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Original Article

Vitality of mycobionts and photobionts after passing through the digestive tract of *Constrictotermes cyphergaster* (Isoptera) workers

Vitalidade de micobiontes e fotobiontes após passagem pelo trato digestivo de operários de *Constrictotermes cyphergaster* (Isoptera)

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Abstract

Termites are among the insects that consume lichens and may be potential dispersers of these symbionts. This study evaluated the vitality of photobionts and mycobionts after passing through the digestive tract of *Constrictotermes cyphergaster*. The percentage of live and dead algae was verified throughout the alimentary canal of 450 workers, originating from five sampled colonies in the Caatinga Dry Forest, NE, Brazil. A progressive growth in algae mortality was observed in the crop, paunch and rectum, however more than 40% of the algae found in faeces presented signs of vitality. Photobiont morphology was different between cells extracted from thallus *in natura* and algae present in termite faeces. The photobiont cells presented more shrunken cytoplasms after passing through the alimentary canal of *C. cyphergaster*. There was also an increase between the cell wall space and the cytoplasm membrane of algae found in the termite faecal pellets. Only four broken spores were found in the intestine, which made the vitality analysis unfeasible for these cells. The record of photobiont vitality in termite faecal pellets is indicative of endozoochoric dispersal, suggesting that this relationship between insects and lichens extends beyond a trophic interaction.

Keywords: caatinga, endozoochory, dispersive mutualism, neotropical region, termites.

Resumo

Os térmitas estão entre os insetos consumidores de liquens e representam um potencial dispersor desses simbiontes. Este estudo avaliou a vitalidade de fotobiontes e micobiontes depois de terem passado pelo trato digestivo de *Constrictotermes cyphergaster*. O percentual de algas vivas e mortas foi verificado ao longo do canal alimentar de 450 operários, oriundos de cinco colônias amostradas em Caatinga Dry Forest, NE, Brazil. Um crescimento progressivo na mortalidade das algas foi observado no sentido papo, pança e reto, porém mais de 40% das algas presentes nas fezes apresentaram sinais de vitalidade. A morfologia dos fotobiontes foi diferente entre células extraídas de talos *in natura* e algas presentes nas fezes dos térmitas. As células do fotobionte apresentaram seu citoplasma mais encolhido, após a passagem pelo canal alimentar de *C. cyphergaster*. Também houve um aumento entre o espaço da parede celular e a membrana do citoplasma das algas encontradas nas pelotas fecais dos térmitas. Apenas quatro esporos quebrados foram encontrados no intestino dos cupins, o que inviabilizou a análise de vitalidade dessas células. O registro da vitalidade de fotobiontes nas pelotas fecais dos térmitas é um indicativo de dispersão endozoocórica, remetendo que a relação entre esses insetos e os liquens pode ir além de uma interação trófica.

Palavras-chave: caatinga, endozoocoria, mutualismo dispersivo, região neotropical, térmitas.

1. Introduction

Lichens represent one of the most successful methods of symbiosis in nature (Lutzoni and Miadlikowska, 2009). They reproduce vegetatively through symbiotic propagules, thallus fragments or by the production of fungal spores, which are mainly dispersed by wind and rain (Nash III, 2008). Many animals including mites, springtails, ants, slugs and snails use lichens as food and/or shelter and can contribute to their dispersion through exozoochory or endozoochory (Gerson, 1973; McCarthy and Healy, 1978; Sharnoff, 1998; Seaward, 2008; Boch et al., 2011).

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Termites are among the organisms that consume lichens, with records for *Hospitalitermes*, *Grallatotermes*, *Longipeditermes* and *Constrictotermes* species (Collins, 1979; Roisin and Pasteels, 1996; Miura and Matsumoto, 1997, 1998; Martius et al., 2000). The presence of spores (mycobiont) and algae (photobiont) originating from lichens were registered in *Hospitalitermes* feeding balls in Malaysia, and in crop food content of *Constrictotermes cyphergaster* (Silvestri), in the Brazilian semi-arid region (Collins, 1979; Barbosa-Silva et al., 2019). Among the lichen structures found in *C. cyphergaster* intestines, algae with apparent vitality were present (Barbosa-Silva et al., 2019). However, how these structures resist the effects of intestinal pH and the digestive enzymes of these insects and evolve into a new lichen thallus is unknown.

