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The Significance of the Fungus *Aspergillus penicilloides* to the House Dust Mite *Dermatophagoides pteronyssinus*

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Abstract

The digestive tract of the European dust mite *Dermatophagoides pteronyssinus* Trouessart in laboratory culture contains the xerophilic fungus *Aspergillus penicilloides* Spegazzini and bacteria that cannot be cultured on nutrient agar. The eggs of the mites are fungus-free and produce mites that develop to adulthood under aseptic conditions. The developmental rate of these fungus-free mites was, however, significantly reduced on diet supplemented either with living fungus 33 days prior to sterilization of the diet and introduction of the mites, or with killed fungus at the start of the experiment. These data suggest that *A. penicilloides* is of nutritional value to the mites but do not support the hypothesis that the principal function of the fungus is modification of the diet to a form more nutritious to the mites.

Keywords: *Dermatophagoides pteronyssinus*, symbiotic fungi, house dust, *Aspergillus penicilloides*

1. Introduction

Mites of the suborder Astigmata that infest stored products and house dust are associated with a variety of fungi (Rodriguez and Rodriguez, 1987; Bronswijk, 1981). In stored products mites, the relationship is often mutualistic, in that the mites feed on the fungi and disseminate the fungal

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spores (Griffiths et al., 1964; Rodriguez et al., 1980), but antagonistic interactions have also been documented (Solomon et al., 1964; Rodriguez et al., 1980). The association between house dust mites (most of which belong to the Pyroglyphidae) and fungi has received inadequate experimental study but is generally believed to be a nutritional mutualism. Bronswijk and Sinha (1973) and Saint Georges-Gridelet (1987) have suggested that the fungi may be a source of nutrients for house dust mites, providing sterols and vitamins, respectively. Bronswijk and Sinha (1973) also proposed that fungi associated with the substrate may modify the food of the mites so that it is more nutritious. These authors demonstrated that the population increase of *Dermatophagoides pteronyssinus* Trouessart was enhanced six-fold when the diet of defatted dander and yeast was pre-incubated with the fungus *Aspergillus amstelodami* (Mangin) Thom and Church and concluded that the fungus assimilated lipid components of the dander, thereby reducing the lipid content to a more suitable level for mites. Subsequent studies of Lustgraaf (1978) and Saint Georges-Gridelet (1984) revealed that the relationship between the fungi, dietary lipid content and performance of the mites depends on the composition of the diet. For example, pre-incubation of mite diets with *Aspergillus penicilloides* Spegazzini enhanced the population increase of *D. pteronyssinus* over 8 weeks by 300% when the diet consisted of mattress dust, but depressed it by 35% when the diet contained a mixture of wheat germ, casein and yeast powder (Lustgraaf, 1978). Furthermore, addition of lipid enhanced the population increase of *D. pteronyssinus* on the diet of dried *Daphnia* (which contained 8% lipid) but decreased the population increase on the diet of skin scales and yeast (6.5% lipid) (Saint Georges-Gridelet, 1984).

To date, investigations of the nutritional significance of fungi to house dust mites have failed to apply the standard procedures of symbiosis research (see Smith and Douglas, 1987). Omissions of particular importance are, firstly, the incidence of fungi in the mites and diets and, secondly, the population increase of mites under fungus-free conditions. The study described here was therefore designed to identify the fungi in a laboratory culture of *D. pteronyssinus* and its diet, and to develop methods to obtain fungus-free mites. Using these techniques, the nutritional interactions between dust mites and fungi were investigated.

House dust-fungal associations are not solely of academic interest. House dust mites are regularly found at densities of greater than 500 individuals per g dust (Bronswijk, 1981; Platts-Mills and Chapman, 1987) and they are of considerable medical importance because their bodies and faeces are allergenic (Tovey et al., 1981; Arlian et al., 1987) inducing asthmatic attacks in atopic people (reviewed by Platts-Mills and Chapman, 1987).

