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# Effect of slug mycophagy on *Tuber aestivum* spores

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ABSTRACT

Truffles in the genus *Tuber* produce subterranean fruiting bodies that are not able to actively discharge their spores in the environment. For this reason, truffles depend on mycophagous animals for reproduction. Fungus consumption (mycophagy) is a behaviour typical of both vertebrates and invertebrates. Mammals, especially rodents, are the most studied group of mycophagists and have been found to consume a great variety of fungi. Among invertebrates, mycophagy is documented in arthropods, but rarely in molluscs. In our study we assessed the effect on the morphology and mycorrhizal colonization of *Tuber aestivum* spores after passage through the gut of slugs (*Deroceras invadens*) and, for comparison, of a house mouse (*Mus musculus*). Light, scanning electron and atomic force microscopy revealed that the digestion, especially by slugs, freed spores from the asci and modified their morphology. These are believed to be the reasons why we observed an improvement in oak mycorrhization with the slug and rodent ingested spores in comparison to a fresh spore inoculation. We also demonstrated by molecular barcoding that slugs' guts sampled on a *Tuber melanosporum* truffle ground contain spores from this species and *Tuber brumale*, further suggesting that some invertebrates are efficient *Tuber* spore dispersers.



## 1. Introduction

Fungi produce a wide range of macroscopic fruiting bodies differing in size, morphology and fruiting habit. During evolution, some lineages of epigeous fungi have convergently evolved sequestrate fruiting habits as an adaptive response to selective pressure (Trappe, 1975; Binder and Bresinsky, 2002; Bonito et al., 2013). Since the spores of sequestrate fungi are enclosed within the fruiting body, most species have lost the ability of active discharge (Trappe et al., 2009). This makes many sequestrate taxa rely on features that promote animal dispersal. The production of strong aromas able to attract wild animals (Pacioni et al., 2015; Zambonelli et al., 2017) ensure the ingestion of fruiting bodies and the dispersal of spores in the surrounding environment. Moreover, the structure of spores with durable and thick walls allows them to survive passage through the digestive tract of mycophagous animals (Bonito et al., 2013).

Varying levels of mycophagy has been shown to be widespread among both invertebrates and vertebrates (Hammond and Lawrence, 1989; Nuske et al., 2017; Elliott et al., 2019a,b). Mycophagy can be classified as obligate, preferential, casual, opportunistic or accidental according to an animal's dependence on mushrooms in their diet (Claridge and Trappe, 2005). Most mycophagists also consume other types of food, or opportunistically feed on fungi at times when they are abundant in the environment

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(Maser et al., 1978). Thus, the quantity of fungi eaten varies in every species (Schickmann et al., 2012; Claridge and Trappe, 2005). Mammals are the most studied class of mycophagists, although few of them feed exclusively on fungi (Fogel and Trappe, 1978; Stephenson, 2010). The main knowledge gaps are in the repertoire of mycophagous invertebrates and in their role in fungal dispersion (Vašutová et al., 2019). Insects are the most studied group of mycophagists among invertebrates (Witte and Maschwitz, 2008, Disney et al., 2013). *Leiodes* spp. coleopters and dipterous *Suillia* spp. are the most common insects found in *Tuber* ascomata, but many other genera in Coleoptera and Diptera feed on them (Menta and Pinto, 2016; Rosa-Gruszecka et al., 2017), which may even challenge the future of truffle production. Unlike mammal mycophagists, insects do not completely eat ascoma, but their feeding cavities considerably reduce the economic value of truffles.

Little is known about the mycophagy of molluscs and available studies mainly concern the consumption of epigeous fungi. Keller and Snell (2002) proved the ingestion of fungi by several slug species. True truffles in the genus *Tuber* are also used as food by slugs. Parks (1921) reported that small slugs attack *Tuber californicum* Harkness in its early stages and feed on the gleba, leaving the peridium as an empty shell. It often happens that ascomata of valuable *Tuber* species partially eaten by slugs are found inside natural and cultivated truffle orchards (Fig. S1) causing great economic losses (Mathews et al., 2019). Since it's been proven that mycophagous mammals contribute to spore dispersal, improve spore germinability and might be involved in sexual reproduction (Piattoni et al., 2014; Taschen et al., 2016), we hypothesized that slugs could play a similar role in the biological cycle of truffles as short-distance dispersal agents. In order to test this hypothesis we fed slugs (*Derocera invadens* Reise, Hutchinson, Schunack & Schlitt) with *Tuber aestivum* Vittad. ascomata and compared the effects of gut digestion by these molluscs with ascomata eaten by a house mouse (*Mus musculus* L.) and with uneaten ascomata. Changes in the morphology of ingested and uningested spores were analysed by scanning electronic microscopy (SEM) and atomic force microscopy (AFM) while their ability to germinate and to form ectomycorrhizas was tested by inoculating oak (*Quercus robur* L.) seedlings. Finally, we sampled slugs on a truffle ground to analyse their gut content by molecular barcoding, in order to investigate the spontaneous intake of truffles by slugs.

## 2. Materials and methods

### 2.1. Animal feeding and faecal sampling

A single *T. aestivum* ascoma of 95 g collected in the L'Aquila municipality (Abruzzo, Italy; on May 2018) was selected for analyses and maintained at 4 °C for 2 d before feeding the animals. A fragment of the ascoma was dried and deposited in the Herbarium Mycologicum Aquilanum (AQUI) under the ID code 10,231.

One specimen of *M. musculus* was provided by Charles River Laboratories Italia SRL, while 15 different sized specimens belonging to the *D. invadens* were collected in a private garden in the province of Teramo (Abruzzo, Italy). We applied a DNA barcoding approach to identify the slugs, using as barcode the COI gene fragment. A little fragment of muscle tissue was removed from the foot of three slugs. DNA extraction and PCR reactions with primer pair HCO2198-LCO1490 (Folmer et al., 1994) were carried out after Salvi et al. (2020). Amplicon purification and sequencing were done by Mycrosinth AG (Balgach, Switzerland).

