

# Mycophagous mites and their internal associated bacteria cooperate to digest chitin in soil

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**Abstract** The greater bulk of soil nitrogen is immobilized in chitinous cell walls of fungi. Mycophagous soil mites participate in chitin decomposition and, hence, in the subsequent mobilization of nitrogen. The source of the chitinolytic enzymes was searched in this study. A multi-methodical approach was designed for these studies. Histology, plating and identification of bacteria from mite homogenate and, finally, homogenate and bacterial treatment of the soil fungi were applied. Here the presence and activity of chitinolytic bacteria inside mycophagous mites are reported. These bacteria form an extraintestinal group within the mite's body and pass their enzymes into the mite's gut. Our results demonstrate that true mycophagous mites, defined by their ability to digest chitin (i.e. the fungal cell wall), achieve this through internal "cooperation" with chitinolytic bacteria that provide the necessary chitinolytic enzymes. The nitrogen from chitin is thus made available to other soil organisms and plants.

**Keywords** Soil mites · Mycophagy · Associated bacteria · Chitinolytic enzymes · Destruction of soil fungi

## 1 Introduction

The greater bulk of soil nitrogen is immobilized as chitin in the cell walls of numerous species of micromycetes. Nitrogen mobilization, therefore, must include mycolytic activity and, indeed, this appears to be a common

phenomenon in soil. In grasslands of the U.S.A. and Canada, for example, the dry mass of mycelium has been estimated at from 12.6 to 184 g/m<sup>2</sup> (Kjøller and Struwe 1982). As chitin comprises from 3% to 60% of the dry mass in the cell wall (Hudson 1986), it represents an important source of nitrogen in the soil and chitin decomposers play a substantial role in mobilizing nitrogen and, more generally, in the entire nutrient cycling process. Some soil organisms do appear to be chitinolytic (i.e. able to convert chitin through enzyme activity) and so can be termed as mycolytic (microorganisms) or mycophagous (animals), i.e. as feeding on fungi.

Production of chitinolytic enzymes has been confirmed in literature in some bacteria, including *Bacillus* (Watanabe et al. 1990; Citterio et al. 2001; Aktuganov et al. 2004), *Pseudomonas* (Lim et al. 1991; Wang et al. 1997; Citterio et al. 2001), *Serratia* (Kishore et al. 2005), *Janthinobacterium* (Molloy and Burke 1997), *Brevundimonas* (Jaspers and Overmann 2004) and *Stenotrophomonas* (Zhang et al. 2001; Kobayashi et al. 2002). Autochthonous chitinolytic enzymes are generally absent in all hitherto studied animals including the man, by contrast, except for a few employing pathogenous processes (Aktuganov et al. 2004). Earlier opinion held that saprophagous soil mites exhibit low feeding selectivity, although they transport the microorganisms (bacteria, fungal spores as well as fragments of mycelium) on their body surfaces or in their guts (Wallwork 1976). Several more recent observations, however, have revealed conspicuous food selectivity in some groups of these mites, especially moss mites (Oribatida) and mould mites (Acaridida) (Luxton 1972; Siepel and de Ruyter-Dijkman 1993; Smrž 2002a).

Several methods have been reported in the literature for determining mite food selection. Clearing of mites with lactic acid and then observing them under a light

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microscope can indicate a “swallowing” of fungi. But “swallowed” fungal propagula are also able to pass through the gut as intact particles. Other approaches have included laboratory feeding tests, based on the one-way or cafeteria test (Czajkowska 1970) and recording whether mites make contact with a fungal colony, whether they move their chelicerae, and whether faeces are produced. Again, even all of those phenomena do not together provide conclusive evidence for true mycophagy. Indeed, consumption of fungi might also proceed through piercing of the cell wall and sucking out of its contents, which are rich in trehalose and other substances (Wigglesworth 1974). This form of feeding, however, cannot be termed true mycophagy as the chitinous cell wall remains intact and nitrogen is not mobilized from the cell walls. Further, substances such as trehalose, a so-called fungal sugar, can occur in many organisms (e.g. insects) (Wigglesworth 1974). In contrast, testing of enzyme activity has proven a useful method for assessing mode of feeding, and it has resulted in the classification of mites into a number of feeding groups or guilds (Luxton 1972; Dinsdale 1974; Siepel and de Ruiter-Dijkman 1993; Smrř 1996). Using labelled isotopes of nitrogen also appears to be a very useful method (Schneider et al. 2004).