2. Materials and Methods

Maintenance of house dust mites

This study was conducted exclusively on *D. pteronyssinus* culture Holl/CAB (Hart and Douglas, 1989). Standard cultures of the mites were maintained in continuous darkness at 25°C and 75% relative humidity on a diet of dried yeast (Yestamin) and acetone-washed beard shavings in the ratio of 5:1 (w/w). For experimental studies, replicate groups of 10 eggs were incubated in glass vessels of 12 mm diameter under the same conditions as the standard cultures, except that the diet comprised 10 mg dried yeast and dried liver (Oxoid, L26) (1:1, w/w) supplemented with beard shavings or fungus as indicated in the text. To obtain eggs, adult female mites were incubated in a clean dish for 30 min to allow most of the adhering food particles to drop from their bodies before transfer to ethanol-washed glass slides. The eggs they subsequently deposited over 1–2 days were collected with a sterile needle. These isolated mites also deposited faecal pellets which were used in experiments described below.

Microorganisms associated with mites, mite products and diets

To test the sterility of dietary components, eggs, and faecal pellets of mites, these products were plated separately onto agar with a sterile needle. The internal carriage of microorganisms by *D. pteronyssinus* was tested by piercing individual mites, that has been surface sterilized in 1% sodium hypochlorite (Lustgraaf, 1978), with a sterile needle onto agar. Two agar media were used in all experiments: nutrient agar (containing 2.5% (w/v) nutrient broth no. 2 (Oxoid CM 67)), and malt extract agar (containing 3% (w/v) malt extract (Oxoid L39) and 0.5% (w/v) mycological peptone (Oxoid L40)), solidified with 2% (w/v) agar no. 1 (Oxoid L11) and supplemented with 0–50% sucrose as indicated in text. The plates were incubated in 25°C and scored for microbial growth over 2 weeks.

Aspergillus penicilloides was isolated from *D. pteronyssinus* (see results). This fungus was routinely cultured on malt extract agar with 40% sucrose. To collect spores, mature cultures of *A. penicilloides* were set at an angle of 45°and gently tapped. The spores accumulated along the bottom edge of the plate and were aseptically removed with a sterile spatula.

Sterilization of dietary components

Using the agar media for sterility testing (described above), all dietary components except the fungi were sterilized by incubation in propylene oxide vapour for 2–3 days. The fungi associated with diets and also cultured fungal spores were killed by γ-irradiation at 2.5 Mrad.

Preparation of mites for microscopy

Slide preparations of mites from the development experiments were made with Hoyer's mountant (Krantz, 1978) and the length of the mites was determined at $\times 100$ magnification with an eye-piece graticule.

To investigate the location of microorganisms associated with *D. pteronyssinus*, the mites were prepared for light microscope histology and transmission electron microscopy by the methods of Douglas (1988) developed for homopteran insects. For ease of handling during preparation, the mites were aggregated and orientated in molten low-melting point agarose which, upon setting, was cut into blocks of 10 mm dimension.

3. Results

Microorganisms associated with dietary components of mites

All the dietary components of mites, namely dried yeast, dried liver and acetone-washed beard shavings contained a variety of bacteria that grew on nutrient agar, but the only components that bore mycelial fungi were the acetone-washed beard shavings. In a detailed study of beard shavings from 7 volunteers, *Penicillium, Cladosporium, Wallemia* and the *restrictus, glaucus* and *niger* groups of *Aspergillus* were isolated. With the exception of one volunteer, whose beard shavings were consistently fungus-free, all samples bore at least two taxa of fungi. Most of the fungi were xerophilic (i.e. grew on media containing 30–50% sucrose).

Microorganisms in D. pteronyssinus

As previously reported by Hart and Douglas (1989), the only microorganism isolated from *D. pteronyssinus* culture Holl/CAB was the xerophilic fungus *A. penicilloides*.

