

HIGH CELLULOSE DEGRADATION RATE IN LEAF-CUTTING ANT'S FUNGAL GARDENS

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Abstract. Currently, it is still controversial if the degradation of cellulose in the fungal gardens of leaf-cutting ants is small or large, and whether the resulting products would serve as an important source of energy. In the present study, the cellulose degradation was evaluated in the gardens of the leaf-cutting ant *Atta capiguara*, by means of quantification of the cellulose in the leaves, before the entrance in the colonies and soon after the discarding of these in the garbage. It was found a 74.9% reduction of cellulose in this material. The gardens of leaf-cutting ants and the digestive tract of ruminant animals can be considered analogous systems, because, in both, symbiotic microorganisms degrade cellulose from fresh plant material, to serve as an important source of energy. However, the permanence of the plant material in the fungal gardens is much higher in relation to the rumen, raising the energy demand of the symbiotic microorganisms, which probably leads them to consume most of the energy. On the other hand, considering the fact that the ants do not survive without their cultivated fungus, it is evidenced that, although predominantly indirect, cellulose is very important to leaf-cutting ants.

Keywords: Symbiosis, *Atta*, *Acromyrmex*, fungus, bacteria.

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1. Introduction

Leaf-cutting ants (Hymenoptera: Formicidae) exert important herbivory of the neotropical region. They forage fresh parts of plants taking them inside of the nests where they fragment them into smaller pieces, and use them to grow their mutualistic fungus *Leucocoprinus gongylophorus* (Möller) Heim (Basidiomycota: Agaricaceae) (Bass & Cherrett, 1995; Nygaard *et al.*, 2016; Mueller *et al.*, 2017). Ants obtain most of their nutritional needs through the microbial degradation of biopolymers, particularly carbohydrates and proteins, present in forage plants (Bass & Cherrett, 1995; De Siqueira *et al.*, 1988; Silva *et al.*, 2003; Aylward *et al.*, 2013).

It is a consensus that the degradation of the simplest compounds, such as starch, starts rapidly in the upper portions of the garden, where the ants add the leaves. However, there is controversy whether, in the basal portion, a large (Martin and Weber, 1969; Nagamoto *et al.*, 2011, Moreira-Soto *et al.*, 2017) or small (De Siqueira *et al.*, 1988; Moller *et al.*, 2011; Somera *et al.*, 2015) cellulose degradation occurs.

Firstly, based on *in vitro* results (culture medium) and a preliminary *in vivo* digestibility study, it was proposed that the fungus would be capable of degrading large amounts of biopolymers from plants, including cellulose, which would be an important energy source (Martin & Weber, 1969). Later, De Siqueira *et al.* (1988) showed that this fungus, grown *in vitro*, presents a low cellulase degradation and assimilation when compared to other enzymes such as xylanases, pectinases or amylases.

In addition to the cultivated fungus, there is growing evidence that bacteria and yeasts also have degradation capability of cellulose and other biopolymers of the cell wall, although it is not yet known to what extent this may be important for ants (Bacci *et al.*, 1995; Suen *et al.*, 2010; Aylward *et al.*, 2012a, 2012b; Mendes *et al.*, 2012; Somera *et al.*, 2015; Kooij *et al.*, 2016; Moreira-Soto *et al.*, 2017).

Moller *et al.* (2011) found a high cellulose level in the fungus garden debris and did not find evidence that cellulose is utilized as a significant substrate, although hypothesized that cellulases may have a more significant role under sub-optimal foraging conditions. Additionally, Somera *et al.* (2015) observed low cellulase activity in gardens. Moreover, ultrastructural studies of leaf fragments showed significant degradation of cellulose in the gardens of ants, leaving only the most lignified leaf structures (Nagamoto *et al.*, 2011; Moreira-Soto *et al.*, 2017).

To shed light on this controversy, cellulose degradation was evaluated *in vivo*, quantifying the cellulose in the leaves, before entering the colonies and immediately after discarding them in the garbage chambers. Field colonies of the grass cutter ant *Atta capiguara* Gonçalves 1944, were used.

