

RESEARCH ARTICLE

Dynamics of the microbial community during growth of the house dust mite *Dermatophagoides farinae* in culture

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One sentence summary: The reduction of the mite population size during the late phases of *D. fariane* culture development was correlated with the changes in microbial profiles.

Editor: Rolf Kümmerli

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ABSTRACT

The variation in house dust mite microbial communities is important because various microorganisms modulate the production of allergens by their mite hosts and/or contaminate immunotherapeutic extracts. Temporal changes in mite microbiomes and the mite culture environment occurring at different stages of mite culture development are particularly understudied in this system. Here, we analyzed the dynamics of microbial communities during the culture growth of *Dermatophagoides farinae*. Changes in microbiomes were related to three key variables: the mite population density, microbial microcosm respiration and concentration of guanine (the mite nitrogenous waste metabolite). Mite populations exhibited the following phases: exponential growth, plateau and exponential decline. The intracellular bacterium *Cardinium* and the yeast *Saccharomyces cerevisiae* prevailed in the internal mite microbiomes, and the bacterium *Lactobacillus fermentum* was prevalent in the mite diet. The reduction in the mite population size during the late phases of culture development was related to the changes in their microbial profiles: the intracellular bacterium *Cardinium* was replaced by *Staphylococcus*, *Oceanobacillus* and *Virgibacillus*, and *S. cerevisiae* was replaced by the antagonistic fungi *Aspergillus penicillioides* and *Candida*. Increases in the guanine content were positively correlated with increases in the *Staphylococcus* and *A. penicillioides* profiles

in the culture environment. Our results show that the mite microbiome exhibits strong, dynamic alterations in its profiles across different mite culture growth stages.

Keywords: *Dermatophagoides farinae*; bacteria; yeasts; microbiome; guanine; mite culture; allergens

INTRODUCTION

Allergen production by house dust mites (HDMs; *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) is thought to be modulated via interactions of mites with associated bacteria and fungi (Gregory and Lloyd 2011; Jacquet 2011, 2013). Mites are mass produced in culture as a source of allergens and for therapeutic purposes (Batard et al. 2006; Yang and Zhu 2017). These cultures provide the optimal conditions for mite development, including controlled temperature, humidity and diet (Colloff 2009). The mass cultures are naturally contaminated by microbes, and the extracts from mite cultures contain lipopolysaccharides, β -glucans from microbial cell walls and chitin, a glucosamine polymer of fungal origin (Brown and Gordon 2003). These compounds stimulate the human immune system (Gregory and Lloyd 2011). Mites produce a broad spectrum of proteins, which are modulated by microorganisms and can serve as allergens that are dangerous to sensitive people (Gregory and Lloyd 2011; Jacquet 2011). These include enzymes with hydrolytic activity against structural polysaccharides in microbial cell walls, such as chitinases (group 15) and proteases (allergen groups 3, 6 and 9) (Childs and Bowman 1981; Bowman 1984; Stewart et al. 1994; King et al. 1996; Erban and Hubert 2008, 2015; Erban, Harant and Hubert 2017; Randall et al. 2017). Microbes can induce mites to produce antibacterial proteins, including important allergens, e.g. lipid-binding proteins (group 7) that bind to a bacteria-derived lipid product (Mueller et al. 2010), NlpC/60-like proteins and the LytFM antibacterial protein (Mathaba et al. 2002; Erban et al. 2013; Tang, Stewart and Chang 2015, 2017).

These observations of microbial nutrient importance, immunotherapeutic extract contamination and potential modulation of allergen production have stimulated active research on HDM-associated microbes. Briefly, we know the following: (i) Mites feed on some filamentous fungi, yeasts and bacteria that serve as nitrogen and vitamin sources for mites (Lustgraaf 1978; Tongu, Ishii and Oh 1986; de Saint Georges-Grédelet 1987; Douglas and Hart 1989; Tang et al. 2013). (ii) Mites have a clear preference for some microbial species as food sources (Naegelé et al. 2013). (iii) *D. farinae* and *D. pteronyssinus* differ in their bacterial communities (Hay et al. 1992; Valerio et al. 2005; Kim et al. 2018), and mite microbiome profiles vary among populations of *D. farinae* (Molva et al. 2018). (iv) *D. farinae* is a host of the intracellular symbiotic/parasitic bacterium *Cardinium* (Kim et al. 2018; Molva et al. 2018); however, *D. pteronyssinus* lacks this bacterium (Hubert et al. 2016, 2019; Kim et al. 2018). (v) Every mite culture consists of mites and a mixture of leftover diet, mite feces and debris from mite bodies (i.e. spent growth medium, SPGM). The therapeutic extracts made from mite colonies are prepared from not only mites but also SPGM.

HDM cultures used for therapeutic purposes have specific growth patterns: (i) the latency phase (from the beginning of cultivation to week 10) is characterized by a slow increase in the mite population; (ii) the exponential phase (from weeks 10 to 20) is characterized by exponential growth and (iii) the decline phase (after week 20) is characterized by a major decrease in the mite population size (Eraso et al. 1997a,b).

Differences in the protein (Andersen 1991) and allergen contents of different immunotherapeutic extracts (Eraso et al. 1998) have been observed at different mite culture growth phases. The changes in the microbiomes of mites over time can therefore be connected to the known changes in the allergen contents of culture extracts (Eraso et al. 1998). Similarly, previous studies have shown that the microbiome of HDMs is not stable over time and that the bacterial and fungal profiles of mites can change during mite culture development (Molva et al. 2018; Hubert et al. 2019). However, the sampling design of our previous work using *D. farinae* (Hubert et al. 2019) was not continuous, which may have affected the results. In contrast to this recent study (Hubert et al. 2019), here, we focus on the interactions of culture time, mite population density and guanine content in the culture with microbial composition. We utilized a continuous sampling design for 14 days and analyzed the microbiomes of surface-sterilized mite bodies (henceforth referred to as the 'internal microbial community' or 'the microbiome inside the mites') and from extracts of SPGM (henceforth referred to as 'environmental samples' or 'the microbiome of the spent growth medium').

In this study, we sampled mite microbiomes at different phases of *D. farinae* mite culture development, which mimic the development pattern in industrial immunotherapeutic extract production settings. The microbiome was characterized by barcode sequencing of 16S (bacteria) and 18S DNA (fungi) and by qPCR (quantitative PCR) using group-specific primers. Guanine is a nitrogenous waste product of mite metabolism that is excreted via feces or remains in the bodies of mites (Levinson, Levinson and Muller 1991a,b). Guanine accumulation might have a deleterious effect on mite fitness or the associated microbial community. Therefore, we hypothesized that a measurement of respiration in microcosms provides a direct estimate of microbial metabolic activity and an indirect estimate of microbial growth (Hanlon 1981). Because *Cardinium* can manipulate the sex ratio in its host population (Zchori-Fein and Perlman 2004), we also recorded changes in the male-to-female ratio during mite culture growth. Mite microbial profiles were correlated with mite population growth, guanine, microbial respiration and sex ratio.

MATERIALS AND METHODS

Mites

Dermatophagoides farinae culture was obtained from Prof. Krzysztof Solarz at the Medical University of Silesia, Katowice, Poland, in 2005. The rearing conditions and diet were described previously (Erban and Hubert 2008). The HDM rearing diet (HDMd) consisted of a mixture of dog food/wheat germ/dried fish food/Pangamin dried yeast (*Saccharomyces cerevisiae*) extract/gelatin in a ratio of 10/10/3/2/1 by weight. All the compounds were mill-powdered, sieved (mesh size = 500 μ m) and pasteurized by heating at 70°C for 0.5 h (Erban and Hubert 2008). The colonies were subcultured monthly in the rearing facility. To establish a new culture, ~5000 mites were placed in a new chamber containing 0.3 g of freshly prepared diet.

Experimental and sampling design

The experiments were conducted in six replicates, and each experimental replicate was conducted in a 660 mL flask (Cellstar®, Greiner Bio-One, Frickenhausen, Germany, cat. no. 660-150) containing 36 g of HDMd. The mites were transferred into each experimental flask from six rearing flasks containing 30-day-old mite cultures. Each rearing flask contained ~0.3 g of rearing medium and ~5000 mites. At 14, 28, 42, 56, 70, 84 and 98 days of culture development, live mites and SPGM were sampled separately. For mite DNA extraction, live mites were collected from the chamber plug with a brush, and the mite samples (Table S2, Supporting Information) were then weighed on a microbalance and transferred to a 1.5-mL plastic vial (Eppendorf, Hamburg, Germany) filled with absolute ethanol. After the collection of live mites, the chamber was shaken gently to obtain a homogenous mixture of diet and mites. Thus, the experimental samples included weighed samples of pure mites (internal community, the microbiome inside the mites) and samples of culture medium (environmental samples, the microbiome of the spent growth medium). The samples were then used for the following measurements: (i) microcosm respiration assessment, (ii) estimation of the mite density, (iii) separation of DNA extraction from mites and SPGM, (iv) determination of the guanine concentration and (v) observations of the sex ratio of the mites.

Estimation of mite population density and sex ratio

To estimate changes in the population density during mite culture growth, mites and diet were weighed and transferred to 1.5-mL plastic vials filled with 80% ethanol. The mites were then counted under a dissecting microscope, and the number of mites in each sample was then recalculated based on 0.01 g of diet. We did not distinguish between adults and juveniles, and in accordance with previous studies (Eraso et al. 1998), eggs were not counted. The sex ratio was examined by mounting mites on slides in 60% lactic acid (Voorhorst et al. 1967).

Respiration in microcosms

To determine microcosm respiration, we used a previously described protocol (Molva et al. 2018; Nesvorna, Bittner and Hubert 2019). Respiration was measured using an infrared gas analyzer (IRGA) and respiration apparatus (catalog no. RP1LP, Qubit Systems, Kingston, ON, Canada) based on a Gascard II Infrared Card (Edinburgh Sensors, Livingston, Scotland, United Kingdom) with syringes containing diet collected from the mite cultures after 2 h of incubation at $25 \pm 0.1^\circ\text{C}$. Carbon dioxide (CO_2) concentration in the chamber was determined in units of μL per mg of diet fresh weight per hour ($\mu\text{L}\cdot\text{mg}^{-1}\cdot\text{h}^{-1}$). Based on previous studies, the respiration of SPGM is mostly linked to microbial activity, whereas mite respiration has only a marginal effect (Hubert et al. 2018).

