Microbial Ecology (2019) 77:1048–1066
https://doi.org/10.1007/s00248-018-1294-x

INVERTEBRATE MICROBIOLOGY

Population and Culture Age Influence the Microbiome Profiles of House Dust Mites

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Received: 30 June 2018 / Accepted: 13 November 2018 / Published online: 21 November 2018

Abstract

Interactions with microorganisms might enable house dust mites (HDMs) to derive nutrients from difficult-to-digest structural proteins and to flourish in human houses. We tested this hypothesis by investigating the effects of changes in the mite culture growth and population of two HDM species on HDM microbiome composition and fitness. Growing cultures of laboratory and industrial allergen-producing populations of *Dermatophagoides farinae* (DFL and DFT, respectively) and *Dermatophagoides pteronyssinus* (DPL and DPT, respectively) were sampled at four time points. The symbiotic microorganisms of the mites were characterized by DNA barcode sequencing and quantified by qPCR using universal/specific primers. The population growth of mites and nutrient contents of mite bodies were measured and correlated with the changes in bacteria in the HDM microbiome. The results showed that both the population and culture age significantly influenced the microbiome profiles. *Cardinium* formed 93% and 32% of the total sequences of the DFL and DFT bacterial microbiomes, respectively, but this bacterial species was less abundant in the DPL and DPT microbiomes. *Staphylococcus* abundance was positively correlated with increased glycogen contents in the bodies of mites, and increased abundances of *Aspergillus*, *Candida*, and *Kocuria* were correlated with increased lipid contents in the bodies of mites. The xerophilic fungus *Wallemia* accounted for 39% of the fungal sequences in the DPL microbiome, but its abundance was low in the DPT, DFL, and DFT microbiomes. With respect to the mite culture age, we made three important observations: the mite population growth from young cultures was 5–8-fold higher than that from old cultures; specimens from old cultures had greater abundances of fungi and bacteria in their bodies; and yeasts predominated in the gut contents of specimens from young cultures, whereas filamentous mycelium prevailed in specimens from old cultures. Our results are consistent with the hypothesis that mites derive nutrients through associations with microorganisms.

Keywords Nutrition · Bacteria · Fungi · Yeasts · Gut · Symbiosis · Diet · *Dermatophagoides pteronyssinus* · *Dermatophagoides farinae*

Introduction

The house dust mites (HDMs) *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* are the most common species of mites found in synanthropic environments, including house dust, beds, upholstery, and carpets [1], and are medically important because they produce an array of allergens and cause allergic reactions and respiratory disease [2, 3]. The prevalence of HDM allergen sensitization varies from 65 to 130 million affected individuals worldwide and reaches 50% among asthmatic patients [4]. HDMs are mass-reared for allergen diagnosis and vaccine preparations [5]. These mites reproduce sexually, and their development includes the following stages: egg, prelarva (inactive and

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00248-018-1294-x) contains supplementary material, which is available to authorized users.

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develops inside the egg), larva, protonymph, tritonymph, and adult [6]. Under optimal conditions, the life cycles of *D. farinae* and *D. pteronyssinus* take approximately 17 and 15 days, respectively [7, 8]. When mass-reared, HDMs show specific growth patterns at different times during culture development. Three separate HDM population growth phases have been distinguished: (i) a latency phase (from the beginning of cultivation to the 10th week) characterized by a slow increase in the mite population; (ii) an exponential phase (from the 10th to the 20th week) characterized by exponential population growth; and (iii) a "decline" phase (after the 20th week) characterized by a large decrease in the mite population [9, 10]. Differences in protein content [11] and allergen production [12] have been observed in mites from different culture growth phases.

The ancestors of HDMs are hypothesized to have been permanent parasites of birds and mammals, whereas modern HDMs have secondarily become commensals in vertebrate nests, including human dwellings [13, 14]. Life in anthropogenic habitats, or more generally in vertebrate nests, requires adaptations to not only changing temperatures and low humidity but also specific food sources [1], such as sloughed skin, nails, and hair, that contain difficult-to-digest structural proteins, e.g., keratin, collagen, and elastin [1]. The ability to digest keratins is rare among animals and is limited to permanently parasitic bird lice, dermestid beetles, and a few other taxa [15]. Interaction with microorganisms might explain the ability of HDMs to feed on structural proteins because microorganisms are a natural component of human skin, hair, and nails [16] and co-occur with HDMs in human-made habitats [17].

Several hypotheses have been proposed to explain the ability of HDM to obtain nutrients from structural proteins. According to the nutritional hypothesis, HDMs derive nutrients, vitamins, and sterols from fungi that occur in house dust [18] and act as vectors for microorganisms, transferring them either on the surface of their bodies or in their airborne fecal pellets [19]. Similarly, some xerophilic fungi (*Alternaria alternata*, *Cladosporium sphaerospermum*, and *Wallemia sebi*) are an attractive food source for *D. farinae* [20]. The mites feed on bacteria [21] and the addition of *Micrococcus lysodeikticus* to HDM diets accelerate the growth of *D. farinae* but not *D. pteronyssinus* [22]. The gut bacteria or intracellular symbionts enhance arthropod feeding on unbalanced diets [23, 24], and gut symbionts are present in stored-product mites [25, 26].

Previous studies have mostly focused on the interactions between HDMs and fungi (reviewed by Van Asselt [19]), whereas the interactions of HDMs with bacteria have been understudied. Although some bacterial genes or actinobacterial proteins have been identified in HDMs through omic methods [27–29], a complete and representative description of the HDM microbial community is still lacking. *Bartonella* and *Enterococcus* were recently identified as the main components of the *D. farinae* microbiome through barcode sequencing of the V3-V4 region of 16S RNA [30]. Additionally, a previous study showed that the interactions between mites and microorganisms can change during culture [31], and the known cases of mite culture collapses after a few months of rearing in the laboratory [10] might be explained by an imbalance in symbiotic microorganisms or an outbreak of acaropathogenic bacteria, which suggests that certain microorganisms play very important roles in the lives of mites.

Our study focuses on characterizing the microbiomes of the HDMs *D. pteronyssinus* and *D. farinae* and comparing the differences in these microbiomes caused by different mite populations and culture ages. Bacterial and fungal communities were characterized by barcode sequencing of partial 16S and 18S rRNA sequences, and the microbiome profiles were correlated with data describing mite population growth and the nutrient status of mite population samples.

Materials and Methods

Mites

European house dust mite (*D. pteronyssinus*) and American house dust mite (*D. farinae*) were reared in tissue culture flasks with a 25-cm^2 surface and a 70-mL capacity (IWAKI flasks; Cat. No. 3100-025; Sterilin, Newport, UK) at 75% RH and 25 ± 1 °C in a Secador desiccator (Bel-Art Products, Pequannock, NJ, USA). The laboratory mite cultures have been maintained for more than 10 years at the Crop Research Institute, and industrial mite cultures originated from Trebon (South Bohemia, Czechia) populations. These cultures were maintained for less than 1 year at the Crop Research Institute laboratory. To establish a new culture, approximately 5000 unsexed mites plus their diets were placed in a new chamber containing 0.5 g of a freshly prepared diet; these mites were obtained from cultures in the exponential phase of growth [9, 10]. The diet, which consisted of a mixture of dog food/wheat germ/dried fish food/Pangamin-dried yeast (*Saccharomyces cerevisiae*) extract/gelatin at a ratio of 10/10/3/2/1 *w*/*w*, was mill-powdered, sieved (mesh size of 500 μm), and pasteurized by heating to 70 °C for 0.5 h [22].

Our mite populations included a laboratory population of *D. farinae* (DFL), an industrial allergen-producing population of *D. farinae* (DFT), a laboratory population of *D. pteronyssinus* (DPL), and an industrial allergen-producing population of *D. pteronyssinus* (DPT) (Table 1). Every population was cultured in 30 IWAKI flasks, and the cultures were terminated (six flasks per population) at four different times during consecutive culture development Table S1); the remaining chambers were used as the source of mites for population growth experiment. No new diet was added to the flasks during the experiment. Consecutive culture

Table 1 The origin and rearing conditions for populations of house dust mites

Species	Population	Sampled/obtained description	Year	Collector
Dermatophagoides farinae (Hughes 1961)	Industrial allergen-producing population from Trebon culture	Obtained from Czech facility for allergen production	2012	RNDr. A. Zgarbovsky
	Laboratory	Laboratory population, obtained from Medical University of Silesia, Katowice, Poland	2005	Prof. K. Solarz
Dermatophagoides pteronyssinus (Trouessart 1897)	Industrial allergen-producing population from Trebon culture	Obtained from Czech facility for allergen production	2012	RNDr. A. Zgarbovsky
	Laboratory	Laboratory population, obtained from Fera, UK	1998	Dr. K. Wildey

development differs among species and populations; thus, the sampling times were also different. Samples were collected during the following population growth phases: the latency phase (15 days of cultivation for all populations), the exponential phase (30 and 90 days for DFL and DFT, 30 and 45 days for DPL, and 30 and 60 for DPT), and the "decline" phase (150 days for DFL and DFT, 60 days for DPL, and 120 days for DPT) (Table S1). One replicate was represented by a single rearing in an IWAKI flask. The mites were taken from the inner surface and plugs of the culture flasks using an artistic brush, transferred to 1.5-mL Eppendorf tubes, and weighed on a microbalance (MS Mettler-Toledo, Greifensee, Switzerland) to obtain 0.010 ± 0.005 g per sample. Before sampling, the mites in the flask were visually inspected and determined to be alive based on their movement. The samples were used for DNA extraction or to determine the nutrient contents of the mites (see below).

