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Effect of slug mycophagy on *Tuber aestivum* spores

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Highlights

- The effects of ascoma digestion by slugs and mouse on *Tuber aestivum* spores were compared
- SEM and AMF analyses showed that after slug digestion the spore wall is partially degraded
- Slugs collected on truffle ground contain *Tuber* spores detectable by molecular barcoding
- Slugs digest *Tuber* spores, promoting mycorrhizal infection of *Quercus robur* seedlings

1 **Effect of slug mycophagy on *Tuber aestivum* spores**

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Highlights

- The effects of ascoma digestion by slugs and a mouse on *Tuber aestivum* spores were compared
- SEM and AMF analyses showed that after slug digestion the spore wall is partially degraded
- Slugs collected on truffle ground contain *Tuber* spores detectable by molecular barcoding
- The digestion of spores by slugs promotes mycorrhizal infection of *Quercus robur* seedlings

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 of passage through the gut of a house mouse (*Mus musculus*) and slugs (*Deroceras invadens*) on the morphology and mycorrhizal infectivity of *Tuber aestivum* spores. 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 digested 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 *T. brumale*, further suggesting that some invertebrates are efficient *Tuber* spore dispersers.

Keywords: truffle; ectomycorrhizas; spores; mycophagy; microscopy.

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 feature 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) and improve their ability to germinate (Piattoni et al., 2014).

Varying levels of mycophagy has been shown to be widespread among both invertebrates and vertebrates (Hammond and Lawrence, 1989; Nuske et al., 2017; Elliot 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 feeds on fungi at times when they are abundant in the environment (Maser et al., 1978). Thus, the quantity of fungi eaten varies in every species

78 (Schickmann et al., 2012, Claridge and Trappe, 2005). Mammals are the most studied class of
79 mycophagists, although few of them feed exclusively on fungi (Fogel and Trappe, 1978; Stephenson,
80 2010). Among the more recent phenomena of mycophagy investigated in the last few years, Elliott et
81 al. (2018) documented the feeding of the mammal *Erinaceus concolor* Martin (~~the~~ Eastern European
82 hedgehog) on the hypogeous *Rhizopogon vulgaris* (Vittad.) M. Lange and Ori et al. (2018) reported
83 the consumption of *Tuber aestivum* Vittad. by *Hystrix cristata* L. (North African crested porcupine).
84 *Menura novaehollandiae* Latham (~~the~~ superb lyrebird) was recently found to feed on different species
85 of hypogeous fungi (Elliot and Vernes, 2019). Among invertebrates, insects are the most studied
86 group of mycophagists (Witte and Maschwitz, 2008, Disney et al., 2013). *Leiodes* spp. coleopters and
87 dipterous *Suillia* spp. are the most common insects found in *Tuber* ascomata, but many other genera
88 in Coleoptera and Diptera feed on them (Menta and Pinto, 2016; Rosa-Gruszecka et al., 2017), which
89 may even challenge the future of truffle production. Unlike mammal mycophagists, insects do not
90 completely eat ascoma, but their feeding cavities considerably reduce the economic value of truffles.
91 Little is known about the mycophagy of molluscs and available studies mainly concern the
92 consumption of epigeous fungi. Keller and Snell (2002) proved the ingestion of fungi by several slug
93 species. True truffles in the genus *Tuber* are also used as food by slugs. Parks (1921) reported that
94 small slugs attack *Tuber californicum* Harkness in its early stages and feed on the gleba, leaving the
95 peridium as an empty shell. It often happens that ascomata of valuable *Tuber* species partially eaten
96 by slugs are found inside natural and cultivated truffle orchards (Fig. S1) causing great economic
97 losses (Mathews et al., 2019). Since it's been proven that mycophagous mammals contribute to spore
98 dispersal, improve spore germinability and might be involved in sexual reproduction (Piattoni et al.,
99 2014; Taschen et al., 2016), we hypothesized that slugs could play a similar role in the biological
100 cycle of truffles as short-distance dispersal agents. In order to test this hypothesis we fed slugs
101 (*Derocera invadens* Reise, Hutchinson, Schunack & Schlitt) with *T. aestivum* ascomata and compared
102 the effects of gut digestion by these molluscs with ascomata digested by a house mouse (*Mus*
103 *musculus* L.) and with undigested ascomata. Changes in the morphology of digested and undigested

104 spores were analysed by scanning electronic microscopy (SEM) and atomic force microscopy (AFM)
105 while their ability to germinate and to form ectomycorrhizas was tested by inoculating oak (*Quercus*
106 *robur* L.) seedlings. Finally, we sampled slugs on a truffle ground to analyse their gut content by
107 molecular barcoding, in order to investigate the spontaneous intake of truffles by slugs.
108

109 ## 2. Materials and Methods

110 ### *2.1 Animal feeding and faecal sampling*

111 A single *T. aestivum* ascoma of 95 g collected in the L'Aquila municipality (Abruzzo, Italy; on May
112 2018) was selected for analyses and maintained at 4 °C for 2 days before feeding the animals. A
113 fragment of the ascoma was dried and deposited in the Herbarium Mycologicum Aquilanum (AQUI)
114 under the ID code 10231.

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

122 Both the animal species were fed for 3 days with lettuce (slugs) or commercial rat food (house mouse)
123 and then for 24 h with boiled potatoes (both animal species) to clear the gut as much as possible of
124 recalcitrant plant polymers. Scats produced during these four days were excluded from analyses. On
125 the fifth day, the animals were fed with 60 g of the *T. aestivum* ascoma (30 g per animal). An
126 additional 30 g of the ascoma was stored at -4 °C pending microscope analysis and inoculum
127 preparation of fresh material. Over the following 48 h all the faeces from each animal species were
128 collected and pooled together, immediately placed in sterile distilled water, crushed with a mortar
129 and pestle and blended at high speed for approximately 5 min. The undigested portion of the ascoma

130 was treated in the same way as faecal material. Slug and house mouse digested spores (SDS and RDS,
131 respectively) and undigested spores (UDS) were then maintained at 4 °C until microscopy analyses
132 and inoculum preparation.
133
134 *2.2 Microscopy of digested and undigested spores*
135 In order to quantify the spore density and the ratio of spores free of asci, a drop of spore suspension
136 from each treatment was put on a haemocytometer and examined under a light microscope Axiostar
137 plus (Zeiss, Oberkochen, Germany) at 400 magnifications. Spores' vitality was evaluated with
138 fluorescein diacetate stain (FDA) after Colgan and Claridge (2002). Spores were then viewed with an
139 Eclipse TE 2000-E microscope (Nikon) fitted with a 450-490 nm excitation filter and 515-555 nm
140 barrier filter.
141
142 *2.3 SEM analysis*
143 Fresh and digested spores were also prepared for SEM observation to assess the morphology of spore
144 surfaces and ornamentations. A drop of spore suspension of each treatment was placed on an
145 aluminium SEM stub (diameter 12 mm) and dried. The samples were then sputter-coated with a thin
146 gold film using an AGAR automatic sputter coater (10 mA, 30 s) and examined by use of a FESEM
147 Zeiss Gemini 500.
148
149 *2.4 AFM analysis*
150 Spores were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 h at room
151 temperature and layered on cover glass by centrifugation. After washing with ultrapure water (18.2
152 MU) the samples were layered on fresh muscovite mica and dried by nitrogen flow.
153 The AFM analysis was carried out with an XE-100 Atomic Force microscope (PARK Systems Inc.,
154 Suwon, South Korea). The microscope was equipped with a 50 µm scanner controlled by the XEP
155 1.8 software. The X-Y stages and the Z scan worked in a closed loop manner and in high voltage