The neotropical termite *C. cyphergaster* constructs arboreal nests and has been registered to occur in Brazil, Paraguay, Bolivia and northern Argentina (Mathews, 1977; Torales et al., 2005). This species forages at night on exposed trails and feeds on trunks and sticks at different stages of decomposition, in addition to lichens (Moura et al., 2006; Barbosa-Silva et al., 2019). During the foraging process, individuals mark trails with faecal pellets as a mechanism of social communication (Souto and Kitayama, 2000). These pellets can still contain lichen structures with reproductive viability; thus, termites may be potential dispersers, either through the transportation of these structures in their bodies or through the liberation of spores and/or algae in their faeces. The objective of this study was to examine the vitality of mycobiont and photobiont cells of *Dirinaria confluens* (Fr.) D. Awasthi, *Lecanora* sp., and *Haematomma persoonii* (Fée) A. Massal. after passing through the digestive tract of *C. cyphergaster* and comparing them with the vitality of cells *in natura*.

2. Material and Methods

2.1. Study area

This study was performed in the Reserva Particular do Patrimônio Natural Fazenda Almas (RPPN Fazenda Almas) (7º28'15"S; 36º53'51"W), which covers an area of approximately 3,505 ha. This region presents an average annual precipitation of 560 ± 230 mm, which is concentrated in the months of February, March and April, and a long dry season, often lasting up to eight months. The average annual temperature and relative air humidity are 25 ºC and 65%, respectively (Governo do Estado da Paraíba, 1985; Núcleo de Meteorologia Aplicada, 1987).

2.2. Lichen consumption

Bioassays were prepared to verify the presence and vitality of spores and algae after passing through termite intestines. To achieve this, fragments of five *C. cyphergaster* nests, containing part of its population, were collected. Nine sub-colonies were separated from each nest. Each sub-colonies represented a bioassay that composed of 200 workers and 50 soldiers, according to the average relationship observed between these castes in *C. cyphergaster* nests (Lucena et al., 2019). The individuals were placed into two perforated glass recipients (200 ml), which were connected using glass tubes (10 cm), made from shortened serological pipettes. The termites were placed in one of the recipients, lined with 1 cm of sieved, washed and sterilised sand, covered with 0.5 cm of expanded vermiculite and dampened with 4 ml of sterilised distilled water. The other glass recipient was lined with 1 cm of sand and received the lichen thalli of *D. confluens*, *Lecanora* sp. or *H. persoonii* (Figure 1). The lichens selected in this experiment are part of *C. cyphergaster* diet (Barbosa-Silva and Vasconcellos, 2019). Each thallus was divided into two parts of similar sizes (~ 1.5 cm). One piece was used as food for the termites and the other was used as the control (symbionts that did not pass through the termite intestines). Each lichen species was offered individually, in triplicate, in the bioassays prepared for each nest (3 x *D. confluens* / 3 x *Lecanora* sp./ 3 x *H. persoonii* x per nest). The basal parts of the lichens were wrapped in aluminium paper, with the aim of preventing the termites from eating the wood remains of the plant support for the lichen. The recipients were covered with voile fabric, secured at the corners using rubber bands and placed in the dark for 10 days at room temperature.

2.3. Determination of cell vitality - (schematic representation in supplementary material)

Thirty workers from each set of triplicates were separated for analyses, totaling ninety workers for each nest. The individuals were deposited in Petri dishes for the dissection and removal of the alimentary canal, which was sectioned, and the contents of the crop, paunch and rectum were removed and deposited separately in Eppendorf tubes. The food contents of each isolated region were added to 1 ml of sterilised distilled water. The Eppendorf tubes were manually shaken, and the contents of a capillary were then removed (75 mm long and 1 mm in diameter), which were deposited onto glass slides and recovered with a slip cover for optic microscopic analysis (Coleman, model N-101B with an increase of 400x), verifying the presence of spores and algae.