To obtain the eggs of the mites, separate IWAKI flasks were prepared with approximately 1000 adults, which were separated by sieving (mesh size of 300 μm). The diet was non-powdered wheat germ sieved using a 300-μm mesh to remove small particles. After 1 week, the contents of the flask were sieved (mesh size of 150 μm), and the material that passed through the sieve was transferred to ethanol. Eggs were collected in Eppendorf tubes using a pipet under a dissecting microscope. Each sample consisted of 70 to 100 eggs.

To determine the effect of antibiotics on *Cardinium*, tetracycline (Cat. No. T3258; Sigma-Aldrich, Saint Louis, MO, USA) was diluted in HPLC-grade methanol and incorporated into the diet at a concentration of 1% [26], and a culture of *D. farinae* was maintained on this diet for 1 month. The mites were sampled as described above, and six replicates of *D. farinae* on treated diet were obtained.

DNA Extraction

The surfaces of the mites or eggs were cleaned by washing with bleach (sodium hypochlorite 5%) and ethanol (96%), and the ethanol was then removed using sterile phosphate-buffered saline (PBST - 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, and 135 mM NaCl) with 0.05% *w/w* Tween® 20 detergent (Sigma-Aldrich) [32]. The surface-cleaned mites were homogenized using a plastic pestle homogenizer in PBST, and the homogenate was then extracted using a Wizard® Genomic DNA Purification kit (Cat. No. A1125, Promega, Madison, WI, USA) according to the manufacturer's recommended protocol. The extracted DNA was stored at − 28 °C. The eggs were homogenized as previously described [33], and DNA was extracted using an Exgene™ Genomic DNA MicroKit (GeneAll, Seoul, South Korea).

DNA Amplification and Sequence Processing

The bacteria and fungi in the mite microbiomes were characterized by barcode sequencing of the V4 domain of the 16S rRNA gene (CS1_515F and CS2_806R primers) and the 18S rRNA gene (primers CS1 FF390 and CS2 FR1), respectively (Table S2). PCR amplifications were performed in 25-μL reactions in PCR tubes. For DNA amplification, we used the Takara Ex Taq DNA polymerase master mix (Cat. No. RR001A, Takara Bio, Saint-Germain-en-Laye, France), and the final concentration of CS1_515F and CS2_806R primers was 0.5 ng/μL. The DNA from mite homogenates (80–200 ng) was added to the PCR reaction. The conditions were as follows: CS1 FF390 and CS2 FR1—initial step 95 °C for 8 min, followed by 28 cycles at 95 °C for 30 s, 50 °C for 45 s, 68 °C for 2 min, and a final elongation step at 72 °C for 10 min; CS1_515F and CS2_806R—initial step 95 °C for 5 min, followed by 28 cycles at 95 °C for 30 s, 55 °C for 45 s, 72 °C for 30 s, and a final elongation step at 72 °C for 7 min. For the amplification, we added ddH_2O instead of the extracted DNA into the master mix as a negative control for every PCR and no amplicons were found. The amplicons were visualized on agarose gel electrophoresis. Amplicons were sequenced at the DNA Services Facility of the Research Resources Center at the University of Illinois (Chicago, IL, USA) on a MiSeq platform (Illumina, San Diego, CA, USA) [34]. The sequences were demultiplexed, and the barcodes and primers were removed by the company. The forward and reverse sequences were aligned and processed with

MOTHUR 1.39.5 [35] according to the standard operating procedure (MiSeq SOP [36]) and the UPARSE 10 pipeline including UNOISE algorithms [37, 38] using a protocol that combined both programs [39]. Operational taxonomic units were classified according to the Ribosomal Database Project [40] Training Set No. 15 (available for UPARSE) and SILVA 128 [41] (available for MOTHUR). The representative sequences for each OTU were then compared to those available in GenBank using BLASTn [42]. The DNA sequences were deposited in NCBI SRA: SRP113545: PRJNA395630. In addition, DNA from the eggs and antibiotic-treated mites was used for conventional PCR with *Cardinium*-specific primers (Table S2) using a previously described protocol [26].

We removed 1000 reads collectively representing low-abundance OTUs. This procedure excluded 4% and 0.2% of reads of bacteria and fungi, respectively. Data standardization was based on 5000 sequences in MOTHUR (see Tables S3-S6). Data analyses were performed using PAST [43] and the Vegan package [44] for R [45], and the results were visualized using XLSTAT (Addinsoft, New York, NY, USA). The proportions of bacterial taxa were visualized using KRONA [46] (Figs. S2 and S4), and the profiles were also visualized using bar charts to demonstrate the effect of culture age and sample variability. We tested whether bacterial communities are affected by two factors: population and culture age. Beta diversity was tested using the Bray-Curtis distance via one- and two-way PERMANOVA [47] with 1000 permutations. The dbRDA (redundancy analyses) results were visualized using the Vegan package with LOG(10)-transformed species abundance data [48]; zero abundance values were replaced by 0.00001. The tested variables (population and culture age) were coded as 1 and 0; the mite populations were coded as DFT, DFT, DPL, and DPT; and the sampling times were designated 1, 2, 3, and 4. The forward selection technique [49] was applied using the adespatial library in R (http://bit.ly/anadatr). To describe the effect of the mite nutrient contents, the nutrient content measurements were analyzed together with the population and sampling time variables (young and old mite cultures) by two-way PERMANOVA (Euclidean distance). In the next step, we performed dbRDA with the abundant bacterial and fungal OTUs that were considered species and populations, and the sampling time, nutrient status, and population growth were tested as environmental variables based on the Bray-Curtis distance. The most important environmental variables were identified using the forward selection technique as described above. METASTATS [50] was applied to separately describe the mite population and culture age. The analyses were performed in MOTHUR.

Phylogenetic Analyses

16S rRNA, ITS, and COI sequences obtained from Sanger sequencing were assembled with CodonCode Aligner, v.1.5.2 (CodonCode Corporation, Dedham, MA, USA). The reference sequences originated from GenBank or RDP. Bacterial sequences were assigned to bacterial taxa using the RDP-naïve Bayesian classifier [51], and the sequences were aligned using SILVA Incremental Aligner v.1.2.11 [52]. The best-fitting nucleotide substitution model based on maximum likelihood and Bayesian analyses was found using jModelTest v.2.1.7 [53, 54] and MrModelTest v.2.3 (https://github.com/nylander/MrModeltest2) with PAUP*4.0 (http://paup.phylosolutions.com), respectively, and both of these analyses were based on the AIC. Phylogenetic inference was included in the Bayesian framework in PhyloBayes-MPI v.1.4e [55–57] and MrBayes v.3.2 [58] and the maximum likelihood framework in PhyML v.3.0 [59]. Phylograms were visualized using FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/ [60]).

qPCR

The DNA samples were used to quantify numbers of copies using universal primers for bacteria, fungi, *Actinomycetales*, *Firmicutes*, and specifically for *Cardinium* (Table S2). The standards were prepared from the cloned amplicons from mite homogenate, bacterial, or fungal cultures (Table S2). The amplicons were inserted into pGEM®T vector (Cat. No. A1360, Promega), and plasmids were transformed to competent cells (Cat. No. M180A). The transformed cells were plated to LB (Luria Bertani) medium with ampicillin (100 μg/mL), X-gal (40 mg/mL), and IPTG (100 mM) and incubated overnight. Then, the plasmids were isolated from the cells using Wizard Plus SV Mini preps DNA (Cat. No. A1330, Promega) and isolated DNA was amplified by pUCM13F/R primers [61]. The positive amplicons were sequenced in Macrogen (Seoul, South Korea). The plasmids were linearized by *Sac*I or *Sac*II (*Actinomycetales*) (Cat. No. R0156S, R3138S, Biolabs, New England, Ipswich, MA, USA) according to manufacturer's instructions. The linearized plasmids were purified using a GeneAll® Expin™Combo kit (Cat. No. 112, GenAll, Soul Korea) according to manufacturer's instructions. The concentration of DNA was measured on a NanoPhotometer (Implen, Munich, Germany), and standards were diluted in TE buffers (from the kit); the aliquots were stored at − 80 °C. The amplification was performed on a StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) using 96-well plates with GoTaq qPCR Master Mix (Cat. No. A6001, Promega) containing SYBR Green as a double-stranded DNA-binding dye. The inhibition was tested by serial DNA dilution from each site. As negative controls, the ddH_2O was added to the master mix instead of DNA. Melting curves were recorded to ensure qPCR specificity. Baseline and threshold calculations were performed with the StepOnePLus software. The DNA used in the qPCR analyses originated from the same samples used for the Illumina analyses, and the experimental design included six biological

and two technical replicates per treatment (two samples per flask). The means from these two technical replicates were used, i.e., six biological replicates were analyzed. The data were LOG(10)-transformed before the analyses, and the medians and interquartile ranges were calculated. The Kruskal-Wallis test was applied to compare the differences in mite culture age among the populations.