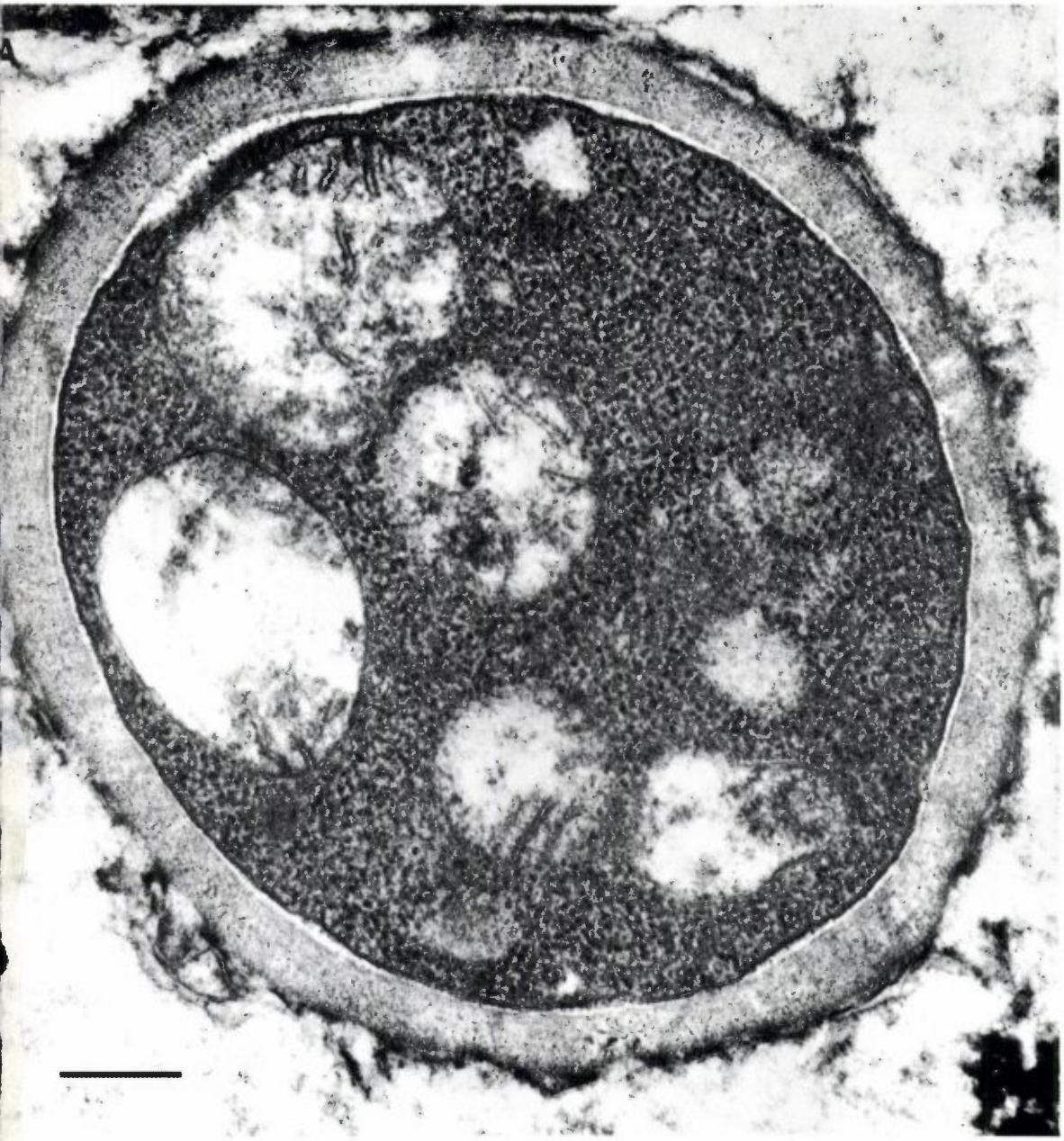

Figure A. Fungal spore in gut lumen of *Dermatophagoides pteronyssinus*. Scale bar = 0.25 μm. Neg 88\6910.