To determine algae vitality, the photobionts were stained with neutral red supra vital dye (0.1%) by adding two drops of dye onto the slides that contained the extracted substrates from the termite alimentary canals. Cell analyses and counts were then performed. A total of 100 photobiont cells were counted randomly for each portion of the worker alimentary canals, with a recount. Among the observed 100 cells, a percentage of live cells was determined, stained red, and dead cells, unstained (Calvelo and Liberatore, 2004). Only a few broken spores were found in the material, which made the vitality analysis unfeasible for these cells.

2.4. Morphological damage to algae after passing through termite alimentary canals - (schematic representation in supplementary material)

To investigate how the photobiont cells were affected morphologically after passing through the digestive tract of *C. cyphergaster,* microscopic slides were prepared with dilutions produced from the worker faecal pellets extracted

from the bioassays (the material used for vitality analyses). The slides were observed and the size of photobiont cells, cytoplasmic content (the average length and width) and the interspace (distance) between the cell wall and cytoplasm membrane were measured using an optic microscope (magnification of 400x). The first 25 algae were measured from each slide. In total, 125 algae of each lichen species were analysed, i.e., 25 algae analysed per nest. The controls of each lichen were scratched with the aid of a stainless-steel blade and the substrate was removed from the thallus and was added to 1 ml of distilled water. A total of 75 cells (evaluated in triplicate) of the material from each lichen were measured following the same methodology described for the analysis of termite faecal material.

2.5. Data analysis

Algae vitality (number of live cells in each 100 counts) in each region of the alimentary canal (crop, paunch and rectum) were compared using an analysis of variance (one-way ANOVA), after evaluating the normality and homoscedasticity of the data. To compare the averages, Tukey's test was used *a posteriori*. The morphological differences between the extracted algae of the lichens *in natura* and after passing through termite alimentary canals were verified using a paired t-test. Analyses were performed using R software version 4.2.2 (R Development Core Team, 2016).

3. Results

Only four spores were found in the analysed material in the paunch region of the workers, but photobiont cells were abundant and were present in 100% of the material. The greatest number of live cells were found in workers crop, followed by the paunch and rectum (Figure 2). Photobiont vitality of *D. confluens* ($F_{2,6}$= 9.2247; P= 0.01), *Lecanora* sp. ($F_{2,6}$= 11.836; P< 0.001) and *H. persoonii* ($F_{2,6}$= 6.9341; P=0.02) was significantly different between the regions

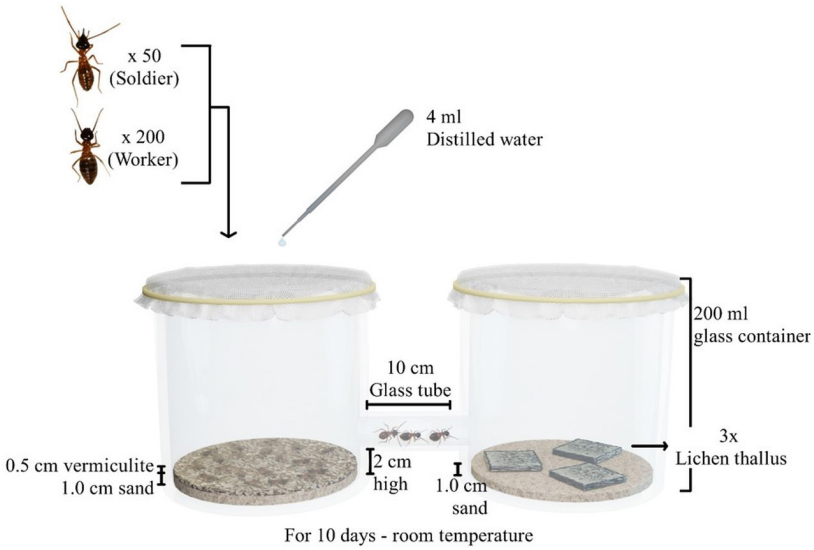

Figure 1. Schematic representation of the bioassays elaborated for consumption of lichens by *Constrictotermes cyphergaster*.