Both the animal species were fed for 3 d with lettuce (slugs) or commercial rat food (house mouse) and then for 24 h with boiled potatoes (both animal species) to clear the gut as much as possible of recalcitrant plant polymers. Scats produced during these four d were excluded from analyses. On the fifth day, the animals were fed with 60 g of the *T. aestivum* ascoma (30 g per animal). An additional 30 g of the ascoma was stored at −4 °C pending microscope analysis and inoculum preparation of fresh material. Over the following 48 h all the faeces from each animal species were collected and pooled together, immediately placed in sterile distilled water, crushed with a mortar and pestle and blended at high speed for approximately 5 min. The uneaten portion of the ascoma was treated in the same way as faecal material. Slug and house mouse ingested spores (SIS and RIS, respectively) and uningested spores (UIS) were then maintained at 4 °C until microscopy analyses and inoculum preparation (3–5 d).

### 2.2. Microscopy of ingested and uningested spores

In order to quantify the spore density and the ratio of spores free of asci, a drop of spore suspension from each treatment was put on a Thoma cell counting chamber (Hawksley, London, UK) and spores counted under a light microscope Axiostar plus (Zeiss, Oberkochen, Germany) at 400× magnification. Spores' vitality was evaluated with fluorescein diacetate stain (FDA) after Colgan and Claridge (2002). Spores were then viewed with an Eclipse TE 2000-E microscope (Nikon) fitted with a 450–490 nm excitation filter and 515–555 nm barrier filter.

### 2.3. SEM analysis

Fresh and ingested spores were also prepared for SEM observation to assess the morphology of spore surfaces and ornamentations. A drop of spore suspension of each treatment was placed on an aluminium SEM stub (diameter 12 mm) after hexamethyldisilazane (HMDS) drying. The samples were then sputter-coated with a thin gold film using an AGAR automatic sputter coater (10 mA, 30 s) and examined by use of a FE-SEM Gemini 500 (Zeiss).

### 2.4. AFM analysis

Spores were fixed in 4 % paraformaldehyde in phosphate buffered saline (PBS; phosphate buffer 0.01 M, NaCl 0.15 M, pH 7.2) for 15 h at room temperature and layered on cover glass by centrifugation. After washing with ultrapure water (18.2 MU) the samples were layered on fresh muscovite mica and dried by nitrogen flow (Plomp et al., 2005).

The AFM analysis was carried out with an XE-100 Atomic Force Microscope (PARK Systems Inc., Suwon, South Korea). The microscope was equipped with a 50 μm scanner controlled by the XEP 1.8 software. The X–Y stages and the Z scan worked in a closed loop manner and in high voltage mode. The speed scan was set between 0.2 Hz and 1.5 Hz. The cantilevers used in this study were Non-Contact High Resonant (NCHR) tips (spring constant between 35 and 42 N $m^{-1}$) with a typical resonant frequency between 200 and 300 kHz. The instrument was set in true non-contact mode for imaging the samples during preliminary investigations and for topography and phase imaging. For nanomechanical surface characterization the instrumentation was set in Force Modulation Mode (FMM) with an amplitude setpoint of 538 nm. All images and data were analysed by XEI software (PARK Systems Inc., Suwon, South Korea). Spores for each treatment (UDS, SDS and RDS) were analysed and imaging was performed in the centre of three different alveoli for each spore (Fig. S2).

Roughness (Rq and RPV), surface phase and FMM variables from the three treatments were statistically analysed using Kruskal–Wallis non-parametric ANOVA followed by Dunn's Multiple Comparison test. Statistics were performed using GraphPad Prism software.

### 2.5. Seedling material and inoculation

Seeds of *Q. robur* were collected in October 2017 from a single plant and stored at 4 °C until sowing. The surface was sterilized in 1 % sodium hypochlorite solution for 1 h and rinsed with distilled water. Oak seedlings were grown in sterile (120 °C for 1 h) peat vermiculite substrate (1:9 v/v) for about 4 weeks before inoculation. Seedlings were maintained at 20 ± 1 °C and in a 14 h photoperiod (5000 lux).

Oak seedlings were separately inoculated with the spore suspensions obtained from mouse scats (RIS), slug scats (SIS) or from the remaining 30 g of uneaten *T. aestivum* ascoma (UIS). Spore suspensions from all treatments were adjusted to $1 \times 10^6$ spores $mL^{-1}$ before being used as inoculum. Three millilitres of spore suspension were injected on each pot directly on seedling roots by sterile micropipettes. Thirty-six seedlings were transplanted into plastic pots (750 mL each) filled with 500 mL of a sterile mixture of peat, vermiculite and sand (2:4:4). At transplant, 9 seedlings were inoculated for each treatment (RIS, SIS and UIS) and an additional 9 uninoculated seedlings were used as controls. All seedlings were grown in a climate room at 20 ± 1 °C under controlled light conditions (14-h photoperiod, 5000 lux) and watered with tap water twice a week. Root colonization was evaluated 1, 2 and 3 months after inoculation, checking three seedlings for each treatment each time. All seedlings were removed from their pots, the root systems were washed with tap water and the ectomycorrhizas were counted under a dissecting microscope (20 × ). From each seedling, 10 root fragments 4–5 cm in length were randomly excised from the whole root system. Ectomycorrhizas of *T. aestivum* were identified by morphotyping after Zambonelli et al. (1993). The identity of young ectomycorrhizas lacking cystidia was molecularly confirmed by species-specific PCR after Mello et al. (2002), in order to exclude any root contamination by other fungal species. A little fragment of the mantels was excided from each mycorrhizas and DNA was amplified by a direct PCR approach (Iotti and Zambonelli, 2006). The degree of root colonization was measured by counting the number of colonized and un-colonized root tips and the results were expressed as a percentage of colonized tips out of the total number of tips examined. Two-way ANOVA was applied to determine significant differences among treatments and monthly root colonization. Means were compared with the Tukey post-hoc test ($P < 0.05$). Statistical analyses were carried out with XL-STAT Software version (Addinsoft Inc., New York, NY, USA).