Measurement of HDM Nutrient Contents (Proteins, Lipids, Glycogen, and Sugars)

To measure the HDM nutrient contents, we used the colorimetric Kaufmann method [62–64] with several modifications [65]. Briefly, the population-level samples of the mites, which were a mixture of adults and juveniles, were homogenized in a FastPrep-24 homogenizer (MP Biomedicals, Santa Ana, CA, USA) in 200 μL of 2% sodium sulfate. Twenty-five microliters of each homogenate was used to estimate the protein contents using the Bradford reagent (Cat. No. B6916, Sigma-Aldrich, Saint Louis, MO, USA), and the optical density (595 nm) was measured with an ELISA reader (Multiskan Ascent, Thermo, Waltham, MA, USA). The reagent was calibrated to a protein standard (Cat. No. P0834, Sigma-Aldrich). The rest of the homogenate was precipitated in 800 μL of a 1:1 (*v*/*v*) chloroform/methanol solution and centrifuged (3000 g, 60 s), and the pellets were used for glycogen analysis. The supernatant was transferred to a new Eppendorf tube and mixed with 600 μL of distilled water. After centrifugation (3000*g*, 60 s), the top fraction (water/methanol) was collected for sugar analysis and the bottom portion (chloroform) was used for lipid analysis. For lipid analysis, chloroform was evaporated on a heat block (110 °C), 200 μL of sulfuric acid (95–98%) was added, and the mixture was incubated for 10 min at 110 °C. One milliliter of vanillin-phosphoric acid reagent, which was prepared by dissolving 0.6 g of vanillin in 100 mL of hot distilled water and 400 mL of 85% phosphoric acid, was added, and the resulting color was measured with an ELISA reader at 405 nm. The reaction was calibrated with a lipid standard. For the sugar and glycogen assays, the tubes were placed on a heat block (110 °C) to evaporate the solvent to a total volume of 100 μL, and 1 mL of anthrone reagent, which contained 150 mL of distilled water, 385 mL of sulfuric acid (95–98%), and 0.75 g of anthrone, was added. The samples were then mixed and heated for 17 min, and the optical density was then measured at 630 nm using an ELISA reader. The reaction was calibrated using a glucose standard (Cat. No. G6918, Sigma-Aldrich). The data were recalculated to milligrams of nutrients per gram of fresh (live) weight of the mites. The analyses were performed such that only two culture ages were measured per population, e.g., DFL 30 and 90 days, DFT 30 and 120 days, DPL 30 and 60 days, and DPT 30 and 120 days. Six biological replicates were obtained from every treatment. The analyses were performed using two technical replicates, and their means were calculated. The data are presented as the medians and interquartile ranges and were analyzed using a Kruskal-Wallis test for all populations with Dunn's post hoc comparison and Bonferroni's correction.

Population Growth

The growth of populations using mites taken from young (30 days of culturing) and old cultures (120-day-old cultures of *D. farinae*, 60-day-old cultures of the laboratory population of *D. pteronyssinus*, and 90-day-old cultures of the industrial allergen-producing population of *D. pteronyssinus*) was measured. We used the remaining chambers for these mites. The mites were sexed under a dissecting microscope, and six females and four males were introduced into an IWAKI chamber with 0.05 ± 0.01 g of a newly pasteurized rearing diet containing *S. cerevisiae*. The chambers were maintained under the same conditions as those used for mite rearing; after 21 days, the chambers were collected and filled with Oudeman's solution (70% ethanol (87 mL), acetic acid (8 mL), and glycerol (5 mL)) [66], and the mites were counted. The data are expressed as the medians and interquartile ranges, and the species, populations per species, and young/old cultures were compared through a nonparametric Mann-Whitney test (Fig. 4).

Histology

For histological analysis, we used the same mite samples that were used for the nutrient content analyses. The mites were fixed in a modified Bouin-Duboscq-Brazil solution [67], transferred to paraffin, and sectioned into 4–6-μm sections [25]. The sections were stained using Masson's triple and Mann-Domenici stains [68] for bacterial visualization and then visualized using an Axioskop compound microscope and AxioVision software (Carl Zeiss, Jena, Germany) (Fig. 5).

Results

HDM Populations Are Genetically Identical Based on ITS and COX1 Sequences

We compared 40 COX1 and 47 ITS (GenBank Accession Nos. MH057546-585 and MH057586-615, respectively) sequences obtained using specific primers whose sequences are available in GenBank (Fig. S1AB). Based on the COX1 sequences, *D. farinae* formed a sister group to *Dermatophagoides* cf. *alexfaini* AD1210 (GenBank Accession No. KF891941) [69, 70]. Our sequences clustered with the *D. farinae* GenBank sequences that originated from specimens that had been carefully identified morphologically and "barcoded" [71], and no genetic separation was observed between the two analyzed

D. farinae populations, i.e., laboratory DFL and industrial allergen-producing DFT. As expected based on morphology, *D. pteronyssinus* formed a sister group with *D. evansi* (KF891942) [69]. The sequences obtained from both populations clustered with *D. pteronyssinus* GenBank sequences [71–73], and no genetic separation was detected among the populations (i.e., laboratory DPL and industrial allergen-producing DPT). Based on our ITS sequences, *D. pteronyssinus* clustered within the reference sequences for this species [74–76] without a substantial separation among the populations. As expected, *D. farinae* formed a sister group to *D. microceras* [74, 77], and all our sequences were unambiguously placed within *D. farinae* [25, 74–76] without forming any obvious phylogenetic structure.

Bacterial and Fungal Microbiomes of HDMs

The identified OTUs (Tables S3 and S4) included sequences with high similarity (99–100%) to the intracellular symbiotic bacterium *Cardinium* (OTU1), which was previously identified in astigmatid mites, and gut-associated *Bartonella*-like bacteria (OTU4 and OTU5). Other abundant sequences belonged to taxa including *Staphylococcus* (OTU2 and OTU23), *Kocuria* (OTU3), *Lactobacillus* (OTU16), *Caulobacter* (OTU6), and *Chitinophagaceae* (OTU9) (Fig. S2).

Cardinium was characterized by phylogenetic analyses of clones obtained with universal primers (GenBank Accession No. MH054392-438), and the results confirmed the similarity of the *Cardinium* sequences to that of *Candidatus* Cardinium hertigii (Fig. 1). *Cardinium* from *D. farinae* was clearly distinct from that of stored-product mites [25, 32], and the *Cardinium* symbionts of stored-product mites (*Acarus siro*, *Tyrophagus putrescentiae*, and *Lepidoglyphus destructor*), the spider mite *Tetranychus cinnabarinus*, and planthoppers (*Sogatella furcifera*, *Euides speciosa*, and *Harmalia sirokata*) [78] formed a sister clade to the symbionts of *D. farinae* (Fig. 1). Conventional PCR using taxon-specific primers and Illumina amplicon analyses confirmed the presence of *Cardinium* in the eggs of all tested populations, and the proportion of *Cardinium* sequences in the eggs was higher in the DFL (97% of cloned sequences) and DFT (17%) microbiomes than in the DPT (9%) and DPL (1%) microbiomes (data not shown). The treatment of mites with 1% tetracycline via their diet did not eliminate *Cardinium*, as demonstrated by the detection of *Cardinium* in the mites from antibiotic-treated diets by conventional PCR using *Cardinium*-specific primers.

Only five OTUs exhibited an abundance higher than 5% of sequences: *Aspergillus* (OTU1), *Saccharomyces* (OTU2), *Wallemia* (OTU4), and *Candida* (OTU5 and OTU6) (Fig. S3). Among the identified fungi, *Saccharomyces cerevisiae* (OTU2) was a component of the rearing diet administered to the mites in all the experiments (Fig. 2).

Mite Populations and Species Show Differences in Their Bacterial and Fungal Microbiomes

A significant difference in the numbers of 16S rRNA copies was observed among the tested HDM populations (Kruskal-Wallis $K_{(3,92)} = 29.55$; $P < 0.001$), and this difference was caused by almost 10-fold lower numbers of bacteria in the DPL microbiome than in the microbiomes of the remaining populations (Table 3). Two-way permutational analyses of variance (PERMANOVA; Bray-Curtis) indicated that all tested factors significantly influenced the distribution of bacterial OTUs in the HDM microbiomes, i.e., population ($F_{(3,80)} = 88.09$; $P < 0.001$), culture age ($F_{(3,80)} = 19.23$; $P < 0.001$), and their interactions ($F_{(9,80)} = 8.27$; $P < 0.001$). A pairwise test with Bonferroni-corrected P values revealed that the OTU distributions showed significant differences between the HDM species (DP and DF) and between the DF populations but not between the DP populations (Table 2). The dissimilarity between samples was visualized by redundancy analyses based on a Bray-Curtis distance matrix (dbRDA) (Fig. 3). After forward selection of environmental variables, the following model was inferred: $F_{(5,90)} = 34.67$; $P < 0.001$ (explaining 62% of the variation). In the model (Fig. 3a), the x-axis separated the symbiont-inhabited DFL from the others. The OTUs with the greatest influence on the model belonged to the intracellular symbiont *Cardinium* (OTU1) infesting the DFL, whereas *Staphylococcus* (OTU2) and *Kocuria* (OTU3) were associated with *D. pteronyssinus*. The differences in bacterial OTU abundances, i.e., the abundances of *Cardinium* (OTU1), *Staphylococcus* (OTU2), *Kocuria* (OTU3), and *Bartonella*-like bacteria (OTU5 and OTU4), among the populations of HDMs were obtained using METASTATS (Table S7). *Cardinium* (OTU1) formed 93% of the DFL microbiome but was found at a lower proportion (32%) in the DFT microbiome (Fig. S2). In contrast, *Staphylococcus* (OTU2) formed almost 50% of the bacterial profile in the DPT microbiome.

