Ultrastructural and microbial analyses of cellulose degradation in leaf-cutter ant colonies

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Abstract

Leaf-cutter ants (Atta and Acromyrmex) use fresh leaves to cultivate a mutualistic fungus (Leucoagaricus gongylophorus) for food in underground gardens. A new ant queen propagates the cultivar by taking a small fragment of fungus from her parent colony on her nuptial flight and uses it to begin her own colony. Recent research has shown that the ants’ fungus gardens are colonized by symbiotic bacteria that perform important functions related to nitrogen fixation and have been implicated in contributing to plant biomass degradation. Here, we combine bacterial culturing in several media for counts and identification using the 16S rRNA gene with electron microscopy to investigate the process of cellulose degradation in the fungus garden and refuse dumps, and to assess the potential role of symbiotic bacteria. We show through electron microscopy that plant cell walls are visibly degraded in the bottom section of fungus gardens and refuse dumps, and that bacteria are more abundant in these sections. We also consistently isolated cellulolytic bacteria from all sections of fungus gardens. Finally, we show by culture-dependent and electron microscopy analysis that the fungus garden pellets carried by recently mated queens are colonized by fungus garden-associated bacteria. Taken together, our results indicate that cellulose is degraded in fungus gardens, and that fungus garden bacteria that may contribute to this deconstruction are vertically transmitted by new queens.

INTRODUCTION

Leaf-cutter ants, subfamily Myrmicinae (tribe Attini) constitute a diverse group of species within the genera Atta and Acromyrmex and are abundant in tropical regions of the New World [1–3]. These ants culture a fungus in specialized gardens, usually kept in underground chambers. The ants’ gardens are composed of the symbiotic fungus Leucoagaricus gongylophorus, the plant material integrated by worker ants and several species of symbiotic bacteria [4–8]. In this mutualistic association, the fungus degrades the plant material and the ants consume the fungus [9–11]. A virgin ant queen vertically transmits the cultivated fungus, taking a pellet of fungal inoculum from the fungus garden of her parent colony when leaving the nest to form a new colony. This fragment is transported within a specialized structure called an infrabuccal pocket and serves as the initial inoculum for the founding of the incipient colony’s fungus garden [12].

Leaf-cutter ants cut and process fresh vegetation to use as substrate for cultivating their fungus [13]. A single mature Atta colony can harvest up to 240 kg (dry weight) of plant material per year, which is comparable to the amount consumed by a large herbivorous mammal [12, 14]. The composition of the fungus gardens differs between upper and lower sections, with recently inoculated leaves on top and partially degraded plant material on the bottom [6, 15]. The partially degraded material is removed from the bottoms of gardens by workers and placed in specialized refuse dumps, either within specific chambers underground or on the surface outside the colony, depending on the ant species [16, 17]. Within these refuse mounds, specialized microbial communities degrade and exploit the residual biomass [6, 15].

Although plant material is the primary energy source for the function of leaf-cutter ant colonies, our understanding of how degradation occurs and the degree in which different plant polymers are used for nutrition is limited. A recent
study comparing the carbohydrate composition of the top and bottom layers of Atta leaf-cutter ant fungus gardens showed a significant decrease in complex polysaccharides within the fungal chambers [6]. However, Moller and collaborators [18] found an enrichment of cellulose in the fungus garden debris and suggest that, as other cell wall polymers are progressively removed, cellulose microfibrils become increasingly exposed, thus concluding that cellulose is not utilized as a significant substrate in the fungus garden. They also show, along with Nagamoto and collaborators [19], that high levels of cellulose and hemicelluloses are still present in the bottom of the fungus gardens.

The degradation and use of different plant polymers for nutrition by L. gongylophorus is still under debate. Past studies found that this fungus has a low cellulase activity when compared to other enzymes such as pectinas or amylases [20], and that it is unable to degrade cellulose in pure culture [21]. However, more recent findings show that it produces a diversity of lignocellulases in ant gardens and has been proposed as the primary driver of plant biomass degradation in these ecosystems [22]. Also, Aylward et al. [22] and Grell et al. [23], provide evidence that cellulases from L. gongylophorus are highly active in the bottom section of the fungus garden. Furthermore, isolates from Atta mexicana were shown to exhibit endoglucanase activity and were able to grow in semi-solid media with α-cellulose and microcrystalline cellulose, as well as in solid media with grass and sugarcane bagasse as the sole carbon source, suggesting that this fungus can indeed utilize lignocellulosic materials for growth [24].

