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► **To cite this version:**

Danival José de Souza, Alain Lenoir, Maria Catarina Megumi Kasuya, Myriam Marques Ramos Ribeiro, Séverine Devers, et al.. Ectosymbionts and immunity in the leaf-cutting ant *Acromyrmex subterraneus subterraneus*. *Brain, Behavior, and Immunity*, 2012, 28, pp.182-187. 10.1016/j.bbi.2012.11.014 . hal-01322600

HAL Id: hal-01322600

<https://hal.science/hal-01322600>

Submitted on 27 May 2016

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Accepted Manuscript

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PII: S0889-1591(12)00500-4
DOI: <http://dx.doi.org/10.1016/j.bbi.2012.11.014>
Reference: YBRBI 2059

To appear in: *Brain, Behavior, and Immunity*

Received Date: 24 July 2012
Revised Date: 21 November 2012
Accepted Date: 22 November 2012

Please cite this article as: de Souza, D.J., Lenoir, A., Kasuya, M.C.M., Ribeiro, M.M.R., Devers, S., Couceiro, J.d.C., Lucia, T.M.C., Ectosymbionts and immunity in the leaf-cutting ant *Acromyrmex subterraneus subterraneus*, *Brain, Behavior, and Immunity* (2012), doi: <http://dx.doi.org/10.1016/j.bbi.2012.11.014>

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17 **Abstract**

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2
3 18 Associations with symbiotic organisms can serve as a strategy for social insects to resist
4
5 19 pathogens. Antibiotics produced by attine ectosymbionts (*Actinobacteria*) suppress the
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8 20 growth of *Escovopsis* spp., the specialized parasite of attine fungus gardens. Our objective
9
10 21 was to evaluate whether the presence or absence of symbiotic actinobacteria covering the
11
12 22 whole ant cuticle is related to differential immunocompetence, respiratory rate and cuticular
13
14 23 hydrocarbons (CH). We evaluated these parameters in three worker groups of *Acromyrmex*
15
16 24 *subterraneus subterraneus*: External workers (EXT), internal workers with actinobacteria
17
18 25 covering the whole body (INB) and internal workers without actinobacteria covering the
19
20 26 whole body (INØ). We also eliminated the actinobacteria by antibiotic treatment and
21
22 27 examined worker encapsulation response. INB ants showed lower rates of encapsulation and
23
24 28 respiration than did the EXT and INØ ants. The lower encapsulation rate did not seem to be a
25
26 29 cost imposed by actinomycetes because the elimination of the actinomycetes did not change
27
28 30 the encapsulation rate. Instead, we propose that actinobacteria confer protection to young
29
30 31 workers until the maturation of their immune system. Actinobacteria do not seem to change
31
32 32 nestmate recognition in these colonies. Although it is known that actinobacteria have a
33
34 33 specific action against *Escovopsis* spp., our studies, along with other independent studies,
35
36 34 indicate that actinomycetes may also be important for the individual health of the workers.

35 **Key words:** Social Immunity; Symbiosis; *Actinobacteria*; Cuticular Hydrocarbons; Energetic
36 Cost

37

38 1. Introduction

39 Symbioses play a central role in the evolution of biological complexity and leaf-
40 cutting ants are a prodigious example of this (Ness et al., 2010). More than a century after
41 Belt's suggestion that leaf-cutting ants use leaves to cultivate a fungus (Belt, 1874), other
42 microorganisms were subsequently added to this complex association, including actinomycete
43 bacteria that confer protection against a specialized parasitic fungi of the fungus garden,
44 *Escovopsis* spp. (Currie et al., 1999; Muchovej and Della Lucia, 1990), and black yeasts that
45 compromise the efficiency of bacteria-derived antibiotic defense in fungus-growing ants
46 (Little and Currie, 2008). Additionally, a very large variety of bacteria with an undefined role
47 is found in the nest and in the dump chambers (Scott et al., 2010).

