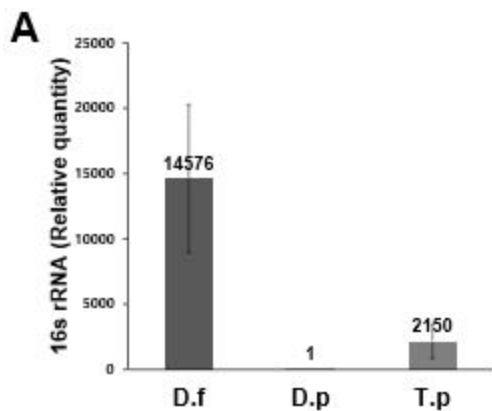


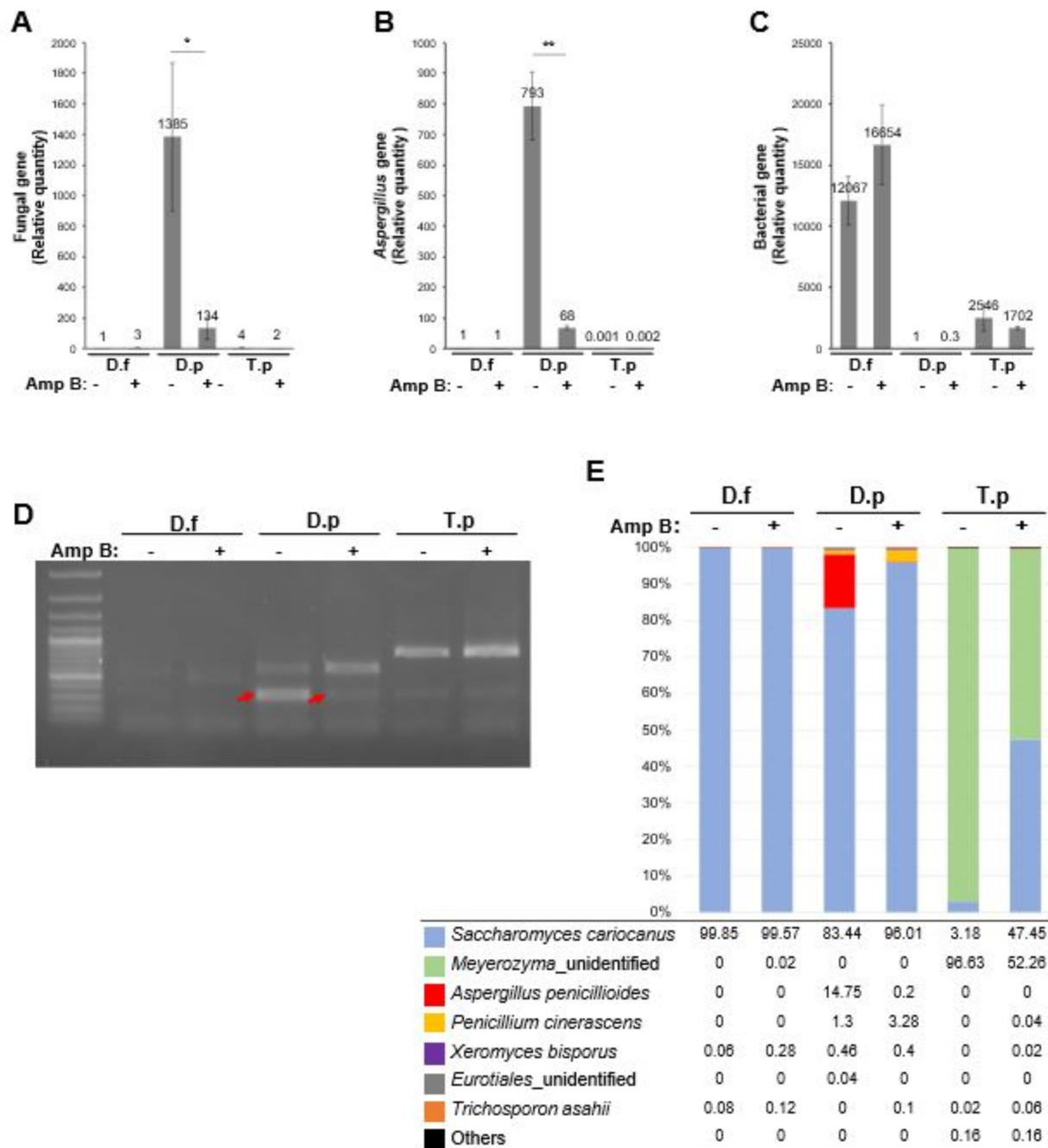
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	Positive regulation of epithelial cell proliferation	0.0006
	Chemokine metabolic process	0.0020