A recent metagenomics-based study found relatively few predicted bacterial cellulases and hemicellulases (0.2–0.6 % of all the predicted proteins involved in activity against carbohydrates) in Atta fungus gardens, significantly less than those found in other well-known lignocellulose-degrading communities, such as the termite hindgut [7]. In contrast, there is evidence that cellulose-degrading bacteria are present in the fungus gardens [4], and another metagenomics-based study found bacterial genes for predicted cellulases in Atta cephalotes fungus gardens [6]. Recently, Kooij and collaborators [25] hypothesized that substrate processing in Atta colonies results in niches for additional bacteria and/or yeast decomposition, similar to the domestication of specialized gut bacteria in large ungulates.

Some of these past studies suggest that bacteria may play a role in the degradation of plant material within the fungus garden of leaf-cutting ants. In the present study, we first analysed different sections of mature Atta sp. fungus gardens and refuse dumps by electron microscopy (EM) techniques to provide evidence of cellulose breakdown and evaluate the presence of bacteria. Then, we employed culture-dependent techniques to assess the amount and distribution of cultivable and cellulolytic bacteria in these fungus garden sections. Finally, to evaluate whether these bacteria could be transmitted vertically, we employed EM techniques to assess the presence and abundance of bacteria in fungus garden pellets used as initial inoculum by Atta sp queens. We were able to isolate bacteria from these pellets that relate to well-established inhabitants of mature fungus gardens, suggesting that these potentially beneficial microorganisms could be transmitted vertically to new colonies.

**METHODS**

**Sample collection**

Fungus gardens and refuse dump samples from Atta colombica and A. cephalotes colonies were collected from different regions in Costa Rica, to incorporate biological variability: Corredores and La Palma de Osa, Puntarenas; Upala, Alajuela; Sarapiquí, Heredia; Turrialba, Cartago and Turrubares, San José. A total of 17 fungus garden samples, all from different colonies, and 12 refuse dump samples also from different colonies were used for the analyses described in this work (Table S1, available in the online Supplementary Material).

Fungus gardens with ants were excavated from underground chambers and the whole garden material was placed in sterile plastic containers using aseptic tools. For each colony we assigned a code corresponding on the initials of the collector, the year, month and day it was collected, and a serial number. The colonies were maintained in the laboratory inside small containers for the fungus garden within a bigger container (Fig. S1) with moistened cotton (which was replaced regularly) and fed three times a week with leaves from yellow trumpetbush (Tecoma stans; Lamiales, Bignoniaceae). Mineral oil was applied to the sides of the large container to help prevent the potential horizontal transfer of microbes by mites. Containers were not airtight, allowing oxygen flow.

Refuse material was collected from external refuse dumps of A. colombica. All samples employed for both EM and bacteria enumeration were processed directly from field collection, except for cellulolytic bacteria isolations and EM analysis of the colony RM090516-03, which was kept in the laboratory (Table S1). Subsamples from the fungus garden were taken from the upper part of the garden (top), the centre (middle) and the lowest part (bottom), similar to the procedure taken by Grell et al. [23]. Also, subsamples from the refuse dumps were taken from the upper part (top) and the lowest part (bottom). To avoid overlap of the fungus garden sections, the top and bottom ones were taken from the extremes.

Thirty-eight queens were collected during nuptial flights and subsequently kept in small sterile containers until they expelled their pellet of fungus. Expelled pellets were immediately collected for analyses. Queens were also collected from different geographical regions, to eliminate the probability that samples came from the same colony. Five of the queens were from La palma de Osa, Puntarenas, collected on 26 May 2010; 20 were from Sarapiquí, Heredia, collected from 5 to 22 June 2010; the other 13 queens were collected on 14 June 2011 from Sarapiquí, Heredia.
Electron microscopy (EM)
To analyse the degradation of plant material in *A. cephalotes* and *A. colombica* fungus gardens (top, middle, bottom) and refuse dumps (top, bottom) as well as to determine the presence of bacteria in the fungus garden pellet carried by *Atta* queens, we employed transmission (TEM) and scanning electron microscopy (SEM) methods (fungus garden: number of samples *n*=4 TEM, *n*=5 SEM; refuse dumps: *n*=2 TEM, *n*=2 SEM; fungus garden pellets from queen ants: *n*=6 TEM, *n*=19 SEM). Samples were fixed in Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde) in 0.1 M phosphate buffer, pH 7.4 for at least 2 h. Samples were post-fixed in 1% osmium tetroxide for 1 h and dehydrated in a gradient of ethanol (SEM: 30, 50, 70, 80, 90, 95, 100%) or acetone (TEM: 30, 50, 70, 90, 100%). SEM samples were placed in ter-butanol, which was then sublimated in a freeze dryer (VFD-20, VD Inc.), mounted on aluminum bases and coated in gold via coater (IB-3, Giko). Eleven pellet samples were dried by critical point with CO₂ (EMS 850, Critical point dryer) and eight in the freeze dryer. TEM samples were included in Spurr epoxy resin. Thin sections (70 nm) were obtained in a PT-PC PowerTome ultramicrotome (RMC products) and stained with uranyl acetate (4% in ethanol 50%) and 2% Sato’s Triple lead.