48 The first studies dealing with *Actinobacteria-Attini-Escovopsis* symbiosis revealed a
49 long history of specific coevolution between actinomycetes and *Escovopsis*. However, recent
50 studies have indicated that actinomycete benefits cannot be restricted to protection against
51 *Escovopsis* because antibiotics derived from actinomycetes have a broad spectrum action
52 (Haeder et al., 2009; Sen et al., 2009; Schoenian et al., 2011; Mueller, 2012). Furthermore,
53 considering the myriad of non-specific parasites in the fungus garden, the specificity of
54 antibiotics produced by actinomycetes is improbable.

55 *Actinobacteria* are easily detected on the cuticle of the workers because they give a
56 whitish appearance; this led Gonçalves (1961) to suggest that this "strange coating", which is
57 easily removed with needles, was most likely a fungus. Later, Currie et al. (1999) isolated and
58 identified these microorganisms as *Actinobacteria*. They are abundant on workers inside the
59 fungus garden where pathogen control is required to prevent symbiotic fungus collapse.
60 Newly emerged major workers do not seem to carry actinomycetes on the cuticle, but
61 actinomycetes appear on callow workers and progressively increase over time, most likely

62 after transmission by old workers or direct contact with the fungus garden (Poulsen et al.,
63 2003a). In this study, there was an observed growth pattern where major workers were
64 progressively covered by the bacterium a few days after emergence and bacterial cover
65 reached a maximum after 10–15 days.

66 Actinomycetes are an interesting group of microorganisms because they are
67 responsible for a considerable portion of commercially important bioactive microbial
68 products. Nevertheless, it is not known how actinomycetes influence the ant immune system,
69 although symbiotic microorganisms influence health and disease in animals, and studies have
70 shown that bacteria contribute to their immune defenses. This symbiosis has been observed in
71 various animal taxa: on the amphibian's skin (Becker and Harris, 2010; Woodhams et al.,
72 2007), in the mammalian intestine (Cash et al., 2006) and in insects (de Souza et al., 2009;
73 Oliver et al., 2003). Ants, as well as all other invertebrates, lack an adaptive immune system
74 and must rely on innate immunity as their primary mechanism of defense against parasites
75 and pathogens (Gillespie et al., 1997). Their immune system is closely associated with
76 hemolymph, which consists of cellular (hemocytes) and liquid components (plasma), where
77 the humoral factors are dissolved. Among cellular responses, encapsulation followed by
78 melanization is an efficient innate immune response against infection by parasites (Gillespie
79 et al., 1997) and has been frequently used to evaluate ant immunity (Sorvari et al., 2008; de
80 Souza et al., 2008; de Souza et al., 2009), including that of leaf-cutting ants (Baer et al., 2005;
81 Ribeiro et al., 2011).

82 Recognition of group members is a critical process to ensure social cohesion within
83 the group. Ants use chemical signatures, composed primarily of cuticular long-chain
84 hydrocarbons, in nestmate recognition (d'Ettorre and Lenoir, 2010). To protect the colony
85 against parasites, it is expected that workers can discriminate nestmates based on individual
86 immunological state. Likewise, odor perception can be affected by immune response. For

87 example, when honeybee immune systems are triggered by the non-pathogenic immunogenic
88 elicitor lipopolysaccharide (LPS), they have a reduced ability to associate an odor with a
89 sugar reward (Mallon et al., 2003). Plenty of bacteria have been shown to play an important
90 role in the production of volatile compounds, some of which may act as chemical messengers
91 within or between species (Leroy et al., 2011). Currently, the role of actinomycetes in
92 chemical communication is unknown and requires more investigation.

93 One general attribute of immune functions is that their operation requires resources
94 that the host might have used for another function (Sheldon and Verhulst, 1996). Immune
95 stimulation increases energy consumption (Freitak et al., 2003; Tyler et al., 2006) and
96 decreases longevity in insects (Armitage et al., 2003). Thus, considering that the immune
97 system is costly to develop, maintain or activate, ants that invest less in immune defense can
98 direct energy to other activities, such as fungus garden care or brood care. If ectosymbiotic
99 bacteria provide immune protection for the ants, the ants can stay protected even with a less
100 active immune system. Inferences on the energetic cost of physiological processes in insects
101 can be made by the evaluation of the oxygen consumption rate, which has been studied in
102 leaf-cutting ants (Hebling-Beraldo and Mendes, 1981; Hebling et al., 1992; Poulsen et al.,
103 2003a).