156 mode. The speed scan was set between 0.2 Hz and 1.5 Hz. The cantilevers used in this study were
157 Non-Contact High Resonant (NCHR) tips (spring constant between 35 and 42 N m^{-1}) with a typical
158 resonant frequency between 200 and 300 kHz. The instrument was set in true non-contact mode for
159 imaging the samples during preliminary investigations and for topography and phase imaging. For
160 nanomechanical surface characterization the instrumentation was set in Force Modulation Mode
161 (FMM) with an amplitude setpoint of 538 nm. All images and data were analysed by XEI software
162 (PARK Systems Inc., Suwon, South Korea). Spores for each treatment (UDS, SDS and RDS) were
163 analysed and imaging was performed in the centre of three different alveoli for each spore (Fig. S2).
164 Roughness (Rq and RPV), surface phase and FMM variables from the three treatments were
165 statistically analysed using Kruskal-Wallis non-parametric ANOVA followed by Dunn's Multiple
166 Comparison test. Statistics were performed using GraphPad Prism software.
167

168 *2.5 Seedling material and inoculation*

169 Seeds of *Q. robur* were collected in October 2017 from a single plant and stored at 4 °C until sowing.
170 The surface was sterilized in 1% sodium hypochlorite solution for 1 h and rinsed with distilled water.
171 Oak seedlings were grown in sterile (120 °C for 1 h) peat vermiculite substrate (1:9 v/v) for about 4
172 weeks before inoculation. Seedlings were maintained at 20 ± 1 °C and in a 14 h photoperiod (5000
173 lux).
174 Oak seedlings were separately inoculated with the spore suspensions obtained from mouse scats
175 (RDS), slug scats (SDS) or from the remaining 30 g of undigested *T. aestivum* ascoma (UDS). Spore
176 suspensions from all treatments were adjusted to 1×10^6 spores mL^{-1} before being used as inoculum.
177 Thirty-six seedlings were transplanted into plastic pots (750 mL) filled with a sterile mixture of peat,
178 vermiculite and sand (2:4:4). At transplant, 9 seedlings were inoculated for each treatment and an
179 additional 9 seedlings were used as controls. All seedlings were grown in a climate room at 20 ± 1°C
180 under controlled light conditions (14-h photoperiod, 5,000 lux) and watered with tap water twice a
181 week. Root infection was evaluated 1, 2 and 3 months after inoculation, checking three seedlings for

182 each treatment each time. All seedlings were removed from their pots, the root systems were washed
183 with tap water and the ectomycorrhizas were counted under a dissecting microscope (20×). From
184 each seedling, 10 root fragments 4-5 cm in length were randomly excised from the whole root system.
185 Ectomycorrhizas of *T. aestivum* were identified by morphotyping after Zambonelli et al. (1993). The
186 identity of young ectomycorrhizas lacking cystidia was molecularly confirmed by species-specific
187 PCR after Mello et al. (2002), in order to exclude any root contamination by other fungal species. A
188 little fragment of the mantels was excided from each mycorrhizas and DNA was amplified by a direct
189 PCR approach (Iotti and Zambonelli, 2006). The degree of root colonization was measured by
190 counting the number of colonized and un-colonized root tips and the results were expressed as a
191 percentage of infected tips out of the total number of tips examined. Two-way ANOVA was applied
192 to determine significant differences among treatments and monthly root colonization. Means were
193 compared with the Tukey post-hoc test ($P < 0.05$). Statistical analyses were carried out with XL-
194 STAT Software version (Addinsoft Inc., New York, NY, USA).
195
196 *2.6 Barcoding of slug gut content*
197 The slugs were collected in a *T. melanosporum* truffle ground located at Chartrier-Ferrière, France
198 (45°04'15"N, 01°27'05"E; truffle ground T6 in Schneider-Maunoury et al., 2018). This choice was
199 made because the presence of this truffle is very clearly delineated by a zone of poor vegetation
200 growth, called the brûlé (*e.g.* Taschen *et al.*, 2016), which is more conspicuous than for other *Tuber*
201 species (Streiblová et al., 2012). In this truffle ground, ten individuals of *Deroceras invadens* were
202 collected in January to February 2017, and genotyped as above. Three of them (S1-S3) were collected
203 away from brûlés, while the seven others (S4-S10) were sampled on independent brûlés. They were
204 carefully surface-washed and dissected in order to extract the whole digestive tract. The DNA was
205 extracted from the gut and the whole fungal community was assessed by amplifying the ITS2 region
206 with the two general primer pairs ITS3/ITS4-OF and ITS86-F/ITS4. After sequencing on an Ion
207 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 XXX-XXX. 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. No residues of ascoma remained uneaten 48 h after starting to feed the animals with truffle.

3.2 Microscope analyses

Almost all spores (97%) of the undigested ascoma (UDS) remained sequestrated within the asci after spore suspension preparation. In contrast, 36% and 100% of spores from rodent (RDS) and slug (SDS) faeces, respectively, were free of the asci. Staining with FDA showed that both fresh and digested spores were all vital (Fig. S3). SEM microscopy revealed an intact episporium in RDS as UDS, while the episporium was slightly degraded and porous in spores digested 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 digested by animals (SDS and RDS) were smoother than undigested ones (UDS), as shown by Rq and RPV variables, which significantly decreased in RDS and SDS (Fig. 2). Simultaneously with the topographic investigation, the surface phase signals (function of chemical properties) revealed an average phase decrease in SDS although