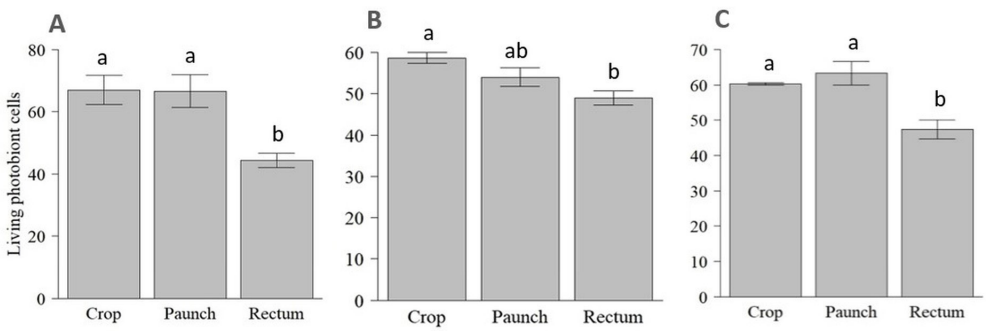

Figure 2. Average (± standard error) of live photobionts in the crop, paunch and rectum of *Constrictotermes cyphergaster* workers fed with (A) *Dirinaria confluens*, (B) *Haematomma persoonii* and (C) *Lecanora* sp. Different letters demonstrate significant differences between averages (P < 0.05) for Tukey's test.

Table 1. Average size (±SE) of photobiont cells, cytoplasmic content and interspace (distance between the cell wall and the cytoplasm) in *Dirinaria confluens*, *Haemmatomma persoonii* and *Lecanora* sp. obtained from thalli *in natura* and after passing through the alimentary canal (Fe) of *Constrictotermes cyphergaster*.

Lichen	Treatment	Cell size (μm)	T test	Cytoplasm size (μm)	T test	Interspace (μm)	T test
Dirinaria confluens	*in natura*	6.74±0.14	t = 3.27	3.87±0.12	t = 12.53	1.90 ± 0.14	t = 3.97
			df = 124		df = 124		df = 124
	Fe	6.16±0.12	P = 0.002	2.23±0.11	P < 0.001	2.61 ± 0.10	P < 0.001
Haemmatomma persoonii	*in natura*	6.57±0.20	t = 0.14	3.71±0.17	t = 6.93	1.94 ± 0.12	t = 2.75
			df = 124		df = 124		df = 124
	Fe	6.46±0.69	P = 0.880	2.48±0.15	P < 0.001	2.62 ± 0.14	P = 0.007
Lecanora sp.	*in natura*	6.46±0.17	t = 3.16	3.62±0.15	t = 4.08	1.93 ± 0.11	t =3.36
			df = 124		df= 124		df = 124
	Fe	5.72±0.17	P = 0.002	2.99±0.16	P < 0.001	2.52 ± 0.16	P = 0.001

of the worker alimentary canals. The percentage of algae vitality in the feces was 49% for workers fed *H. persoonii*, followed by *Lecanora* sp. (47.3%) and *D. confluens* (44.3%).

There was a significant difference in the size of photobiont cells *in natura* and after passage through the digestive tract of *C. cyphergaster* for *D. confluens* and *Lecanora* sp. (Table 1). We observed that the photobiont cells presented smaller cytoplasm sizes after passing through the alimentary canal (Table 1). There was also a difference between the cell wall space and the cytoplasmic membrane of algae extracted from faeces and thalli *in natura* (Table 1). We registered the presence of lichen fragments (piece of thallus with algae and associated hyphae) throughout the alimentary contents (Figure 3).

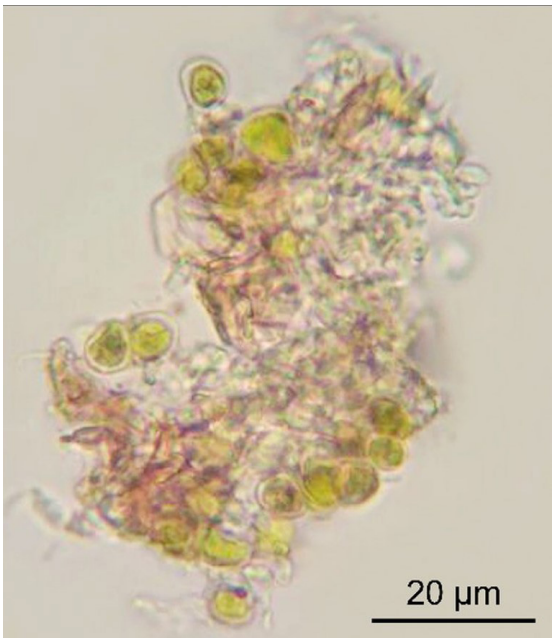

Figure 3. Fragment of lichen thallus found in the paunch of *Constrictotermes cyphergaster* workers.