2. Materials and methods

Five adult *A. capiguara* colonies were found in *Paspalum notatum* pastures located in the city of Botucatu (22°53'09''S and 48°26'42''W), São Paulo, Brazil. These colonies foraged exclusively on *Paspalum notatum* Flüggé (Bahia grass) leaves, a South American native grass.

Whole leaf fragments that were carried by the workers, as well as fragments recently discarded into the garbage chamber, were collected, identified, and stored separately in a freezer. These underground garbage chambers were accessed by digging with hand tools. Prior to processing, those fragments were dried in a lab oven at 50 °C until constant weight was achieved. The fragments were then ground in a mill and sifted through a fine mesh sieve. Samples (1 g) from each treatment were submitted to a chemical composition analysis for cellulose percentage quantification, according to the Van Soest method, 1967, described by Silva (1991).

In order to correct the effect of biomass loss due to assimilation and respiration (Martin & Weber, 1969; Oliveira *et al.*, 2012; Reis *et al.*, 2017), the conversion factor of 2.10 obtained for *Atta capiguara* by Garcia *et al.* (2003) was used. Statistical analysis (paired t-test) was performed comparing the fresh material with the garbage samples after the application of the conversion factor. The conversion factor, traditionally used in leaf-cutting ants, is the ratio of forage material incorporated into the garden and the disposal of this material in the trash, and its value usually varies around 2.0 for grass cutting ants (Jonkman, 1977; Fowler *et al.*, 1990).

3. Results and Discussion

The cellulose determinations on the *P. notatum* leaf fragments, before and after being used by the symbiotic fungus, resulted in concentrations of 30.4% (0.304 g per sample) and 16.0% (0.160 g) respectively. Such difference in concentration is not a good measure of how much cellulose has been degraded since it does not take into account the reduction of plant mass through processes such as microbial respiration and assimilation by the animal symbiont. Thus, it is necessary to apply a correction method to correctly evaluate the degradation of a given polymer (Gray, 1947; Martin & Weber, 1969; Barry & Manley, 1984; Reis *et al.*, 2017; Oliveira *et al.*, 2012). In the present work, we corrected through the conversion factor (Garcia *et al.*, 2003), resulting in mean reduction from 0.3040 to 0.0762 g (Fig. 1) (74.9% cellulose degradation). The difference between treatments is statistically significant (paired t-test; p value = 0.0000000333, df = 4).

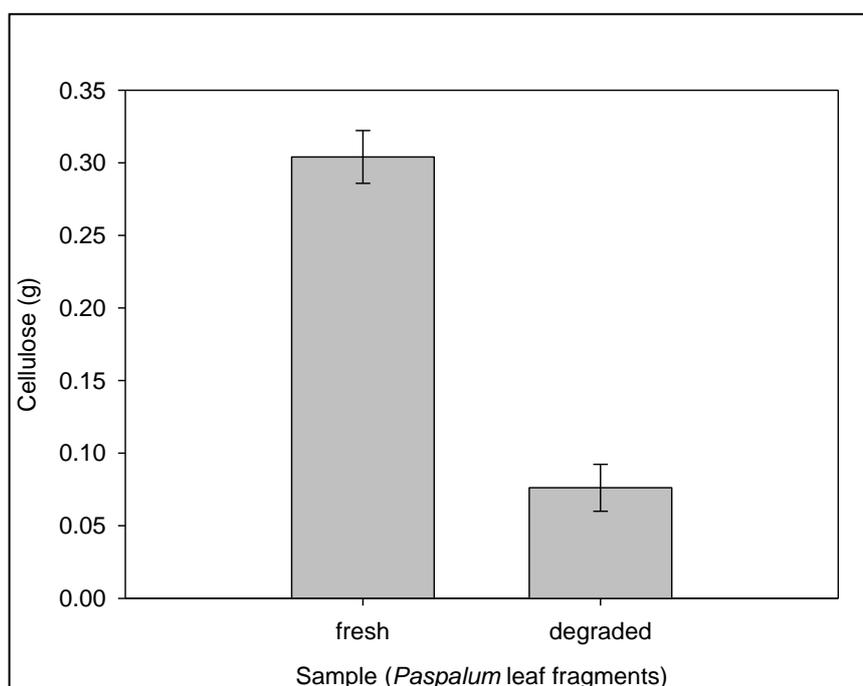


Fig. 1. Cellulose amount per *Paspalum notatum* sample (\pm SD), before and after the permanence in ant's garden