### 2.6. Barcoding of slug gut content

The slugs were collected in a *Tuber melanosporum* Vittad. truffle ground located at Chartrier-Ferrière, France (45°04′15″N, 01°27′05″E; truffle ground T6 in Schneider-Maunoury et al., 2018). This choice was made because the presence of this truffle is very clearly delineated by a zone of poor vegetation growth, called the brûlé (e.g. Taschen et al., 2016), which is more conspicuous than for other *Tuber* species (Streiblová et al., 2012). In this truffle ground, ten individuals of *D. invadens* were collected in January to February 2017, and genotyped as above. Three of them (S1–S3) were collected away from brûlés, while the seven others (S4–S10) were sampled on independent brûlés. They were carefully surface-washed and dissected in order to extract the whole digestive tract. The DNA was extracted from the gut and the whole fungal community was assessed by amplifying the ITS2 region with the two general primer pairs ITS3/ITS4-OF and ITS86-F/ITS4. After sequencing on an Ion Torrent sequencer (Life Technologies, Carlsbad, USA), exactly as in Schneider-Maunoury et al. (2018), the raw sequence data were processed bioinformatically as in this previous study to establish the list of fungal operational taxonomic units (OTUs). Raw sequence reads are available in GenBank under accession numbers MZ287266-289. The ecology of each OTU, that is, saprotroph, saprotroph-pathotroph, endophyte, or unknown, was inferred from that of closest relatives in GenBank and UNITE databases.

## 3. Results

### 3.1. Slug identification

Slugs used in this trial were identified as *D. invadens* after a BLASTN search against the GenBank database. The three COI amplicons showed no nucleotide polymorphisms and were deposited in GenBank under the accession number MW065802.

### 3.2. Microscope analyses

No residues of ascoma remained uneaten 48 h after starting to feed the animals with truffle. Almost all spores (97 %) of the uneaten ascoma portion (UIS) remained sequestrated within the asci after spore suspension preparation. In contrast, 36 % and 100 % of spores from rodent (RIS) and slug (SIS) faeces, respectively, were free of the asci. Staining with FDA showed that both fresh and ingested spores were all vital (Fig. S3). SEM microscopy revealed an intact episporium in RIS as UIS, while the episporium was slightly degraded and porous in spores ingested by slugs (Fig. 1).

AFM investigations also confirmed the variation in spore surface morphology among treatments. The local topographic roughness was considered as a surface descriptor since the raw topographic signal itself was uninformative due to spore morphology. The spores ingested by animals (SIS and RIS) were smoother than uningested ones (UIS), as shown by Rq and RPV variables, which significantly decreased in RIS and SIS (Fig. 2). Simultaneously with the topographic investigation, the surface phase signals (function of chemical properties) revealed an average phase decrease in SIS although it was not statistically significant ($P = 0.053$, Fig. 3). However, the phase roughness (function of chemical inhomogeneity) was significantly different among the treatments, indicating that the surface is chemically altered after slug digestion. In detail, the phase roughness of SIS was higher than UIS ($P < 0.05$) and RIS ($P < 0.001$).

The AFM investigation was also carried out in FMM mode to study the nano-mechanical properties of the surfaces. The signals recorded in FMM amplitude (describing the local stiffness of surfaces) and FMM phase (used to record the elastic properties of samples) are represented by the images of Fig. 4A. No significant variations in local stiffness were detected among treatments (Fig. 4B), but differences were recorded in roughness along the hard and soft FMM amplitude regions characterising the samples.

The hard regions (bright areas of FMM amplitude images of Fig. 4A) showed a significantly higher FMM amplitude roughness in UIS *vs* RIS ($P < 0.05$) and SIS *vs* RIS ($P < 0.001$) (Fig. 4C), while the soft regions (dark areas of FMM amplitude images of Fig. 4A) revealed significantly higher roughness medians in SIS *vs* RIS ($P < 0.05$, Fig. 4D).

The surface elasticity of the samples by FMM phase signals was statistically lower in SDS than in RIS ($P < 0.01$) and in UIS ($P < 0.001$) (Fig. 4E). The FMM phase signal, which was completely different in SIS, indicated that somehow the surfaces were altered by digestion processes, as also occurred in RIS, but to a lesser extent.

### 3.3. Plant mycorrhizal colonization

One month after inoculation, UIS and RIS seedlings showed no signs of root colonization by *T. aestivum*, while SIS seedlings had