Guanine concentration assay

Two types of samples were obtained after 14 and 98 days: pure mites and diet + mites. The mite (0.05 ± 0.02 g) samples were lyophilized; then, we used three biological and three technical replicates (14-day mite samples) or seven biological and three technical replicates (98-day mite samples). The contents of the rearing chambers were lyophilized in 15-mL centrifuge tubes covered with filters in a PowerDry LL3000 (Thermo; Waltham, MA, USA), and 0.1 g of the sample was homogenized in 3 mL of

ammonium acetate buffer (pH 3.5) with 0.1 g of an equal mixture of garnet sharp particles (0.3/1.0 mm diameter particles; BioSpec, Bartlesville, OK, USA; cat. nos. 11079103 and 11079110) and vortexed. The samples were then treated in an ultrasonic bath for 30 min (30°C). After this treatment, 0.12 g of sorbent mixture (1:1 C_{18} (EC) and Diamino sorbents, Macherey-Nagel, Duren, Germany) was added to the sample, and the mixture was mixed by vortexing. The extraction efficiency of guanine was 73%. The samples were centrifuged for 10 min at $10\,232 \times g$, and 1 mL of each sample was then filtered through regenerated cellulose syringe filters with a pore size of $0.45 \mu\text{m}$ (TR-200 435; diameter 13 mm, Teknokroma, Barcelona, Spain) into a 2-mL glass vial. The analysis was conducted using an Agilent high-performance liquid chromatography system (1200 series; Agilent Technologies Deutschland, Waldbronn, Germany) equipped with a degasser, quaternary pump, autosampler, thermostat and diode-array detector. The chromatographic separation was performed on a Kinetex C18 analytical column ($150 \text{ mm} \times 4.6 \text{ mm}$, $2.6 \mu\text{m}$; Phenomenex, Torrance, CA, USA) protected by a guard column with a C18 cartridge ($4 \text{ mm} \times 3.0 \text{ mm}$; Phenomenex). The column temperature was maintained at 24°C throughout the analysis. Separation was performed with a flow rate of 0.5 mL/min using an injection volume of 5 μL and the following gradient of mobile phases A (20 mM ammonium acetate buffer, 3.5 pH) and B (methanol): 5 min, 100% A; 10–11 min, 80% A and 17 min, 100% A. The instrument control and data evaluation were performed using OpenLab software (Agilent). The calibration and extraction efficiency were evaluated based on a guanine standard (cat. no. G6779, Sigma-Aldrich, St. Louis, MO, USA). Non-matrix-matching quantitation was performed because all the samples contain guanine, and the final concentration of guanine was expressed in units of $\mu\text{mol/L}$.

DNA extraction

Mite samples were surface cleaned by replacing 100% ethanol after centrifugation ($13\,000 \times g$ for 1 min) with bleach, vortexing for 5 s and removing the bleach after centrifugation ($13\,000 \times g$ for 1 min). The samples were then washed twice in 100% ethanol to remove the rest of the bleach by centrifugation. DNA was extracted using the Exgene Genomic DNA micro kit (Cambio, Cambridge, UK, cat. no. GA-118-050) by replacing the ethanol with 300 μL of CL buffer (a component of the kit), and the solution was removed under a sterile hood. The mites were transferred to a 2.0-mL Screwcap MCT conical NS (cat. no. CP5912, Alpha Laboratories, Eastleigh, Hampshire, UK) with 0.5 g of an equal mixture of 0.3-mm and 1.0-mm garnet sharp particles (cat. nos. 11079103gar and 11079110gar, BioSpec) and one glass bead (3-mm diameter, cat. no. R155761, BioSpec). The samples were homogenized in a Mini-BeadBeater (BioSpec) for 5 min. The homogenates were centrifuged ($10\,000 \times g$ for 2 min), the supernatant was transferred to a new sterile 1.5 mL vial, and 20 μL of proteinase K was added. The sample was incubated overnight at 56°C . The rest of the isolation was performed according to the manufacturer's instructions.

To characterize the mite culture microbial community, the SPGM from the experimental flask was mixed with 50 mL of 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) (Hubert et al. 2012), and the mixture was vortexed for 5 s. The mixture was filtered through a 40- μm mesh Cell Strainer (cat. no. 27305, Stemcell Technologies, Cambridge, MA, USA) to remove the remaining diet and any mite bodies. The filtered supernatant was centrifuged ($845 \times g$, 5 min),

the supernatant was discarded, and the pellets were cleaned by washing in PBST and centrifugation. The clean pellets were finally resuspended in 10 mL of PBST and frozen (-40°C). One milliliter of the supernatant was transferred to a 1.5-mL Eppendorf vial, and the Exgene Genomic DNA micro kit was used for DNA extraction. The tubes were centrifuged ($6785 \times g$; 3 min), the supernatant was discarded, and the pellets were suspended in 200 μL of CL buffer. Subsequently, 20 μL of proteinase K was added, and the samples were incubated overnight at 56°C . The rest of the procedure was performed according to the manufacturer's instructions. The isolated DNA was stored at -20°C until analysis. The quality of the isolated DNA was examined by PCR with universal primers (Table S1, Supporting Information) and using a NanoDrop One instrument (Thermo-Fisher Scientific, Waltham, MA, USA) (Table S2, Supporting Information).

Barcode sequencing

For fungal identification, the 18S rRNA gene fragment was amplified (Table S1, Supporting Information) (Chemidlin Prevost-Boure et al. 2011; Caporaso et al. 2012). For bacterial barcoding, because chloroplast DNA present in the diet can influence profiling of arthropod bacterial communities (Hanshew et al. 2013), we used a protocol that prevents the chloroplast DNA from amplifying (Sakai et al. 2004). Bacterial barcoding was performed with the primer F27 and an equimolar mixture of 783r-aL, 783r-bL and 783r-cL modified reverse primers (Table S1, Supporting Information) (Sakai et al. 2004). The modification enables subsequent PCR amplification by the CS1.515F and CS2.806R primers (Table S1, Supporting Information) (Caporaso et al. 2012). Takara Ex Taq DNA polymerase and master mix (cat. no. RR001A, Takara Bio, Saint-Germain-en-Laye, France) were used. The negative control was ddH_2O . The amplicons were sequenced at the DNA Services Facility, Research Resources Center, University of Illinois (Chicago, IL, USA) on a MiSeq platform (Illumina, San Diego, CA, USA) (Earley et al. 2015). The sequences (2×250) were demultiplexed, and the barcode and primer sequences were removed by the company. Raw 16S and 18S DNA sequences are available in GenBank under the SRA study accession number SRP150479 (Table S2, Supporting Information). The forward and reverse sequences were aligned and processed using the combination of MOTHUR 1.40.0 (Schloss et al. 2009) and UPARSE 10 (Edgar, 2013, 2016) according to the standard operation procedure (Kozich et al. 2013). The mismatches/ambiguous sequences and the chimeras, chloroplast and mitochondria sequences were then discarded. Operational taxonomic units (OTUs) were identified at 97% similarity and described according to the Ribosomal Database Project (Cole et al. 2014) using the training set no. 15 and Silva128 (Quast et al. 2013). The representative sequences for each OTU were then compared to those in GenBank using BLASTn (Tables S3 and S4, Supporting Information). In the bacterial dataset, all OTUs with read counts lower than 1000 were removed from further analyses. Both datasets were subsampled by recalculating 5000 total reads/sample.

PCR, cloning and Sanger sequencing

For characterization of the fungal taxa, the internal transcribed spacer (ITS) gene was amplified from the mites and SPGM extract samples collected from 84- and 98-day-old cultures using fungi-specific primers (Table S1, Supporting Information). The products were cloned according to a previously described protocol (Kopecky et al. 2014) and sequenced by Macrogen (Seoul,

South Korea). The sequences ($n = 38$) were assembled with CodonCode Aligner v.1.5.2 (CodonCode Corporation, Dedham, MA, USA). The cloned sequences were compared to the closest sequences retrieved by a BLAST search from the GenBank nucleotide database, to the sequences used for yeast description (Ihrmark et al. 2012; Schoch et al. 2014; Vu et al. 2016; Yurkov, Dlačny and Peter 2017) and to sequences of yeast symbionts of *Sinodendron cylindricum* (Tanahashi and Hawes 2016). The sequences were aligned in Seaview v.4 (Gouy, Guindon and Gascuel 2010) using MUSCLE (Edgar 2004b,a); a phylogenetic tree was constructed in PHYML 3.0 (Guindon et al. 2010) and visualized in (Rambaut 2014).

qPCR

A previously described protocol (Kopecky et al. 2014; Nesvorna, Bittner and Hubert 2019) was applied, and the primers and PCR profiles are detailed in Table S1 (Supporting Information). Fragments of 16S and 18S rDNA were used as markers. The DNA templates used in the qPCR analyses originated from the same samples used for barcode sequencing to identify the taxonomic composition and abundance of selected microbial groups. The analyses included two technical replicates, which were then averaged. The DNA copy numbers were recalculated for each mite or extract sample. The data were $\log_{10}$ -transformed prior to analysis.

Data analyses

The vegan (Oksanen 2018), adespatial (Dray et al. 2018) and mgcv (Wood 2006) packages of PAST (Hammer 2018) and R software (R Development Core Team 2016) were used. The temporal changes in the mite population size, amount of guanine, diversity, respiration and proportion of reads were modeled using generalized additive mixed models (GAMMs) because most responses (i) were strongly non-linear (not normally distributed) and (ii) contained temporal replications arising from repeated measurements. The mite rearing chamber was considered as a single random effect in each model. The linear predictor included a thin-plate spline function for the time, which was specified as a covariate. In some cases, a factor was included in the predictor. The change in the sex ratio over time was modeled by a GAMM with binomial errors (GAMM-b) and the experimental chamber as a random effect. The population size and respiration were assumed to have gamma errors (GAMM-g), the guanine content and diversity indices were assumed to have normal errors (GAMM) and the proportion of reads were assumed to have beta errors (GAMM-b). GLM with Poisson error (GLM-p) was used to compare the mite densities among treatments. The inverse Simpson index, which is an alpha diversity estimate, was calculated from the OTUs in the standardized datasets. For the analysis of beta diversity, the barcode sequencing data were analyzed via Bray–Curtis dissimilarity and the qPCR data using Manhattan dissimilarity. The medians per rearing chamber or medians per sampled time were used to observe the effects of the population density and SPGM respiration on the microbial profiles in the *D. farinae* microbiome, and these statistics were then analyzed through permutation analysis of variance (PERMANOVA) (Clarke 1993) using the *adonis2* function in R (10 000 permutations). Redundancy analyses (RDA) using the *capscale* function were then performed for visualization. The 'best' RDA models were selected based on forward selection of the tested variables using the *forward.sel* function (Zeleny 2017).