104 Our objectives were to evaluate whether the presence or absence of symbiotic bacteria
105 covering the ant cuticle is related to differences in (1) the encapsulation responses between
106 workers, (2) the level of metabolic activity, which is determined by measuring individual
107 respiratory rates, and (3) the cuticular hydrocarbons pattern. We also eliminated the bacteria
108 using an antibiotic treatment and examined worker encapsulation response after the treatment.

111 2. Material and Methods

112 2.1. Colony maintenance

113 In this study, we used adult colonies of *Acromyrmex subterraneus subterraneus* that
114 had been collected three years before in Viçosa, Minas Gerais State, Brazil. The colonies were
115 maintained in the laboratory at the Animal Biology Department Insectary at the Federal
116 University of Viçosa, according to the methodology developed by Della Lucia et al. (1993).
117 They consisted of a vial (3 L) with the fungus garden connected to a foraging arena and were
118 maintained at 25 ± 2 °C with a relative humidity of $75 \pm 5\%$ and a 12:12 light:dark regime. On
119 a daily basis, the ants received fresh leaves of *Ligustrum japonicum* Thumb, *Tecoma stans* L.,
120 *Acalypha wilkesiana* Müll Arg and *Rosa* spp., in addition to clean water.

122 2.2. Workers selection and encapsulation rate assay

123 The encapsulation response depends on humoral and cellular factors, and the cellular
124 defense system is coupled with humoral defense in the melanization of pathogens. Thus, the
125 encapsulation rate assay provides an accurate measure of immunocompetence, which is
126 defined as the ability to produce an immune response (Ahtiainen et al., 2004; Rantala and
127 Kortet, 2004). We used three three-year-old colonies (A, B, and C) for measuring the
128 encapsulation rate of *A. subterraneus subterraneus* workers. Three groups of workers of
129 similar size (approximately 2.4 mm of head capsule width) were defined based on their nest
130 location (internal/external) and the extent of actinomycetes covering their cuticle (clearly
131 visible/not visible): (1) external workers without visible bacteria covering the body (EXT), (2)
132 internal workers with bacteria covering the whole body (INB) and (3) internal workers
133 without visible bacteria covering the whole body (INØ). Considering the wide variation in
134 bacterial coverage of the ants, we have chosen two distinct worker classes. INB workers

135 referred to those whose head, thorax and gaster were entirely covered with bacteria from a top
136 view. This pattern corresponds to ‘score 12’ (maximum) established and used by Poulsen et
137 al. (2003a). From a top view, the EXT and INØ workers exhibited no coverage of bacteria on
138 the head, thorax and abdomen. Insertion of an artificial antigen in the hemocoel provokes its
139 encapsulation, and this method has been frequently used to evaluate insect immunity (de
140 Souza et al., 2009; de Souza et al., 2008; Fytrou et al., 2006; Lu et al., 2006; Sorvari et al.,
141 2008; Vainio et al., 2004). We measured the encapsulation response by inserting an inert
142 antigen, a 1.5 mm-long piece of a sterile nylon monofilament (0.12 mm diameter), into each
143 ant’s thorax between the second and third leg pairs. After introduction of the antigen, the
144 workers were individually placed in glass test tubes. The tubes were maintained in an
145 incubator at 25 °C, 75% R. H., in the dark. This procedure was carried out on 10 workers
146 from each colony, with a total of 30 workers for each group. Twenty-four hours later, the
147 implants were removed from the hemocoel and placed on a glass slide to be mounted in
148 Entellan© medium. Nylon monofilament was examined under a light microscope and
149 photographed using a digital camera (Axioskop 40 Zeiss microscope). The mean gray value
150 of the whole implant was measured using the ImageJ 1.37v software. It was assumed that the
151 darkest gray received the highest encapsulation rate (total black). The background gray value
152 was subtracted to correct the gray values of the implants. The colony was included as a
153 random factor and treatments were analyzed by an ANOVA followed by an Unequal N HSD
154 test at 5% probability.