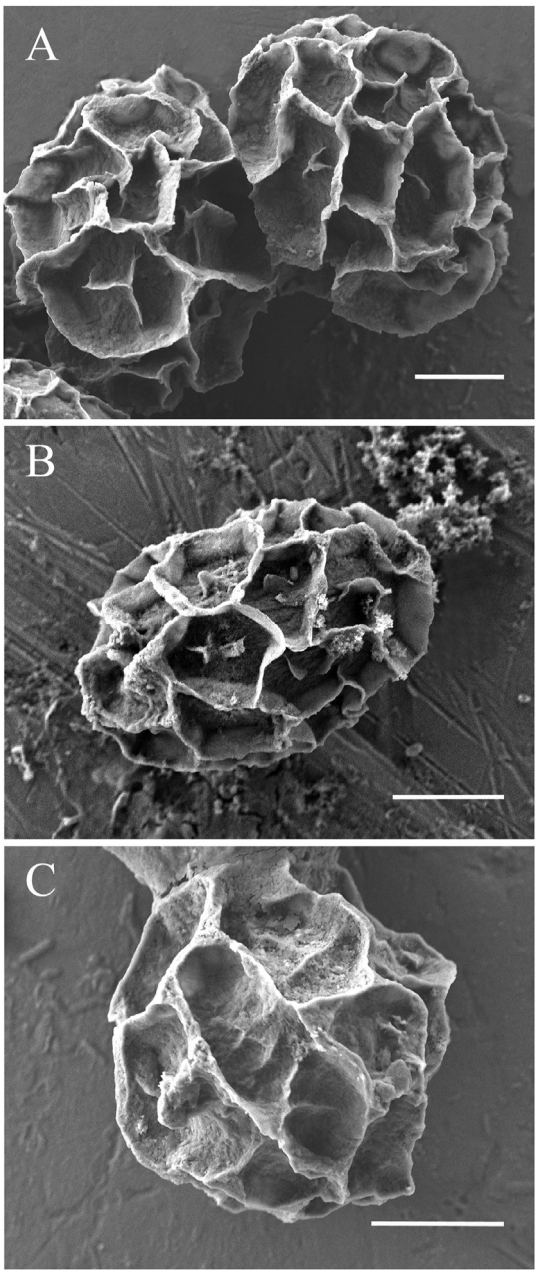


**Fig. 1. Scanning electron micrographs of spores**
*T. aestivum* spores from fresh ascoma (A), mouse scats (B), slug scats (C). Scale bar = 10 µm.

some young mycorrhizas (<1 % in total) with a thin mantle, without cystidia (Fig. S4). Molecular analyses confirmed their identity. Two and three months after inoculation, seedlings were all mycorrhized, but the colonization level in SIS seedlings was significantly higher than in UIS seedlings. Root colonization of RIS was lower than that of SIS seedlings two or three months after inoculation, but no significant differences were found between the root colonization of RIS *vs* UIS and SIS *vs* RIS (Fig. 5). No contamination with other ectomycorrhizal fungi was found during the period of investigation, since (i) all ectomycorrhizas showed the typical morphology and anatomy of *T. aestivum* and (ii) the uninoculated controls were free of any ectomycorrhizas.

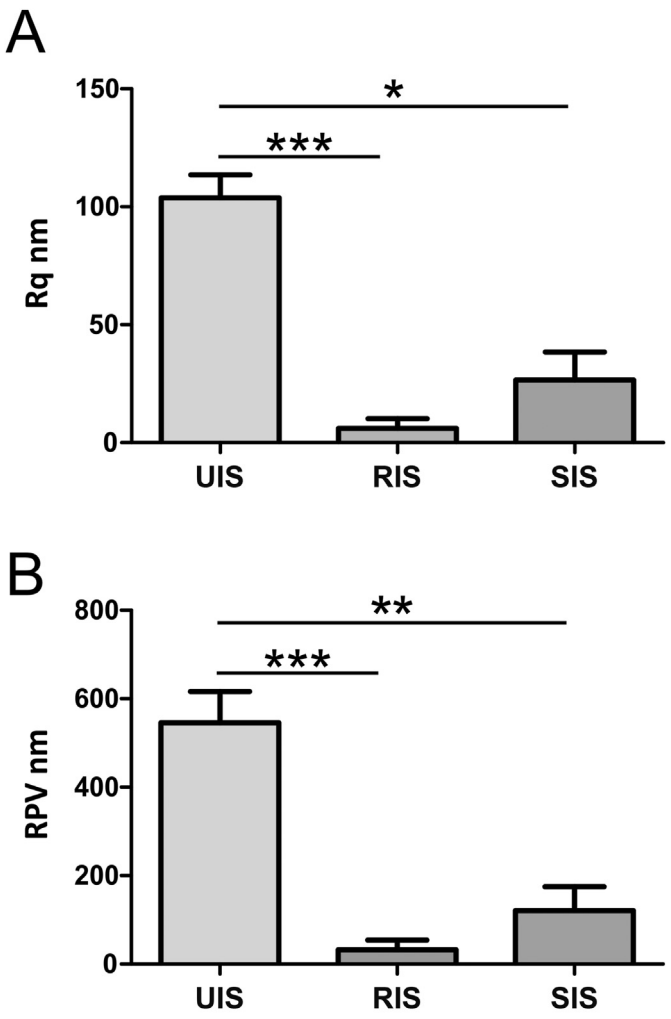


**Fig. 2. Topographic roughness of spore surface**
Topographic roughness outcomes, Rq in (A) and RPV in (B), recorded by NCM imaging of surfaces. Statistically significant differences were noted between RIS (mouse-ingested spores) and SIS (slug-ingested spores), respectively compared to UIS (uningested spores).

### 3.4. Barcoding of slug gut content

Fungal barcoding of the digestive tract of ten slugs from a *T. melanosporum* truffle ground revealed from 309 to 2,981 reads per sample (11,708 reads in all) that belonged to Asco- and Basidiomycota from groups of plant saprotrophs, plant sapro/pathotrophs and plant endophytes, as well as two ectomycorrhizal *Tuber* species, *T. melanosporum* and *Tuber brumale* Vittad. (Fig. 6A). Fungal diversity was unexpectedly low (as compared to root samples that processed in the same Ion Torrent sequencing batch, and that are reported in Schneider-Maunoury et al., 2018): in all, 1 to 5 OTUs was found per gut (versus >100 OTUs per samples in Schneider-Maunoury et al., 2018, Fig. 6B). Two of the slugs found away from the *T. melanosporum* brûlé displayed no *Tuber*, while the third revealed exclusively *T. brumale* and *T. melanosporum* reads (respectively 99.9 % and 0.1 %; Fig. 6B). The seven slugs sampled on *T. melanosporum* brûlé all displayed a dominance of reads of this species, from 70.0 % in S8 to 100 % in S6, S9 and S10, plus few *T. brumale* reads in S7 (0,005; Fig. 6B). Accordingly, direct light microscopy observation of faeces of slugs S4, S5 and S8 displayed apparently intact *T. melanosporum* spores (not shown).