Several authors observed bacteria inside the mites. Wolf and Rockett (1984) isolated several bacterial genera from two oribatids (*Rhysotritia* and *Pergalumna*). According to those authors, the effect of habitats was supposed to be important for the bacterial internal communities. The bacterial production of enzymes, however, was not assumed, although *Bacillus* or *Pseudomonas* were isolated. The participation of the gut bacteria was considered in the digestion processes by the Stefaniak and Seniczak (1976; 1981) and Seniczak and Stefaniak (1978), but without any identification of bacteria or specification of those processes. The type of food was assumed to be the substantial factor for the structure of the gut bacterial community. Again, the digestion of grazed food was not observed at the grazed food in gut.

To confirm whether mycophagy including chitinolytic activity is really occurring, a series of tests was undertaken in order to answer three questions: 1) are animals really able to digest chitin; if so, 2) how does the animal digest the chitin and utilize it for nutrition; and 3) are chitinolytic enzymes available to animals? 4) are we able to define the actual mycophagy?

## 2 Materials and methods

**Tested mites and fungi** Several species of mites were examined, all previously considered as mycophagous: *Damaeus onustus* C.L.Koch, *Damaeus riparius* Nicolet, *Damaeus auritus* C. L. Koch, *Metabelba pulverosa*

Strenzke, *Belba pseudocorynopus* Märkel & Mayer, or as occasional mycophagous - *Scutovertex minutus* (C.L.Koch) (all Oribatida sampled in forest and steppe biotopes) (see Pauly 1956; Luxton 1972; Smrř 1992, 2006). These mites were compared with three control oribatid species known to avoid fungi (non-mycophagous) sampled in forest and steppe soils: *Platynothrus peltifer* (C. L. Koch), *Phthiracarus* sp., and *Trichoribates trimaculatus* (C. L. Koch) (see Luxton 1972; Dinsdale 1974; Smrř 1992, 2006, 2007). The latter oribatids rejected any fungi, and therefore they were reared for the short time of the tests on algae covering tree bark (*Protococcus* sp.). The acaridid mycophagous mite *Tyrophagus putrescentiae* (Schrank) (Czajkowska 1970; Smrř and řatská 1987; Smrř and Jungová 1989; Smrř 2003) was reared in the laboratory on the tested fungi. All experiments were performed in 220 cm<sup>3</sup> glass boxes with a plaster of Paris/charcoal bottom.

The tested fungi were applied from our reference collection (*Alternaria* sp.—strain M 100, *Fusarium oxysporum*—strain M 26, *Mucor racemosus*—strain M 6, *Penicillium claviforme*—strain M 83, *Penicillium griseofulvum*—strain M 66, and *Verticillium* sp.—strain M 21). All those fungi were consumed by mites as sporulating ones on malt agar. Only the algal cover on the tree bark (*Protococcus* sp.) was invaded in rearing boxes by the sterile mycelium and the identification of those mycelium was not possible.

**Histology** Mites (20 individuals per experimental group) were fixed in modified Bouin-Dubosque-Brasil fluid (Smrř 1989) and embedded in Paraplast (Polysciences). The samples were then sectioned on a Leica 2155 rotation microtome (5 µm thickness), stained using Masson’s triple stain, and observed under an AX-70 Provis (Olympus) light microscope, which included using a Nomarski DIC prism for unstained objects (Smrř 1989). Ten mites from each group were fixed in cacodylate-buffered glutaraldehyde (4%), embedded in Poly/Bed epoxy resin (Polysciences), and sectioned on a Leica 2155 rotation microtome by tungsten carbide knife (1 µm thickness).