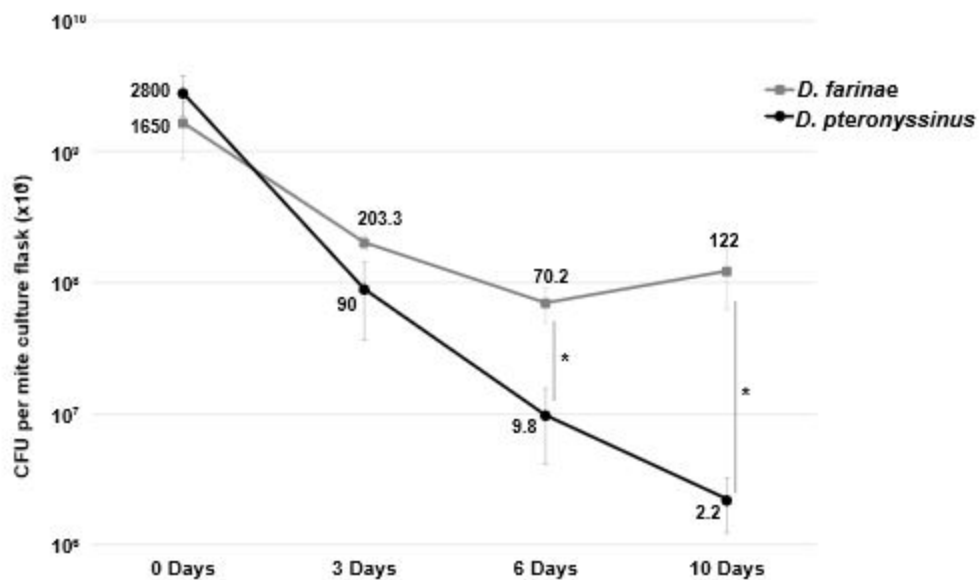
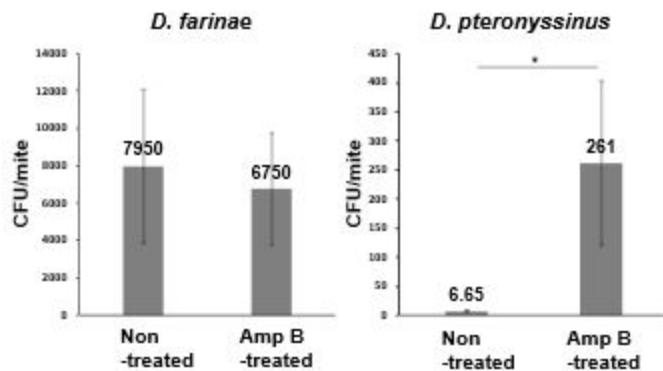
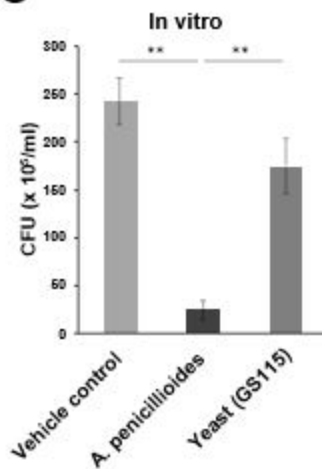
②	Term name	FDR
	Type I interferon signaling pathway	< 0.0001
	Response to bacterium	0.0067
	Pattern recognition receptor signaling pathway	0.0085

③	Term name	FDR
	Xenobiotic catabolic process	0.0044
	Antibiotic metabolic process	0.0205

A**B**





A**B****C**

Microbiome and mycobiome interaction in house dust mites and impact on airway cells