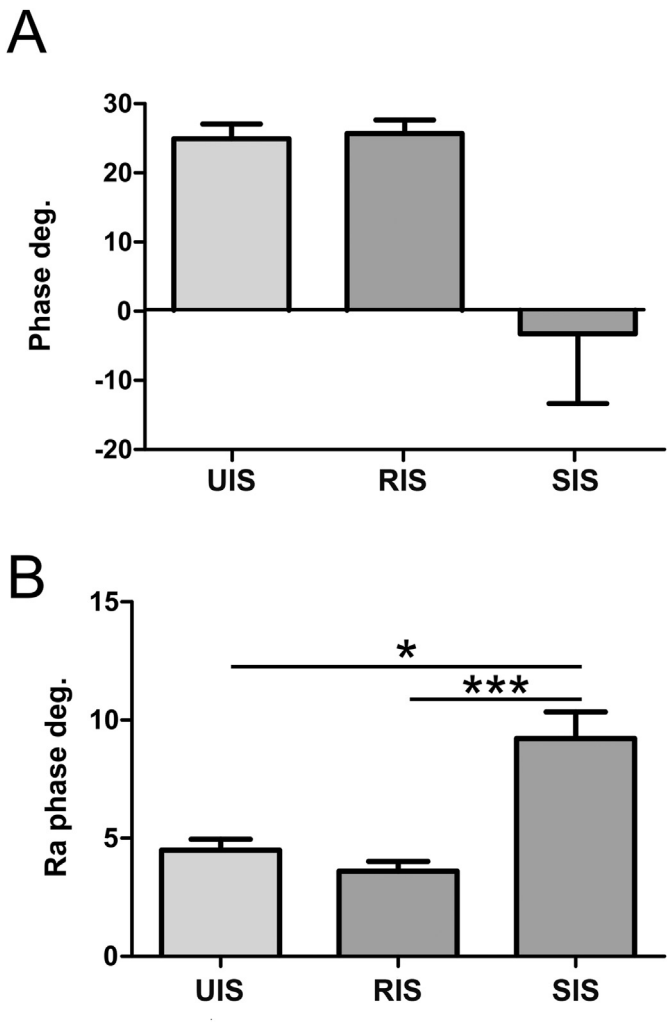


**Fig. 3. Phase signal analysis of spore surface by NCM imaging**
Phase signal analysis by NCM imaging reveals a limited overall alteration of the chemical properties of the surfaces. The plotted decrement in SIS (slug-ingested spores) was not statistically significant $P = 0.053$ (A); for the phase signal roughness analysis, SIS were statistically different from RIS (mouse-ingested spores) and UIS (unigested spores) (B).

## 4. Discussion

Studies of the effects of digestion by animals on *Tuber* spores are scarce and mainly focus on mammals. The viability of *Tuber* spores after digestion was previously studied on *Sus scrofa domesticus* L (pig) (Piattoni et al., 2014; Livne-Luzon et al., 2017), and the rodent species *Peromyscus leucopus* Rafinesque (white footed deermouse) (Miller, 1985). Germinated *T. aestivum* spores were found in scats of *Hystrix cristata* L (crested porcupine) (Ori et al., 2018). In this work, we tested for the first time a *Tuber* species (*T. aestivum*) as food for slugs and analysed the effects on the morphology and viability of spores. Our barcoding data on the gut of *D. invadens* collected from the wild, which revealed *T. brumale* and *T. melanosporum* DNA, with spores even observed for the latter species, point to the ecological relevance of our *ex-situ* analyses.

### 4.1. *Slug processing of* Tuber *spores*

In our feeding study, the highest number of free spores was found in the slug faeces, while more than half of spores were still contained within the asci in the rodent faeces. The mechanical action applied to fresh material for the inoculum preparation only freed 3 % of spores. Notwithstanding these differences, all the spores recovered from faeces, within or outside the asci, were found to be vital. Many variables can influence the effects of the passage through the digestive tract, such as differences in digestive tract anatomy, body temperature, gut microbial composition and gastrointestinal transit times (Colgan and Claridge, 2002; Danks, 2012). For instance, the percentage of free spores found in *M. musculus* faeces is lower than that observed by Piattoni et al. (2014) after passage through the pig gut. This lower value may be justified by the short gut retention time typical of this rodent species (8–18 h, Karasov et al., 1986), in comparison to the longer transit time of pigs (over 18 h, Clemens and Stevens, 1980). Some studies conducted on different slug species demonstrated that retention time may vary from 11 to 48 h and that three d of starvation may be needed to clear the gut of food (Roach, 1966; Lyth, 1982).

After microscopic analyses, the slugs appeared more efficient also in the degradation of the episporium. In particular, SEM highlighted corrosion of spore ornamentation in ascoma fragments ingested by slugs. In turn, AFM analyses revealed changes in spore wall architecture and topography, induced not only by their passage through the digestive tract, but also depending on the animal species. Slug and mouse ingested spores showed a significant increase of the roughness signal of spore surface stiffness. The slug samples behaved in the same manner as pig ingested spores (Piattoni et al., 2014), with an increase of the roughness signal of local stiffness. However, the most evident phenomenon caused by slug digestion was the chemical alteration of the episporium, which probably modified the surface elasticity of spores. Beyond the gut retention time, changes in episporium surface can be caused by the different digestive enzymatic repertoire of each animal. Given current knowledge, it is hazardous to hypothesize which polymers are mostly degraded by the digestive activities of slugs, because the chemical composition and structural organization of truffle spore walls is unknown. However, the main components of the fungal cell wall are chitin (Balestrini et al., 2000), mannan, α- and β-glucan (Noothalapati et al., 2016). Chitin is specifically degraded by chitinases (Sun et al., 1999) and acidic mammalian chitinase, classified as endochitinase, has been identified in *M. musculus* (Boot et al., 2001).