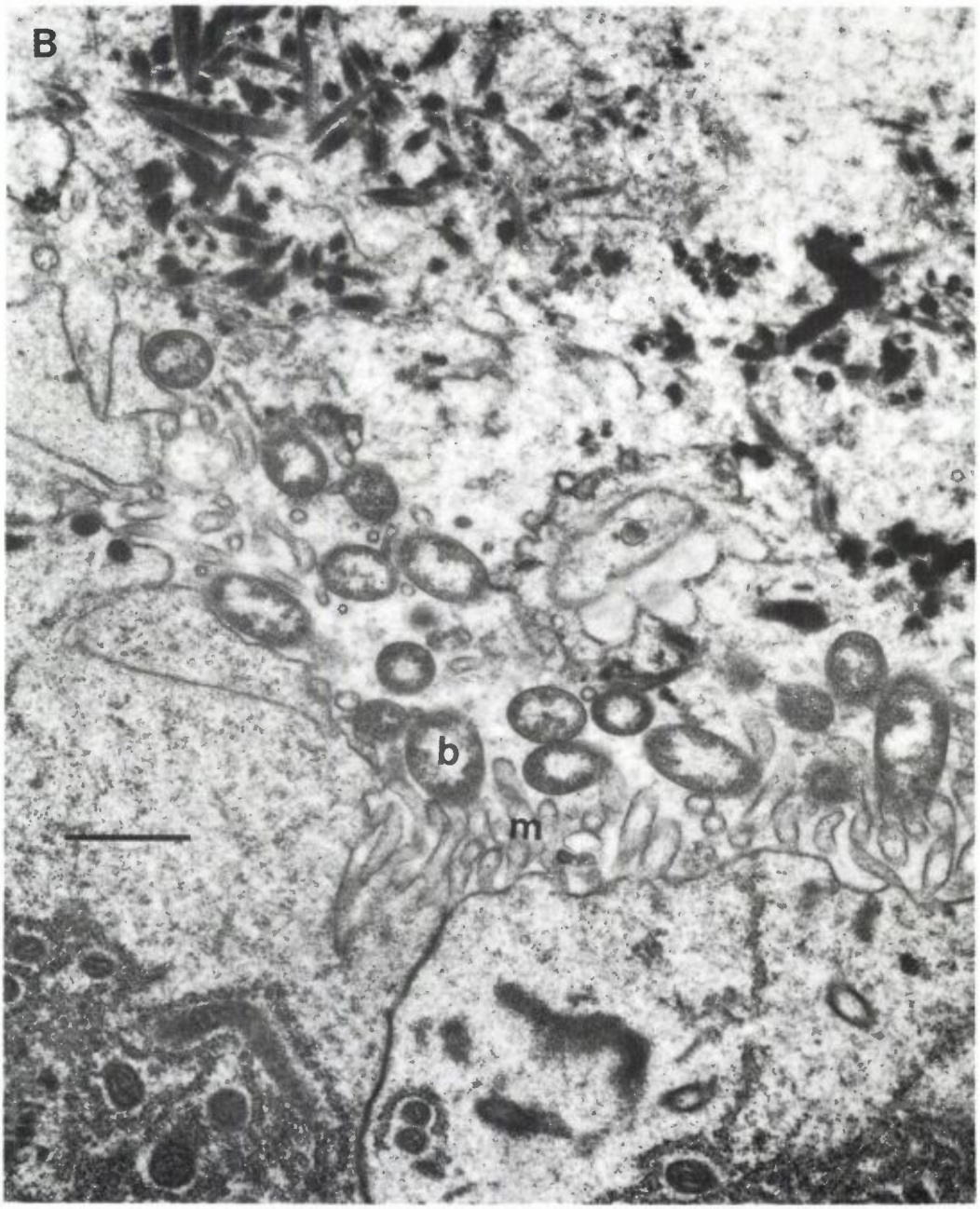

Figure B. Bacteria (b) associated with microvilli (m) of midgut cells of *D. pteronyssinus*. Scale bar = 0.7 μm. Neg 88\6903.

In light and electron microscopical studies of *D. pteronyssinus* from standard cultures, fungal spores were identified in the lumen of the digestive tract (Fig. 1a), but not the gut epithelial cells or any other region of the mite body. Fungal hyphae were not observed. Serial transverse paraffin-wax sections of the entire body were obtained for 26 adult females. The gross organisation of the digestive tract was closely similar to that of *Dermatophagoides farinae* Hughes (Brody et al., 1972) and fungal spores were scored in 18 (69%) individuals. Of a total of 74 spores, 49 were in the anterior midgut, 17 in the posterior midgut and 6 in the broad cuticle-lined anterior portion of the hindgut. No fungi were evident in the paired caeca, the lumina of which were filled with amorphous, densely-staining material.