Supporting Information

Supplementary figures

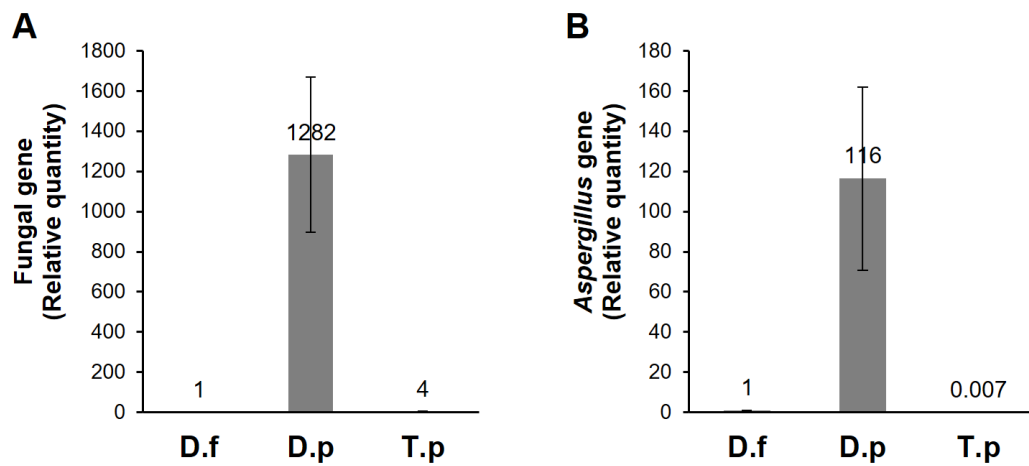


Figure S1. Relative amounts of (A) total fungi and (B) *Aspergillus* in *D. farinae* (D.f), *D. pteronyssinus* (D.p), and *T. putrescentiae* (T.p).

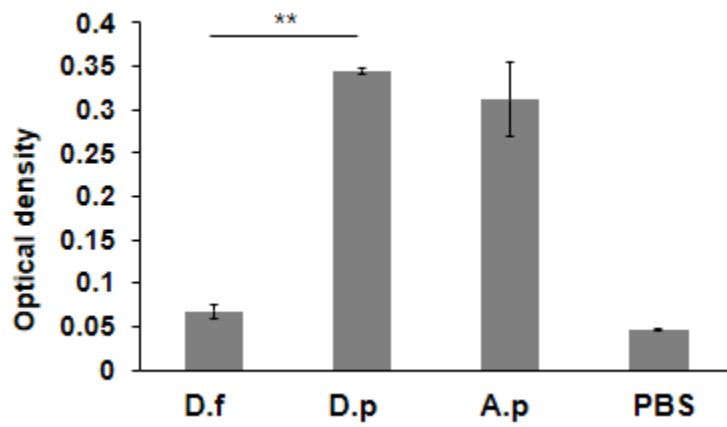


Figure S2. Levels of *A. penicillioides* protein in *D. farinae* (D.f), *D. pteronyssinus* (D.p), and *A. penicillioides* (A.p) extracts. PBS is the negative control. *D. pteronyssinus* was found to have more *A. penicillioides* than *D. farinae*. The *A. penicillioides* strain was isolated from *D. pteronyssinus*.

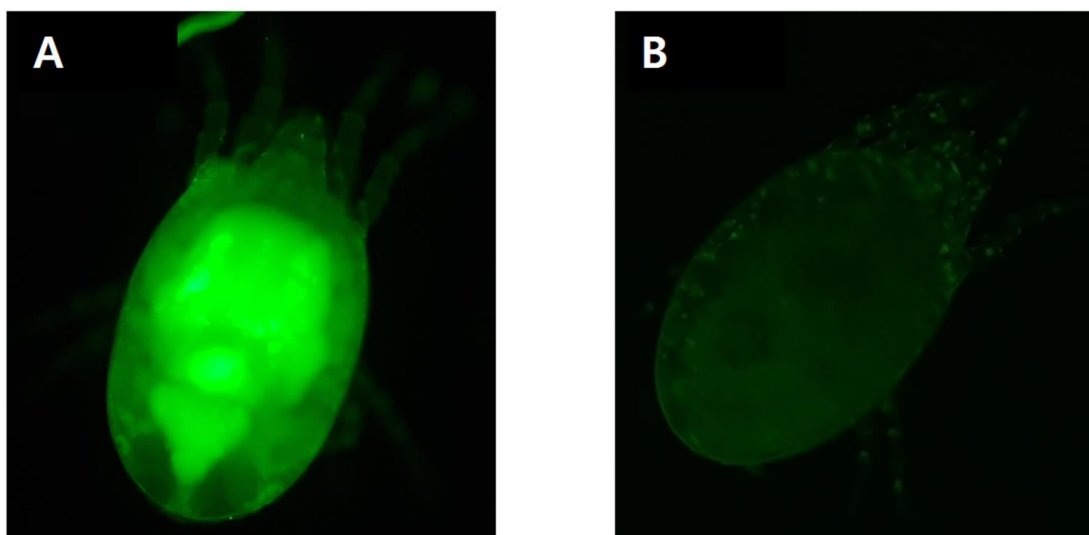


Figure S3. Fluorescence microscopy of (A) *E. faecalis* expressing GFP in *D. farinae* 10 days after inoculation and (B) negative control. *E. faecalis* settled in the gut of *D. farinae* 10 days after inoculation.