The explanation for the significant presence of cellulose in the discarded material (16.0%) is that the thick lignin layer in the cells with secondary wall performs as a physical barrier (Suen *et al.*, 2010; Nagamoto *et al.*, 2011; Nygaard *et al.*, 2016), protecting them from the degradation promoted by the carbohydrate-active enzymes present in the fungus garden (Suen *et al.*, 2010; Khadempour *et al.*, 2016). It is noted that even after passage into the digestive tract of herbivorous animals, such as sheep and horses, well known to be capable of using cellulose, a substantial amount of such polymer is also found (Gray, 1947; Barry & Manley, 1984; Oliveira *et al.*, 2012).

This result is not inconsistent with that of Martin and Weber (1969), where the garbage material was compared with the partially degraded material (incorporated in the garden), and thus an underestimated value of 45% was obtained. Also underestimated

are 30% of Suen et al. (2010), because in this case, it is uncorrected degradation. Thus, these quantitative evidences support the works of Nagamoto et al. (2011) and Moreira Soto et al. (2017), which, based on ultra-structural evidence, concluded that there is great degradation of cellulose in the gardens of leaf-cutting ants.

This high rate of degradation that was found is similar to the ruminant animals (Barry & Manley, 1984) (for more details, see Tab. 1). In contrast, in humans (omnivorous), the reduction is lower (Southgate & Durmin, 1970); this fact, together with the much lower fiber intake (McGill & Devareddy, 2015), results that less than 10% of human energy needs come from this source (McNeil, 1984). On the other hand, ruminants derive about 70% of their energy requirements from plant fiber through microbial degradation (Flint & Bayer, 2008). Leaf-cutting ants, as well as ruminants, primarily use fresh fiber-rich plants, and, in partnership with their symbiotic microorganisms, degrade cellulose in large amounts. Therefore, in these terms, they can be considered systems that have become analogous through evolutionary convergence. On the other hand, the permanence of plant material in fungus gardens is much greater than in the digestive tract of herbivorous or omnivorous animals (Table 1).

Table 1. Comparison of cellulose degradation, fibers consumption, permanence, and energy contribution, in different systems

	cellulose degradation rate	fibers input	time of permanence	Energy (%) from fibers
Sheep (ruminant)	high (63-78%) ^(a)	high (252-293 g/day) ^(a)	short (few days) ^(a)	high (up to 70%) ^(b)
Man (omnivorous)	lower (15.4-55.1%) ^(c)	low (16.1 g/day) ^(d)	short (few days) ^(c)	low (<10%) ^(e)
<i>Atta capiguara</i> garden	high (74.9%) ^(f)	high (grass leaves) ^(f)	long (2-3 months) ^(g)	see discussion

(a) Barry and Manley (1984); (b) Flint and Bayer (2008); (c) Southgate and Durmin (1970); (d) McGill and Devareddy (2015); (e) McNeil (1984); (f) present work; (g) Fisher et al. (1996), and Sousa-Souto et al. (2007).

The gongylidia (inflated hyphal tips) are the exclusive food of the young forms of leaf-cutting ants, and are richer in protein compared to hyphae (Quinlan & Cherrett, 1979; Bass & Cherrett, 1995). The literature reports that the degradation of cellulose is concentrated mainly in the basal portion of the gardens of leaf-cutting ants, where fewer gongylidia occur. These facts have been used as indirect evidence that cellulose degradation is not of great importance to ants (Grell *et al.*, 2013; Somera *et al.*, 2015; Kooij *et al.*, 2016).

However, the consumption of gongylidia is not the only way for worker ants to obtain nutrients. For instance, Somera et al. (2015) demonstrated that the fungus, secretes polyols in the gardens, and the ants ingest them, probably via grooming. In addition, hyphae, although less rich in protein, are also consumed by them (Quinlan & Cherrett, 1979; Bass & Cherrett, 1995) and thus may also be important as an energy source for adult ants. Grell et al. (2013) hypothesized that the energy from cellulose serves for the hyphae respiration in the basal portion of the garden, preventing its disintegration. Then, cellulose, indirectly, can be useful to the workers, obtaining some energy through the consumption of these structures (hyphae in the basal portion) in periods of scarcity.