234 it was not statistically significant (P = 0.053, Fig. 3). However, the phase roughness (function of
235 chemical inhomogeneity) was significantly different among the treatments, indicating that the surface
236 is chemically altered after slug digestion. In detail, the phase roughness of SDS was higher than UDS
237 ($P < 0.05$) and RDS ($P < 0.001$).
238 The AFM investigation was also carried out in FMM mode to study the nano-mechanical properties
239 of the surfaces. The signals recorded in FMM amplitude (describing the local stiffness of surfaces)
240 and FMM phase (used to record the elastic properties of samples) are represented by the images of
241 Figure 4A. No significant variations in local stiffness were detected among treatments (Fig. 4B), but
242 differences were recorded in roughness along the hard and soft FMM amplitude regions
243 characterising the samples.
244 The hard regions (bright areas of FMM amplitude images of Figure 4A) showed a significantly higher
245 FMM amplitude roughness in UDS *vs* RDS ($P < 0.05$) and SDS *vs* RDS ($P < 0.001$) (Fig. 4C), while
246 the soft regions (dark areas of FMM amplitude images of Figure 4A) revealed significantly higher
247 roughness medians in SDS *vs* RDS ($P < 0.05$, Fig. 4D).
248 The surface elasticity of the samples by FMM phase signals was statistically lower in SDS than in
249 RDS ($P < 0.01$) and in UDS ($P < 0.001$) (Fig. 4E). The FMM phase signal, which was completely
250 different in SDS, indicated that somehow the surfaces were altered by digestion processes, as also
251 occurred in RDS, but to a lesser extent.
252

253 *3.3 Plant mycorrhizal colonization*

254 One month after inoculation, UDS and RDS seedlings showed no signs of root colonization by *T.*
255 *aestivum*, while SDS seedlings had some young mycorrhizas (< 1% in total) with a thin mantle,
256 without cystidia (Fig. S4). Molecular analyses confirmed their identity. Two and three months after
257 inoculation, seedlings were all mycorrhized, but the colonization level in SDS seedlings was
258 significantly higher than in UDS seedlings. Root colonization of RDS was lower than that of SDS
259 seedlings two or three months after inoculation, but no significant differences were found between

the root colonization of RDS *vs* UDS and SDS *vs* RDS (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.

3.4. Barcoding of slug gut content

Fungal barcoding of the digestive tract of ten slugs from a *T. melanosporum* truffle ground revealed from 420 to 3921 reads per sample (15209 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 *T. brumale* (Fig. 6A). Fungal diversity was unexpectedly low (as compared to root samples processed in the same run in Schneider-Maunoury et al., 2018): in all, 1 to 10 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 six slugs sampled on *T. melanosporum* brûlé all displayed a dominance of reads of this species, from 72.0% in S8 to 100% in S9 and S10, plus few *T. brumale* reads in S4 (0.4%; Fig. 6B). Accordingly, direct light microscopy observation of faeces of slugs S4, S5 and S8 displayed apparently intact *T. melanosporum* spores (not shown).

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

286 the morphology and viability of spores. Our barcoding data on the gut of *D. invadens* collected from
287 the wild, which revealed *T. brumale* and *T. melanosporum* DNA, with spores even observed for the
288 latter species, point to the ecological relevance of our *ex-situ* analyses.
289
290 4.1. *Slug processing of* Tuber *spores*
291 In our feeding study, the highest number of free spores was found in the slug faeces, while more than
292 half of spores were still contained within the asci in the rodent faeces. The mechanical action applied
293 to fresh material for the inoculum preparation only freed 3% of spores. Notwithstanding these
294 differences, all the spores recovered from faeces, within or outside the asci, were found to be vital.
295 Many variables can influence the effects of the passage through the digestive tract, such as differences
296 in digestive tract anatomy, body temperature, gut microbial composition and gastrointestinal transit
297 times (Colgan and Claridge, 2002; Danks, 2012). For instance, the percentage of free spores found in
298 *M. musculus* faeces is lower than that observed by Piattoni et al. (2014) after passage through the pig
299 gut. This lower value may be justified by the short gut retention time typical of this rodent species (8-
300 18 h, Karasov et al., 1986), in comparison to the longer transit time of pigs (over 18 h, Clemens and
301 Steven, 1980). Some studies conducted on different slug species demonstrated that retention time
302 may vary from 11 to 48 h and that three days of starvation may be needed to clear the gut of food
303 (Roach, 1966; Lyth, 1982).
304 After microscopic analyses, the slugs appeared more efficient also in the digestion of the episporium.
305 In particular, SEM highlighted corrosion of spore ornamentation in ascoma fragments digested by
306 slugs. In turn, AFM analyses revealed changes in spore wall architecture and topography, induced
307 not only by their passage through the digestive tract, but also depending on the animal species. Slug
308 and mouse digested spores showed a significant increase of the roughness signal of spore surface
309 stiffness. The slug samples behaved in the same manner as pig digested spores (Piattoni et al., 2014),
310 with an increase of the roughness signal of local stiffness. However, the most evident phenomenon
311 caused by slug digestion was the chemical alteration of the episporium, which probably modified the

312 surface elasticity of spores. Beyond the gut retention time, changes in episporium surface can be
313 caused by the different digestive enzymatic repertoire of each animal. Given current knowledge, it is
314 hazardous to hypothesize which polymers are mostly degraded by the digestive activities of slugs,
315 because the chemical composition and structural organization of truffle spore walls is unknown.
316 However, the main components of the fungal cell wall are chitin (Balestrini et al., 2000), mannan, α-
317 and β-glucan (Noothalapati et al., 2016). Chitin is specifically degraded by chitinases (Sun et al.,
318 1999) and acidic mammalian chitinase, classified as endochitinase, has been identified in *M. musculus*
319 (Boot et al., 2001).
320 We are unaware of any studies of the enzymatic activity of slug digestive juices, but there are several
321 studies among snails (Myers and Northcote, 1958; Holden and Tracey, 1950; de Terra and Tatum,
322 1961). Snails and slugs both belong to the non-achatinoid clade within the *Stylommatophora*
323 infraorder of the order *Pulmonata* (Wade et al., 2006). The digestive juice of snails has been widely
324 used for the lysis of the cell walls of yeast (Eddy and Williamson, 1957) and moulds (Aalders and
325 Hall, 1962) because of its remarkable number of enzymes able to attack many substrates, such as
326 chitin, mannans and glucans (Myers and Northcote, 1958; Holden and Tracey, 1950). The greater
327 efficiency of spore wall degradation by slugs may also be due the anatomy of their upper digestive
328 tract. Indeed, the mouth of Agriolimacidae has a complex radula apparatus with hundreds of teeth
329 which is situated on a stiffening cartilaginous plate. The movements of the radula crumble food before
330 ingestion and digestion (Wiktor, 2000). The fact that three time more *T. aestivum* spores were freed
331 from asci in slugs than in the house mouse may be due to the slug radula, whereas larger fragments
332 of ascoma may persist after mouse chewing, which may protect asci and spores from enzymatic lysis.
333

334 *4.2 Slugs as* Tuber *spore dispersers*

335 Results obtained by microscope analyses are supported by the mycorrhization trial. The SDS
336 inoculum was more efficient than the other two inocula both in terms of time and amount of root
337 colonized. The first ectomycorrhizas occurred just one month after inoculation in SDS plants while