Six microanatomical characteristics were examined (Smrř 1996, 2002a).

- content of the gut (type of food consumed and stage of digestion),
- activity of gut walls,
- metabolite deposits (guanine crystals). Guanine, a metabolite deposit, is typically found following digestion of large amounts of nitrogenous food (e.g. chitin). Viewed under great magnification, these crystals are birefringent, conspicuously silver, and appear as concentric circles. They are whitish when seen in the mite’s body. The great bulk of those crystals is able to

damage the internal mite organ (Smrž and Čatská 1987, 1989; Smrž et al. 1991).

- nutrient deposits (glycogen granules),
- conspicuous free cells (haemocytes) between the internal organs,
- bacteria-containing bodies inside the intestinal lining.

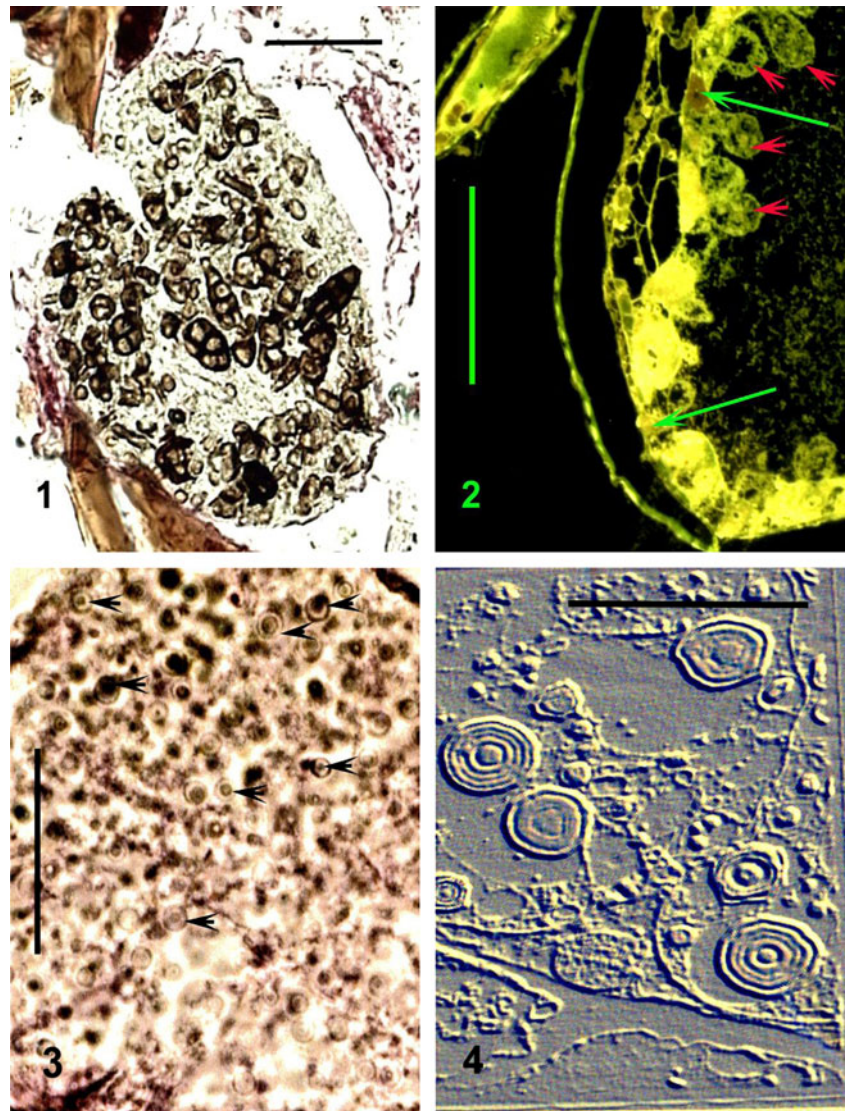
**Mite faeces** Mite faeces were smeared, stained by acridine orange, and observed under an AX-70 fluorescence microscope (Olympus). The presence of fungal propagula and bacterial cells were ascertained through differential staining, i.e. dead fungal cells would show up red while living bacteria would show up luminous green (Smrž 2002b).