The ability of fungi to degrade lignin was lost along co-evolution with the ants (Nygaard *et al.*, 2016), but recent studies have consolidated that *L. gongylophorus* is fully capable of degrading the other polymers of the cell wall and the middle lamella (Aylward *et al.*, 2013; Grell *et al.*, 2013; Lange & Grell, 2014; Khadempour *et al.*, 2016; Nygaard *et al.*, 2016). Cellulolytic capacity has probably been preserved because it is adaptive, so it is unlikely that this fungus will not substantially degrade cellulose, or that the metabolites of this cellulose have no significant utility. In addition to probably degrading most of the cellulose under normal conditions (present study), *L. gongylophorus* cellulases may have an even more increased role under sub-optimal foraging conditions, as for example in dry seasons, when fresh leaf material, richer in starch and poorer in cellulose, is less available (Moller *et al.*, 2011; Grell *et al.*, 2013).

Even though bacteria are found mostly in the older portion of the garden (Moreira Soto *et al.*, 2017), which correlates them with the fact that cellulose degradation also concentrates on this site (Grell *et al.*, 2013; Somera *et al.*, 2015; Moreira Soto *et al.*, 2017), they seem to contribute less effectively to the degradation (Aylward *et al.*, 2013; Khadempour *et al.*, 2016). On the other hand, Aylward *et al.* (2012a) have shown that these bacteria are likely to participate in amino acid biosynthesis, B-complex vitamins and other nutrients which potentially increase the growth or efficiency of the biomass processing of the mutualistic fungus.

Taking into account the results obtained, considering the time of permanence of the plant material in the fungus garden of the ants, and the current literature, it is concluded that there is a great degradation of the cellulose in the fungus gardens of the leaf-cutting ants, serving primarily as a source of energy for the metabolism of symbiotic microorganisms, particularly the mutualistic fungus. Thus, taking the current knowledge into consideration, and that the ants depend on their cultivated fungus, cellulose has great importance, albeit indirectly, for the survival of leaf-cutting ants.

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References

- Aylward, F.O., Burnum, K.E., Scott, J.J., Suen, G., Tringe, S.G., Adams, S.M., Barry, K.W., Nicora, C.D., Piehowski, P.D., Purvine, S.O., Starrett, G.J., Goodwin, L.A., Smith, R.D., Lipton, M.S. & Currie, C.R. (2012a). Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus gardens. *The ISME Journal*, 6(9), 1688-1701.
- Aylward, F.O., Currie, C.R. & Suen, G. (2012b). The evolutionary innovation of nutritional symbioses in leaf-cutter ants. *Insects*, 3(1), 41-61.
- Aylward, F.O., Burnum-Johnson, K.E., Tringe, S.G., et al. (2013). *Leucoagaricus gongylophorus* produces diverse enzymes for the degradation of recalcitrant plant polymers in leaf-cutter ant fungus gardens. *Applied and Environmental Microbiology*, 79, 3770-3778.
- Bacci Jr, M., Ribeiro, S.B., Casarotto, M.E.F. & Pagnocca, F.C. (1995). Biopolymer-degrading bacteria from nests of the leaf-cutting ant *Atta sexdens rubropilosa*. *Brazilian Journal of Medical and Biological Research*, 28(1), 79-82.