338 no signs of root colonization were observed with RDS or UDS inocula. Three months after
339 inoculation, UDS plants had 7% less ectomycorrhizas than RDS plants and 25% less than SDS plants.
340 Miller (1985) reported that the passage through the rodent gut did not stimulate germination of *Tuber*
341 spp. spores found in rodent faeces, unless they were refrigerated or dried after defecation. On the
342 contrary, the plants inoculated with spores digested by the pig showed 17% more root colonization
343 compared to the plants inoculated with undigested spores five months after inoculation (Piattoni et
344 al., 2014). In our study, we confirmed that mouse spore digestion does not significantly stimulate
345 ectomycorrhizal formation with respect to the control plants whereas the slugs already significantly
346 promoted plant mycorrhization 1 month after inoculation.
347 Slug mycophagy could have an important, and until now overlooked, role in the dispersion and
348 reproduction of many hypogeous as well as epigeous mushrooms. As far back as the 19th century,
349 Voglino (1895) found that slugs feed on the hymenium of many agaricoid epigeous mushrooms
350 (*Russula* spp., *Lactarius* spp., *Tricholoma* spp., *Inocybe* spp., etc.). He found a high number of
351 germinated basidiospores of these fungal species in the gut and scats of slugs. Later, several authors
352 reported many slug species feeding on epigeous basidiomycetes, but they usually ignored the role of
353 slugs in spore dispersal (Maunder and Voitk, 2010). To our knowledge, studies on slug mycophagy
354 involving truffles are very rare. Sappa (1940) didn't see changes in episporium morphology of *T.*
355 *magnatum* spores after digestion by the snail *Helix pomatia* L. and considered this mollusc not
356 relevant for spore germination of this truffle.
357 *Deroceras invadens* (used in this study) and other slug species such as *Milax gagates* Draparnaud,
358 *Lehmannia nyctelia* Bourguignat, *Deroceras reticulatum* Muller and *Arion intermedius* Normand are
359 considered pests for *Tuber* ascomata, in which form holes or large cavities (Mathews et al., 2019).
360 However, their feeding activity could be as or even more relevant for truffle dispersal than that of
361 mammals.
362 Our data show that slugs from *T. melanosporum* brûlés, but also sometimes away from brûlés, contain
363 a high level of truffle DNA: this likely reflects truffle spore resistance to digestion, rather than the

364 true proportion of truffle in nutrition. Yet, it suggests a potential for dispersion, as shown by slug S1,
365 which may have dispersed *T. melanosporum* and *T. brumale* away from existing brûlés.
366
367 *4.3 Relevance of slugs for* Tuber *life cycle*
368 Finally, dispersal by slugs may explain two main features of the *Tuber* life cycle. Firstly, the
369 individuals from the two mating types are spatially separated within a truffle ground and *Tuber* ECMs
370 occurring in a soil patch generate the maternal tissues of ascomata found in this patch (Rubini et al.,
371 2011; Murat et al., 2013; Taschen et al., 2016; De la Varga et al., 2017; Leonardi et al., 2019;
372 Schneider-Maunoury et al., 2019). This raised the question of how strains of opposite mating types
373 can encounter each other in the field and initiate the fertilization leading to ascomata (Selosse et al.,
374 2013). Vegetative mycelia or propagules could have a role in this process but the most recent studies
375 have led many authors to speculate that germinating spores are the main source of paternal genotypes
376 (Selosse et al., 2013; Murat et al., 2013; Le Tacon et al., 2016; Taschen et al., 2016; De la Varga et
377 al., 2017; Leonardi et al., 2019). For this reason, mycophagous animals may represent the vectors by
378 which spores of different mating types can reach their sexual partner. In particular, slugs move over
379 short distances within a truffle ground and deliver the spores in a form that is more likely to germinate
380 and act as male partner, facilitating mating events and, consequently, supporting the production
381 potential of truffle grounds. The loss of truffles intended for sale due slug feeding activity could
382 represent a reasonable loss of income compared to the beneficial effects on truffle production.
383 Secondly, the populations of *Tuber* spp. are characterized by a strong isolation by distance, *i.e.*
384 physically close individuals are genetically close (Murat et al., 2013; Taschen et al., 2016; De la
385 Varga et al., 2017). Such a situation is a bit unexpected in view of the large-scale foraging abilities
386 of wild mammals (see discussion in Taschen et al., 2016), which should mix genotypes over long
387 distances, but it fits a dispersal by invertebrates with a shorter foraging range, such as slugs.
388 Especially, slugs may actively help to scatter the spore bank sequestered in the large number of
389 ascomata that remain unremoved by mammals in truffle grounds (Schneider-Maunoury et al., 2019).

390 The fact that spores dispersed by slugs form faster mycorrhizas, as we observed, may provide them
391 with a first-occupant advantage over those dispersed by some larger animals. The development of
392 *Tuber* spp. populations by short-distance invertebrate dispersal may entail the observed isolation by
393 distance. Conversely, mammals could be relevant for medium- and long-distance colonization of new
394 areas (Murat et al., 2004; Piattoni et al., 2014).
395 Our results (re)open the debate about the respective roles of different mycophagous animals
396 (molluscs, insects, rodents, mammals) in the biological cycle of *Tuber* species. Many mammals may
397 be opportunistic, poorly efficient feeders on an interaction that first coevolved between truffles and
398 smaller invertebrates. However, such considerations deserve further, more specific investigations.
399
400
401
402 **Declaration of competing interest**
403 The authors state that there are no conflicts of interest related to this publication.
404
405 **Acknowledgments**
406 The use of snails and slugs for the production of truffle spore inoculum for plant mycorrhization has
407 been protected by a patent application. We thank Marcin Jakalski for help with bioinformatics.
408
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564
565
566 **Figure captions**
567
568 **Figure 1 – Scanning electron micrographs of spores**
569 *T. aestivum* spores from fresh ascoma (A), mouse scats (B), slug scats (C). The figure backgrounds
570 were removed by Adobe Photoshop® Software (original images are supplied in supplementary file
571 Fig. S5). Scale bar = 10 µm
572
573 **Figure 2 – Topographic roughness of spore surface**
574 Topographic roughness outcomes, Rq in (A) and RPV in (B), recorded by NCM imaging of surfaces.
575 Statistically significant differences were noted between RDS (mouse-digested spores) and SDS (slug-
576 digested spores), respectively compared to UDS (undigested spores)
577
578 **Figure 3 - Phase signal analysis of spore surface by NCM imaging**
579 Phase signal analysis by NCM imaging reveals a limited overall alteration of the chemical properties
580 of the surfaces. The plotted decrement in SDS (slug-digested spores) was not statistically significant
581 $P = 0.053$ (A); for the phase signal roughness analysis, SDS were statistically different from RDS
582 (mouse-digested spores) and UDS (undigested spores) (B)
583
584 **Figure 4 – Force Modulation Mode amplitude and phase of spore surface**