Samples were observed using Hitachi H-7000 and H-7100 TEMs, and in Hitachi S-570, S-2360N and S-3700N SEMs. To estimate bacterial density in the pellet samples (*n*=8) using SEM, we measured and calculated the area of each pellet, generated 15 micrographs at 4000× at random, counted the number of bacteria and estimated the field area. From this we calculated the mean bacterial count per field and estimated the total number of bacteria in each pellet, as described by Raymond and collaborators [26].

Fungus garden and dump bacteria density counts
Samples of each section (top, middle and bottom) of *A. cephalotes* (*n*=5) and *A. colombica* (*n*=3) fungus gardens were taken separately for comparison (total *n*=8), as well as samples from two refuse dump sections, top and bottom, from *A. colombica* (*n*=9). Bacteria were extracted to perform two different kinds of count, one for cultivable bacteria (general and cellulolytic) and the other for direct enumeration.

For bacterial extraction, we separated the ants from the fungus garden and used 0.2 g of garden material. We added 10 ml of 10 mM phosphate buffered saline (PBS) with 0.1% (v/v) tween 20 followed by vortexing for 1 min at full speed (Barnstead Thermolyne 37600 Mixer, Thermos Fisher Scientific, Inc.), agitation for 30 min at 120 r.p.m. (orbital shaker, HOTECH Instruments Corp.) and sonication for 1 min (50/60 Hz, FS6 Sonic Cleaner, Fisher Scientific). The material was then filtered with sterile gauze to remove large particles.

From this bacterial extract, serial dilutions were made and plated on LB agar and carboxymethyl cellulose agar medium 2 (CMC 2 [27]). Plates were incubated at room temperature for at least 48 h (LB) or 5 days (CMC 2), and those with approximately 30–300 colonies were counted. In the CMC 2 media, cellulolytic activity was detected by incubation with Congo red (1 mg ml⁻¹) stain for 15 min, and then de-stained for 10–15 min with 1M sodium chloride solution, both with manual agitation at room temperature. Clear zones around the bacterial colonies indicated cellulolytic activity, and only the colonies that presented activity were counted.

The bacterial extract was also filtered and used for direct enumeration of total bacteria using DAPI fluorochrome, as described by Raymond et al. [26]. In all cases, the number of bacteria per gram of fungus garden or refuse dump section (fresh) was compared by ANOVA, using SPSS Statistics for Windows, Version 17.0 (SPSS Inc. Released 2008. Chicago: SPSS Inc.).

Bacterial isolation from fungus garden and pellets
To further understand the role of bacteria in the fungus gardens and complement microscopy findings, we searched for cultivable bacteria capable of degrading cellulose in the three sections of mature fungus gardens of two *A. cephalotes* colonies (Upala, Alajuela and Osa, Puntarenas) using three different media: CMC 3 and 2 (with and without malt extract, respectively [27]), and cellulose agar [28]. Bacteria were extracted as stated in the previous section, plates were incubated at room temperature for at least 5 days and cellulase activity was detected in CMC media with Congo red as described above, and in cellulose agar employing the Graff C reagent as described by Pinto-Tomás et al. [28]. Morphologically different cellulolytic bacteria (according to colony shape, size and colour) were identified by 16S sequencing.

To culture bacteria from the fungus garden pellets, we employed 13 pellet samples (collected 14 June 2011 in La Selva Biological Station, Sarapiquí). These samples were homogenized by hand-grinding in 500 µl sterile water, and 100 µl of this solution was plated in each of the five different media used: LB agar, potato dextrose agar (PDA), CMC agar medium 2 [27], nitrogen-free (NF) agar [29] and chitin agar [30]. All morphologically different bacteria (according to colony shape, size and colour) were purified and classified by 16S rRNA sequencing.