155 2.3. Antibacterial treatment and encapsulation response

156 In this experiment, we used a fourth colony (colony D) to test the effects of removing
157 bacteria on worker immunity. To kill the bacteria, we followed the methodology described by
158 Poulsen et al. (2003a). We established six experimental treatments using workers with
159 bacteria covering the whole body: (1) 22 without treatment, (2) 20 treated with a dry brush to

160 remove their bacterial cover, (3) 20 treated with a wet (water only) brush, (4) 20 treated with a
161 brush containing a solution of penicillin G (622 mg/L), (5) 20 with a brush containing a
162 solution of streptomycin sulfate (1230 mg/L) and (6) 20 treated with a brush containing a
163 mixture of the two antibiotics. Ant workers were all about the same size (~ 2.4 mm HW) and
164 the brushing operation lasted approximately 10 s. Afterwards, all ants were marked with a dot
165 of paint and placed in mini-colonies established in plastic pots containing 100 mL of fungus
166 garden and approximately 100 nestmate workers without visible bacteria coating. Ten days
167 later, the marked workers were removed for an encapsulation assay, as described in section
168 2.2. We verified that these marked workers did not show a visible white coating of bacteria in
169 the integument, confirming that the treatments were effective. The groups were compared by
170 an ANOVA followed by an Unequal N HSD test at 5% probability.

171 2.4. Respirometry assay

172 The aim of this study was to assess the metabolic rate and to infer a possible energetic
173 cost of maintaining ectosymbiotic bacteria. The production of carbon dioxide was measured
174 in a carbon dioxide analyzer (TR 2; Sable System International, Las Vegas, Nevada, USA)
175 using methods adapted from Hebling et al. (2000) and Guedes et al. (2006). A series of 25 mL
176 flasks was used, each flask containing three workers (2.4 mm head capsule width) from each
177 group (EXT, INB, and INØ) in a completely closed system. Carbon dioxide-free air was
178 injected into the flasks for 2 min at 600 mL/min. An infrared reader was connected to the
179 outlet of the system to quantify carbon dioxide (μmol). The test tubes were connected to the
180 system for three hours before measurement of CO_2 production from the workers, which was
181 achieved by injection of CO_2 -free air into the vials for two minutes at a flow rate of 600
182 mL/min. This air flow directs CO_2 to an infrared reader connected to the system and allows
183 rapid quantification of the amount of CO_2 produced on an hourly basis (in μmol). There were
184 14 replicates for each group, which were taken at the same proportion from three colonies (A,

185 B, and C). In total, we took 42 workers from each colony. The value of CO₂ production for
186 each vial was divided by three to calculate the mean respiratory rates, which were analyzed by
187 an ANOVA followed by a Tukey test at 5% significance and using Statistica 7.0. The colony
188 was included as a random factor.

189 2.5. Chemical analyses

190 In this experiment, we used the same three colonies (A, B and C). The head-thorax
191 with the legs taken from the three groups of media workers (EXT, INB and INØ); 6 workers
192 per group per colony were immersed in 1 mL of pentane and removed after 30 min. Before
193 analysis, the solvent was evaporated and redissolved with 5 µL of pentane; we then added 2
194 µL of pentane containing 200 ng of eicosane (C₂₀) as an internal standard. Two microliters
195 were injected into a FID gas chromatograph (VGM250Q system, Perkin-Elmer) using a DB-5
196 fused silica capillary column. The temperature was maintained at 150 °C during the splitless
197 initial two minutes, raised from 150 °C to 310 °C at 5 °C/min and held at 310 °C for the last
198 10 min. The cuticular hydrocarbons were previously identified (Viana, 1996; Viana et al.,
199 2001), and to verify the names of the peaks, including the smaller peaks, we analyzed in more
200 detail the cuticular profile with the same GC coupled to a Perkin-Elmer MS operating 70 EV.
201 We used a high-temperature column (DB-5HT, 30 m, 0.251 mm x 0.10 µm) with the same
202 temperature program. The areas of the peaks were estimated by peak integration using a
203 TurboChrome Workstation. From the area, we calculated the quantities and relative
204 proportions of substances using the internal standard area (ng per sample). The relative
205 proportions of CHs were used to construct a dendrogram. The total quantities of hydrocarbons
206 were compared with a Kruskal-Wallis test. The profiles between the three groups were
207 compared with a dendrogram using the single-link Ward method and Euclidian distance. We
208 also verified that there were no differences between the colonies.