In addition to fungal spores, the digestive tract bore flocculent masses of food, free cells (presumably sloughed off from the gut epithelium (Brody et al., 1972)) and, in the midgut, an extensive bacterial microflora. In every mite examined, the bacteria were closely associated with the microvilli of the epithelial cells (Fig. 1b). They were up to 3 μm long, bounded by a cell wall and had a distinct nucleoplasmic region, but no inclusions were apparent.

When faecal pellets of *D. pteronyssinus* were incubated on nutrient agar or malt extract agar, the sole microorganism that grew was *A. penicilloides*. The percentage of contaminated pellets varied widely between different experiments, for example, between 30% and 80% for pellets of adult females from standard culture.

No microorganisms grew from any of the freshly-deposited eggs incubated on nutrient agar or malt extract agar containing 0, 10 or 40% sucrose in four separate experiments, each with at least 10 replicate eggs for each agar medium. Eggs pierced on the agar were also apparently free of microorganisms (one experiment, 35 replicates). To investigate further their sterility, eggs were allowed to develop under aseptic conditions and the larvae they produced were reared to adulthood on the sterile 'experimental' diet of dried yeast and dried liver (see Materials and Methods). All the mites tested (a total of more than 100 in a series of experiments throughout the study) bore no microorganisms that grew on nutrient agar or malt extract agar.

Ingestion of A. penicilloides by D. pteronyssinus

Individuals of *D. pteronyssinus* from the standard culture were introduced to the experimental diet of dried yeast and dried liver supplemented with 1–50% (w/w) spores of *A. penicilloides* and observed under a dissecting microscope at $\times$100 magnification. The mites ingested the fungus, whether the spores were freshly collected from cultures of *A. penicilloides* or γ-irradiated.

Mites that had been incubated in diet containing 10–50% spores bore clumps of fungus both in their guts and adhering to the surface of their bodies and these mites moved less freely through the diet than those in diets with 0 and 1% fungal spores.

The performance of fungus-free mites

The observations that mites fed on *A. penicilloides*, and that both the eggs laid by the mites and the beard shavings from one volunteer were consistently fungus-free, provided the opportunity to investigate the influence of *A. penicilloides* on the survival and growth of *D. pteronyssinus*. The first experiment (see table) was designed primarily to assess the proposed role of *A. penicilloides* in modifying beard shavings to a more suitable form for the mites (see Introduction). The fungus-free eggs hatched between 7 and 9 days after deposition and many empty egg cases but no shrivelled or aborted eggs were observed, suggesting that most, or all, of the mites had developed to hatching. However, many mites died as larvae or nymphs, nearly 80% on media supplemented with γ-irradiated fungal spores (treatment 4) and 45–55% on the other diets (treatments 1–3). The variation in mortality on the different diets was significant (see table) and it was concluded that the fungal spores in treatment 4 deleteriously affected the mites.

A different indication of the quality of the various diets was provided by the second index of performance, the duration of development to adulthood. Adults were first observed on day 17 of the experiment and all living mites reached adulthood by day 29. The mean development time varied significantly between treatments and by Scheffé's multiple range test, mites on fungus-free diets (treatments 1 and 2) took significantly longer to develop than those on media containing *A. penicilloides* (treatments 3 and 4) ($p<0.05$).

No significant difference between the size of adults raised on the various media was observed; in all treatments, the lengths of females were 0.36–0.40 mm and males were 0.30–0.34 mm. These values are comparable to those for adults obtained from routine cultures.

The performance of mites on the diet of sterile dried yeast, dried liver and fungus-free beard shavings supplemented with fungal spores freshly collected from cultures of *A. penicilloides* was also investigated. Larval and nymphal mortality was high (72% of 40 mites) and the developmental time to adulthood (20.27 ± 0.45 days, $n=11$) was within the range of values for mites reared on fungus-free diets and diets with γ-irradiated fungi.