Based on qPCR data, the numbers of 18S rRNA copies of various fungal species showed no differences among the populations (Kruskal-Wallis $K_{(3,92)} = 1.40$; $P = 0.713$). A two-way PERMANOVA using the Bray-Curtis distance revealed that the distribution of fungal OTUs was influenced by both the population ($F_{(3,80)} = 59.09$; $P < 0.001$) and culture age ($F_{(3,80)} = 98.55$; $P < 0.001$) as well as by the interaction between these two variables ($F_{(3,80)} = 14.79$; $P < 0.001$). A pairwise test with Bonferroni correction showed that the DPL microbiome significantly differed from those of the other populations ($P < 0.05$). No other differences were found (Table 2). The dbRDA model ($F_{(6,89)} = 32.21$; $P < 0.001$) supported these findings and explained 62% of the variability (Fig. 3b). The x-axis separated the fungal microbiomes of the *Dermatophagoides* populations and the microbiomes of young mite cultures from the remaining categories. The

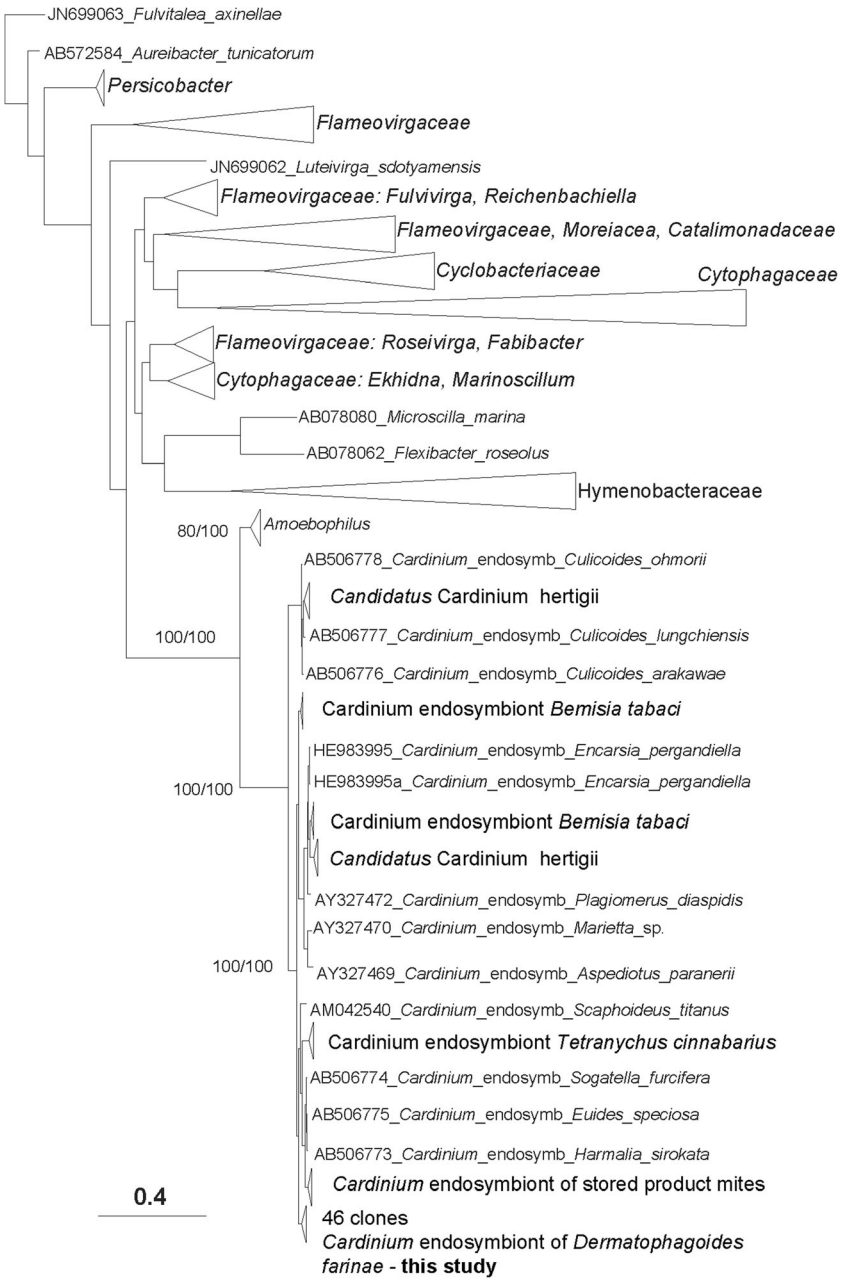

Fig. 1 Bayesian phylogenetic analysis of *Cardinium* symbiont clones isolated from *Dermatophagoides farinae*. A total of 30 partial 16S rRNA gene sequences and the reference sequences from RDP were used. The branch lengths correspond to the mean posterior estimates of evolutionary distances (scale bar, 0.4). The branch labels indicate the Bayesian posterior probabilities and bootstrap support values from a separate maximum likelihood analysis. *Bacteroides fragilis* (NR074784) was used as the outgroup. Legend: DF *Dermatophagoides farinae*; DP *Dermatophagoides pteronyssinus*; T industrial allergen-producing population from Trebon culture; L laboratory populations

microbiomes of the young cultures had a higher abundance of *Saccharomyces* (OTU2), whereas *Aspergillus* (OTU1) and *Candida* (OTU5) were more abundant in older cultures. The *y*-axis separated the DPL microbiome from the others, and these samples were correlated with *Wallemia* (OTU4). Based on METASTATS analyses, the OTUs did not significantly differ among the populations, but there were differences between mite species (Table S7) due to *Wallemia* (OTU4), which represented 39% of the sequences in the DPL microbiome, and *Candida* (OTU6), which represented 18% of the sequences in the DFL microbiome. Both these OTUs occurred at much lower proportions than the other fungal OTUs. No significant differences in the abundance of *Saccharomyces* (OTU2) were observed among the mite populations (Table S7).

Comparison of Bacterial and Fungal Microbiomes in HDM Cultures of Different Ages

The Bray-Curtis and Jaccard matrices revealed that the culture age affected the composition of bacterial and fungal OTUs in the microbiomes of all mite populations (Table 2), and the dissimilarity among the samples was visualized by dbRDA (Fig. 3a, b). *Culture age* was an important environmental variable in the models for both bacterial and fungal microbiomes

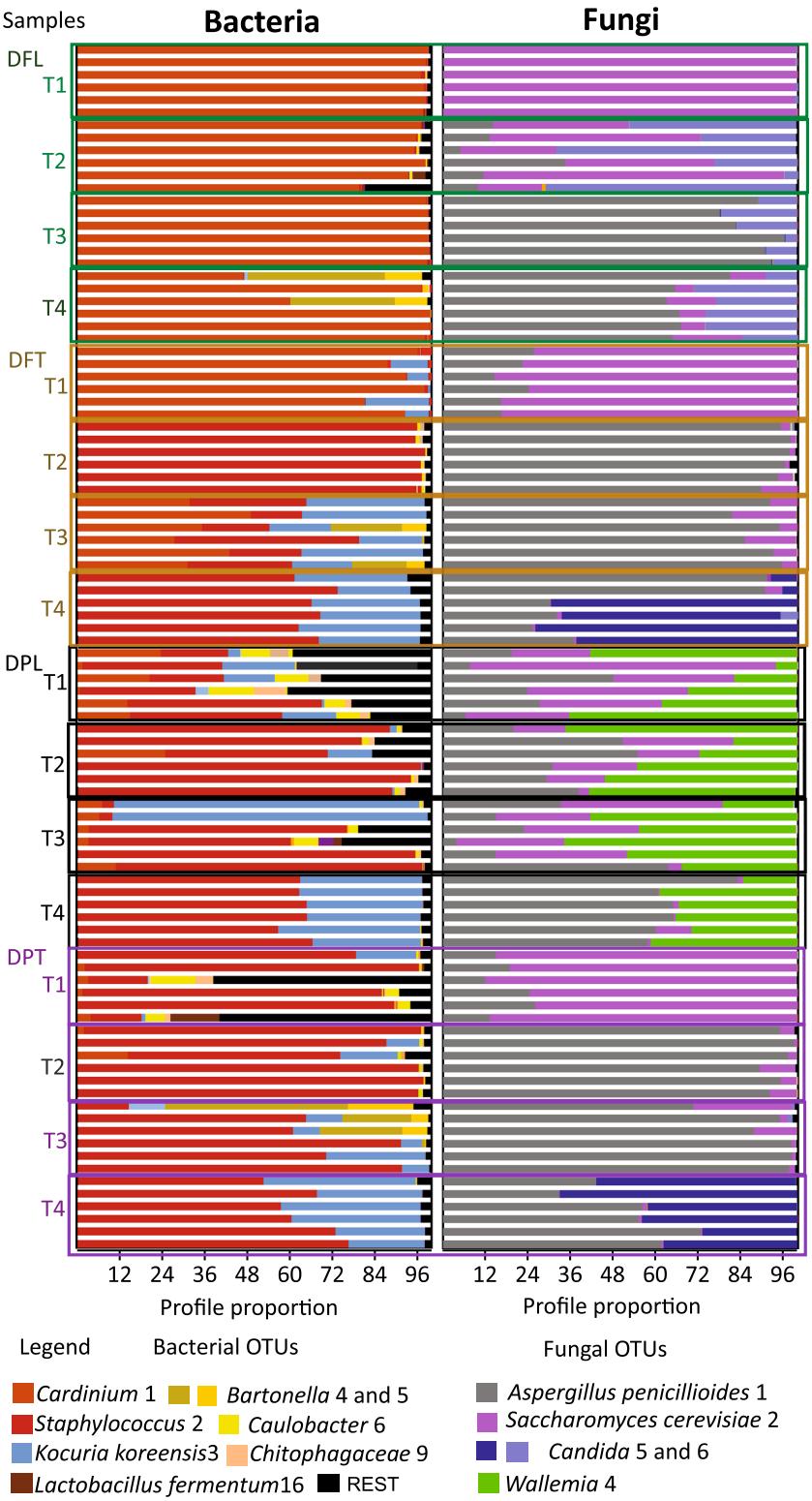

Fig. 2 Microbiome profiles of house dust mites (*D. pteronyssinus* and *D. farinae*) in samples from cultures of different ages (days). Legend: DF *D farinae*; DP *D. pteronyssinus*; T industrial allergen-producing population from Trebon cultures; L laboratory populations; culture development time: T1–T4 indicate four categories of the culture age, where T1 is the youngest culture and T4 is the oldest culture

(see above) and was responsible for the differences between the younger and older mite cultures.