Bacterial identification by 16S rRNA sequencing
We selected bacterial colonies to identify cultivable populations (general and cellulolytic). We performed a direct-colony PCR with 27F and 1492R primers [31] to obtain full-length 16S sequences. PCR reactions included Dream taq 2X Master Mix (Thermo Fisher Scientific Inc.) or Econotaq Plus Green 2X Master Mix (Lucigen Corp.). For 25 µl reactions, 1 µl of each primer (10 µM) was employed. Initial denaturation was conducted for 5 min at 95 °C, followed by 29 cycles consisting of denaturation at 94 °C for 45 s, annealing at 51 °C for 50 s and extension at 72 °C for 2 min, and then a final extension step for 10 min at 72 °C. The PCR products were cleaned and sequenced by the DNA sequencing facility in the Biotechnology Center at the University of Wisconsin-Madison.
Sequencing results were edited and assembled with DNA baser (Heracle BioSoft S.R.L.) and compared against the NCBI database using BLAST [32]. We confirmed the identification of the isolates using the classifier tool from the ribosomal database project (Table S2 [33]). For phylogenetic analyses, sequences were aligned using GUIDANCE [34], employing the MAFFT algorithm and a bootstrap of 100 repeats, and no columns were removed. Both ends of the alignment were trimmed with MEGA 5 [35] and, from this, the model of nucleotide substitutions was selected using jModelTest [36, 37]. We used the Kimura 2-parameter model, and the phylogenetic tree was constructed using MrBayes 3.2 software [38, 39]. For constructing the tree, four chains were run, with a temperature of 0.2, for a maximum of 107 generations, discarding the initial 25%, and the standard deviation (SD) of split frequencies at the end of the run was 0.002. Final editions of the tree were made using MEGA 5. Sequences were deposited in GenBank under accession numbers KT025880–KT025926.

**RESULTS**

**Ultrastructure of leaf-cutting ant fungus gardens and refuse dumps**

SEM observations of fungal garden sections showed that in the top section, the ants’ symbiotic fungus does not penetrate the cuticular and epidermal layers of the leaves (Fig. 1a), but rather penetrates the leaves through the cuts made by the ants prior to leaf inoculation into the fungus garden (Fig. S2a). These observations were also confirmed by TEM analyses, which demonstrated that in the top section of the fungal gardens, most of the plant cell walls were intact (Fig. 1b), while only some plant cells had lost their cell wall integrity and liberated their cell contents (Fig. S2a). This is also likely due to the chewing and cutting performed by the ants when preparing the leaf substrate for inoculation into the fungal gardens [12].

SEM observation of the fungal garden middle section revealed initial degradation of plant material. There were evident ruptures in the cuticle and epidermal layers, where the penetration of the symbiotic fungus hyphae into the leaf material is clearly seen (Fig. 1c). TEM showed that leaf material was partially degraded in certain areas, as evidenced by alterations in the internal structure as well as in the cell wall of several plant cells. However, these alterations in some cases were minor and the majority of the cells were still intact (Fig. 1d).

In contrast, the bottom of the fungus garden had mostly degraded plant cells. TEM observation showed few intact cells, while the majority showed cell wall degradation and altered internal structures (Fig. S2b); in some cases the cell contents were liberated. Moreover, in some areas of the bottom section, we were unable to locate a single plant structure, it being composed exclusively of the symbiotic fungus hyphae and plant cell residues (Fig. 1f). SEM analyses showed similar results of leaf structure loss and plant cell wall degradation, along with xylem fibres, trichomes and cuticular material in some areas of this layer (Fig. S3a). Bacterial cells were evident only in the bottom of the fungus garden, as we could not observe their presence in the top or middle sections within the samples analysed. These bacteria were mainly rod-shaped, and were grouped over the surface of leaf fragments (Fig. 1e).

Our ultrastructural analyses of the A. colombica refuse dump, employing both SEM and TEM, showed a similarity in composition between the bottom section of the fungus garden and the top section of the refuse dump, including the degradation of plant cell walls and the presence of xylem fibres, trichomes and cuticular material (Fig. S4a). Some of these residues were also present in the lower part of the refuse dumps, in which the presence of undegraded trichomes was also evident (Fig. 2c). SEM also showed the presence of numerous rod-shaped bacteria over plant material residues located in both the top (Fig. 2a) and bottom sections, although these were more evident in the former. Observation of both sections by TEM revealed high levels of bacteria (Fig. 2b, d), as well as a small number of intact plant cells with starch granules (Figs 2d and S4b).

**Bacterial quantification and distribution in fungus gardens and refuse dumps**

The top of the fungus garden had an average of $5 \times 10^5$ cultivable bacteria per gram (fresh). The number of cellulolytic bacteria per gram in this section had an average of $3 \times 10^5$. We performed a direct enumeration of total bacteria using DAPI, and the average number of bacteria per gram was equivalent to the number of cultivable bacteria, corresponding to $5 \times 10^5$ (Fig. 3).