4. Discussion

The current study has overcome the chief limitation to the experimental study of the house dust mite-fungus relationship by the development of a method to generate and maintain fungus-free mites. The mites that hatch from isolated eggs are fungus-free. The first generation of fungus-free mites can be reared to adulthood and subsequently produce offspring on sterile diets of either dried liver and dried yeast, or dried yeast and fungus-free beard shavings, although their developmental rate is slower than that of untreated mites (14.3 ± 0.5 days) (Hart and Fain, 1988). The persistence of fungus-free mites over several generations is currently being investigated.

The improved developmental rate of fungus-free *D. pteronyssinus* cultures with *A. penicilloides* confirms the suggestions in the literature (see Introduction) that fungi in the diet are advantageous to house dust mites, probably by the provision of nutrients. Our data provide information on the means by which putative nutrients are obtained by the mites. Firstly, nutrients are not acquired biotrophically by release of substances from living fungal cells because fungi killed by γ-irradiation promote mite developmental rate. Secondly, the close similarity to developmental rate of mites on diets with killed fungi and preincubated with fungi prior to sterilization (treatments 3 and 4 in table) does not support the view that the primary role of fungi is modification of the diet. The very poor survival of mites on diets containing killed or living fungal spores without preincubation (treatment 4 and text) probably arose from the adhesion of the spores to the bodies of the mites, interfering with their motility and possibly feeding. It may be advantageous to use lower concentrations of fungus in future experiments.

Pertinent to the conclusion that *A. penicilloides* represents a source of nutrients but does not significantly modify the diet of house dust mites is the design of experiments on which the dietary modification hypothesis is based. In the original study of Bronswijk and Sinha (1973), the population increase of *D. farinae* was greater if the diet was pre-incubated with *A. amstelodami* than in the absence of this fungus. The critical data on the performance of mites on diet supplemented with *A. amstelodami* without pre-incubation were not obtained. The data of Lustgraaf (1978) on the population increase of *D. pteronyssinus* on mattress dust with and without *A. penicilloides* are more readily explained by the hypothesis that *A. penicilloides* provides more nutrients than by the dietary modification hypothesis that Lustgraaf espouses. The population increase of mites was enhanced three-fold by addition of *A. penicilloides* to the diet, independent of the duration of pre-incubation from 0–24 weeks. It is clear that no experimental

Table 1. Performance of fungus-free *Dermatophagoides pteronyssinus* reared on various media.

Five or six replicate groups of 10 eggs deposited over 2 days by female *D. pteronyssinus* from standard culture were transferred (on day 0 of the experiment) to the experimental diet of dried liver and dried yeast, supplemented with 5% (w/w) fungus-free beard shavings and 5% (w/w) spores of *A. penicilloides* as indicated, and incubated under standard conditions. The cultures were checked daily until all living individuals reached adulthood. Closely similar results were obtained for males and females and values for the two sexes are amalgamated.

Treatment	Dietary supplement	(No. mites survived to adulthood)/ (total no. mites)[1]	Development time mean ±s.e. (no. reps)[2]
1	none	22/50 (44)	21.73±0.91 (22)
2	γ-irradiated fungus-free beard shavings	27/60 (45)	21.74±0.77 (27)
3	fungus-free beard shavings & fungal spores incubated together for 33 days prior to γ-irradiation	33/60 (55)	19.52±0.33 (33)
4	fungus-free beard shavings & fungal spores γ-irradiated separately & combined aseptically	14/60 (23)	19.29±0.61 (14)
		$X^2 = 13.15$ (3 df) $p<0.005$	$F_{3.93} = 5.92$ $p<0.001$

[1]% survival in parenthesis

[2]Development time is taken from day 0 of the experiment (see legend to table).

studies to date have provided unambiguous evidence that fungi contribute to the performance of house dust mites by modification of dietary components.

A final issue is the discovery of a substantial bacterial microflora in the midgut of *D. pteronyssinus*. These bacteria were not isolated onto nutrient agar, probably because they are culturally fastidious; as with many gut bacteria, they maybe obligate anaerobes. Bacteria have seldom previously been reported in association with house dust mites (Bronswijk and Sinha, 1973) and their significance to the mites remains to be established.

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