**Transmission electron microscopy** Mites were fixed in cacodylate-buffered glutaraldehyde (4%), postfixed in 1% osmium tetroxide, embedded in Poly/Bed medium (Poly-

sciences), and sectioned using a Reichert Ultracut E ultramicrotome. Sections were stained in lead citrate and uranyl acetate and observed under a Philips EM 300 transmission electron microscope.

**Internal bacteria (Smrž 2000)** Living mites were rinsed twice in 96% ethyl alcohol followed by 10% JAR (Procter & Gamble) domestic detergent (to remove microorganisms from the body surface) and finally washed twice in distilled water. The mites were subsequently homogenized in Ringer's solution. The internal bacteria from the mite homogenate (homogenate from 1 larger mite of *Damaeus*, or from 10 smaller individuals of the other mites) were plated on MPA (meat peptone agar) (pH=7) and identified at the internationally certified microbiological institute of the Czech Collection of Microorganisms (Brno, Czech Republic). Bacteria were only determined as present or absent, no quantitative

**Figs. 1–4** Alimentary tract: 1—Mesenteron of *Belba pseudocorynopus* crowded by fungal spores (*Alternaria* sp. dominated), 2—Apocrine secretion (red arrowheads) in the mesenteric caecum wall and the incorporated hemocytes (green arrows), a nymph of the mite *Belba pseudocorynopus*, 3—*Scutovertex minutus*, the mesenteric wall with guanine crystals (arrowheads), 4—*Tyrophagus putrescentiae*, guanine crystals within mesenchymous tissue between the internal organs. Masson's trichrome (1), autofluorescence under a confocal microscope (2), Nomarski DIC prism (3), TEM, pseudorelief in PC (4). Scales: 0.1 mm (1,2), 0.02 mm (3), 0.003 mm





correlations were established. During bacteria plating, species dominance was very clear. The dominant bacterial species formed around 8 colony forming units (CFU) per dish. The maximum number of CFU was 12 per dish.

**Enzyme activity (Smrž 2000)** Mite homogenate (for method, see previous paragraph) was tested on a thin layer of carboxymethyl-chitin on a microslide, with subsequent staining using basic fuchsin. The same test was performed on a bacterial suspension isolated from the homogenate that had been previously identified at the Czech Collection of Microorganisms