RESULTS

Mite population size, guanine production and microbial respiration in microcosms during mite culture growth

The growth of the mite populations in the six culture chambers showed a significantly non-linear (GAMM-g, $F_{5,9} = 192$, $P < 0.0001$; Figure S1A, Supporting Information), hump-shaped pattern. Exponential growth occurred during the first 42 days, and this increase was followed by a plateau, which corresponded to a population size of ~1200 individuals, lasted for 40 days and contained two small peaks, followed by an exponential decline. The proportion of males in the population declined significantly over the course of the experiment (GAMM-b, $\chi^2_1 = 32.2$, $P < 0.0001$; Figure S2, Supporting Information) from nearly 50% in the beginning to 28% after 98 days.

The content of guanine in the SPGM was also significantly non-linear (GAMM, $F_{5,3} = 49.3$, $P < 0.0001$; Figure S1B, Supporting Information): it increased exponentially during the first 56 days, reached a maximum at $1344 \mu\text{mol L}^{-1}$ shortly before day 56 and then declined to $1128 \mu\text{mol L}^{-1}$ after day 84. The microbial respiration in the SPGM showed a strongly non-linear pattern (GAMM-g, $F_{5,9} = 23.4$, $P < 0.0001$; Figure S1C, Supporting Information) characterized by regular fluctuations and reached maxima at the beginning of culture and after 40 and 85 days. The first peak in respiration corresponded to the exponential growth of mites in the cultures, whereas the second peak corresponded to changes in the bacterial and fungal profiles in the SPGM before the culture decline phase (i.e. after 84 days).

Structure and diversity of the microbiome in the mite culture

The microbial community was composed of 94 bacterial and 29 fungal OTUs (Figs 1 and 2) with at least 3% similarity to previously described taxa. A dedicated effort was made to better describe the yeast *Candida* (FOTU1). The ITS regions from mite DNA samples collected after 80 and 84 days of culture growth were amplified, cloned and Sanger sequenced. These sequences ($n = 38$) formed a single clade, indicating that there might have been little divergence between them. This clade was highly similar (96%) to *Candida glucosophila*, GenBank accession number MF314336 (Yurkov, Dlačny and Peter 2017) (Figure S3, Supporting Information). The fungal microbiome contains barcode sequences with high similarity to *Malassezia restricta* (FOTU10), a yeast of medical importance, which were found at low abundance in the internal mite community.

Estimates of the alpha diversity of the microbiomes, expressed as the inverse Simpson index, showed an increasing, non-linear pattern with increasing culture age. This index also showed significant differences in the bacterial microbiome between the internal community and environmental samples (GAMM, $F_1 = 10.4$, $P = 0.0019$; Fig. 3A and B). In both of these samples, the alpha diversity increased as the mite population declined in the culture at the end of the experimental period. Similarly, the inverse Simpson index of the fungal microbiome showed significant differences between the internal community and environmental samples (GAMM, $F_1 = 36.8$, $P < 0.0001$; Fig. 3C and D). The fungal alpha diversity increased from the start of culture to after 70 days of culture, and this increase was followed by a decline in the SPGM and no change in the community in the mite body samples obtained after 84 and 98 days.

For both bacterial and fungal datasets (barcode sequencing) and the qPCR dataset, PERMANOVA showed significant effects of the mite culture growth time and the sample source (mite and SPGM) and the interaction of these two variables (Table 1). As expected, the effect of the origin of the samples from a particular rearing flask was not significant, which indicated that the microbial communities in all the flasks tested during the experiment were similar. The internal and environmental communities were then separated, and the effects of culture time, mite population density, microbial respiration in microcosms, and guanine concentration in the culture and mite sex ratio were tested as environmental variables. All microbial profiles showed significant effects (Bray–Curtis matrix, $P < 0.05$) of mite culture time, mite density and their interaction with both fungal and bacterial microbial communities in both the internal community and environmental samples (Table 2). In addition, the guanine content and the interaction between the experiment time and guanine content significantly ($P < 0.05$) influenced the bacterial and fungal profiles in the environmental samples. The microbial respiration in microcosms (microbial metabolism) and the mite sex ratio had no significant effect on the microbial profiles, as demonstrated by the PERMANOVA models. Redundancy analysis (RDA) triplots showed strong separation between the samples from the exponential and declining phases of mite culture growth with respect to the microbial profiles and DNA copy numbers of selected bacterial taxa (Fig. 4A and E).

Changes in the profiles and copy numbers of selected microbial taxa during mite culture growth

As detected by qPCR, the DNA copy numbers of the bacterial and fungal taxa in both the internal community and SPGM samples increased with increasing culture age, with the exception of the fungal taxa, which showed a decrease in the 84–98-day-old cultures (Fig. 5). In mite bodies, *Cardinium* (OTU1) was clearly dominant until the declining phase, when its abundance strongly decreased. As demonstrated by qPCR with taxon-specific primers, *Firmicutes* exhibited a 100-fold increase in the mite samples at the decline phase of culture growth. During the decline phase, *Staphylococcus* (OTU3 and OTU465), *Virgibacillus* (OTU6) and *Oceanobacillus* (OTU7) were the main groups replacing *Cardinium* (OTU1) in the mite microbiome (Fig. 5).

The mite diet and SPGM samples contained *Lactobacillus fermentum* (OTU4); however, as the mite cultures developed, this bacterium was replaced by *Staphylococcus* (OTU3), *Oceanobacillus* (OTU7) and *Virgibacillus* (OTU6). The low numbers of reads of *L. fermentum* (OTU4) in the mite samples indicated that this bacterium was not consumed by mites (Fig. 6). The internal community and environmental samples showed similar bacterial profile patterns, and in these samples, the final stage showed a 10-fold increase in *Firmicutes* and a >100-fold decrease in *Actinomyces* compared with the levels observed at the initial stage of mite culture (Fig. 6). The most common *Actinomyces* included *Propionibacterium* (OTU10), *Nocardia* (OTU16) and *Corynebacterium* (OTU32).

The number of fungal DNA copies increased by a factor of 10 with increasing culture time in both internal mite and environmental samples (Fig. 5). With increases in the mite culture time, the proportion of *S. cerevisiae* (FOTU3) decreased, whereas those of *Aspergillus penicillioides* (FOTU4) and *Candida* (FOTU1) increased (Fig. 7). Interestingly, the *Candida* (FOTU1) profile in the mite microbiome after 14 days of culture was higher than that after 28 days of mite culture growth.

Table 1 Effect of the culture growth time, source of the samples (internal community and environmental culture samples) and flask on the composition of the microbiome of *D. farinae*. The effects of these factors were compared by PERMANOVA using 10 000 permutations. Significant factors are indicated in bold. Several datasets were tested: qPCR dataset and fungal and bacterial datasets obtained by barcode sequencing. The qPCR dataset was tested using the Manhattan distance, and the barcode bacterial and fungal datasets were tested using the Bray–Curtis distance.

Factor	Df	Bacteria	Fungi	qPCR						
				Pr (>F)	R2	F	Pr (>F)	R2	F	Pr (>F)
Time	1	0.10	20.540	0.000	0.45	78.935	0.0001	0.04	17.764	0.000
Source	1	0.41	80.102	0.000	0.05	8.7594	0.0004	0.75	355.673	0.000
Flask	1	0.01	1.157	0.295	0.00	0.5758	0.6113	0.00	0.863	0.362
Time:Source	1	0.08	16.159	0.000	0.05	8.8659	0.0003	0.04	18.036	0.000
Time:Flask	1	0.00	0.488	0.768	0.00	0.3823	0.8181	0.00	1.249	0.254
	1	0.01	0.985	0.382	0.00	0.3086	0.8983	0.00	1.317	0.235
Time:Source:Flask										
Residual	77	0.39			0.44			0.16		
Total	83	1.00			1.00			1.00		

Table 2 Effect of the culture growth time, mite population density in the culture, guanine contents, microbial respiration in microcosms and sex ratio on the composition of the microbiome of *D. farinae*. The effect of these factors was compared by PERMANOVA using 10 000 permutations. Significant factors are indicated in bold. Several datasets were tested: qPCR dataset and fungal and bacterial datasets obtained by barcode sequencing. The qPCR dataset was tested using the Manhattan distance, and the barcode bacterial and fungal datasets were tested using the Bray–Curtis distance.

Samples	Internal community			Environmental culture			
	Df	R2	F	Pr (>F)	R2	F	Pr (>F)
Bacteria							
Time	1	0.27	28.13	0.000	0.35	41.82	0.000
Density	1	0.23	24.22	0.000	0.13	15.69	0.000
Respiration	1	0.02	1.88	0.128	0.05	5.43	0.001
Guanine	1	0.03	2.69	0.061	0.05	6.21	0.001
Sex_ratio	1	0.02	2.57	0.071	0.02	1.78	0.137
Time:Density	1	0.05	5.47	0.005	0.09	10.76	0.000
Time:Respiration	1	0.04	4.06	0.019	0.02	2.54	0.052
Time:Guanine	1	0.02	1.80	0.140	0.01	1.26	0.269
Residual	33	0.32			0.28		
Total	41	1			1		
Fungi							
Time	1	0.43	36.61	0.000	0.63	122.64	0.000
Density	1	0.04	3.32	0.032	0.06	11.13	0.001
Respiration	1	0.01	1.03	0.327	0.01	1.26	0.258
Guanine	1	0.03	2.95	0.043	0.06	11.68	0.000
Sex_ratio	1	0.02	1.67	0.156	0.01	2.80	0.075
Time:Density	1	0.05	4.16	0.015	0.05	9.33	0.002
Time:Respiration	1	0.02	1.62	0.165	0.01	1.25	0.252
Time:Guanine	1	0.01	1.04	0.316	0.01	1.00	0.324
Residual	33	0.39			0.17		
Total	41	1			1		
qPCR							
Time	1	0.43	36.61	0.000	0.63	122.64	0.000
Density	1	0.04	3.32	0.032	0.06	11.13	0.001
Respiration	1	0.01	1.03	0.327	0.01	1.26	0.258
Guanine	1	0.03	2.95	0.043	0.06	11.68	0.000
Sex_ratio	1	0.02	1.67	0.156	0.01	2.80	0.075
Time:Density	1	0.05	4.16	0.015	0.05	9.33	0.002
Time:Respiration	1	0.02	1.62	0.165	0.01	1.25	0.252
Time:Guanine	1	0.01	1.04	0.316	0.01	1.00	0.324
Residual	33	0.39			0.17		
Total	41	1			1		