585 FMM amplitude and phase representative images (A) The overall FMM amplitude signals remained
586 unaltered among the samples (B); the amplitude roughness of the hard subzones of surfaces decreased
587 in RDS (mouse-digested spores) compared to UDS (undigested spores) and increased in SDS (slug-
588 digested spores) compared to RDS (C); in the soft regions, only a roughness increment in SDS
589 compared to RDS was observed (D); surface elasticity was altered only in SDS, as indicated by FMM
590 phase signal analyses (E)
591
592 **Figure 5 – Ectomycorrhizal colonization of *Quercus robur* seedings**
593 Percentage of ectomycorrhizal colonization obtained by inoculation with *T. aestivum* UDS
594 (undigested spores, white columns), RDS (mouse-digested spores, grey columns) and SDS (slugs-
595 digested spores, black columns). Bars indicate standard deviation, and different letters indicate
596 significant differences between treatments ($P < 0.05$)
597
598 **Figure 6 – Molecular barcoding of fungal communities from slug guts**
599 Summary of the fungal community found in 10 slug guts (A); fungal community of the guts of the
600 ten slugs found away from (S1 to S3) and on (S4 to S10) a *T. melanosporum* brûlé (B)
601
602 **Captions of supplementary material**
603
604 **Table S1 –** Fungal OTUs recovered from slug gut.
605
606 **Figure S1 –** *Tuber aestivum* ascomata, found in a private truffle orchard, partially eaten by slugs.
607
608 **Figure S2 -** The picture shows a *T. aestivum* spore imaged in NCM AFM and the arrows indicate the
609 regions employed for all AFM investigations.

610

611 **Figure S3 –** Vitality assessment of *T. aestivum* spores stained with fluorescein diacetate (FDA) of (a)
612 fresh truffle, (b) digested by the mouse and (c) by slugs.

613

614 **Figure S4 –** Young mycorrhiza of *T. aestivum* obtained in seedlings inoculated with slug-digested
615 spores, one month after inoculation.

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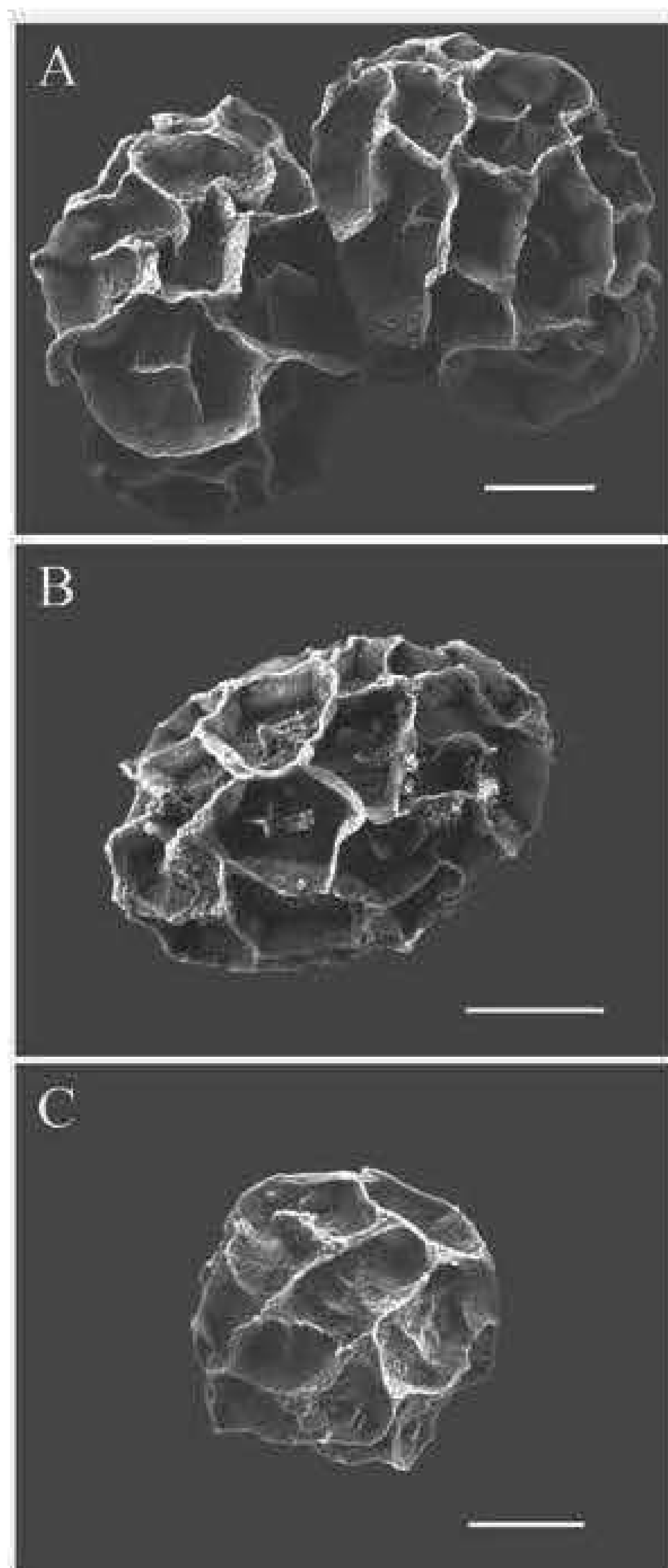