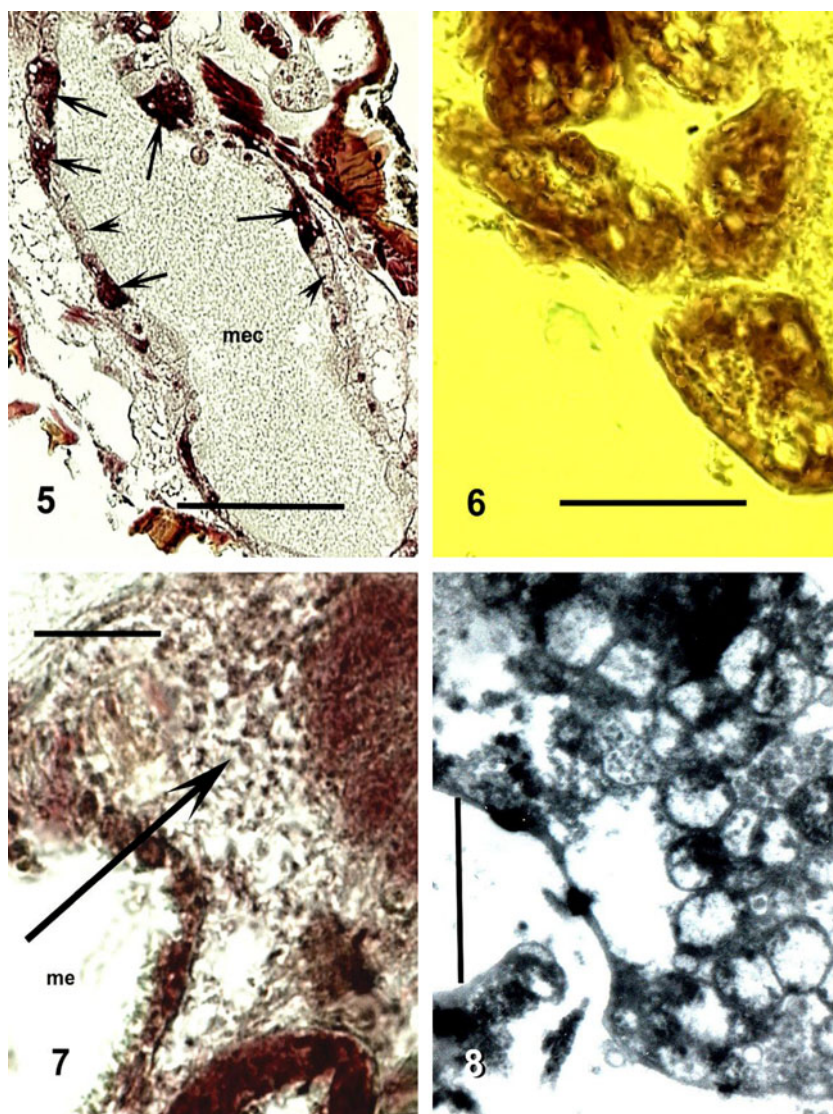
### 3 Results

In the real mycophagous mite species (*Damaeus onustus*, *D. riparius*, *D. auritus*, *Metabelba pulverosa*, *Belba*

*pseudocorynopus*), the alimentary system was observed to be crowded with fungal propagula (Fig. 1) (spores and fragments of mycelium) in all three gut parts (mesenteron, colon, rectum). These corpuscles displayed increasingly high levels of destruction, especially as regards their cell walls, as they passed through the system. Cell damage was clearly visible in all three gut parts. The cell walls were nearly intact in the mesenteron (the first part of the entodermal gut), became thinner in the colon (the second gut section), and an amorphous mass with almost no wall structures, or with only scarce fragments of the walls, was visible at the third gut section (i.e. the rectum, including the anal atrium). “Swallowed” fungal propagula were scarce in non-mycophagous mites, and usually absent. When observed, they were never seen in more than one gut section at the same time and were passed through all three gut sections as intact corpuscles.

Cells in the gut wall of mycophages turned voluminous and contained dark enzyme granules and vacuoles. They

**Figs. 5–8** Alimentary tract and mesenchymal tissue between the internal organs: 5—mesenteral caecum with incorporated hemocytes (arrows), arrowheads point to the wall cells, 6—the vacuolized hemocytes adjacent the mesenteral caecum, 7—a cluster of associated bacteria (arrow) near mesenteron inside oribatid *Damaeus onustus*, 8—bacteria associated with *Tyrophagus putrescentiae*. Masson’s trichrome (5), Nomarski DIC prism (6,7), TEM (8). Scales: 0.050 mm (5), 0.02 mm (6), 0.01 mm (7), 0.003 mm (8). Abbreviation used: me—mesenteron, mec—mesenteric caecum



exhibited apocrine secretion (Fig. 2). The gut walls of non-mycophages were thin, without any of the conspicuous micromorphological characteristics mentioned above.

Conspicuous crystals of guanine were observed within the gut walls (Fig. 3) of mycophages, as well as around the gut (Fig. 4). No guanine crystals were observed in non-mycophages.

Nutrient deposits, in the form of red-stained granules of glycogen (a polysaccharide without nitrogen), indicated a response to a high intake of palatable food and a high rate of digestion in mycophages. Red-stained glycogen granules were also deposited in non-mycophagous mites (*Platynothrus peltifer*; *Trichoribates trimaculatus*). Indeed, glycogen granules appeared to be deposited in the presence of any palatable food (in this case, plant litter) and, therefore, were not a good indicator for mycophagy.