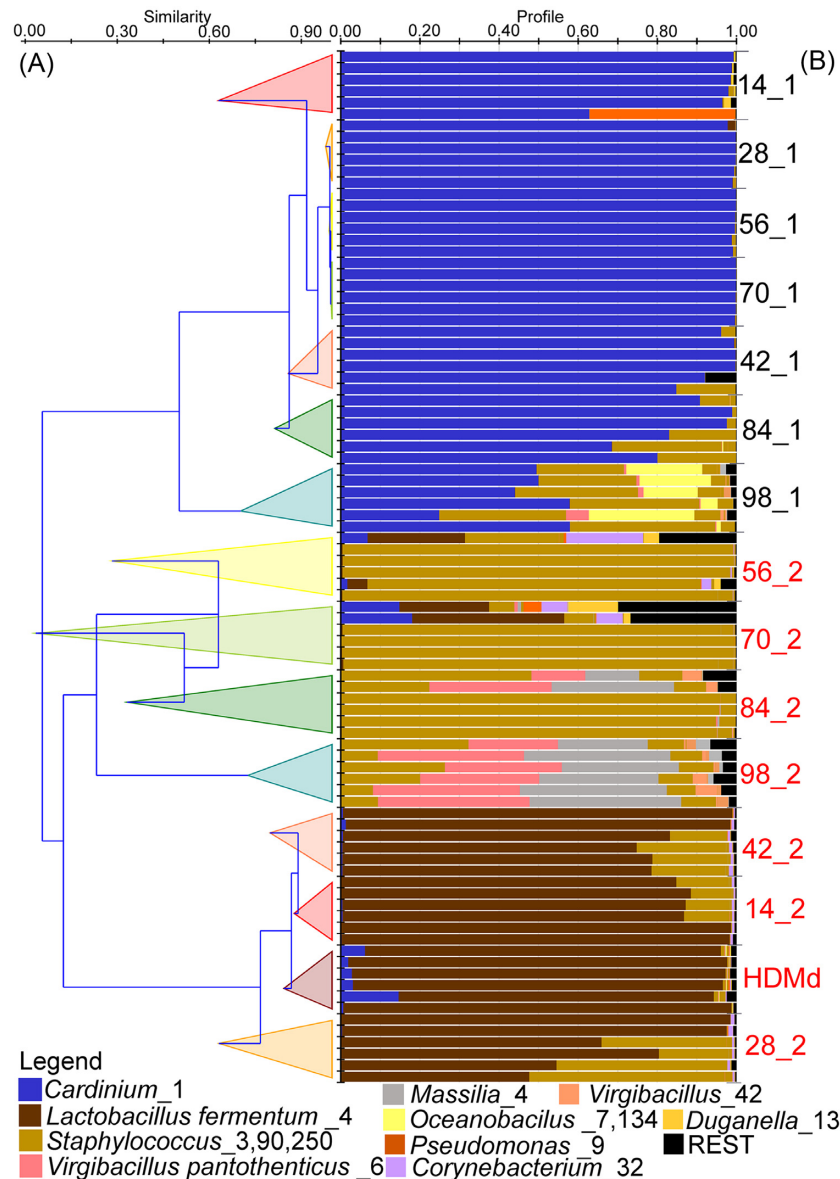


Figure 1. Changes in the bacterial profile at different times of *D. farinae* culture development. (A) Sample similarity based on clustering using the Bray-Curtis matrix; (B) profile of the bacterial microbiome. Prefixes indicate the mite culture age (days); the suffix '.1' indicates the internal mite bacteriome, while '.2' indicates the SPGM microbiome.

DISCUSSION

Patterns and correlations of microorganism profiles and mite culture development phases

The microbiome of *D. farinae* showed temporal changes in the profiles of the dominant taxa inside the mites and in the mite culture environments during mite culture growth. These changes were related to the age of the mite culture, mite population density, microbial respiration in the microcosms from the mite cultures, and concentration of the nitrogenous waste metabolite guanine. Major changes in the bacterial microbiome occurred during the decline phase when *Cardinium* (an obligate internal bacterial endosymbiont) was replaced by bacteria belonging to the phylum *Firmicutes*; a similar succession occurred in the culture medium (SPGM), where *Oceanobacillus* and *Virgibacillus* replaced *L. fermentum*. The temporal changes in the fungal microbiomes are characterized by replacement of

S. cerevisiae by *C. glucosophila* and *A. penicillioideis* in both microbiomes (mite and SPGM) with increasing culture age. It is interesting to speculate that the decline phase of house dust mite cultures is linked to the disappearance of *Cardinium*. This bacterium has a complete biotin synthesis pathway, and it has been hypothesized to be a mutualist essential for mite nutrition (Penz et al. 2012; Santos-Garcia et al. 2014).

Microcosm respiration is considered an indirect estimate of bacterial/fungal metabolism and can be stimulated by microarthropod (mites and springtails) grazing on the microbes (Hanlon 1981; Bengtsson and Rundgren 1983). Overgrazing of microbes by microarthropods decreased microbial respiration, while optimal grazing increased microbial growth (Seastedt 1984; McGonigle 1995). Although SPGM respiration did not generally change with increases in mite abundance during culture growth, two fluctuations in the respiration level and a decrease during the declining phase of mite culture growth were

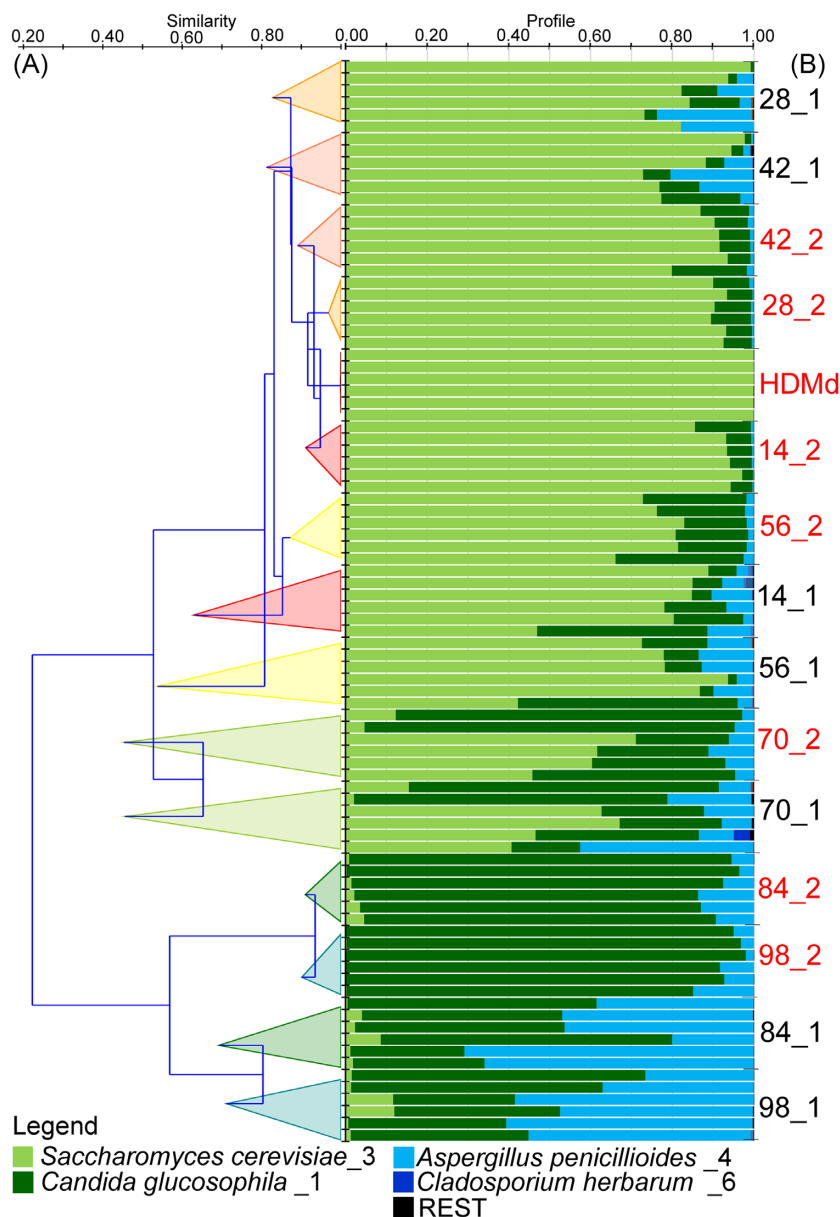


Figure 2. Changes in the fungal profile at different times of *D. farinae* culture development. (A) Sample similarity based on clustering using the Bray–Curtis matrix; (B) profile of the bacterial microbiome. Prefixes indicate the mite culture age (days); the suffix ‘.1’ indicates the internal mite bacteriome, while ‘.2’ indicates the SPGM microbiome.

observed. These changes in microcosm respiration could be explained by differences in mite grazing activity and changes in the microbial species composition. However, microcosm respiration had no effect on the microbiome composition. The first fluctuation was linked to the disappearance of *L. fermentum*, which was the main bacterial taxon present in the environmental sample medium during the exponential phase of mite population growth but then virtually disappeared at the end of the exponential phase (Fig. 1). The second change in SPGM respiration was linked to the disappearance of *S. cerevisiae* from the SPGM. After day 42, the abundance of *S. cerevisiae* started to decrease, and at the end of the decline phase, this yeast almost completely disappeared and was replaced by *A. penicillioides* and *Candida* (Fig. 2). Notably, *S. cerevisiae*, which is well known to be beneficial for mite growth, has been used as part of the mite rearing diet in this and other studies (Andersen 1991; Batard et al. 2006). The decline

in the mite populations was clearly related to the disappearance of this yeast from the mite internal community and SPGM samples. Interestingly, a similar transition from yeasts to filamentous fungi was previously detected in old cultures of *D. farinae* (Hubert et al. 2019). During the decline phase, the total fungal abundance in the SPGM increased, and the decrease in respiration at this phase can be explained by fungistasis, i.e. inhibition of the germination of fungal spores (Lockwood 1964).