The variable *Culture age* significantly influenced the abundances of 16S and 18S rRNA copies of mite-associated microorganisms (Table 3). In general, the numbers of both bacteria and fungi increased with increasing culture age, but this finding was not obtained for the DPT microbiome, for which culture age had no significant effect on the abundance of SSU fungal rRNA copies (18S). Another exception was the SSU bacterial rRNA (16S) in the DFL microbiome, in which

Table 2 The effect of culture age on the distribution of bacterial and fungal $OTUs_{97}$ in the microbiome of house dust mites. The standardized Illumina amplicon data were compared by one-way PERMANOVA separately. The data sets were evaluated with a Bray-Curtis matrix. The differences between groups of Illumina data from the Bray-Curtis distribution were evaluated by pairwise comparison, and the results are organized diagonally; above the diagonal, *F* values are presented, and below the diagonal, *P* values after Bonferroni's correction are presented. Italics indicates statistically significant differences. The culture development times T1–T4 indicate four categories of the culture age, where T1 is the youngest culture and T4 is the oldest culture

Population		Bacterial microbiome				Fungal microbiome			
		Bray-Curtis		Jaccard		Bray-Curtis		Jaccard	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
DFL		*2.52*	*0.024*	*3.43*	*0.001*	*61.06*	*0.001*	1.03	0.43
DFT		*128.50*	*0.001*	*5.86*	*0.001*	*36.68*	*0.001*	*1.95*	*0.007*
DPL		*3.85*	*0.001*	*2.39*	*0.001*	*5.79*	*0.001*	0.82	0.686
DPT		*3.50*	*0.001*	*6.35*	*0.001*	*114.50*	*0.001*	*2.44*	*0.001*
Population	Culture age	Bacteria: Bray-Curtis				Fungi: Bray-Curtis			
		T1	T2	T3	T4	T1	T2	T3	T4
DFL	T1		2.135	*3.747*	2.58		*20.57*	*1242*	*591*
	T2	0.074		2.791	2.39	*0.014*		*35.48*	*17.97*
	T3	0.133	*0.017*		2.608	*0.019*	*0.017*		*18.91*
	T4	0.564	0.966	0.132		*0.013*	*0.012*	*0.032*	
DFT	T1		*1176*	*41.89*	*584.2*		*984.9*	*539.3*	*29.54*
	T2	*0.014*		*81.51*	*157.2*	*0.014*		4.288	*12.42*
	T3	*0.019*	*0.017*		*30.72*	*0.015*	0.41		*12.24*
	T4	*0.013*	*0.012*	*0.02*		*0.012*	*0.005*	*0.021*	
DPL	T1		*7.894*	2.311	*12.35*		3.035	0.562	*15.29*
	T2	*0.04*		1.953	*10.16*	0.276		0.703	*9.074*
	T3	0.485	1		0.485	1	1		*13.54*
	T4	*0.013*	*0.026*	1		*0.013*	*0.011*	*0.046*	
DPT	T1		1.934	2.442	*4.365*		*736.2*	*203.5*	*158.9*
	T2	1		3.761	*14.9*	*0.011*		0.516	*56.49*
	T3	0.418	0.1836		*4*	*0.018*	1		*35.66*
	T4	*0.013*	*0.046*	*0.047*		*0.014*	*0.018*	*0.012*	

DF D. farinae, *DP D. pteronyssinus*, *L* laboratory population, *T* industrial-allergen producing population from Trebon population

the prevailing taxon, *Cardinium*, fluctuated during the sampling time with the same minima and maxima, as detected by universal 16S rRNA primers. The increase in bacterial copy number occurred at the same time as an apparent increased abundance of *Actinobacteria* and *Firmicutes* (Table 3), corresponding to the increasing proportions of *Staphylococcus* (OTU2) and *Kocuria* (OTU3) in the Illumina amplicon dataset (Fig. 2).

A METASTATS analysis revealed a notable fluctuation in the *Cardinium* profile and the number of 16S DNA copies in the DFT microbiome. The appearance of *Bartonella*-like bacteria (OTU4 and OTU5) was detected in some samples, but this finding was irregular and not related to culture age (Fig. 2). The profile of the fungal microbiome was characterized by a decreasing proportion of *Saccharomyces* (OTU2) and an increasing proportion of *Aspergillus* (OTU1) with increasing culture age (Fig. 2), and *Candida* (OTU5) appeared in mites from older cultures.

The Relationship Among Microbiome Composition, Population Growth, and Nutrient Status

A two-way PERMANOVA (Euclidean distance matrix) revealed that the nutrient status of mites significantly differed among all recorded time periods ($F_{(1,72)} = 399$; $P < 0.001$) and among populations ($F_{(3,72)} = 57$; $P < 0.001$), and the interaction of these two factors was also significant ($F_{(3,72)} = -26.48$; $P < 0.001$). Population growth differed between species ($U_{(1,26)} = 0.000$; $P < 0.001$): young cultures of *D. pteronyssinus* showed twofold higher growth rates than young cultures of *D. farinae*. Significant differences were also detected between both populations of *D. farinae* and *D. pteronyssinus* ($U_{(1,14)} = 49.0$; $P = 0.037$ and $U_{(1,10)} = 31.00$; $P = 0.040$, respectively): DFT and DPT exhibited approximately 10% higher growth than DFL and DPL. In addition, mites from young cultures exhibited five- to eightfold higher population growth than mites from old cultures (Fig. 4).

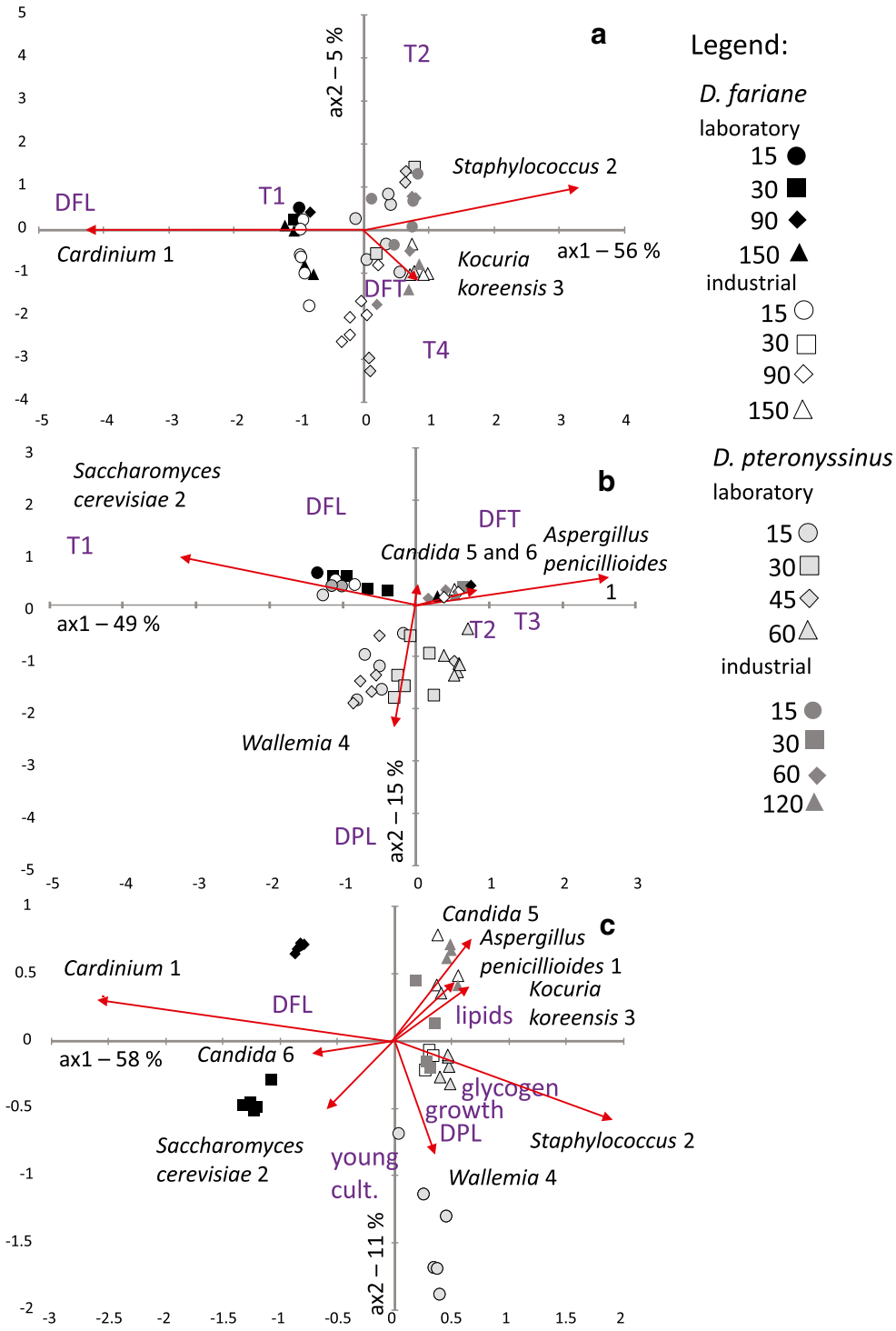

Fig. 3 Comparison of the bacterial and fungal microbiomes among house dust mite species, populations, and culture development times based on dbRDA models using the Bray-Curtis matrix. **a** Bacterial microbiome. **b** Fungal microbiome. **c** Fungal and bacterial microbiome and nutrient status of mites and mite population growth of young and old laboratory cultures. Legend: DF *D. farinae*; DP *D. pteronyssinus*; T industrial allergen-producing population from Trebon culture; L laboratory populations. The numbers after the mite population names indicate the culture development time; T1–T4 indicate four categories of the culture age, where T1 is the youngest culture and T4 is the oldest culture