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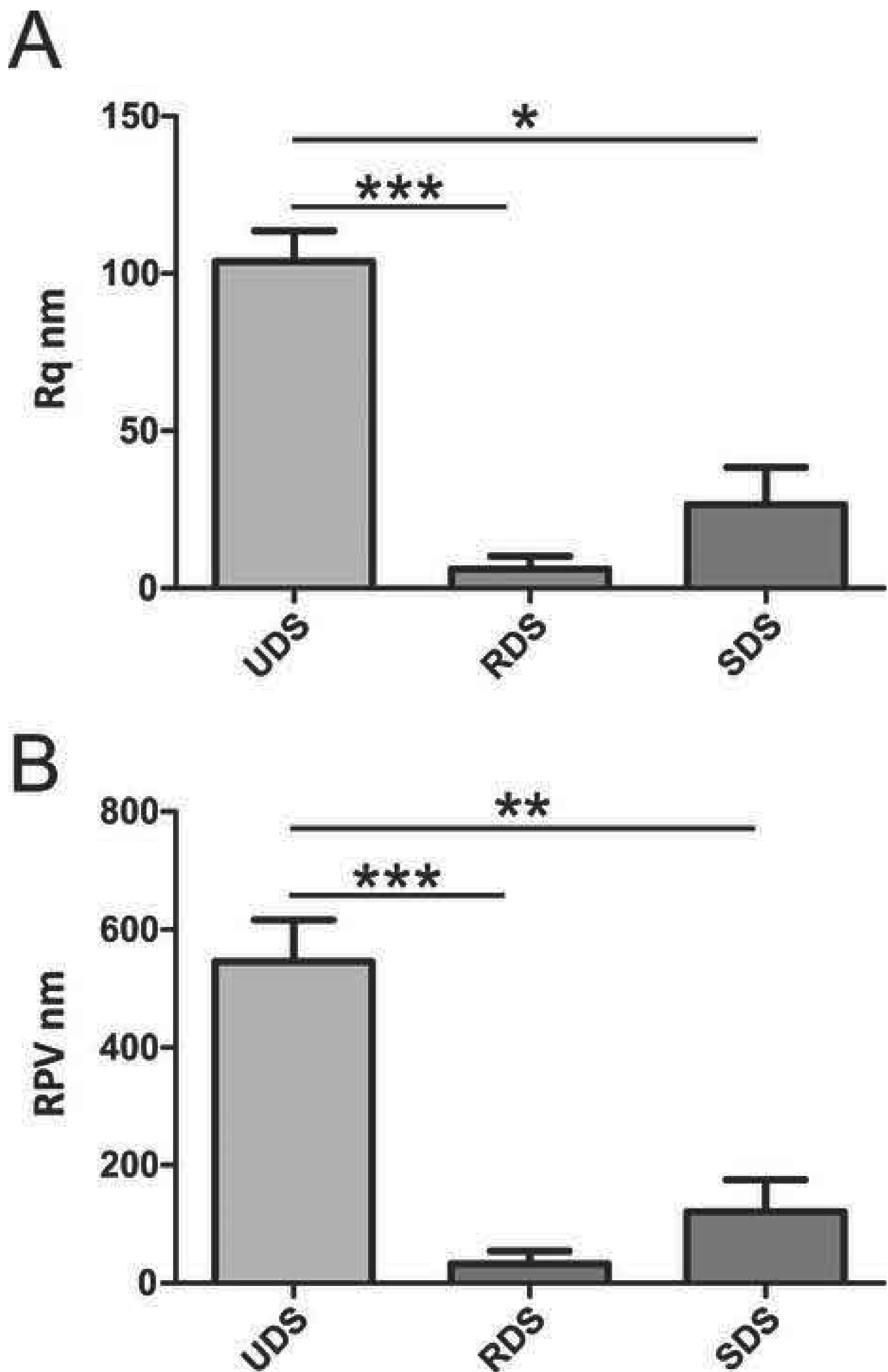

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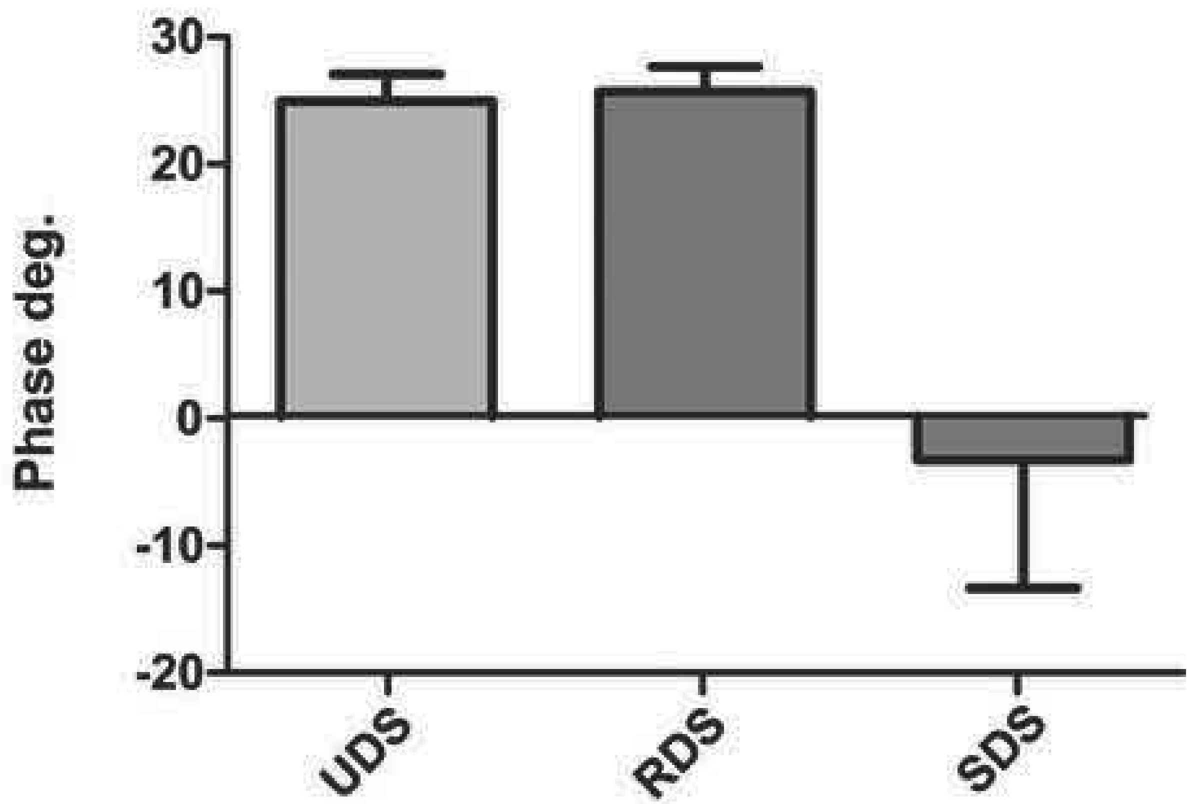