Possible factors affecting microorganism dynamics

The observed age-related changes in the mite microbiome can be explained by the following partially non-exclusive hypotheses: (i) the mite microorganism communities naturally change during the mite lifespan (in other words, the ages of individual mites and population demographics are factors); (ii) there

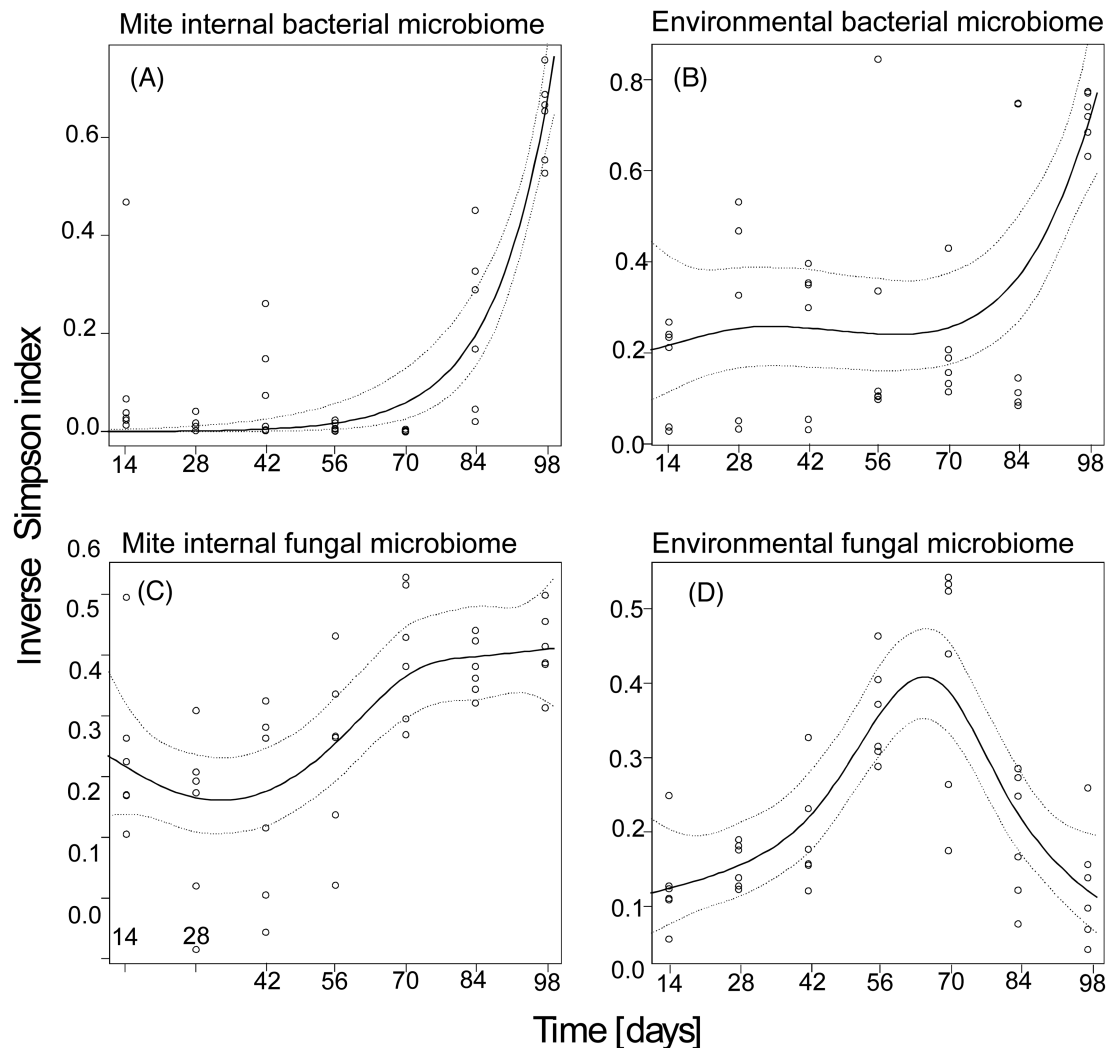


Figure 3. Changes in the alpha diversity of the *D. farinae* microbiome at different times of mite culture development. (A) Mite internal bacterial microbiome; (B) SPGM (environmental) microbiome; (C) mite internal fungal microbiome; (D) SPGM fungal microbiome. The alpha diversity was estimated by the inverse Simpson diversity index. The experiments were performed in six replicates in six rearing chambers; Illumina datasets were generated for each replicate and then standardized. The smoothed curve (solid line) was estimated by a GAMM analysis, and the dotted lines show 95% confidence intervals.

are differences between males and females, and successional changes in microorganism communities occur because males become less abundant in older cultures and (iii) guanine concentrations in the mite culture affect the microbiome.

Individual age as a factor influencing the microbiome composition is well established in mammals, including humans (Vuong et al. 2017); however, different patterns have been observed in arthropods. In insects, bacterial profiles differ among developmental stages as the result of diet or habitat adaptation and social behavior (Duguma et al. 2015; Jones et al. 2018). Little is known about the effect of the individual age of insects on microbiome composition. For example, the microbiomes of teneral and 15-day-old adults of tsetse flies (*Glossina palpalis gambiensis*) were almost identical (Doudoumis et al. 2018). In contrast, overwintering of the field cricket (*Gryllus veletis*) resulted in permanent changes in the *Pseudomonas* and *Wolbachia* profiles (Ferguson et al. 2018). In *D. farinae* females, the average time from the beginning to the end of egg production is 37 ± 11 days, and the lifespan is 112 ± 10 days and is characterized by continuous mating; in addition, each female produces 95 ± 6 eggs (Alexander, Fall and Arlian 2002). These data suggest that the

individuals surviving at the end of the experimental period likely originated from eggs deposited at the beginning of the experimental period. Therefore, if microbial profiles are linked to the ages of individual mites, the presence of a large number of 'senescent' mites in the culture may affect the microbiome profile of the aging culture. This argument, however, is questionable because at the decline phase, there is a much larger proportion of young mites from the exponential phase.

The sex ratio of *D. farinae* is usually 1/1 (females/males) in young cultures, but in 6-month-old cultures, the sex ratio is 2/1 (Hodgson 1976). Here, we observed a significant decrease in the number of males after 84 and 98 days of culture growth. However, the sex ratio did not show any significant effect on the microbiome profile changes in this study (Table 2).

The guanine contents corresponded to changes in the mite population density, i.e. the guanine contents decreased with decreasing mite population density. The decreasing amount of guanine in old cultures indicates that guanine does not accumulate in mite cultures because it can be degraded by microorganisms (Rouf and Lomprey 1968). In this study, we found only a correlation between guanine content and changes in the mite

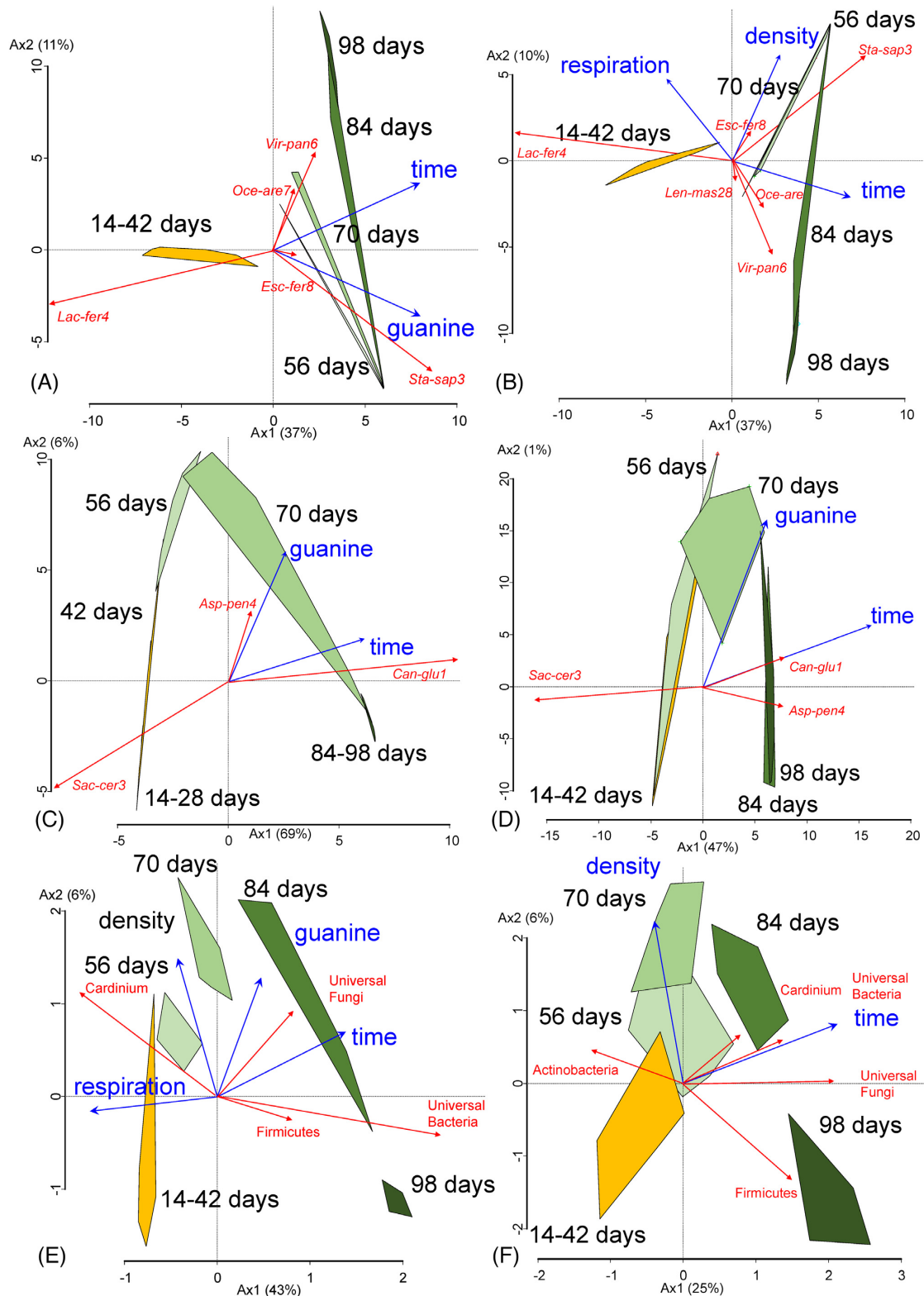


Figure 4. Triplot visualization of the principal coordinates obtained from the redundancy analysis of the *D. farinae* bacterial and fungal microbiomes. The samples are presented as orthogonal objects. Only the variables that had significant effects ($P < 0.05$) on the OTU distribution after forward selection are shown: (A) bacterial microbiome in mite bodies (internal mite communities); (B) bacterial microbiome in SPGM (mite culture environment communities); (C) fungal microbiome in mite bodies; (D) fungal microbiome in SPGM; (E) qPCR results for the mite bodies; (F) qPCR results for SPGM. (A–D) Standardized datasets from barcode sequencing; (E–F) qPCR datasets.