The inclusion of the nutrient status of mite bodies (Fig. S4) and population growth variables in the dbRDA models and the combined analysis of the abundant bacterial and fungal OTUs yielded a significant model ($F_{(6,41)} = 48.832$; $P < 0.001$) that explained 78% of the total variation in the dataset (Fig. 3c). After forward selection, population growth, glycogen, and lipid status were found to have a significant effect on the OTU distribution, but no effect was observed for proteins and saccharides. *Staphylococcus* (OTU2) was correlated with increased glycogen contents in mites, whereas *Aspergillus* (OTU1), *Candida* (OTU5), and *Kocuria* (OTU3) were linked to increased lipid contents. Similar to the previous dbRDA model (Fig. 3b), the younger cultures had *Saccharomyces* (OTU2), whereas the older cultures did not (Fig. 3c).

Table 3 The numbers of copies of the 16S and 18S rRNA genes amplified by universal and specific primers from the samples of the house dust mite microbiome. The data were recalculated as the numbers of copies per mite and then LOG10-transformed and presented as medians and interquartile ranges in the brackets. The effect of culture age was tested by the Kruskal-Wallis nonparametric test; the letters indicate differences in the Dunn post hoc comparison after Bonferroni corrections

Population	Culture age	Fungi (univ)	Bacteria (univ)	Actinobacteria	Firmicutes	Cardinium
DFL	T1	1.25 (1.13–1.32)a	5.91 (5.79–5.96)ab	2.01 (1.89–2.14)	2.53 (2.39–2.57)ab	3.52 (3.51–3.55)ab
	T2	1.98 (1.88–2.10)ab	5.53 (5.49–5.56)a	2.21 (2.15–2.21)	2.15 (1.97–2.46)ab	3.27 (3.18–3.33)a
	T3	2.96 (2.92–2.98)b	6.27 (6.29–6.26)b	2.31 (2.21–2.62)	1.58 (1.35–1.69)a	3.83 (3.75–3.86)b
	T4	3.06 (2.98–3.15)b	5.96 (5.89–5.96)ab	2.05 (2.03–2.12)	2.85 (2.71–3.38)b	3.60 (3.44–3.64)ab
		$K = 20.11$; $P < 0.001$	$K = 18.75$; $P < 0.001$	$K = 6.104$; $P = 0.104$	$K = 13.67$; $P = 0.001$	$K = 16.23$; $P < 0.001$
DFT	T1	2.33 (2.11–2.50)ab	5.95 (5.85–5.96)a	1.82 (1.75–2.01)ab	2.59 (2.40–2.62)a	3.57 (3.49–3.68)b
	T2	2.04 (1.91–2.22)a	5.66 (5.53–5.66)a	0.08 (− 0.06–0.12)a	3.64 (3.50–3.72)ab	0.11 (− 0.04–0.26)a
	T3	3.22 (2.89–3.34)b	6.18 (6.16–6.21)ab	3.38 (3.33–3.40)b	4.13 (4.11–4.15)ab	3.43 (3.36–3.46)ab
	T4	3.34 (3.33–3.38)b	6.81 (6.54–6.97)b	3.84 (3.51–3.90)b	4.87 (4.72–4.99)b	0.04 (− 0.06–0.37)a
		$K = 16.53$; $P < 0.001$	$K = 18.16$; $P < 0.001$	$K = 18.89$; $P < 0.001$	$K = 19.41$; $P < 0.001$	$K = 16.81$; $P < 0.001$
DPL	T1	2.36 (2.19–3.08)ab	4.06 (3.89–4.14)a	2.89 (2.75–3.00)ab	2.81 (2.73–2.84)a	1.21 (0.57–1.32)
	T2	2.32 (1.89–2.54)a	4.38 (3.93–5.05)a	2.70 (1.68–2.78)a	2.76 (2.20–3.30)a	0.49 (0.34–1.04)
	T3	2.51 (2.31–2.70)ab	4.52 (4.13–4.80)ab	2.74 (2.63–3.02)ab	2.93 (2.66–3.18)a	1.25 (0.53–1.66)
	T4	3.02 (2.92–3.06)b	5.95 (5.73–6.07)b	3.35 (3.24–3.42)b	3.98 (3.86–4.12)b	0.47 (0.32–0.91)b
		$K = 7.67$; $P = 0.044$	$K = 13.21$; $P = 0.001$	$K = 14.03$; $P = 0.001$	$K = 11.43$; $P = 0.004$	$K = 3.706$; $P = 0.336$
DPT	T1	2.32 (2.14–2.67)	4.67 (3.86–4.94)a	0.01 (− 0.11–0.55)a	3.42 (3.25–3.50)a	− 0.08 (− 0.55–0.41)ab
	T2	2.78 (2.53–2.78)	5.36 (5.32–5.71)ab	0.45 (0.23–1.60)a	3.53 (3.49–3.18)ab	1.17 (0.81–1.43)c
	T3	2.61 (2.35–2.71)	6.21 (6.11–6.33)b	2.54 (2.43–2.64)b	4.33 (4.23–4.48)ab	0.03 (− 0.4–0.17)a
	T4	2.77 (2.42–3.14)	6.20 (6.16–6.32)b	3.26 (3.05–3.54)b	4.41 (4.32–4.49)b	0.95 (0.78–1.12)bc
		$K = 3.50$; $P = 0.33$	$K = 16.08$; $P < 0.001$	$K = 19.45$; $P < 0.001$	$K = 11.89$; $P = 0.003$	$K = 15.85$; $P < 0.001$

DF D. farinae, *DP D. pteronyssinus*, *L* laboratory population, *T* industrial-allergen producing population from Trebon population

Microorganisms Participate in Food Bolus Formation in the Gut of HDMs

A food bolus is formed in the ventriculus when ingested material is mixed with excreted particles from ventricular and cecal cells [79], and this food bolus is then enveloped by the peritrophic membrane before passing through the intercolon and colon to the postcolon (Fig. 5a, b) [80]. Three different types of food boli could be distinguished in our samples, and these consisted of (i) mucoid particles (Fig. 5b), (ii) fragmented plant material (Fig. 5b), and (iii) fungal hyphae (Fig. 5f). Combinations of fragmented plant material with yeast cells (Fig. 5c, g) or fungal hyphae (Fig. 5d) were also detected. The fungal mycelium had no cell contents, i.e., there were hollow rings inside the fungal cell walls (Fig. 5c). A food bolus was never formed by bacteria alone, but some bacteria were found attached to the ingested fragmented plant material on mite chelicerae (Fig. 5f). No differences in the food bolus content were observed between *D. farinae* and *D. pteronyssinus*. Fungal hyphae (mold) were found in the food boli of specimens from older cultures, whereas specimens from young cultures mostly contained yeasts.

Discussion

Symbiotic or nutritive interactions between mites and microorganisms are believed to enable HDM survival in anthropogenic environments. HDMs can either directly digest structural proteins (keratin, elastin, and collagen) or feed on and/or interact with microorganisms that co-occur in house dust to metabolize these proteins (nutritional hypothesis) [1, 19, 22, 81–83]. Our data supported the latter and identified *Kocuria*, *Staphylococcus*, *Aspergillus*, and *Saccharomyces* as possible nutrient sources for the investigated mites. HDMs can utilize nutrients from bacteria or fungi following disruption of their cell walls by bacteriolytic/lysozyme-like activity [22, 29, 83, 84]. In this study, we observed digestion of the fungal cell contents, as evidenced by hollow spaces inside the mycelium, whereas food boli formed exclusively from bacteria were not observed. The symbiotic hypothesis (*Cardinium* and *Bartonella*-like) was generally not supported due to the low abundance of *Cardinium* in the *D. pteronyssinus* microbiome and the absence *Bartonella*-like bacteria in some of the consecutive samples obtained during mite population growth. In this study, we did not observe transient passage of