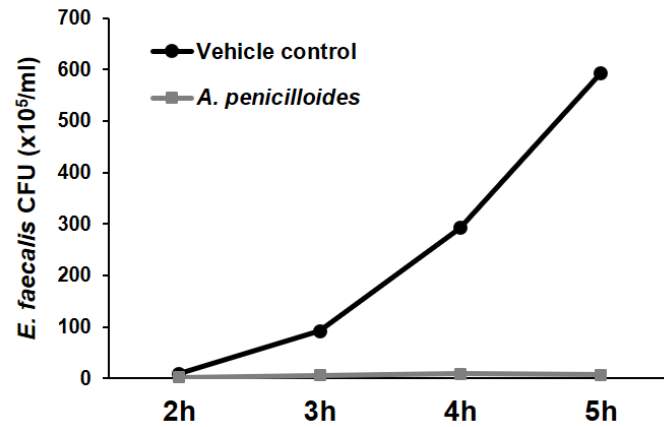


Figure S4. *E. faecalis* growth is suppressed after treatment with *A. penicillioides* extract *in vitro*.

E. faecalis grown in BHI broth medium was treated with the extract of *A. penicillioides* isolated from *D. pteronyssinus*.

Methods (A full description of the methods)

Ethical aspects

An institutional review board (IRB) approval was not required. No humans or laboratory animals other than mites were used in this study.

Mite cultivation

Inactive dry yeast (Choheung Co., Gyeonggi, South Korea) was mixed with fish food (KKW1Q0040; Jeil Feed Co., Daejeon, South Korea) at a ratio of 1:1, and the mixture was autoclaved. *D. farinae* and *D. pteronyssinus* were previously collected from beds in a Korean household (1998). *T. putrescentiae*, a storage mite, was previously collected from a rice barrel in a Korean house (2005). Mites were cultivated in the autoclaved medium in T75 cell culture flasks (70075, SPL Life Sciences, Gyeonggi, South Korea) at 25°C for six weeks. Mites (*D. farinae* and *D. pteronyssinus*) were also cultivated in the presence of an antifungal agent, amphotericin B powder, at a 10:1 ratio (Cayman Chemical, Ann Arbor, MI, USA) in autoclaved medium for four weeks.

Isolation of DNA and preparation of mite body extracts

Autoclaved saturated salt water was used to harvest mites separated from the culture medium and debris. DNA was then extracted from mites using a NucleoSpin DNA Insect Kit (Macherey-Nagel, Duren, Germany). The rest of the harvested mites were used to prepare a

homogenate by sonication with a sonicator and centrifuged at 10,000 *g* for 30 minutes. Foreign substances, including bacteria, were removed from the extracts using a sterile 0.22- μ m pore-sized Millex-GP syringe filter (EMD Millipore, Burlington, MA, USA).

LPS measurement

The concentration of LPS in mite extracts was measured using an LAL QCL-1000 Kit (Lonza, Basel, Switzerland).

Cell culture and mite extract treatment

Human bronchial epithelial cells (BEAS-2B, ATCC® CRL-9609™, Rockville, MD, USA) were obtained from the American Type Culture Collection. BEAS-2B cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F40 medium at 37°C and 5% CO₂. HDM was used to treat cells seeded at a concentration of 1×10^6 cells/well onto 6-well plates. Cells were sampled 24 hours after a single exposure to 100 μ g/mL of extract from each mite. Four independent samples were examined following exposure to each mite extract or phosphate-buffered saline (PBS) as a control. Total RNA was extracted using a Trizol extraction kit (Qiagen, Hilden, Germany) and eluted in RNase free-water according to the manufacturer's instructions.