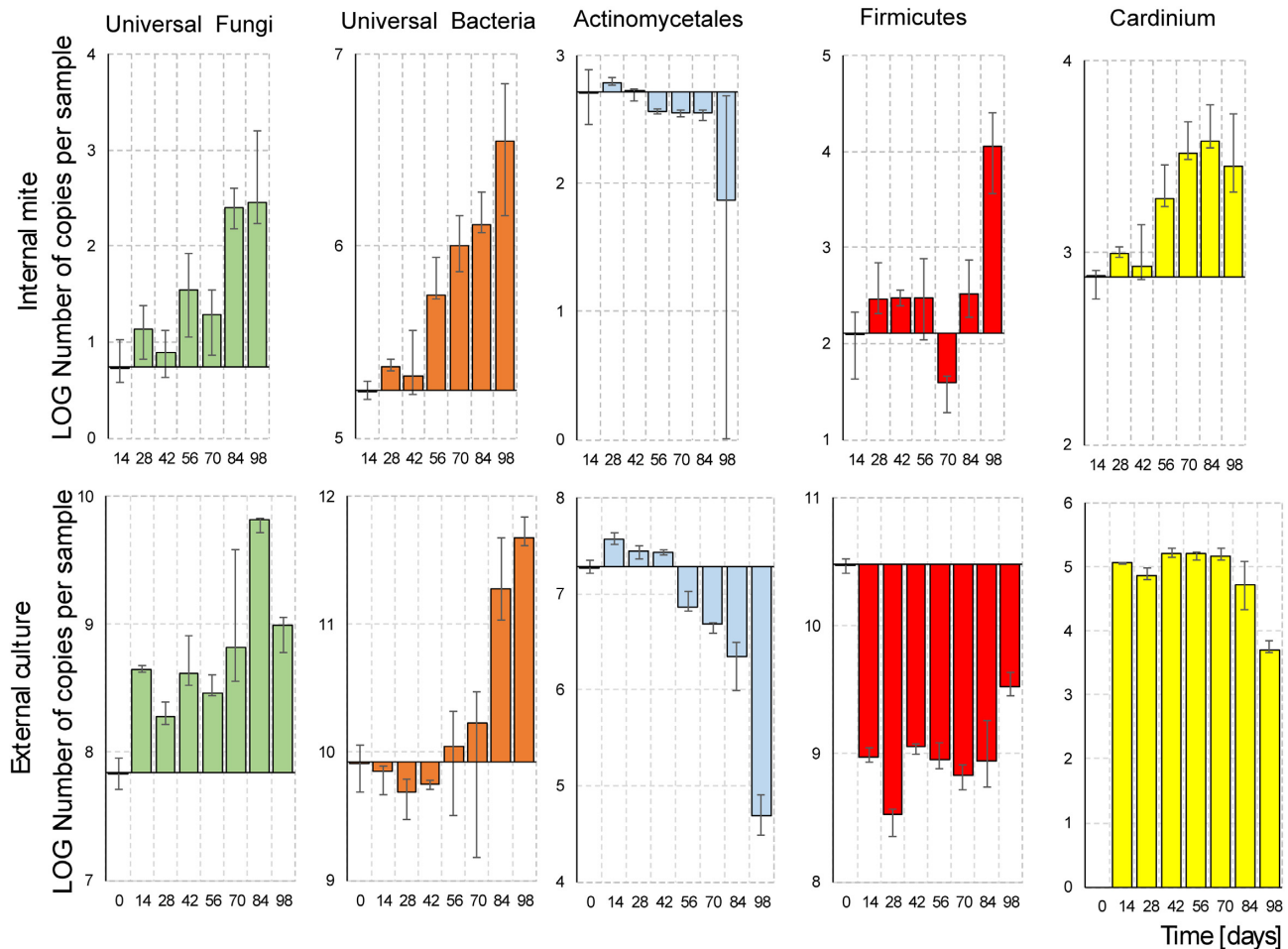


Figure 5. Changes in the DNA copy numbers (estimated by qPCR) for selected microorganisms in *D. farinae* cultures at different ages. Data are shown for mite internal microorganisms (upper row) and SPGM (lower row). The copy number values are log₁₀-transformed.

internal bacterial community. The accumulation of guanine in the parenchymal tissues of mites, the so-called white-body syndrome, is considered a suppressive factor for the astigmatid mite *Tyrophagus putrescentiae* (Smrz and Catska 1989), but white-body syndrome was not observed in this study.

Spores of several bacterial species can be toxic, possibly affecting mite population growth. For example, in *D. pteronyssinus*, tritonymphal mortality can be induced by adding spores of *Lysinibacillus* (*Bacillus*) *sphaericus* and *Bacillus thuringiensis* to the mite diet (Saleh, Kelada and Shaker 1991). The effect of *B. sphaericus* was more pronounced than that of *B. thuringiensis* (Saleh, Kelada and Shaker 1991). The addition of *B. thuringiensis* var. *tenebrionis* to the mite diet decreased population growth in *D. farinae*, but the inhibition effect was 10–100 times weaker than it was in beetles and ticks (Erban et al. 2009). Similarly, addition of *Bacillus cereus* to the diet suppressed population growth in *T. putrescentiae* (Erban et al. 2016) and other organisms (Erban et al. 2009). Feeding *D. pteronyssinus* on the fungus *A. penicillioideus* reduces survival, development rate and fecundity in this mite (Hay, Hart and Douglas 1993), and similar suppressive effects have been reported for other microorganisms isolated from HDM cultures (Molva, Nesvorna and Hubert 2019). We believe that these microorganisms inhibit mite culture growth indirectly, without necessarily being pathogenic to the mites, which agrees with previous results (Hubert et al. 2019).

The demise of gram-negative bacteria at the decline phase of mite cultures—implications for immunotherapeutic extract production

The HDM cultures used for the production of immunotherapeutic extracts contain detrimental endotoxins, i.e. toxic, heat-stable lipopolysaccharide substances of the cell walls of gram-negative bacteria. Endotoxins can interact with the epitopes of immunogenic molecules, which decreases the overall amounts of allergens that are useful for medical applications. The levels of these endotoxins can differ among different mite cultures and can also depend on the mite diet (Avula-Poola, Morgan and Arlian 2012). The composition of the bacterial microbiome (*Cardinium*, *Kocuria* and *Staphylococcus*) in our samples was different from those found in other *D. farinae* strains. In our culture, three bacterial species were dominant (*Cardinium*, *Kocuria* and *Staphylococcus*), and this was consistent with the results of our previous screening, indicating a high stability of the microbiomes during a period of almost 10 years (Hubert et al. 2016; Molva et al. 2018; Hubert et al. 2019). The only exception was the lack of *Bartonella*-like bacteria. For example, *Enterobacter* (63% of all bacterial sequences), *Staphylococcus* (17%), *Escherichia* (5%) and *Bartonella*-like bacteria (2%) were reported in the microbiome of a Chinese population of *D. farinae* (Chan et al. 2015), although *Cardinium* was also

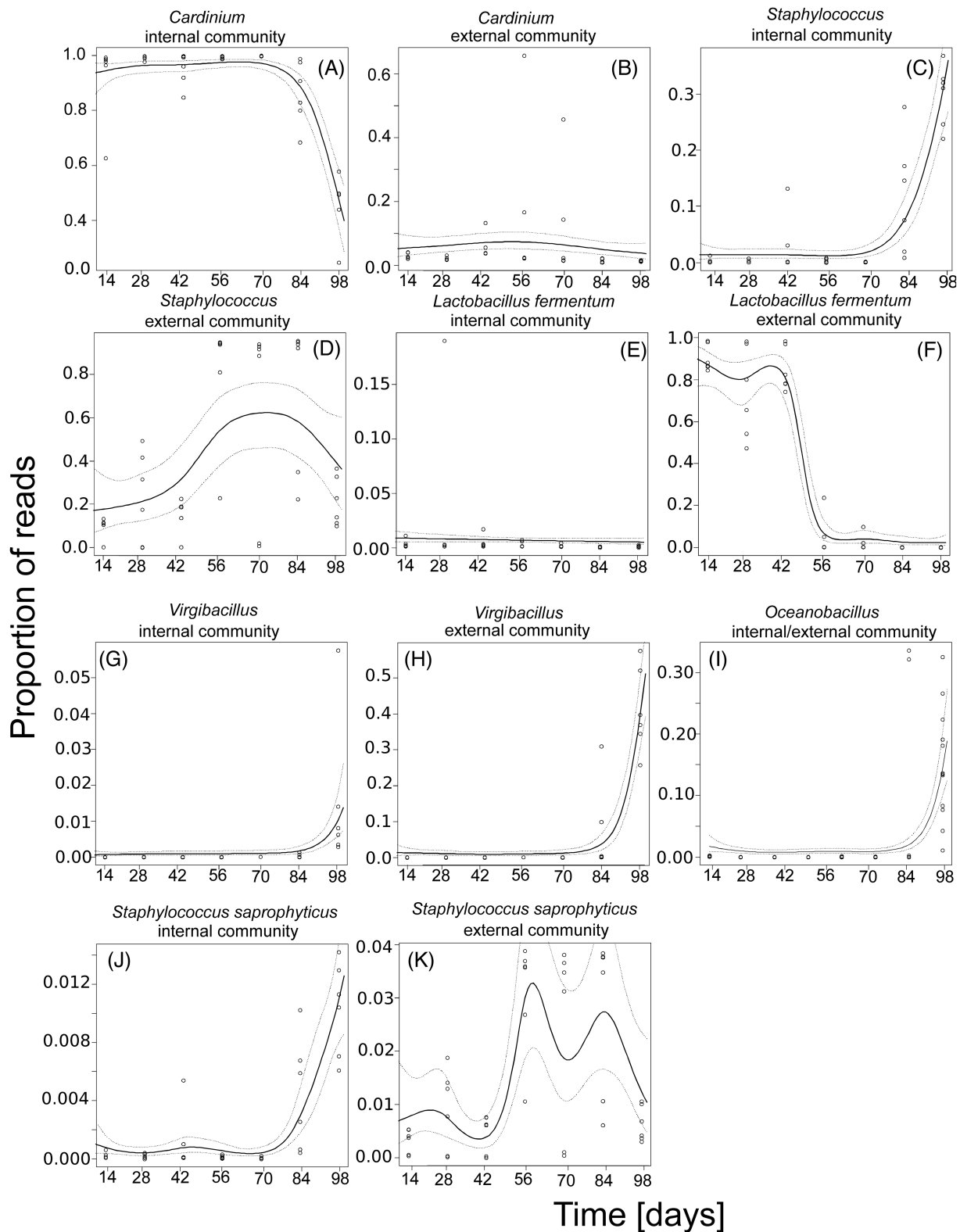


Figure 6. Changes in the proportion of bacterial reads in the *D. farinae* microbiome during mite culture growth. (A) *Cardinium* (OTU1) in internal community samples; (B) *Cardinium* (OTU1) in environmental samples; (C) *Staphylococcus* (OTU3) in mite samples; (D) *Staphylococcus* (OTU3) in SPGM samples; (E) *L. fermentum* (OTU4) in mite samples; (F) *L. fermentum* (OTU4) in SPGM samples; (G) *Virgibacillus* (OTU6) in mite samples; (H) *Virgibacillus* (OTU6) in SPGM samples; (I) *Oceanobacillus* (OTU7) in combined samples from both mite and SPGM samples; (J) *Staphylococcus saprophyticus* (OTU465) in mite samples; (K) *S. saprophyticus* (OTU465) in SPGM samples. The experiments were performed with six replicates in six rearing chambers; Illumina datasets were generated for each replicate and then standardized. The smoothed curve (solid line) was estimated by a GAMM, and the dotted lines show 95% confidence intervals.