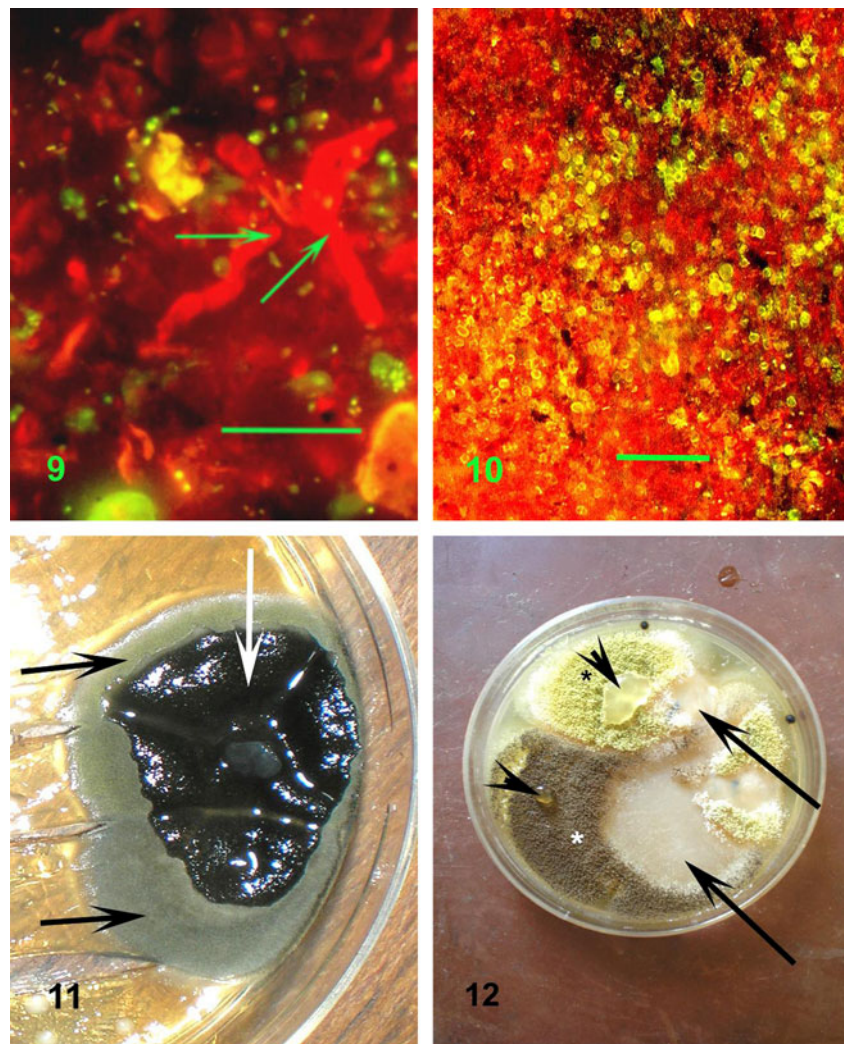
Both the presence and number of free cells within mites feeding on fungal food were conspicuous. Haemocytes were observed penetrating into the gut walls between wall

cells (Figs. 2, 5 and 6). In spite of the palatability of non-fungal food, free cells were very rarely observed in non-mycophagous mites.

Bacterial bodies were especially observed around the posterior part of the mesenteron and around the blind mesenteric caeca (a projection of the mesenteron) of mycophages (Fig. 7). The structure of the bacterial bodies (density of bacteria and size of body) varied according to the fungus consumed and digested. Their presence and shape were confirmed by transmission electron microscopy (TEM—Fig. 8). No such bodies were recorded in non-mycophages.