RNA-seq and bioinformatics

The RNA samples were sequenced by Macrogen (Seoul, South Korea) using a NovaSeq 6000 System (USA). The generated reads were mapped against the genomic DNA reference (UCSC hg19) using the HISAT2 program (Johns Hopkins University, Baltimore, Maryland, USA). After read alignment, relative transcript abundance was measured using String Tie software (Johns Hopkins University, Baltimore, Maryland, USA), which determines abundance as fragments per kilobase of transcript per million mapped reads (FPKM). Differentially expressed gene analysis was performed using DESeq2, six combinations were compared (*D. farinae*/PBS, *D. pteronyssinus*/PBS, *T. putrescentiae*/PBS, *D. farinae*/*D. pteronyssinus*, *D. pteronyssinus*/*T. putrescentiae*, and *D. farinae*/*T. putrescentiae*), and 912 genes exhibiting $\geq$ two-fold change and nbinomWaldTest raw p-values < 0.05 for at least one combination were selected for further study. Three samples for each group were analyzed as a single cluster to determine the level of similarity within the group. The numbers of clusters were independently assessed using hierarchical clustering (Euclidean method, complete linkage) to construct a heatmap. Genes upregulated at least two-fold after treatment with each mite extract compared to PBS treatment and showing a false discovery rate (FDR) < 0.05 were used to construct a Venn diagram. Gene Ontology analysis was performed with G:Profiler (<https://biit.cs.ut.ee/gprofiler/gost>) using the term “GO biological process.” Terms with an FDR < 0.1 are shown in Table S1.

Real-time polymerase chain reaction (PCR)

Quantitative reverse transcription PCR was performed by monitoring the synthesis of double-stranded DNA during 40 PCR cycles using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The sequences of the primers used were as follows:

GAPDH, 5'-CAACTACATGGTTTACATGTTC-3' (forward) and 5'-GCCAGTGGACTCCACGAC-3' (reverse); IL-8, 5'-GACCACACTGCGCCAACAC-3' (forward) and 5'-CTTCTCCACAACCCTCTGCAC-3' (reverse); IL-6, 5'-AATTCGGTACATCCTCGACGG-3' (forward) and 5'-GGTTGTTTTCTGCCAGTGCC-3' (reverse); CXCL1, 5'-GCCAGTGCTTGCAGACCCT-3' (forward) and 5'-GGCTATGACTTCGGTTTGGG-3' (reverse); CXCL2, 5'-TCCAAAGTGTGAAGGTGA-3' (forward) and 5'-GGTTGAGACAAGCTTTCTG-3' (reverse); CYP1A1, 5'-CACCTCCAAGATCCCTACACTGA-3' (forward) and 5'-ACCAGACAGAAGATGACAGAGGC-3' (reverse); and ABCC2, 5'-TGTGGCCAGCCTGCAACT-3' (forward) and 5'-CCTCTGGCCTATGCTCAGGTT-3' (reverse). Relative levels of mRNA were determined using the $\Delta\Delta C_t$ method with normalization to those of GAPDH.

HDM elongation factor 1- α genes were amplified using forward primer 5'-ACCCGTGAACATGCTTTGCT-3' and reverse primer 5'-CACCATTCTCTCAAGCTCGT-3'. Bacterial 16S rRNA was amplified using forward primer 5'-CGGTGAATACGTTCYCGG-3' (BACT1369F) and reverse primer 5'-GGWTACCTTGTTACGACTT-3' (PROK1492R) from XenoTech and the AMPIGENE qPCR Mix (ENZO Life Sciences, Plymouth Meeting, PA, USA). *Aspergillus* ITS2 genes were amplified using forward primer 5'-GTCCGGTCCTCGAGCGTAT-3' and reverse primer (ITS2) 5'-GTTTCAGCGGGTATCCCTACCT-3'. Fungal genes were amplified using forward primers 5'-TCCGTAGGTGAACCTGCGG-3' (ITS1) and 5'-TCCTCCGCTTATTGATATG-3' (ITS4). Each sample was analyzed in triplicate.

Cytokine measurement

BEAS-2B human bronchial epithelial cells (American Type Culture Collection, Manassas, VA, USA) were cultured in a 6-well cell culture plate (SPL Life Sciences, Gyeonggi, Korea) in DMEM/F12 (1:1) supplemented with 10% fetal bovine serum and 1% penicillin (Gibco, Grand Island, NY, USA) for 24 hours at 37°C in a 5% CO₂ incubator. A single extract (100 µg/mL) was applied to each well, and the supernatant was collected after 24 hours of culture. The concentrations of cytokines secreted from BEAS-2B cells were measured in supernatants using DuoSet enzyme-linked immunosorbent assay (ELISA) human IL-6 and IL-8 kits (R&D Systems, Minneapolis, MN, USA).