- Barry, T.N. & Manley, T.R. (1984). The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep: 2. Quantitative digestion of carbohydrates and proteins. *British Journal of Nutrition*, 51(3), 493-504.
- Bass, M. & Cherrett, J.M. (1995). Fungal hyphae as a source of nutrients for the leaf-cutting ant *Atta sexdens*. *Physiological Entomology*, 20(1), 1-6.
- De Siqueira, C.G., Bacci, M., Pagnocca, F.C., Bueno, O.C. & Hebling, M.J.A. (1998). Metabolism of plant polysaccharides by *Leucoagaricus gongylophorus*, the symbiotic fungus of the leaf-cutting ant *Atta sexdens* L. *Applied and Environmental Microbiology*, 64, 4820-4822.
- Fisher, P.J., Stradling, D.J., Sutton, B.C. & Petrini, L.E. (1996). Microfungi in the fungus gardens of the leaf-cutting ant *Atta cephalotes*: a preliminary study. *Mycological Research*, 100(5), 541-546.
- Flint, H.J. & Bayer, E.A. (2008). Plant cell wall breakdown by anaerobic microorganisms from the mammalian digestive tract. *Annals of the New York Academy of Sciences*, 1125(1), 280-288.
- Fowler, H.G., Forti, L.C. & Di Romagnano, L.F.T. (1990). Methods for the evaluation of leaf-cutting ant harvest. In R.K. Vander Meer, K. Jaffe & A. Cedeno (Eds.), *Applied myrmecology: a world perspective* (pp. 228-241). Boulder, Colorado: Westview Press.
- Garcia, M.G., Forti, L.C., Nagamoto, N.S., Lopes, J.F., Noronha Jr, N.C. & Verza S.S. (2003). Estimativa do fator de conversão entre o material forrageado e o depositado no lixo, para *Atta capiguara* Gonçalves e *Atta bisphaerica* Forel. *Anais do XVI Simpósio de Mirmecologia, UFSC, Florianópolis, Santa Catarina, Brazil*, 181-183.
- Gray, F.V. (1947). The digestion of cellulose by sheep the extent of cellulose digestion at successive levels of the alimentary tract. *Journal of Experimental Biology*, 24(1-2), 15-19.
- Grell, M.N., Linde, T., Nygaard, S., Nielsen, K.L., Boomsma, J.J., Lange, L. (2013). The fungal symbiont of *Acromyrmex* leaf-cutting ants expresses the full spectrum of genes to degrade cellulose and other plant cell wall polysaccharides. *BMC Genomics*, 14(1), 928.
- Jonkman, J.C.M. (1977). Determination of the vegetative material intake and refuse production ratio in two species of grass-cutting ants (Hym.: Attini). *Zeitschrift für Angewandte Entomologie*, 84(1-4), 440-443.
- Khadempour, L., Burnum-Johnson, K.E., Baker, E.S., Nicora, C.D., Webb-Robertson, B.-J.M., White III, R.A., Monroe, M.E., Huang, E.L., Smith, R.D. & Currie, C.R. (2016). The fungal cultivar of leaf-cutter ants produces specific enzymes in response to different plant substrates. *Molecular Ecology*, 25(22), 5795-5805.
- Kooij, P.W., Pullens, J.W., Boomsma, J.J. & Schiøtt M. (2016). Ant mediated redistribution of a xyloglucanase enzyme in fungus gardens of *Acromyrmex echinator*. *BMC Microbiology*, 16(1), 81.
- Lange L. & Grell M.N. (2014). The prominent role of fungi and fungal enzymes in the ant-fungus biomass conversion symbiosis. *Applied Microbiology and Biotechnology*, 98(11), 4839-4851.
- Martin, M.M. & Weber, N.A. (1969). The cellulose-utilizing capability of the fungus cultured by the attine ant *Atta colombica tonsipes*. *Annals of the Entomological Society of America*, 62(6), 1386-1387.
- McGill, C.R. & Devereedy, L. (2015). Ten-year trends in fiber and whole grain intakes and food sources for the United States population: National Health and Nutrition Examination Survey 2001–2010. *Nutrients*, 7(2), 1119-1130.
- McNeil, N.I. (1984). The contribution of the large intestine to energy supplies in man. *The American Journal of Clinical Nutrition*, 39(2), 338-342.
- Mendes, T.D., Rodrigues, A., Dayo-Owoyemi, I., Marson, F.A., Pagnocca, F.C. (2012). Generation of nutrients and detoxification: possible roles of yeasts in leaf-cutting ant nests. *Insects*, 3(1), 228-245.