In the middle section of the fungus garden, the average number of cultivable bacteria per gram was $7 \times 10^4$. The number of cellulolytic bacteria/gram was $5 \times 10^4$ in average, while direct enumeration showed the total number of bacteria per gram as $6 \times 10^5$ (Fig. 3).

Regarding the bottom section of the fungus garden, this had the highest number of bacterial cells when compared to the other sections. The number of cultivable bacteria per gram was $1 \times 10^6$, and the number of cellulolytic bacteria per gram was $1 \times 10^5$. Direct enumeration of cultivable bacteria yielded a lower result than quantification, detecting only $8 \times 10^5$ bacteria per gram (Fig. 3).

Refuse dump sections had a higher number of bacteria than fungus garden samples in all three quantification methods used. The average number of cultivable bacteria per gram was the same in the top and bottom sections: $2 \times 10^7$. The number of cellulolytic bacteria per gram was $3 \times 10^6$ and $5 \times 10^6$ on average for the top and bottom sections, respectively. Direct enumeration yielded, on average, a total number of bacteria per gram of $3 \times 10^6$ for the top section and $2 \times 10^6$ for the bottom section (Fig. 3).

Statistical analyses of these results showed that the difference in the average number of bacteria per gram, as assessed...
by both cultivation (LB media) and direct counts (DAPI), was significant ($P<0.05$) between both refuse dump sections and the fungus garden sections. Cellulolytic counts were significantly different between the bottom section of the refuse dump and the fungus garden sections, while the top section of the refuse dump was not significantly different from the other four samples. Finally, our results show an almost ten-fold increase, and sometimes even greater, regarding the

**Fig. 1.** Ultrastructure of fungus gardens from leaf-cutter ant colonies. Scanning electron microscopy (SEM; Panels a, c and e) and transmission electron microscopy (TEM; panels b, d and f) analysis from the top layer of the fungus garden (panels a and b [arrows indicate fibres]), middle layer (panels c and d) and bottom layer [panels e and f (arrows indicate fungal cells)]. The top layer revealed leaf segments cut by the ants prior to inoculation, surrounded by hyphae. Degradation of leaf cells and cell walls was evident in the bottom of the fungus garden, and only in this section we could observe a group of bacteria over the leaf surface. In certain areas of this section we found only the cell contents of degraded plant material and fungal cells, showing that plant material was fully degraded (panel f).
number of bacteria present in the refuse dumps when compared to the fungus gardens.

**Identification of cellulytic bacteria in laboratory-maintained leaf-cutting ant fungus gardens**

Nineteen cellulolytic isolates were obtained from different fungus garden sections. The majority of these isolates (11) were obtained from the bottom section of the fungus garden, while four were isolated from the top section and the remaining four from the middle section. These 19 isolates were classified in three phyla according to their 16S rRNA gene. The isolates from the top section were all Firmicutes, related to *Staphylococcus* and *Lactococcus* (Fig. 4). The four isolates from the middle section were affiliated with Firmicutes and Actinobacteria, and they appeared to be closely related to both *Bacillus* sp. and *Microbacterium* strains. Finally, the bottom section had representatives of all three phyla, including two isolates that grouped with Proteobacteria in our phylogenetic analyses, and those appear to be closely related to *Pseudomonas* and *Achromobacter* strains. Another isolate grouped with Actinomycetales, also classified as *Microbacterium*, while the remainder of the isolates (8) were included in the Firmicutes phyla, with two of them being grouped with *Lactococcus*, two with *Lysinibacillus* and the rest (4) being closely related to *Bacillus* strains (Fig. 4 and Table S2).

**Presence of potentially symbiotic bacteria in fungus garden pellets carried by leaf-cutting ant queens**

Employing electron microscopy techniques, including SEM (Fig. 5a) and TEM (Fig. 5b), we detected the presence of bacterial cells within the pellet in all samples analysed (*n*=19 SEM, 6 TEM). Morphologically, most of these cells were rod-shaped, and in some cases had evident flagella. It was evident that the bacteria were alongside the hyphae over the plant material (Fig. S5). The number of bacteria in eight individual pellets, with sizes ranging from $7 \times 10^2$ to $1 \times 10^3 \mu$m in diameter, was quantified employing SEM, showing a high variability in bacterial abundance in each pellet. The sample with the lowest number of bacteria per pellet had $5 \times 10^2$ cells. In contrast, the sample with the highest estimated number of bacteria per pellet had $5 \times 10^4$ cells. Thus, the differences in bacterial abundance were up to a hundred-fold between one pellet to another. The average number of bacterial cells per pellet was $1 \times 10^4$ (Table 1).