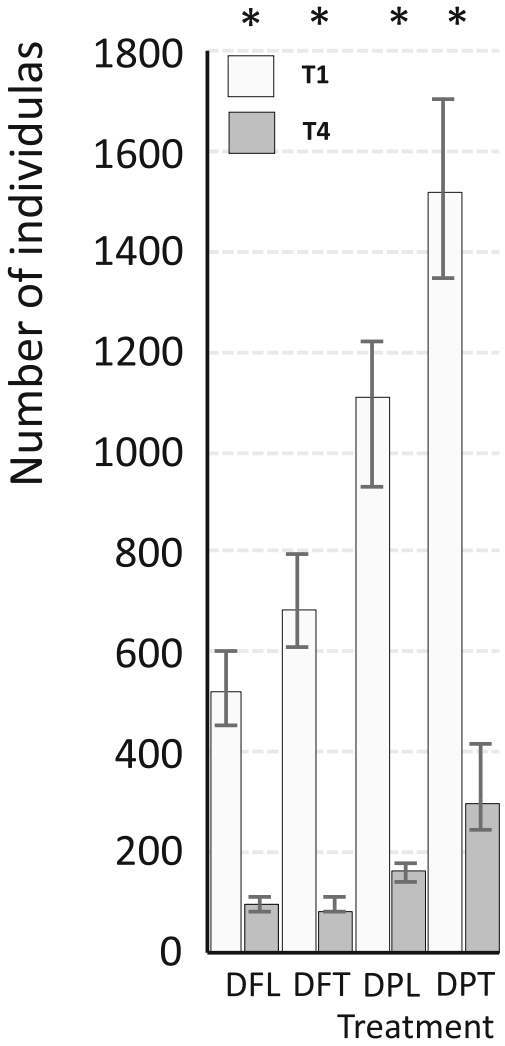

Fig. 4 Comparison of the growth of laboratory and industrial allergen-producing populations of house dust mites. Population growth is expressed as the number of individuals after 21 days of growth starting from 10 individuals. The specimens from young (T1) and old cultures (T4) were compared separately for each population using a Mann-Whitney test; an asterisk indicates a significant difference ($P < 0.05$). The columns are the medians, and the bars represent the interquartile ranges. Legend: DF *D. farinae*; DP *D. pteronyssinus*; T industrial allergen-producing population from Trebon culture; L laboratory populations. The numbers after the mite population names indicate the culture development time; T1 is the youngest culture, and T4 is the oldest culture

microorganisms in HDM with the exception of the *Cardinium*; therefore, we are not able to distinguish between the nutritional and symbiotic importance of microorganisms for HDM. The microorganisms can either be used as food or establish a symbiotic relationship with the HDM host and provide nutrients.

Microbiomes in Different HDM Populations

In this study, no substantial genetic differences (ITS and COX1) were found between the conspecific HDM populations, indicating that the observed differences in the bacterial microbiomes between the HDM populations were not influenced by different phylogenetic origins. We found differences in the bacterial profiles of *D. farinae* and *D. pteronyssinus* and between *D. farinae* populations. An intracellular symbiont, *Cardinium*, has been identified in both *D. farinae* and *D. pteronyssinus* [32, 85]. In this study, *Cardinium* showed different profiles and quantitative changes between the studied HDM populations. In addition, *Cardinium* was detected in mite eggs, indicating the maternal transfer of this bacterium, which has been well documented in other host organisms [86]. Our phylogenetic analyses showed that *Cardinium* infecting *Dermatophagoides* mites forms a sister group to a cluster of bacteria from stored-product mites [85] and bacteria from the predatory mite *Cheyletus eruditus* [87] and several planthoppers and leafhoppers. Published genome-scale analyses of *Cardinium* symbionts have suggested nutritive interactions with their hosts, such as through biotin biosynthesis [88, 89], as well as cytoplasmic incompatibility and feminization in insects and mites [89–91]. Although the mites investigated in this study reproduce sexually, some sex ratio bias might still exist, such as the male/female ratio of 0.38/0.539 reported previously [92]. The presence of *Cardinium* in HDM microbiomes can differ among cultures. Although in this study, we observed a high number of sequences of *Cardinium* in the *D. farinae* microbiome, *Cardinium* was not reported in a Korean population of *D. farinae* [30]. The instability of the *Cardinium* among different samples of the industrial allergen-producing population of *D. farinae* can be explained by the influence of sampling and experimental design. We used population-scale samples in this design. *Cardinium* is probably not present in the all mites, and the presence of a few infested specimens can strongly influence the results due to stochasticity [93].

We observed that 1 mg of tetracycline per gram of diet did not eliminate *Cardinium*. The choice of this concentration was based on previous experiments with stored-product mites, which showed that higher concentrations of the antibiotic suppressed mite development. Previous studies have also indicated that adding antibiotics to the food given to mites reduced but did not completely eliminate *Cardinium* [26, 94]. In contrast, tetracycline treatment of the false spider mite *Brevipalpus californicus* resulted in the production of male offspring following the elimination of *Cardinium* [95]. Thus, the effect of antibiotic addition on mites should be further researched by screening for *Cardinium* in individual males and females.

The presence of *Bartonella*-like bacteria in our samples was consistent with previous observations [30, 96]. Representative sequences from both OTUs (OTU4 and OTU5) showed 99% similarity to sequences from the red poultry mite, *Dermanyssus gallinae* [97]. Previous analyses of *Bartonella* sequences from *D. gallinae* suggested a phylogenetic similarity of the sequences of *Bartonella* from stored-product mites and *Bartonella apis* [98, 99] from the honey bee. The next most prevalent

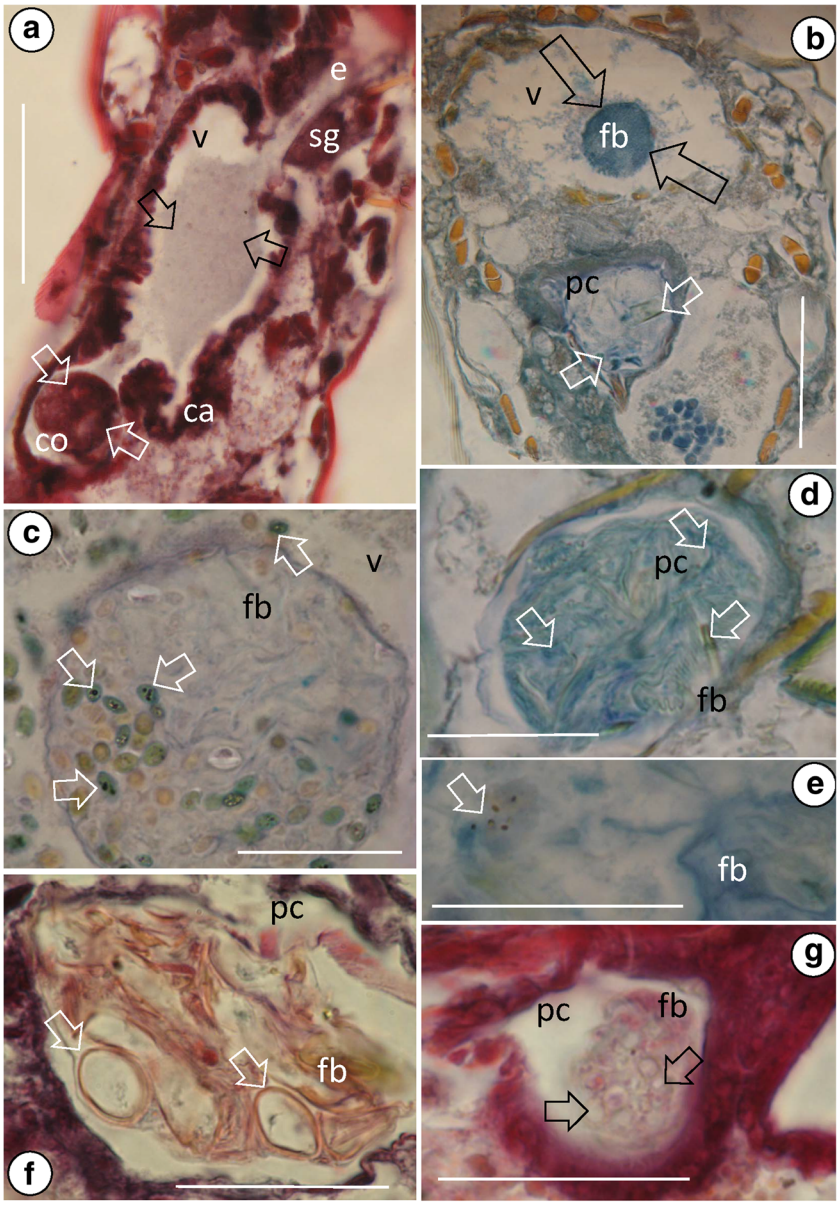

Fig. 5 Gut contents and food boli of the house dust mites *D. pteronyssinus* and *D. farinae*. **a** Total view of a parasagittal section of the midgut of a mite from a 60-day-old population of the laboratory population of *D. pteronyssinus*; the ventriculus contains ingested mucoid particles indicated by black arrows, and white arrows point to the food bolus. **b** Total view of a horizontal section of the mesodeum of a mite from a 30-day-old population of the industrial allergen-producing population of *D. farinae*; the food bolus in the ventriculus consists of mucoid particles (black arrows), while the food bolus in the postcolon contains fragmented particles (white arrows). **c** A food bolus in the ventriculus of a mite from a 30-day-old population of the laboratory population of *D. pteronyssinus*; yeasts (arrows) are present in both the food bolus and the ventricular contents. **d** The postcolon of a mite from a 90-day-old population of the laboratory population of *D. farinae*; the food bolus is composed of fragmented particles (white arrows) and mycelium (black arrow). **e** Detailed view of the ventricular contents of a mite from a 30-day-old population of the industrial allergen-producing population of *D. farinae*; the arrow points to bacteria on the fragmented food. **f** Detailed view of a fungal food bolus in the postcolon of a mite from a 120-day-old population of the laboratory population of *D. farinae*; fungal hyphae without cell contents are indicated by arrows. **g** Detailed view of the postcolon of a mite from a 60-day-old population of the laboratory population of *D. pteronyssinus*; the food bolus in the postcolon contains small fragmented mycelium (arrows). Scale bars: **a**, **b** 50 μm; **c**–**g** 25 μm. e esophagus, ca cecum, co colon, fb food bolus, pc postcolon, sg synganglion, v ventriculus

alpha-proteobacterium, *Caulobacter*, which had a relative proportion of 2% of the total DFL microbiome profile, showed 100% sequence identity to an unidentified bacterium from human skin (GenBank Accession No. JF107935) [100].