We are unaware of any studies of the enzymatic activity of slug digestive juices, but there are several studies among snails (Holden and Tracey, 1950; Myers and Northcote, 1958; de Terra and Tatum, 1961). Snails and slugs both belong to the non-achatinoid clade within the *Stylommatophora* infraorder of the order *Pulmonata* (Wade et al., 2006). The digestive juice of snails has been widely used for the lysis of the cell walls of yeast (Eddy and Williamson, 1957) and moulds (Aalders and Hall, 1962) because of its remarkable number of enzymes able to attack many substrates, such as chitin, mannans and glucans (Holden and Tracey, 1950; Myers and Northcote, 1958). The greater efficiency of spore wall degradation by slugs may also be due the anatomy of their upper digestive tract. Indeed, the mouth of Agriolimacidae has a complex radula apparatus with hundreds of teeth which is situated on a stiffening cartilaginous plate. The movements of the radula crumble food before ingestion and digestion (Wiktor, 2000). The fact that three times more *T. aestivum* spores were freed from asci by slugs than by the house mouse may be due to the slug radula, whereas larger fragments of ascoma may persist after mouse chewing, which may protect asci and spores from enzymatic lysis.

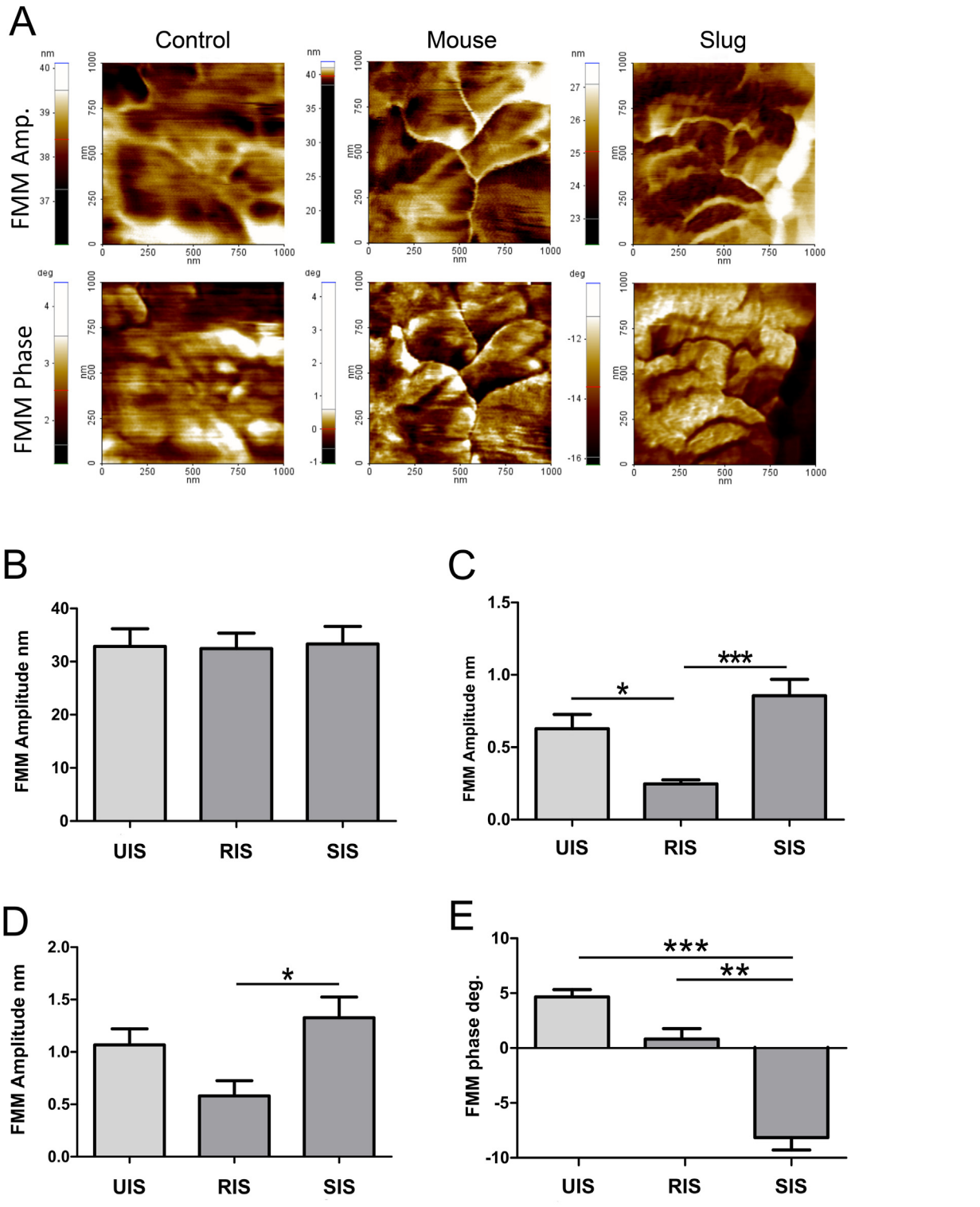


**Fig. 4. Force Modulation Mode amplitude and phase of spore surface**
FMM amplitude and phase representative images (A) The overall FMM amplitude signals remained unaltered among the samples (B); the amplitude roughness of the hard subzones of surfaces decreased in RIS (mouse-ingested spores) compared to UIS (uningested spores) and increased in SIS (slug-ingested spores) compared to RIS (C); in the soft regions, only a roughness increment in SIS compared to RIS was observed (D); surface elasticity was altered only in SIS, as indicated by FMM phase signal analyses (E).