Mycophage faeces contained numerous digested fragments of fungal propagula. Many luminous green (living) bacterial cells were also present (Fig. 9). Mite digestion did not appear to be rigorous. No digested fungal fragments were observed in non-mycophage faeces. In addition, markedly fewer or no bacterial cells were observed there in comparison with the mycophagous mites (Fig. 10).

**Figs. 9–12** Consumption and digestion of food: 9–10 Faecal smears: 9—*Damaeus riparius* under fluorescent light—digested (dead) fungal mycelium (arrows) and living bacteria (green points), 10—*Trichoribates trimaculatus*, living algae (green spots); 11–12: Fungal colonies on the Petri dish with malt agar treated by bacterial suspension, restricted spots: 11—*Alternaria* sp., treated with *Serratia liquefaciens*—black spot (white arrow), intact fungus (black arrow), 12—*Penicillium griseofulvum* (white asterisk) and *P. claviforme* (black asterisk) treated by *Pseudomonas fluorescens*. Arrows point to treated spot, arrowheads to the control water drops. Acridine orange under fluorescent light (9, 10). Scales: 0.01 mm (9, 10)





Clear areas were formed in a basic fuchsin purple-blue layer when mycophage homogenate was applied to a carboxymethyl-chitin film on a microscopic slide, clearly indicating the digestion of chitin. The homogenate of non-mycophagous mites did not produce clear areas. The bacteria isolated and identified from the mite homogenate were found to be chitinolytic (Table 1).

Drops of a suspension of the chitinolytic bacteria isolated from the mycophagous mites digested the test fungi (*Alternaria* sp., *Penicillium griseofulvum*, *Penicillium claviforme*, *Mucor racemosus* and *Fusarium oxysporum*). Areas of digested fungus were very clearly delineated and visible, and they were surrounded by intact fungus (Figs. 11 and 12). The treatment of fungus by homogenate of mycophagous mites resulted in the same phenomenon. The population of *Tyrophagus putrescentiae*, chosen because of its rapid rate of reproduction, was introduced onto *Alternaria* sp. established on a malt agar Petri dish. After three weeks, an amorphous “mud” covered the dish, with no fungus visible. Mites introduced onto *Fusarium oxysporum*, and *Penicillium claviforme* produced the same results.

**Table 1** Chitinolytic bacteria isolated from soil mites in our laboratory and identified at CCM Brno

Bacterial species	Mite species	Origin of mite
field sampling:		
<i>Bacillus badius</i> <sup>a</sup>	DR,A, Ch	NS, forest
<i>Bacillus cereus</i> <sup>a</sup>	DR,A, Ch	NS, forest
<i>Bacillus megaterium</i> <sup>a</sup>	DR, Ch	NS, forest
<i>Janthinobacterium lividum</i> <sup>a</sup>	DR	NS, forest
<i>Pseudomonas fluorescens</i> <sup>c</sup>	DO,DA, BP	NS, forest
laboratory rearing:		
		offered food in laboratory
<i>Pseudomonas stutzeri</i> <sup>d</sup>	TP	ISM,
<i>Brevundimonas vesicularis</i> <sup>d</sup>	TP	ALT
<i>Stenotrophomonas maltophilia</i> <sup>d</sup>	TP	V
<i>Serratia liquefaciens</i> <sup>b,d,e</sup>	TP	PC,PSP
<i>Serratia marcescens</i> <sup>b,d,e</sup>	TP	ISM,PC
<i>Serratia rubidea</i> <sup>c</sup>	AL	ISM

Abbreviations used: *A* *Achipteria coleoptrata*, *AL* *Archegozetes, longisetosus*, *ALT* *Alternaria* sp., *BP* *Belba pseudocorynopus*, *DA* *Damaeus aurius*, *Ch* *Chamobates voigtsii*, *DO* *Damaeus onustus*, *DR* *Damaeus riparius*, *FO* *Fusarium oxysporum*, *ISM* invasive sterile mycelium on algae, *NS* natural soil, *PC* *Penicillium claviforme*, *PSP* *Penicillium* sp., *TP* *Tyrophagus putrescentiae*, *V* *Verticillium* sp.,