Firmicutes (*Staphylococcus*) and *Actinobacteria* (*Kocuria*) accounted for the significant differences in the bacterial communities among the HDM mite populations in this study. Both bacterial taxa occur frequently in stored-product mites [32]. Analyses of the human microbiome have shown a large

proportion of *Actinobacteria* (more than 50% of bacteria) on human hairs and skin [101]; thus, the *Actinobacteria* that naturally occur on human skin, hairs, and nails may represent a food source for HDMs. This view is supported by the feeding of HDMs on diets enhanced with *Micrococcus lysodeikticus* [22] and by the presence of *Actinobacteria* proteins in *D. pteronyssinus* feces [29]. The differences in the numbers of copies of *Actinobacteria* and *Firmicutes* among the populations tested in this study did not support the possibility of random ingestion because their numbers increased with increasing culture age. *Staphylococcus*, *Bacillus*, and *M. luteus* were identified in an industrial allergen-producing population of *D. pteronyssinus* in Australia [102], which corresponds to the presence of the groups observed in this study. The next most prevalent member of *Firmicutes* was *Lactobacillus fermentum* (OTU16), which had a relative proportion of 2% in the microbiome of the laboratory population of *D. pteronyssinus*. This bacterium also occurs on human mucous membranes [103]. These data support the possibility that HDMs preferentially utilize nutrients from *Actinobacteria* and *Firmicutes* growing in house dust rather than digesting structural proteins. *Kocuria* was recently described to cause complications in atopic keratoconjunctivitis (Inada et al.), and thus, this bacterium is capable of growing on sloughed skin and serving as a food source for HDMs [104].

The low numbers of fungal OTUs in our samples are consistent with the low diversity of fungi (nine species) previously isolated from *D. pteronyssinus* [105]. The identified genera included *Saccharomyces* (OTU2), which was a component of the rearing diet for all culture ages during the experiment. Other abundant fungal taxa, *Aspergillus* and *Wallemia*, are known to be associated with the European HDM *D. pteronyssinus*, which is found in house dust [105]. *Aspergillus* has been proposed as a nutrient source for *D. pteronyssinus* [106] and *D. farinae* [31]. *Wallemia sebi* does not affect the growth of *D. pteronyssinus* in experimental settings [106] but is the preferred diet of *D. farinae* [20]. Our observations suggest that the detected fungi originate *from* the ingested diet, indicating that these microorganisms might have nutritive value. We detected a fungus of allergenic importance, *Candida* [107], and our results indicated its transmission by *Dermatophagoides* mites.

Microbiome Changes at Different Mite Culture Ages

Previous studies showed that diet significantly influences the population growth of mites and the production of allergens [108], and as confirmed in this study, culture age is the key factor influencing the number of HDMs in a culture [10], the mite fitness [109], and the production of allergens [9]. As was also confirmed in the present study, which included the administration of a single diet, mite population growth is influenced by the species and the population [110, 111], and the quantity of fungi in the mite culture also depends on the age of the mite culture [31]. For example, the quantity of *Aspergillus penicillioides* was 10-fold higher in 7- to 14-month-old cultures of *D. pteronyssinus*. In laboratory experiments, the presence of *Aspergillus* had a suppressive effect on mite population growth; however, the second generation of the *Aspergillus*-free mites did not survive [81, 106]. Thus, it is not surprising that both the bacterial and fungal microbiomes of the tested populations differed with different culture ages. Both successional and neutral changes in the microbiome are expected. The neutral changes caused by *Cardinium* were discussed above, and the lower population growth of DFL compared with that of the other mite populations is likely associated with *Cardinium* infestation. However, we are unable to explain how the differences in the growth of *D. pteronyssinus* populations are associated with the microbiome composition. The experimental design included pasteurized diets and a large number of rearing chambers at the beginning of the experiment, but, as mite samples were collected, we decreased this number. This design could introduce stochasticity because the mites can randomly introduce microbiota that develop independently inside the rearing chambers. However, this stochasticity would also cause intersample variability, which was found to be low. Here, we employed our regular protocol for maintaining mite cultures and thus expect that a similar stochastic effect is also present in the industrial mite cultures.

Our novel finding is that mites from young cultures showed five- to eightfold higher population growth than mites from old cultures. The population growth of mites has been proposed as an indirect indicator of fitness [112]. In the growth experiments, the mites were introduced to new diet containing yeasts (*S. cerevisiae*). Newly established, 15-day-old cultures contained a random combination of bacterial profiles and almost no fungi with the exception of *Saccharomyces* (OTU2), which was present in the diet used in all the experiments. Our observations of the gut contents indicated that the mites in young cultures feed on fragmented plant material and yeasts (*S. cerevisiae*), which were the components of rearing diets. In older cultures, no yeast cells in the gut were observed and mycelium food boli prevailed. *Aspergillus* sequences were the most abundant in the fungal profile from the mites and should be present in the gut of mites. These findings suggest that mites switch from yeasts to filamentous fungi during the aging of mite culture.

However, high glycogen contents in fat tissues and the absence of guanine deposits are indicative of a suitable diet [113–115]. The relative abundances of *Aspergillus*, *Staphylococcus*, and *Kocuria* increased with increasing culture age, supporting the nutrient hypothesis based on mite-microorganism interactions. Although old colonies in the decline phase were characterized, no acaropathogenic bacteria were identified. These findings support a nutritive function for mite-associated microorganisms, which consequently grow

during the lives of mite colonies. The shift in the gut microbiota linked to an increase in culture age might not drive changes in nutrient acquisition but could simply be due to changes in the quality of the culture conditions. In other words, because the yeast was eliminated from the diets by intensive mite feeding, nutrient availability could drive both the mite quality and the microbiota composition. Further study is necessary to separately compare these effects.

Correlation of Mite Nutrient Status with Microbiomes

The artificial addition of proteins, lipids, and carbohydrates to the diets of *D. pteronyssinus* resulted in differences in mite fitness and different levels of allergen expression [5]. We detected a similar effect in previous experiments, in which differences in the mite nutrient status were observed [5]. Population-level samples include mite individuals of mixed ages; thus, the nutrient status cannot be correlated with fitness because a rapidly growing population containing juveniles can differ from a slowly growing population that mainly consists of adults. Here, we observed correlations for *Aspergillus* with *Candida* and for *Kocuria* with increased lipid contents in mites, which confirm that fungi serve as a lipid source for mites [18, 19]. The positive correlation between increased lipid contents and the presence of *Kocuria* could be the result of the high lipid content of this bacterium or preferential feeding by this bacterium on lipid-rich components of the diet. Similarly, the presence of *Staphylococcus* was correlated with increased glycogen contents in mites, probably because this bacterium can selectively grow on glycogen-rich substrates.

Medical Importance

House dust mites produce a broad spectrum of proteins that act as allergens, including allergens associated with digestion or antibacterial functions, but various microorganisms also form an important part of the bodies of mites. Here, we report the differences in the microbiomes of different *D. farinae* and *D. pteronyssinus* populations and the variations between old and young mite cultures. Although laboratory cultures of mites have limitations [111], they represent a good model for observing mite-microbe interactions. HDMs are industrially reared to produce immunotherapy drugs and as a standardized source of allergens for accurate allergy testing [5]. Unless directly related to the immunogenic properties of natural mite populations, microorganisms are unwanted contaminants in such production [96]. The associated microorganisms include the intracellular symbiont *Cardinium*, a putative *Bartonella*-like symbiont, gut/environmental bacteria (*Staphylococcus* and *Kocuria*), and fungi (*Aspergillus*, *Saccharomyces*, *Wallemia*, and *Candida*). Although we used industrial allergen-producing mite populations, we did not apply a cleaning protocol to separate mites from their diets before medical use. We showed that these mites are inhabited by a limited number of bacterial species, and industrially prepared samples of mites contain bacteria, fungi, and yeasts.

Acknowledgements The authors are grateful to Barry OConnor and the anonymous reviewers for providing useful comments on earlier drafts of this manuscript. We thank Marie Bostlova, Martin Markovic, Bc. Vit Molva, and Jan Hubert Jr. for their technical assistance; Prof. Krzysztof Solarz for the laboratory mite culture; and RNDr. Alexandr Zgarbovsky for the industrial allergen-producing mite cultures.

Funding Information JH, TE, and MN were supported by the Czech Science Foundation (GACR) as part of Project No. 17-12068S. PBK was supported by the Russian Science Foundation (Project No. 16-14-10109), the Ministry of Education and Science of the Russian Federation (No. 6.1933.2014/K Project Code 1933), and the Russian Foundation for Basic Research (No. 15-04-0s5185-a).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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