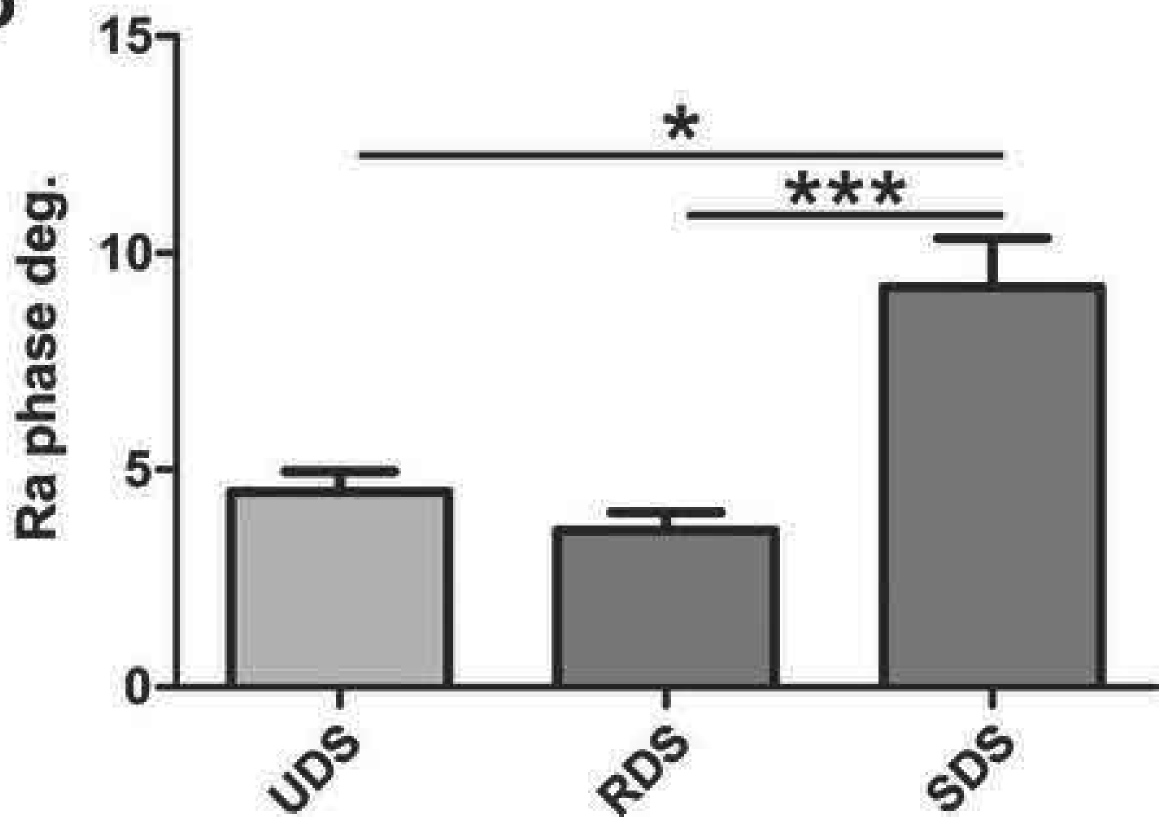

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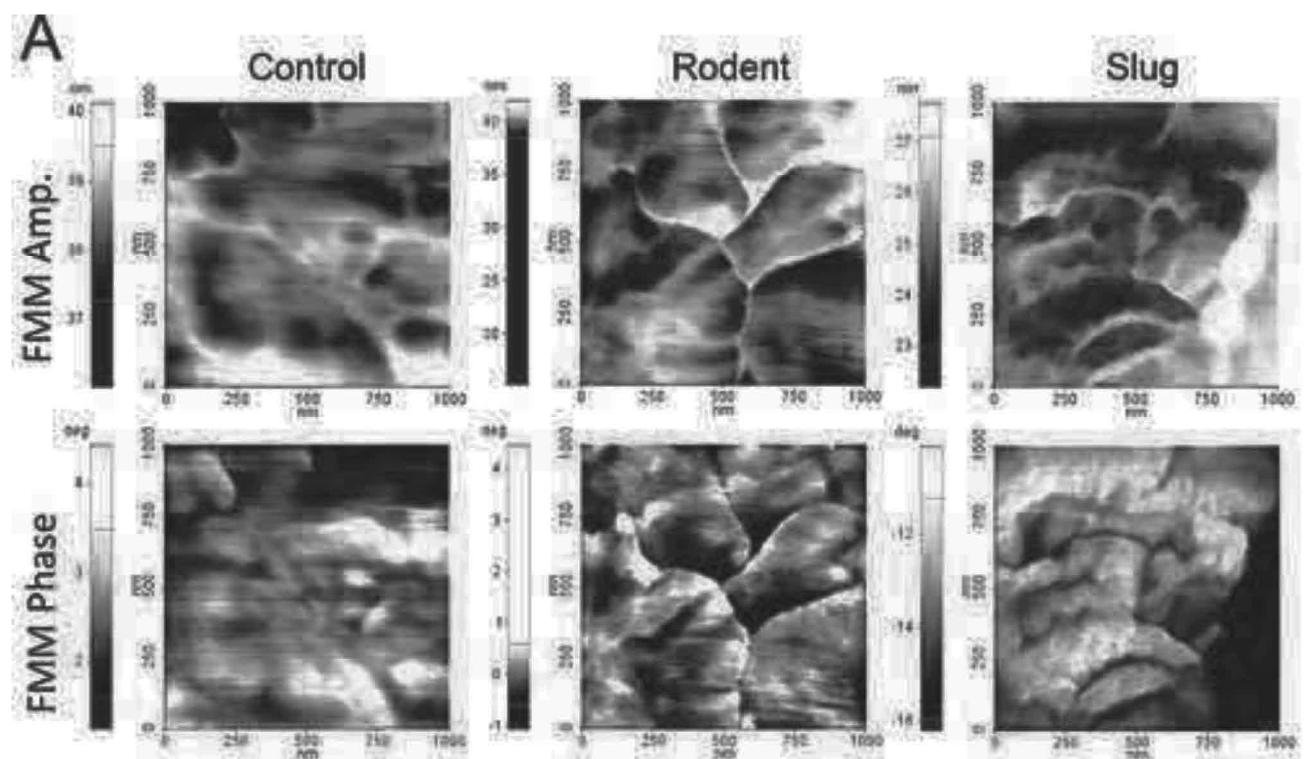