### 4.2. *Slugs as* Tuber *spore dispersers*

Results obtained by microscope analyses are supported by the mycorrhization trial. The SIS inoculum was more efficient than the other two inocula both in terms of time and amount of root colonized. The first ectomycorrhizas occurred just one month after inoculation in SIS plants while no signs of root colonization were observed with RIS or UIS inocula. Three months after inoculation,

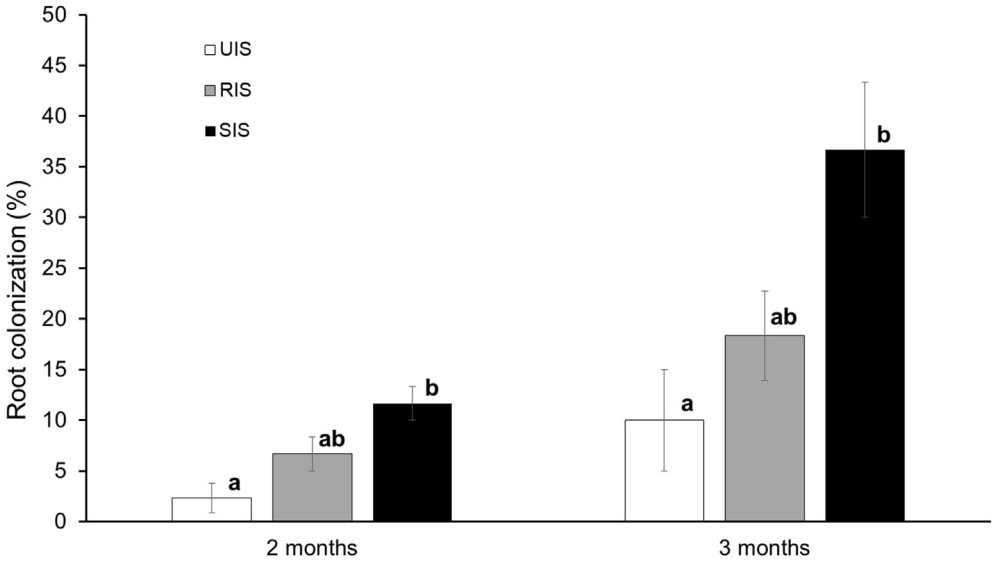


**Fig. 5. Ectomycorrhizal colonization of *Quercus robur* seedings**
Percentage of ectomycorrhizal colonization obtained by inoculation with *T. aestivum* UIS (uningested spores, white columns), RIS (mouse-ingested spores, grey columns) and SIS (slugs-ingested spores, black columns). Bars indicate standard deviation, and different letters indicate significant differences between treatments ($P < 0.05$).

UIS plants had 7 % less ectomycorrhizas than RIS plants and 25 % less than SIS plants. Miller (1985) reported that the passage through the rodent gut did not stimulate germination of *Tuber* spp. spores found in rodent faeces, unless they were refrigerated or dried after defecation. On the contrary, the plants inoculated with spores ingested by the pig showed 17 % more root colonization compared to the plants inoculated with uningested spores five months after inoculation (Piattoni et al., 2014). In our study, we confirmed that mouse spore digestion does not significantly stimulate ectomycorrhizal formation in comparison to the control plants whereas the slugs already significantly promoted plant mycorrhization 1 month after inoculation.

Slug mycophagy could have an important, and until now overlooked, role in the dispersion and reproduction of many hypogeous as well as epigeous mushrooms. As far back as the 19th century, Voglino (1895) found that slugs feed on the hymenium of many agaricoid epigeous mushrooms (*Russula* spp., *Lactarius* spp., *Tricholoma* spp., *Inocybe* spp., etc.). He found a high number of germinated basidiospores of these fungal species in the gut and scats of slugs. Later, several authors reported many slug species feeding on epigeous basidiomycetes, but they usually ignored the role of slugs in spore dispersal (Maunder and Voitk, 2010). To our knowledge, studies on slug mycophagy involving truffles are very rare. Sappa (1940) didn't see changes in episporium morphology of *Tuber magnatum* Picco spores after digestion by the snail *Helix pomatia* L. and considered this mollusc not relevant for spore germination of this truffle.

*Deroceras invadens* (used in this study) and other slug species such as *Milax gagates* Draparnaud, *Lehmannia nyctelia* Bourguignat, *Deroceras reticulatum* Muller and *Arion intermedius* Normand are considered pests for *Tuber* ascomata, in which form holes or large cavities (Mathews et al., 2019). However, their feeding activity could be as relevant for truffle dispersal than that of mammals.

Our data show that slugs from *T. melanosporum* brûlés, and also sometimes away from brûlés, contain a high level of truffle DNA: this likely reflects truffle spore resistance to digestion, rather than the true proportion of truffle in nutrition. Yet, it suggests a potential for dispersion, as shown by slug S1, which may have dispersed *T. melanosporum* and *T. brumale* away from existing brûlés.

### 4.3. *Relevance of slugs for* Tuber *life cycle*

Finally, dispersal by slugs may explain two main features of the *Tuber* life cycle. Firstly, the individuals from the two mating types are spatially separated within a truffle ground and *Tuber* ECMs occurring in a soil patch generate the maternal tissues of ascomata found in this patch (Rubini et al., 2011a; 2011b; Murat et al., 2013; Taschen et al., 2016; De la Varga et al., 2017; Leonardi et al., 2019; Schneider-Maunoury et al., 2019). This raised the question of how strains of opposite mating types can encounter each other in the field and initiate the fertilization leading to ascomata (Selosse et al., 2013). Vegetative mycelia or conidia could have a role in this process (Selosse et al., 2013; Le Tacon et al., 2016) but conidia were never found so far. The most recent studies on male gene dispersal have led many authors to speculate that germinating ascospores are the main source of paternal genotypes (Selosse et al., 2013; Murat et al., 2013; Le Tacon et al., 2016; Taschen et al., 2016; De la Varga Pastor et al., 2017; Leonardi et al., 2019). For this reason, mycophagous animals may represent the vectors by which spores of different mating types can reach their sexual partner. In particular, slugs move over short distances within a truffle ground and deliver the spores in a form that is more likely to germinate and act as male partner, facilitating mating events and, consequently, supporting the production potential of truffle grounds. The loss of truffles intended for sale due slug feeding activity could represent a reasonable loss of income compared to the beneficial effects on truffle production.