<sup>a</sup> Smrž and Trelová (1995)

<sup>b</sup> Smrž (2003)

<sup>c</sup> Smrž and Norton (2004)

<sup>d</sup> Smrž and Soukalová (2008)

<sup>e</sup> Smrž, this study

## 4 Discussion and conclusions

Many authors have reported the consumption of fungi by soil saprophagous mites (Coleman and McGinnis 1970; Pande and Berthet 1973; Mitchell and Parkinson 1976; Stefaniak and Seniczak 1981; Schneider et al. 2004). On that basis, these mites were classified into feeding guilds based on different forms of consumption (sucking, cell walls grazing) (Siepel and de Ruiter-Dijkman 1993). The digestion of the substrates, especially chitin in the fungal cell walls, however, has been neglected. Therefore, the autochthonous or allochthonous origin of the chitinases in mites was not defined or considered. Overgaard Nielsen (1962) assumed the occurrence and origin of enzymes and he reported the bacterial production of chitinolytic enzymes in pulmonate snails. Shimano and Matsuo (2002) discussed the production of enzymes by the microorganisms with respect to their microanatomical results. The biochemical and immunological definition of enzyme activities, classified as allergens Der p 15 and Der p 18, were reported in *Dermatophagoides pteronyssinus* (O'Neil et al. 2006). They were considered as potentially important allergens for humans as well as dogs. The source of two allergens, however, were not identified.

The multimethodical approach used to examine chitinolysis and mycophagy in soil organisms (i.e. bacteria and mites) indicated that while many soil mites consume parts of fungi only some can be considered as truly mycophagous, i.e. consuming all parts of the fungus cell, including the chitinous cell wall (Smrž 2007).

Microanatomical methods clearly showed not only “swallowing” of fungi fragments but also progressive digestion of fungal cells through the guts of mites considered as mycophages, while their faeces contained a large proportion of dead fungal fragments and many live bacteria. In the 1990 s, several chitinolytic bacteria (*Bacillus badius*, *B. cereus*, *B. megaterium*, *Janthinobacterium lividum*, *Serratia marcescens*, *S. liquefaciens* and *Pseudomonas fluorescens*) had been isolated (Smrž et al. 1991; Smrž and Trelová 1995) from mites known to be mycophagous (*Damaeus riparius*, *D. auritus*, *Achipteria coleoptrata*, *Chamobates voigtsii*, and *Tyrophagus putrescentiae*) (Smrž et al. 1991; Smrž and Trelová 1995). A multimethodical approach, especially enzyme activity and homogenate tests, had not been applied in those studies, however, and it had been possible only to hypothesize a process of “cooperation” between the isolated bacteria and mites for chitinolysis resulting in mycophagy.

The tested mycophagous mites were able to digest the whole fungal propagula, i.e. including the chitinous cell walls, through “cooperation” with internal extraintestinal bacteria that produced chitinolytic enzymes. These passed into the gut lumen and participated in chitin digestion.

Chitinolysis by some bacterial species isolated from these (*Serratia marcescens*, *S. liquefaciens*, *S. rubidea* and *Pseudomonas fluorescens*, *P. stutzeri*, *Brevundimonas vesicularis*, *Stenotrophomonas maltophilia*) was confirmed on thin carboxymethyl-chitin film and on a test fungus sample (Smrř and Čatská 1987; Smrř et al. 1991; Smrř 2003; Smrř and Norton 2004; Smrř and Soukalová 2008).

The fungal digestion differed whether bacteria were in suspension or were “cooperating” inside the mycophagous mites. The only bacterial digestion without mites resulted in restricted spots or area in fugal cover. The association of chitinolytic bacteria inside mites resulted in the destruction of complete fungal colonies in the Petri dish due to the mite’s ability to move and graze (Smrř and Čatská 1987; Smrř et al. 1991; Smrř and Norton 2004; Smrř and Soukalová 2008).

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