To evaluate the cultivable bacterial diversity associated with these fungus garden inocula (queen pellets), we employed
Regarding differences among the various culture media employed, the majority of our 29 isolates were obtained in LB agar (17), including seven genera from all three phyla. All isolates obtained in PDA media (6) belonged to Proteobacteria, encompassing four genera: Pseudomonas, Acinetobacter, Herbaspirillum and Pantoa. The remaining six isolates were obtained with CMC 2 media (two isolates), classified as Kocuria and Acinetobacter, and with nitrogen-free media (four isolates), identified as Pseudomonas, Acinetobacter (2) and Bacillus (Fig. 4 and Table S2).

**DISCUSSION**

Leaf-cutting ants and their mutualistic fungus *Leucoagaricus gongylophorus* depend on leaf material as their only source for nutritional intake. In this association, the leaf material is degraded within the fungus garden and the ants feed on the fungus [9–11]. Therefore, this dependence makes plant biomass degradation a process critical to the survival of these ant species. Several publications address the issue of cellulose degradation in the fungus gardens of leaf-cutting ants, aiming at the question of whether the symbiotic fungus or the ants’ microbial associates are able to degrade this complex polymer [6, 19, 22, 23]. Employing electron microscopy, we provide visual evidence that plant cell walls are degraded as the plant tissues progress through the fungus garden structure, thereby confirming the ability of the system to decompose this recalcitrant material.

Our ultrastructural analyses suggest that cellulose is being degraded throughout the fungus garden, and it appears to be more fully degraded in some areas of the bottom section. In the top section, the fungal hyphae do not seem to be able to break down cuticular and epidermic layers, but instead penetrate the leaves through the cuts made by the ants. These results are consistent with previous reports indicating that the fungal symbiont has difficulty penetrating intact leaf surfaces [43], as well as limited capability to degrade cellulose in pure culture [4, 21, 44]. These results are also in accordance with the findings of Moller et al. [18], who demonstrated that in *Acromyrmex echinatior* fungus gardens pectin is degraded very early in the process through fungal enzymes that cross the ant digestive system undegraded and reach the leaves in faecal droplets, providing access to protein and starch inside plant cells. Our observations also offer evidence that the symbiotic fungus is probably absorbing nutrients that are quickly released from the plant cells cut open by the ants [14, 20]. It has been proposed that pectin renders hemicellulose inaccessible by enzymes, and therefore pectin degradation at the top of the fungus garden would help degradation of other plant cell wall components downstream [18]. As our results show, plant cell wall degradation is evident in the bottom part of the fungus garden (Fig. 1f), indicating that the catalytic machinery needed to break down its complex polymers is present within this system. Moller et al. [18] state that significant quantities of cellulose and xylan are not employed as energy sources in *A. echinatior* fungus gardens, but our microscopic analyses demonstrate a high level of degradation in some areas of the

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**Fig. 3.** Levels of bacteria at different sections of *Atta* sp. fungus gardens and *A. colombica* refuse dumps. Data represent the mean bacterial counts ± SEM, per gram of sample (n=8 fungus gardens, 9 refuse dumps). Samples were compared using ANOVA (statistically different groups denoted by the letters a and b above the bars). In all cases the refuse dump samples had higher numbers of bacteria than the fungus garden samples (P<0.05). However, the average number of bacteria in the refuse dump top section using CMC-2 was not significantly different to found in fungus garden samples (FG, fungus garden; RD, refuse dump). Direct counts were performed with DAPI staining, while total cultivable bacteria were counted on LB media and cellulolytic bacteria on CMC-2 media.

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Additional research on the fungus garden of *A. echinatior* [23] showed an enrichment of transcripts coding for cellulases, hemicellulases, xylanases, acetyl xylan esterases and xyloglucanases in the bottom of the fungus garden

*Fig. 4.* Phylogenetic tree based on the 16S rRNA gene of isolates from fungus garden and fungus garden pellets. Isolates in blue belong to the fungus garden and those in red to the fungus garden pellet. Red triangles indicate isolates that share a cluster with *Klebsiella*, *Pantoea*, Enterobacteriaceae and *Serratia*, known symbionts of leaf-cutting ants. Underlined strains were isolated from fungus gardens in other studies where they were established as the main members of the fungus garden bacterial community. Codes in the isolates indicates the colony of origin, then the type of sample, section (fungus garden) and the media used for isolation.

bottom section, suggesting that those sources are at least partially degraded before being discarded in the refuse.
compared to the top and middle sections. This is also consistent with our findings, suggesting that other nutritional sources are preferred in the top section.