Secondly, the populations of *Tuber* spp. are characterized by a strong isolation by distance, *i.e.* physically close individuals are genetically close (Murat et al., 2013; Taschen et al., 2016; De la Varga et al., 2017). Such a situation is a bit unexpected in view of the large-scale foraging abilities of wild mammals (see discussion in Taschen et al., 2016), which should mix genotypes over long distances, but it fits a dispersal by invertebrates with a shorter foraging range, such as slugs. Especially, slugs may actively help to scatter the spore bank sequestered in the large number of ascomata that remain unremoved by mammals in truffle grounds (Schneider-Maunoury et al., 2019). The fact that spores dispersed by slugs form faster mycorrhizas, as we observed, may provide them with a first-

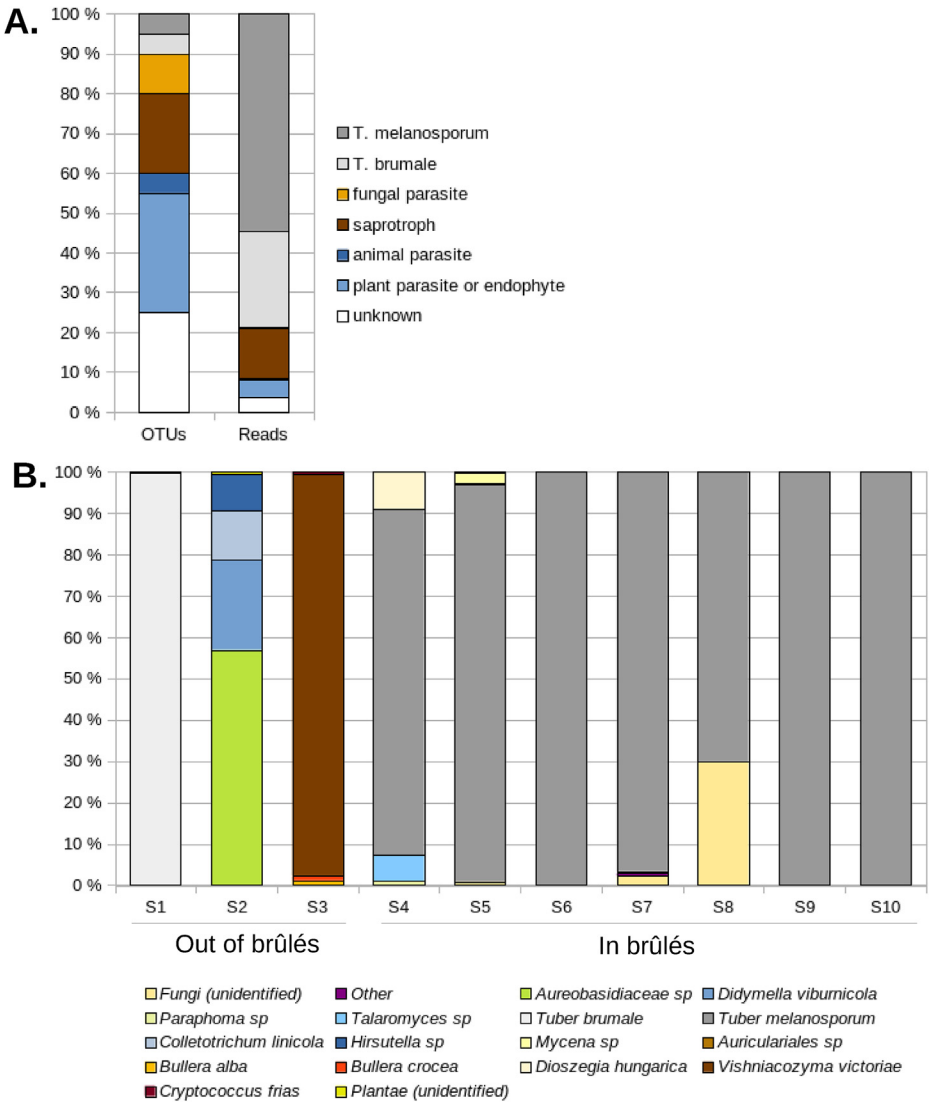


**Fig. 6. Molecular barcoding of fungal communities from slug guts**
Summary of the fungal community found in 10 slug guts (A); fungal community of the guts of the ten slugs found away from (S1 to S3) and on (S4 to S10) a *T. melanosporum* brûlé (B).

occupant advantage over those dispersed by some larger animals. The development of *Tuber* spp. populations by short-distance invertebrate dispersal may entail the observed isolation by distance. Conversely, mammals could be relevant for medium- and long-distance colonization of new areas (Murat et al., 2004; Piattoni et al., 2014).

Our results (re)open the debate about the respective roles of different mycophagous animals (molluscs, insects, rodents and other mammals) in the biological cycle of *Tuber* species. Many mammals may be opportunistic, poorly efficient feeders on an interaction that first coevolved between truffles and smaller invertebrates. However, such considerations deserve further, more specific investigations.

## Declaration of competing interest

The authors state that there are no conflicts of interest related to this publication.

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The use of snails and slugs for the production of truffle spore inoculum for plant mycorrhization has been protected by a patent application. We thank Marcin Jakalski and Sebastien Leclerc for help in bioinformatics.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2021.05.002.

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