Fungus-specific TA cloning

Fungal ITS1 and ITS2 genes in mite DNA samples were amplified using PCR with the following primer pair: forward primer, 5'-TCCGTAGGTGAACCTGCGG-3' and reverse primer, 5'-GCTGCGTTCTTCATCGATGC-3'.²⁵ DNA bands in agarose gel were excised and extracted using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and the TOPcloner™ TA Kit (Enzymomics, Daejeon, South Korea) was used for TA cloning. Cloned colonies were sequenced, and homology was determined through NCBI BLAST searches.

Mycobiome deep sequencing and bioinformatics

From the mite DNA, the ITS region of fungal DNA was amplified by PCR using the following primer pair: forward primer (fITS9), 5'-GAACGCAGCAAAATGCGA-3' and reverse

primer (ITS4), 5'-TCCTCCGC TTATTGATATGC-3'.²⁶ 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) following the manufacturer's instructions. Raw reads were processed using QIIME v1.9.1 and UNITE v18.11.2018, the fungal database, as indicated in previous literature.²⁷

Inoculation of E. faecalis (green fluorescence protein) into mites

E. faecalis OG1RF harboring pMV158GFP and pAM β 1 was grown on brain heart infusion (BHI) broth and agar medium with tetracycline (1 μ g/mL) and erythromycin (1 μ g/mL) to maintain the plasmids.²⁸ Mites (*D. farinae* and *D. pteronyssinus*, 1 g) were inoculated with 1 mL of *E. faecalis* solution (2×10^{11} CFU/mL) with or without amphotericin B (3 mg/mL). At 3, 6, and 10 days after inoculation, the mites (1 g) were homogenized and spread on BHI agar with antibiotics to count the remaining *E. faecalis*.

Culture of Aspergillus penicillioides and extract preparation

D. pteronyssinus homogenate was spread on BHI agar and cultured at 25°C for 7 days. Identified *A. penicillioides* colonies using fungal ITS1 and ITS2 gene sequencing were transferred to yeast extract sucrose agar medium and cultured at 25°C for 7 days.²⁹ To obtain an extract containing compounds that exhibit antibacterial activity, *A. penicillioides* extracts were prepared using a modified maceration method with CH₂Cl₂ and MeOH.³⁰ *A. penicillioides* (10 mg) was added to 1 mL of CH₂Cl₂: MeOH (1:1 v/v), and bead-beating was performed for 20

minutes, followed by mixing in a shaking incubator (70 rpm) for 4 hours. After centrifugation at 8,000 g for 5 minutes, the supernatant was obtained and incubated overnight to dry all solutions. The remaining powder was dissolved in 500 µL Tween 20 (0.05%) in PBS (PBST) to obtain an extract. Control extract groups were prepared by applying the above method to *Pichia pastoris* (GS115) or PBS (vehicle control).

E. faecalis growth inhibition test with *A. penicilliioides* extract

GFP_*E. faecalis* was cultured in BHI broth containing ampicillin overnight at 37°C with shaking at 200 rpm. Overnight culture broth was diluted to 1:1000 with BHI broth. Extracts (50 µL) of *A. penicilliioides*, *P. pastoris*, or vehicle control were added to 500 µL of the diluted bacterial broth and incubated for 4 hours. After incubation, each bacterial broth was spread on BHI agar containing ampicillin and cultured overnight, following which colonies were counted.

ELISA against Aspergillus

ELISA plates were coated with 100 µL of extracts from *D. farinae*, *D. pteronyssinus*, and *A. penicilliioides* in coating buffer at a concentration of 2 µg/mL and incubated at 4°C overnight. A 1:1000 diluted rabbit anti-*Aspergillus* antibody (ab20419, Abcam, Cambridge, UK) was used. HRP-conjugated goat anti-rabbit IgG (1:5000 dilution) (Cell Signaling Technology, Danvers, MA, USA) was used. Streptavidin-peroxidase (Sigma-Aldrich, St. Louis, MO, USA) was used. 3,3',5,5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) was added as a substrate solution. The reaction was stopped by H₂SO₄.

Statistical analysis

Student's t-test and analysis of variance, with Bonferroni correction as a post-hoc analysis, were used. Differences with p values ≤ 0.05 were considered statistically significant.

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