Similarly, recent research has documented the expression of fungal cellulases in *Atta* fungus gardens [22], suggesting that the symbiotic fungus produces the enzymes needed to degrade the major components of plant cell walls. While our results do not provide a complete picture of cellulose degradation in leaf-cutting ant fungus gardens, they do suggest that symbiotic bacteria may also be involved in this process. Several lines of evidence support this statement. First, we consistently isolated cellulolytic bacteria (mostly Firmicutes) from the different sections of the fungus garden, in agreement with previous culture-independent studies indicating the presence of cellulase-encoding genes of bacterial origin [6, 7]. Second, cellulolytic bacteria could be transferred vertically within the fungus garden pellets utilized by recently mated queens to start a new colony; our isolates from these initial inocula included several Gamma-Proteobacteria, described by Suen et al. [6] as an important source of cellulase genes found in the fungus garden metagenome. Third, the number of bacteria in the different sections of the fungus gardens and refuse dumps, as demonstrated by culture-dependent and EM techniques, is consistent with the degree of plant cell wall degradation. It is worth mentioning that most bacteria observed by SEM were rod-shaped, in both fungus gardens and queen pellets, which is consistent with the majority of our isolates belonging to Proteobacteria and Firmicutes, two phyla comprising mainly rod-shaped bacteria [45].

Cellulose degradation is even more evident in the refuse dumps, which also harbour a higher number of associated bacteria that are probably exploiting the partially degraded nutrients constantly brought by the ants from the fungus garden. It is possible that the combination of different nutrient availabilities, as well as the sanitary measures that the ants continuously apply to the gardens, contribute to create a spatial structuring of different specialized microbial communities associated with fungus gardens and refuse dumps; this is supported by previous studies using culture-independent analyses [6, 15]. Our images also show some residues of plant material in the dumps, which is consistent with previous reports [43, 46] indicating that hemicellulose and cellulose are partially degraded throughout the fungus garden, while lignin appears to remain mostly intact until it reaches the refuse dumps.

The high number of bacteria in the refuse dump sections, when compared to the fungus garden sections, indicates that material taken to these dumps is not fully degraded, and the bacteria present in refuse dumps exploit these leftover nutrients derived from the fungus garden. The estimated number of bacteria in all samples ranged from $10^4$ per gram in the middle section of the fungus garden to $10^7$ per gram in refuse dump samples. This average number of bacteria in the dumps was very similar to the number of bacteria in soil estimated in another study, which ranged from $10^6$ to $10^8$ per gram [47]. The estimated number of bacteria in the garden sections was significantly lower than the number in these soil samples, suggesting that the ants’ activities have a sanitizing effect resulting in the lower bacterial abundance seen in the fungus gardens. The higher abundance of cultivable and cellulolytic bacteria in the top compared to the middle section could suggest that bacteria from faecal droplets that reach the garden when the ants mix them with the freshly deposited leaf material [48] grow more easily in culture, and have cellulose-degrading capabilities. Hence, our results reinforce the findings that ants pretreat leaves prior to inoculation in the fungus garden [17], and this may be selecting for certain, potentially beneficial, microbial community members. This selection may favour the presence of cellulose-degrading bacteria. Our counts for cellulolytic bacteria appear to follow a similar trend to the counts for cultivable bacteria. Despite these results, differences were not significant and it is likely that not all cellulolytic bacteria will grow on CMC 2 agar, and therefore the observed trend must be tested further. Bacterial abundances in each fungus garden section may also vary in response to diet shifts, as seen in the microbial community of

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**Table 1.** Estimated number of bacteria per fungus garden pellet using scanning electron microscopy. Samples were collected from *Atta cephalotes* queens on 22 June 2010 at La Selva Biological Station (Organization of Tropical Studies).

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<th>Sample code*</th>
<th>Estimated number of bacteria</th>
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<td>LSBM 150</td>
<td>$3 \times 10^4$</td>
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<tr>
<td>LSBM 308</td>
<td>$3 \times 10^3$</td>
</tr>
<tr>
<td>LSBM 152</td>
<td>$8 \times 10^3$</td>
</tr>
<tr>
<td>LSBM 307</td>
<td>$3 \times 10^3$</td>
</tr>
<tr>
<td>LSBM 317</td>
<td>$6 \times 10^2$</td>
</tr>
<tr>
<td>LSRV 57</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>LSBM 303</td>
<td>$9 \times 10^3$</td>
</tr>
<tr>
<td>LSRV 52</td>
<td>$5 \times 10^2$</td>
</tr>
</tbody>
</table>

*LS, La Selva; BM or RV, collector initials.

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*Fig. 5.* Electron micrographs of the fungal pellet carried by ant queens to establish new colonies. All samples analysed by SEM (panel a) and TEM (panel b) showed the presence of rod-shaped bacteria colonizing the pellet.
Sericomymex ants [48], but the variations found in our study were not significant.