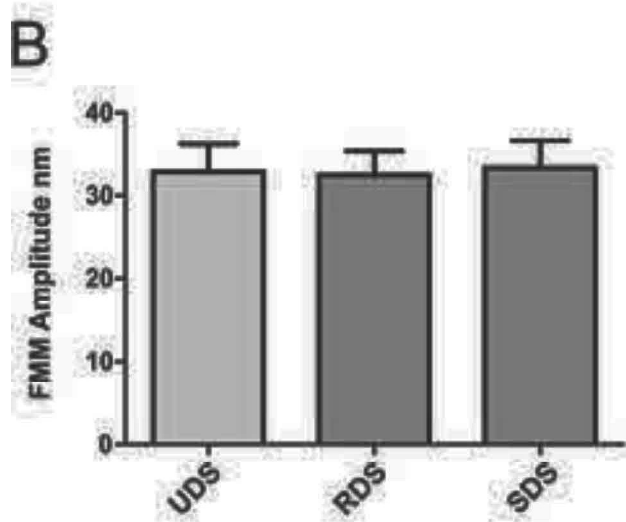

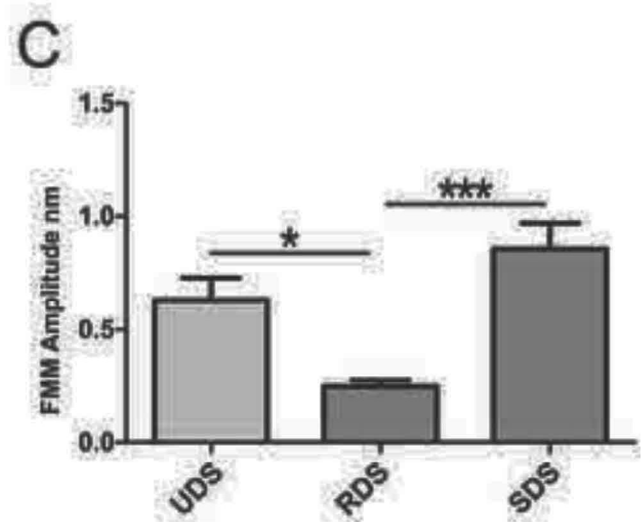

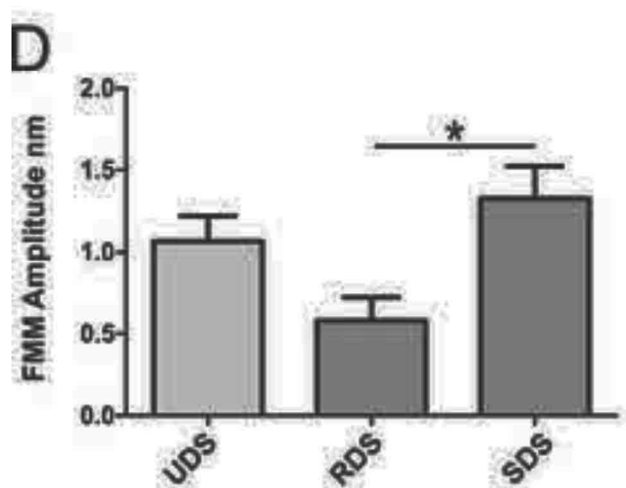

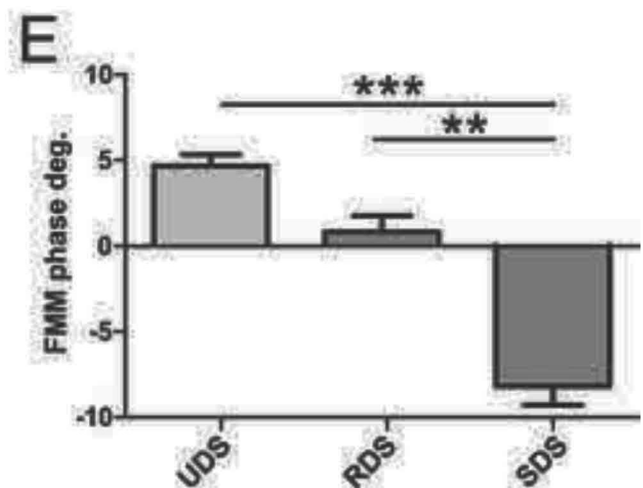

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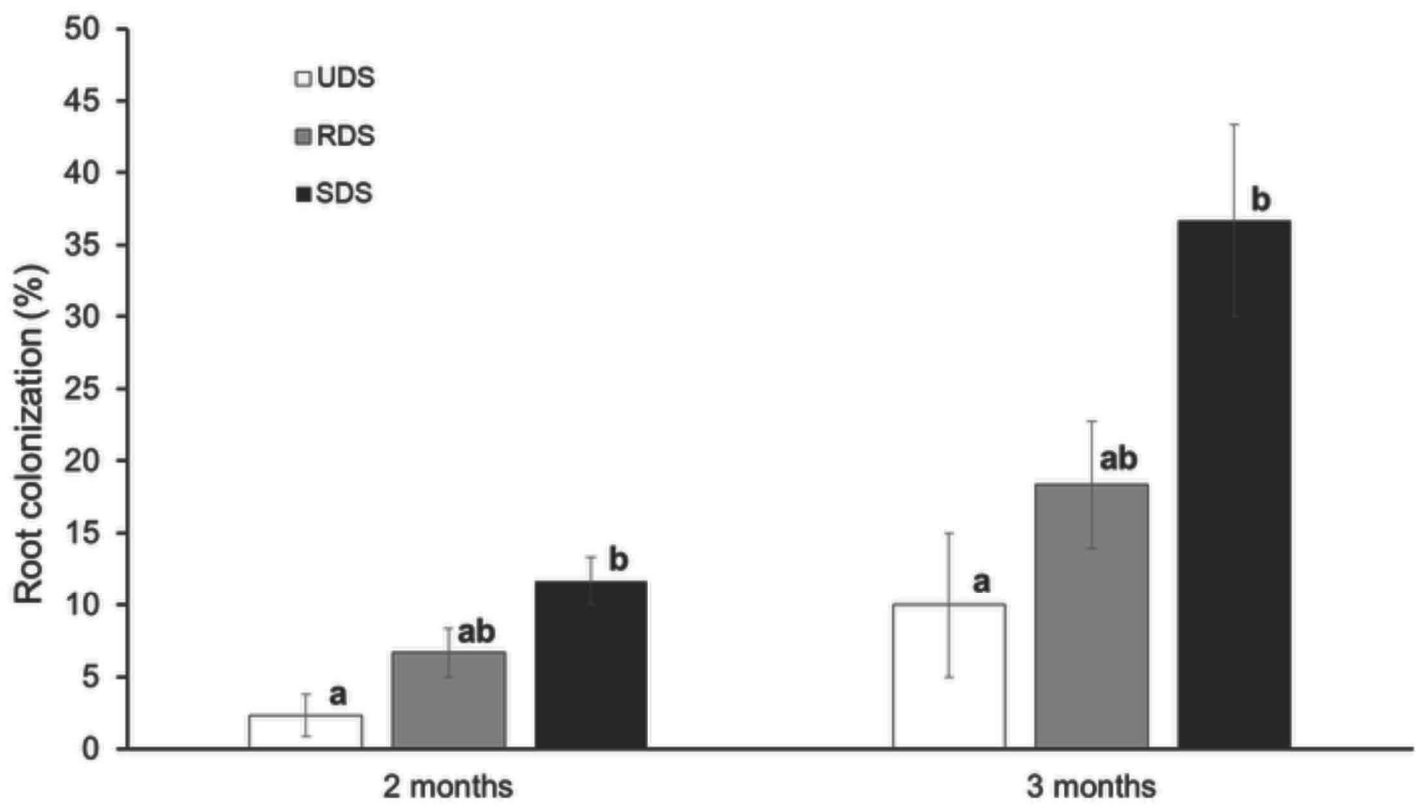

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