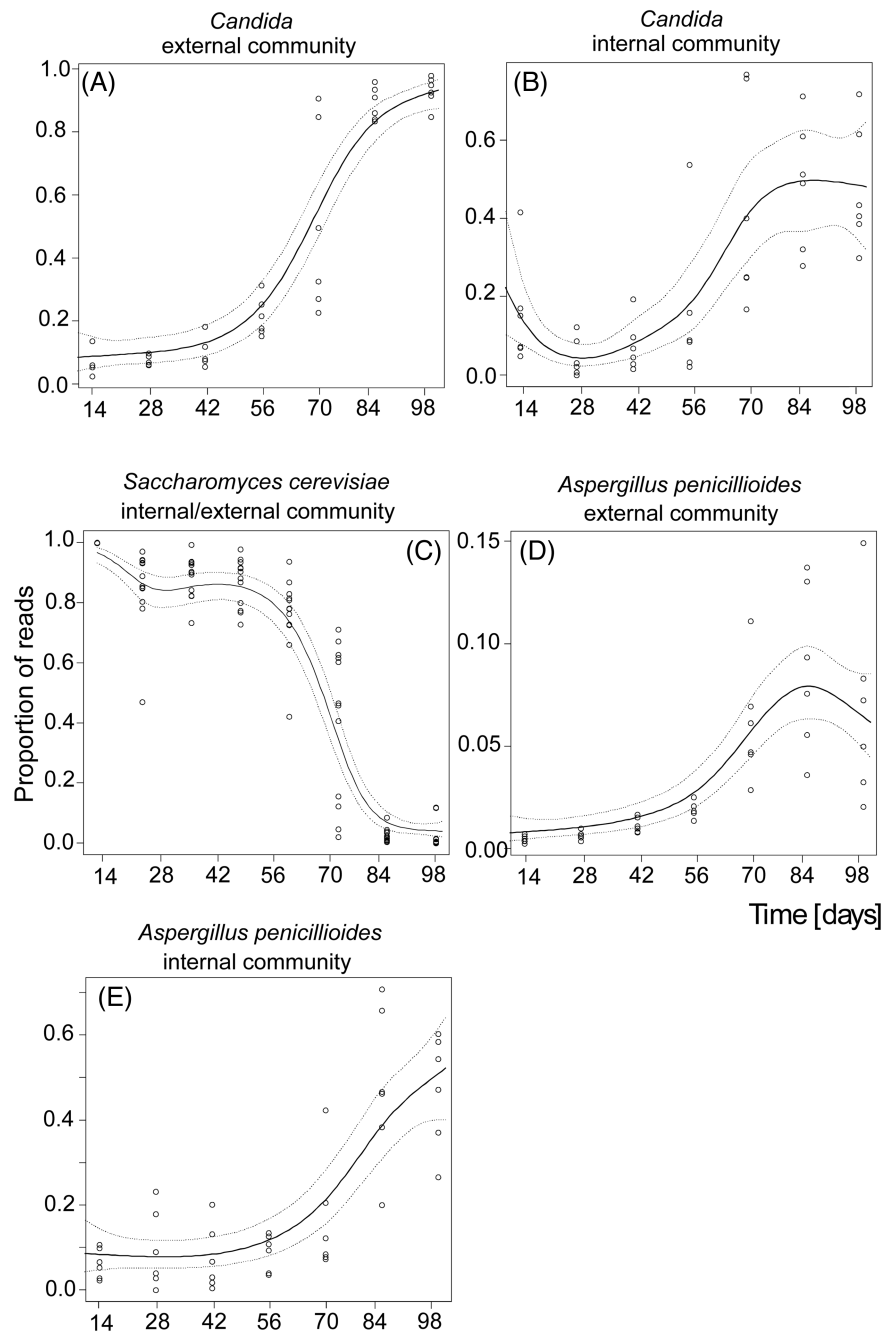


Figure 7. Changes in the proportion of fungal reads in the *D. farinae* microbiome during mite culture growth. (A) *Candida* (FOTU1) in SPGM samples; (B) *Candida* (FOTU1) in mite samples; (C) *S. cerevisiae* (FOTU3) in pooled SPGM and mite samples; (D) *A. penicillioides* (FOTU4) in SPGM samples; (E) *A. penicillioides* (FOTU4) in mite samples. The experiments were performed with six replicates in six rearing chambers; Illumina datasets were generated for each replicate and then standardized. The smoothed curve (solid line) was estimated by a GAMM, and the dotted lines show 95% confidence intervals.

present at a high abundance but was not detected by these researchers (our data). In contrast, the microbiome of the Korean population was dominated by *Bartonella*-like bacteria (98%), followed by *Enterococcus faecalis* (2%) (Lee et al. 2019). In summary, different gram-negative bacteria (*Cardinium*, *Bartonella*-like, *Escherichia* or *Enterobacter*) can be responsible for endotoxins in immunotherapeutic extracts from *D. farinae*. However, in our study, we observed a regular succession of microorganisms during *D. farinae* culture growth, and the bacterial succession was linked to an increase in the concentrations of gram-positive bacteria (*Firmicutes*), which are not considered

endotoxin producers and do not contaminate allergen immunotherapeutic extracts.

CONCLUSION

We show that mite microbiomes exhibit strong, dynamic alterations at different stages of mite culture growth. These changes can be dramatic, with one abundant microorganism being nearly replaced by other microorganisms at the later stages of mite culture development. These changes seem to be consistent across independent cultures, hinting at the possibility that they

may be mediated by a common factor or a combination of several factors. Here, we measured several potential variables that can affect mite microbiomes (such as mite demographics or guanine contents) and found a strong correlation between some of these variables and the changes in the mite microbiomes. Particularly, the decline in bacteria of the genus *Cardinium* (a possible internal mutualist providing a biotin synthesis pathway for the mite host), *L. fermentum* (an external bacterium in the diet) and the yeast *S. cerevisiae* (external and internal, providing nutritive benefits) may cause the decline phase of mite culture development. However, these relationships may be correlational rather than causal. Because the biological relationships between mites and bacteria are still poorly understood, we consider our study to be a useful starting point in this area rather than a work that provides a definitive answer to this complex question. Certainly, more research is needed to elucidate the factors affecting the dynamics of microbial communities during HDM culture growth.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](https://femsec.oup.com/femsec/article-abstract/95/11/fz153/5581497) online.

ACKNOWLEDGMENTS

We thank Marie Bostlova and Martin Markovic for the technical assistance provided and Jan Kopecky for the helpful advice. We also thank Barry O'Connor (University of Michigan) for providing comments on the earlier draft of the manuscript.

Author Contributions: MN, VM—experiments; ES—guanine assay; SP—statistical analyses; JH—bioinformatics, RDA and ANOSIM analyses; SP, JH, TE, PK—scientific writing; all authors—commented on the final draft.

FUNDING

JH, TE, VM and MN were supported by the Czech Science Foundation (GACR) as part of project no. 17-12068S. PK was supported by the Russian Science Foundation (grant no. 16-14-10109).

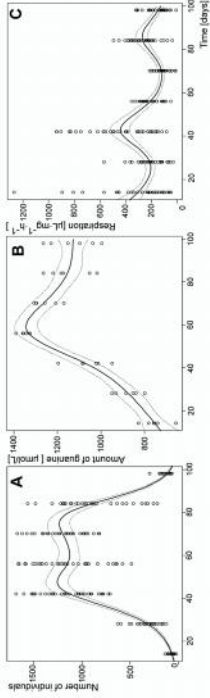
Conflicts of interest. None declared.

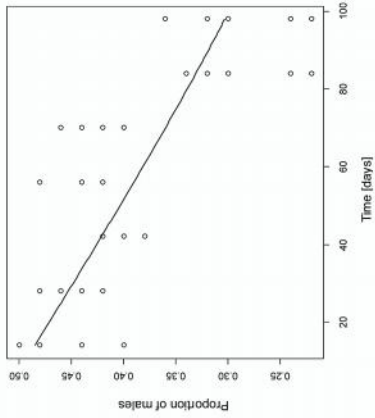
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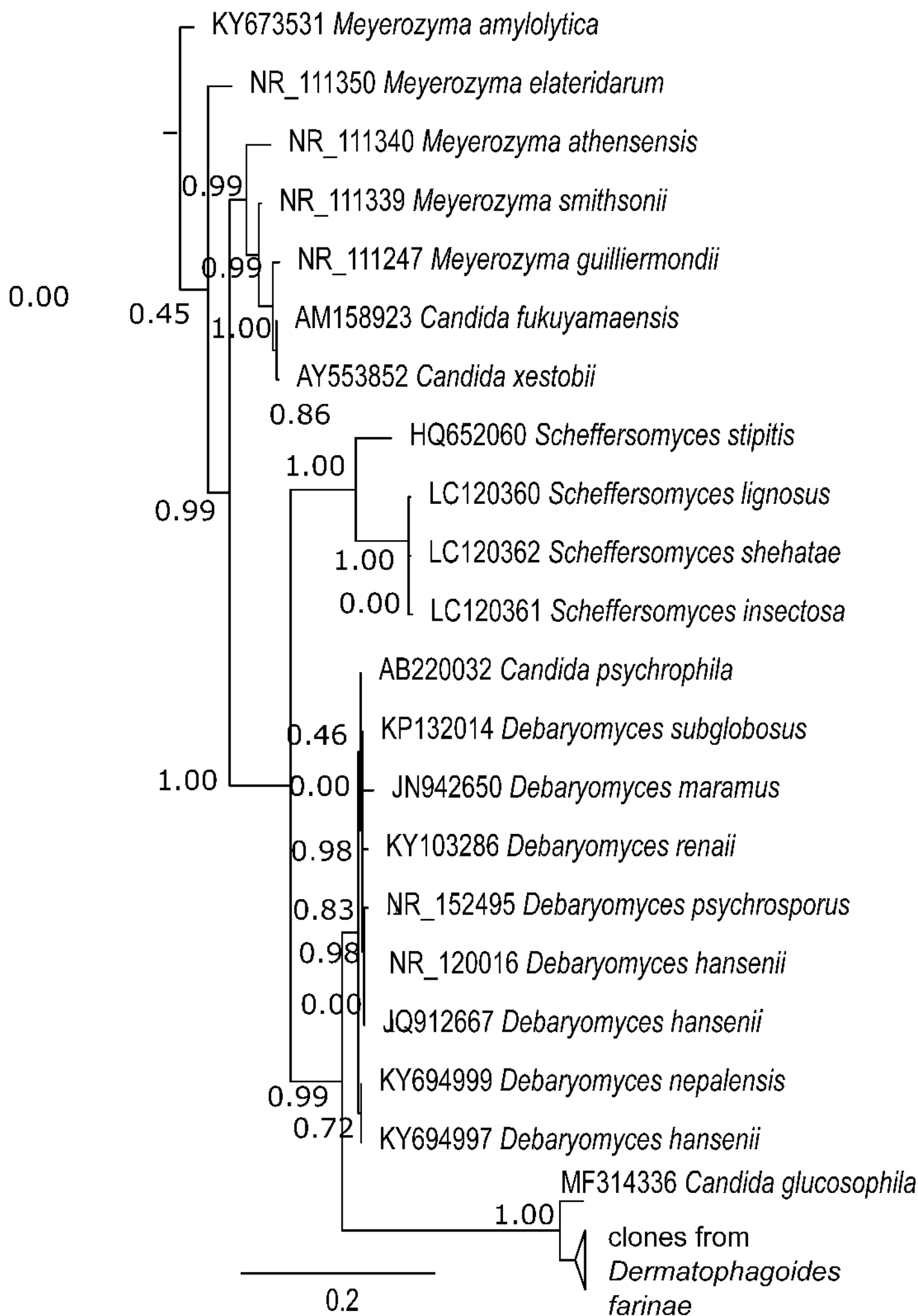
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Supplementary material