Even though they do not contribute to colony nutrition directly, bacteria that degrade lignin and other complex polymers in refuse dumps are contributing towards efficient waste management. In dominant species with internal dumps, these also contribute towards efficient nest ventilation by generating metabolic energy that leads warm air to exit by specialized tunnels while bringing in colder air towards the fungus garden chambers [49]. In the present study, the direct enumeration of total bacteria yielded in some cases lower abundance than the culture-dependent methods, contrary to what was expected. Kirchman and collaborators [50] determined that direct enumeration introduces high variation due to the selection of fields in the microscope, since the bacteria may not be evenly distributed on the filter. The fields counted in this study were selected at random, but the presence of areas with low bacterial abundance may have caused the underestimation shown.

The importance of bacteria for leaf-cutting ant colonies may justify their vertical transmission, as we detected the presence of bacteria in all fungus garden inocula examined. Using culture-dependent methods, we identified isolates that were related mostly to Proteobacteria, which have been determined by previous culture-dependent and independent analyses to be major bacterial community members in mature fungus gardens [5–7]. Our electron microscopy analyses showed mainly rod-shaped bacteria, with a high variation in the number of bacteria per fungus garden pellet. Further research is needed to determine whether there is a correlation between the number of bacteria in this garden inoculum and colony fitness, as well as to help us understand other aspects related to colony founding, including the role of the queen in selecting the appropriate piece of garden with the right combination of beneficial microbes before leaving the colony for the nuptial flight.

In the present study, bacterial diversity associated with fungus garden pellets was evaluated by culture-dependent methods. It is well known that this approach underestimates diversity, since more than 99% of the bacteria are not cultivable [51]. Additional experiments involving deep-sequencing metagenomics of fungus garden pellets are needed to fully establish vertical transmission. However, the main bacterial groups found in our study within these pellets are consistent with the most prevalent phylotypes detected in leaf-cutter ants’ fungus gardens employing culture-independent techniques [6, 7, 15].

Some of our isolates from fungus garden pellets and cellulolytic isolates from fungus garden sections have previously been reported as members of the bacterial community associated with other ant species, providing key services to their insect host. For example, van Borm et al. [52] found nitrogen-fixing Pseudomonas in the gut of Tetraponera ants. Additional studies detected Pseudomonadales, Actinomycetales and Enterobacteriales associated with Megalomomyrce (a social parasite of attine ants) and their hosts Sericomymex, Trachymyrmex and Cyphomyrce, suggesting that Megalomomyrce ants acquire these bacteria by consuming the fungus garden maintained by the attine ants [48]. Moreover, bacterial members from these three orders have also been found associated with the ants and fungus garden of the lower attine Mycocepurus, Pseudomonas and Pantoea being two of the most common genera found in their garden [53], similar to what has been described for Atta sp. [6, 7, 15]. Another study, analysing the gut-associated bacteria of Cephalotes varians turtle ants [51] by standard cultivation methods, detected bacteria in the genera Bacillus, Enterobacter, Microbacterium, Pantoea, Pseudomonas and Serratia, which correspond to some of the bacterial genera obtained in the present work. Taking these results together, it is evident that the bacterial diversity in the fungus garden inocula found in our study relates not only to Atta fungus gardens analysed by culture-independent methods but also to the microbial community of other ant species and their fungus gardens, suggesting that they may perform important functions for the hosts. These findings also raise the question of whether the cellulolytic bacteria found in fungus gardens from our study were introduced from the ants’ digestive tract, since they may reach the garden by the faecal droplets the ants deposit on leaf material [48]. Further research beyond the scope of the current work is needed to fully evaluate this possibility.

Our results provide consistent evidence that cellulose degradation occurs in the fungus gardens of leaf-cutting ants. The distribution of bacteria throughout the different fungus garden sections, their activity in culture, and the ultrastructural changes observed in plant material, all suggest that bacteria in the fungus gardens of leaf-cutter ants could play an important role in cellulose degradation. These findings also support current evidence proposing synergistic biomass degradation by the leaf-cutting ants’ symbiotic fungus and the bacteria associated with the fungus gardens. Our work also provides evidence for vertical transmission of bacteria through the fungus garden pellet used as the initial inoculum, and shows that the community associated with this pellet is highly similar to mature fungus gardens microbiota reported in previous studies [6, 7, 15]. Therefore, our findings confirm the importance of the fungus garden-associated microbial community for leaf-cutting ants, and enhance our knowledge regarding the crucial role played by microbial symbionts in the ecology and evolution of social insects.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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