- Moller, I.E., De Fine Licht, H.H., Harholt, J., Willats, W.G. & Boomsma, J.J. (2011). The dynamics of plant cell-wall polysaccharide decomposition in leaf-cutting ant fungus gardens. *PLoS One*, 6, e17506.
- Moreira-Soto, R.D., Sanchez, E., Currie, C. R. & Pinto-Tomás, A.A. (2017). Ultrastructural and microbial analyses of cellulose degradation in leaf-cutter ant colonies. *Microbiology*, 163(11), 1578-1589.
- Mueller, U.G., Ishak, H.D., Bruschi, S.M., et al. (2017). Biogeography of mutualistic fungi cultivated by leafcutter ants. *Molecular Ecology*, 26(24): 6921-6937.
- Nagamoto, N.S., Garcia, M.G., Forti, L.C., Verza, S.S., Noronha, N.C. & Rodella, R.A. (2011). Microscopic evidence supports the hypothesis of high cellulose degradation capacity by the symbiotic fungus of leaf-cutting ants. *Journal of Biological Research-Thessaloniki*, 16, 308-312.
- Nygaard, S., Hu, H., Li, C., Schiøtt, M., Chen, Z., Yang, Z., Xie, Q., Ma, C., Deng, Y., Dikow, R.B., Rabeling, C., Nash, D.R., Wcislo, W.T., Brady, S.G., Schultz, T.R., Zhang, G. & Boomsma, J.J. (2016). Reciprocal genomic evolution in the ant–fungus agricultural symbiosis. *Nature Communications*, 7, 12233.
- Oliveira, K.D., Costa, C., Bittar, C.M.M., Santos, V.P.D., Oliveira, V.A.B. & Sá, J.C.D. (2012). Indigestible cellulose and lignin in determining feces production and apparent digestibility in horses. *Acta Scientiarum. Animal Sciences*, 34(3), 267-272.
- Quinlan R.J. & Cherrett J.M. (1979). The role of fungus in the diet of the leaf-cutting ant *Atta cephalotes* (L.). *Ecological Entomology*, 4, 151-160.
- Reis, M.J., Santos, S.A., Prates, L.L., Detmann, E., Carvalho, G.G.P., Santos, A.C.S., Rufino, L.M., Mariz, L.D., Neri, F. & Costa, E. (2017). Comparing sheep and cattle to quantify internal markers in tropical feeds using in situ ruminal incubation. *Animal Feed Science and Technology*, 232, 139-147.
- Silva, A., Bacci Jr, M., De Siqueira, C.G., Bueno, O.C., Pagnocca, F.C. & Hebling, M.J.A. (2003). Survival of *Atta sexdens* on different food sources. *Journal of Insect Physiology*, 49, 307-313.
- Silva, D.J. (1991). *Análise de alimentos (métodos químicos e biológicos)*. Viçosa, MG: UFV, Imprensa Universitária.
- Somera, A.F., Lima, A.M., Dos Santos-Neto, Á.J., Lanças, F.M., Bacci, M. (2015). Leaf-cutter ant fungus gardens are biphasic mixed microbial bioreactors that convert plant biomass to polyols with biotechnological applications. *Applied and Environmental Microbiology*, 81(13), 4525-4535.
- Sousa-Souto, L., Guerra, M.B.B., Schoederer, J.H., Schaefer, C.E.G.R. & Da Silva, W.L. (2007). Determinação do fator de conversão em colônias de *Atta sexdens rubropilosa* (Hymenoptera: Formicidae) e sua relação com a qualidade do material vegetal cortado. *Revista Árvore*, 31, 163-166.
- Southgate, D.A.T. & Durnin, J.V.G.A. (1970). Calorie conversion factors. An experimental reassessment of the factors used in the calculation of the energy value of human diets. *British Journal of Nutrition*, 24(2), 517-535.
- Suen, G., Scott, J.J., Aylward, F.O., Adams, S.M., Tringe, S.G., Pinto-Tomás, A.A., Foster, C.E., Pauly, M., Weimer, P.J., Barry, K.W., Goodwin, L.A., Bouffard, P., Li, L., Osterberger, J., Harkins, T.T., Slater, S.C., Donohue, T.J. & Currie, C.R. (2010). An insect herbivore microbiome with high plant biomass-degrading capacity. *PLoS Genetics*, 6(9), e1001129. doi:10.1371/journal.pgen.1001129