Title: Dynamics of the microbial community during growth of the house dust mite *Dermatophagoides farinae* in culture

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Journal: FEMS Microbiology Ecology

Supplementary Figures: Figures S1–S6

Supplementary tables: Table S1

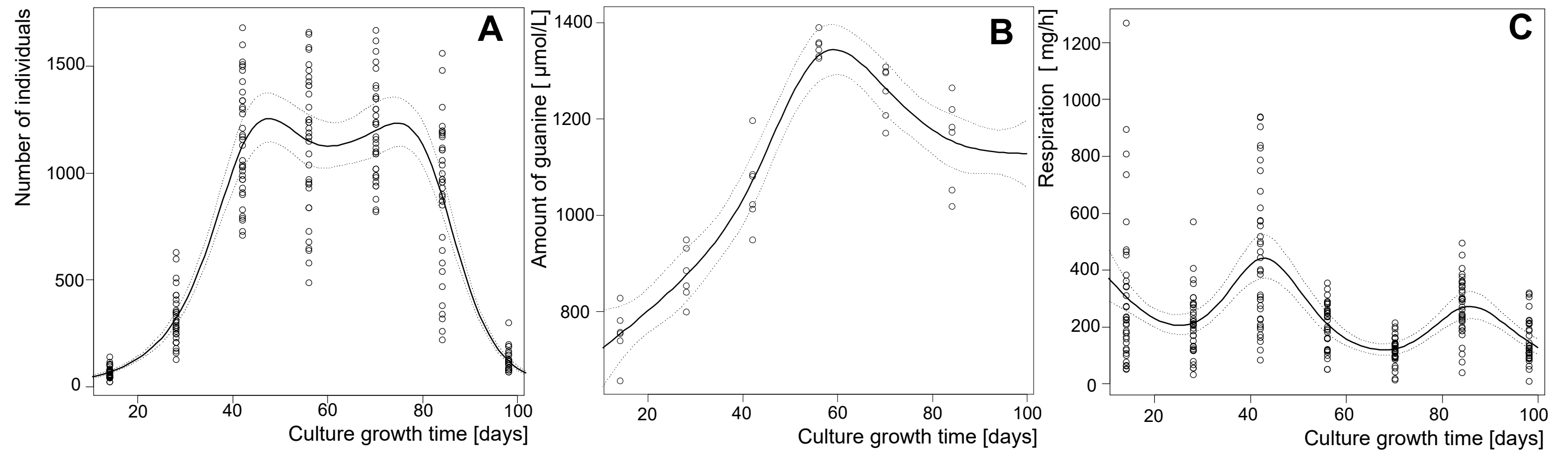


Figure S1. Changes in *Dermatophagoides farinae* cultures during culture growth. **A** - Changes in the numbers of mite individuals during mite culture growth; **B** - Changes in the amount of guanine in SPGM from *D. farinae* cultures during mite culture growth; and **C** - Changes in the respiration of SPGM from *D. farinae* cultures during mite culture growth. The experiments shown in **A–C** were performed in six replicates. The smoothed curve (solid line) was estimated by a GAMM analysis, and the dotted lines show 95% confidence intervals.

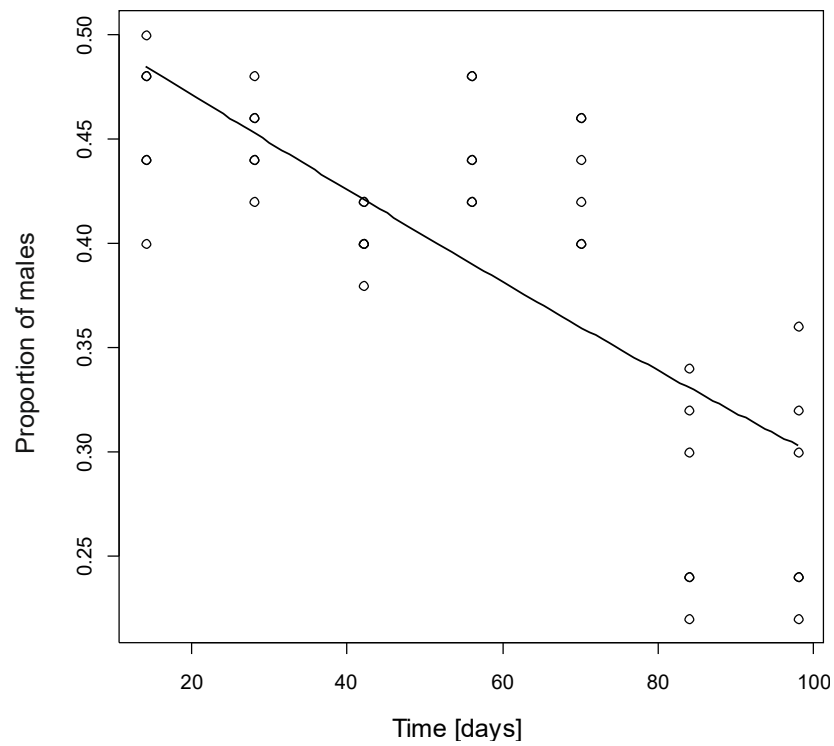


Figure S2. Change in the *D. farinae* sex ratio (male/female) over the course of the experiment. The estimated logit model is shown.

Table S1. Oligonucleotide primers for PCR and qPCR quantification of 16S and 18S DNA sequences in *D. farinae* samples.

Group	Name	Primer/probe (5'-3')	AT (°C)	Tm	Size (bp)	Reference	Note
Universal 18SrRNA, qPCR	FR1	ACACTGACGACATGGTTCTACA CGWTAACGAACGAGACCT	50	87.5	390	Chemidlin Prevost-Boure <i>et al.</i> (2011)	1
	FF390	CGATAACGAACGAGACCT					
Illumina	CS1_FF390	ACACTGACGACATGGTTCTACA CGWTAACGAACGAGACCT	50		390	Chemidlin Prevost-Boure <i>et al.</i> (2011)	
	CS2_FR1	TACGGTAGCAGAGACTTGGTCT A/I/C CAT TCA ATC GGT A/I/T					
	F27	AGAGTTTGATCMTGGCTCAG					
Universal, 16S rRNA -V4, Illumina	783r-a	GGACTACCAGGGTATCTAATCCTG	57				
	783r-bL	GGACTACCGGGTATCTAATCCCG					
	783r-cL	GGACTACCGGGTATCTAATCCGG				Sakai <i>et al.</i> (2004)	
	CS1_515F	ACACTGACGACATGGTTCTACAGTGCCAGCMGCCGCGGTAA	55		275	Caporaso <i>et al.</i> (2012)	
	CS2_806R	TACGGTAGCAGAGACTTGGTCTGGACTACHVGGGTWTCTAAT					
Universal, 16S rRNA, PCR	F27	AGAGTTTGATCCTGGCTCAG	50		1500	Lane (1991)	
	R1492	TACGGYTACCTTGTTACGACTT					
Universal, 16S rRNA, qPCR	Com1	CAGCAGCCGCGGTAATAC	59	82.49	270	Dorn-In <i>et al.</i> (2015)	2
	769R	ATCCTGTTTGMTMCCCVCR					
<i>Cardinium</i> , 16S rRNA, qPCR	Card_6QF	TGCAAATCTCAAAGCATGT	56	83.76	160	Hubert <i>et al.</i> (2017)	3
	Card_6QR	TCAAGCTCTACCAACTCCCA					
<i>Firmicutes</i> , qPCR	Firm934F	GGAGYATGTGGTTTAATTCTGAAGCA	60	82.13	126	Guo <i>et al.</i> (2008)	4
	Firm1060R	AGCTGACGACAACCATGCAC					
<i>Actinobacteria</i> , qPCR	Actino235F	CGCGGCCTATCAGCTTGTTG	60	89.04	283	Stach <i>et al.</i> (2003)	5
	Eub518R	ATTACCGCGGCTGCTGG					
Universal ITS, PCR	ITS1	CTTGGTCATTTAGAGGAAGTAA	55		600	White <i>et al.</i> (1990)	
	ITS4	TCCTCCGCTTATTGATATGC					

Notes – standard preparation: 1 – source: *Fusarium* fungal culture, amplification: FR1/FF390; 2 – source: *Tyrophagus putrescentiae* DNA, amplification: WpF/WpR (Calvitti *et al.* 2015); 3 – source: *Dermatophagoides farinae* DNA; amplification Card4/Card6 (Kopecky *et al.* 2013) ; 4 – source: *Melissococcus plutonius* bacterial culture; amplification: Firm934F/Firm1060R; 5 – source: *Kocuria* isolate; amplification F27/R1492

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