4. Discussion

Termite digestion is divided between the mechanical grinding of food in the foregut (crop), enzymatic production in the midgut (paunch) and microbial and enzymatic activity in the hindgut (Ni and Tokuda, 2013). Our analyses showed that the greater compromise in algal vitality occurred in the interval between the paunch and the rectum (hindgut) of the *C. cyphergaster* workers, compromising the morphology and even resulting in the death of some individuals. However, there was a partial tolerance of these photobionts to enzymatic and microbial action found in this portion of termite intestines.

Another factor that may have affected the structure and vitality of algae is the pH of the *C. cyphergaster* digestive tract. Termites of the subfamily Nasutitermitinae, to which *C. cyphergaster* belongs, present an intestinal pH between 6.5 and 10.5 varying between the foregut (pH~8.0 and 10.5), midgut (pH ~6.5 and 9) and hindgut (pH ~6.5 and 7.5) (Brune et al., 1995; Bignell and Eggleton, 1995). For *Trebouxia* spp., the photobiont of most lichens with green algae, the optimal pH for maintaining vitality is between 4 and 7 (Ahmadjian, 1993). The pH interval between 8 and 9 is not adequate for this photobiont (Bačkor et al., 1998), indicating that these algae confront stressful conditions in termite intestines, which may explain the observed morphological alterations.

The majority of algae found in *C. cyphergaster* faeces presented shrunken cytoplasms but were also found in non-damaged cells. Similar results have been found for *Xanthoria parietina* (L.) Th. Fr. algae after passing through snail intestines, where it was found that these photobionts presented a decrease in their photosynthetic potential compared to photobionts extracted from the same lichen *in natura* (Froberg et al., 2001). The determination of photosynthetic potential can also be used as a measure to indicate photobiont cell viability (Schreiber et al., 1986; Sonesson et al., 1995). The knowledge morphological

condition of the algae after passing through the digestive tract of termites is important to indicate the potential viability of these organisms, ruling out plasmolysis processes. Changes in the morphology of these photobionts can compromise their functionality and physiology. However, these impairments can be transitory and the cell can recover, or it can lead to the death of the alga (Stadelmann and Kinzel, 1972; Leblanc and Rao, 1973).

The scarcity of *D. confluens*, *Lecanora* sp. and *H. persoonii* spores in the food contents of *C. cyphergaster* results from the rejection of the apothecia (region that contains the thallus spores) of these lichens during consumption (Barbosa-Silva, 2019). Mechanical damage during lichen ingestion may explain the broken spores found in the termite intestines. However, viable spore dispersion is recognised for species of the subfamilies Macrotermitinae. The consumed fungal spores passed through the intestines of these termites and remained intact, completing their life cycle by germinating in fresh faeces (Batra and Batra, 1979; Wood and Thomas, 1989; Rouland-Lefèvre, 2000).

Even in the absence of spores, the record of thallus fragments (associated algae and hyphae) in *C. cyphergaster* intestines is a good indication of dispersion. This type of structure was also observed in snail intestines, where both symbionts presented vitality and were eliminated in the faeces, suggesting that a lichen dispersal mechanism is more efficient than isolated symbionts (Boch et al., 2011). Fungal hyphal vitality was not evaluated in this study and the germinal viability of the identified fragments could not be affirmed.

The record of photobiont cell vitality and lichen fragments present in the faecal pellets of *C. cyphergaster* indicate that endozoochoric dispersal by these insects is possible, suggesting that the relationship between termites and lichens may extend beyond a trophic interaction. In the field, the next step would be to analyse the germinal viability of photobionts, with the aim of showing dispersal effectiveness.

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Supplementary Material

Supplementary material accompanies this paper.

Figure S1. Schematic representation of the procedures performed to determine the vitality of photobionts along the alimentary canal of Constrictotermes cyphergaster.

Figure S2. Schematic representation of the procedures performed to investigate how the photobiont cells were affected morphologically after passing through the digestive tract of Constrictotermes cyphergaster.

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