209 Because products of bacterial metabolism may contribute to the colony odor and play
210 an important role in nestmate recognition (see for termites (Matsuura, 2001) and Minkley et
211 al. (2006)), we analyzed whether the hydrocarbons could have originated from actinobacteria.
212 A *Pseudonocardia* strain (GenBank accession code JF514546; the other two isolates were
213 JX543365 and JX543366) was isolated from *A. subterraneus subterraneus* workers (see
214 Appendix A for the isolation and identification of the bacterium), and we performed a pentane
215 extraction from a small piece of a 1 cm diameter of an agar pure culture that was analyzed as
216 previously described. We also analyzed the hydrocarbons on the gelose used for bacteria
217 culture in the same chromatographic conditions.

219 3. Results

220 3.1. Bacteria and encapsulation rate

221 Variation was observed in the encapsulation rate among the three groups of workers
222 ($F_{2, 81} = 35.66$, $P < 0.001$), i.e., there was a significant effect of treatment on the encapsulation
223 response. Internal workers with bacteria (INB) had the lowest encapsulation rate compared
224 with internal workers without bacteria (INØ) and external workers (EXT) (Unequal N HSD, P
225 < 0.05). The colonies showed a variation in the degree of encapsulation ($F_{2, 81} = 16.62$, $P <$
226 0.001), but no interaction between treatments and colonies was verified ($F_{4, 81} = 0.82$, $P =$
227 0.52); the three colonies exhibited the same pattern of encapsulation rate variation (Fig. 1).

228 3.2. Bacteria removing and encapsulation rate

229 The encapsulation rates of workers whose actinobacteria were removed by
230 streptomycin or a combination of streptomycin + penicillin were reduced in comparison with
231 control workers, brush-treated or penicillin-treated workers (Fig. 2). Ten days after treatment,
232 we could verify that the treatment had a highly significant effect ($F_{5, 72} = 8.92$, $P < 0.001$). We

233 compared the survival proportion of the ants undergoing the bacteria removal treatments
234 against that observed in the control groups. The hypothesis tested was $H_0: P_{\text{control}} = P_{\text{treatment}}$
235 vs. $H_1: P_{\text{control}} > P_{\text{treatment}}$ (one-sided test). The p-value is computed based on
236 the t-value for the following comparisons: Control vs. Dry brush, $P = 0.0042$; Control vs. Wet
237 brush, $P = 0.0001$; Control vs. Pen. G, $P = 0.0021$; Control vs. Strep., $P = 0.0021$; Control vs.
238 Pen. G + Strep., $P = 0.0002$. As all treatments provoked mortality in treated ants, including
239 the Dry brush, it appears that ant mortality is due to the stress of the ant removal from the nest
240 and its manipulation. It is possible that the treatments to eliminate actinobacteria cause
241 selective survival; therefore, we would be sampling the encapsulation response of a subset of
242 the ants. However, we have no evidence of differential mortality associated with the level of
243 encapsulation response because similar mortality occurred in groups with higher
244 encapsulation response (Wet brush) and in groups with lower encapsulation response (Pen. G
245 + Strep.), as verified in Fig. 2.

3.3. Respiratory rate

248 The individual metabolic rate of the workers, measured in terms of CO_2 production,
249 showed a pattern of increase as workers lost their bacterial coating and switched to external
250 activities (Fig. 3; Kruskal-Wallis, $H(2, n = 42) = 6.94, P = 0.03$). Individuals living inside the
251 nest, with or without a whitish coat of bacteria, had significantly lower respiration rates
252 compared with individuals performing external activities.

3.4. Hydrocarbons

255 Hydrocarbon quantities on the thorax did not vary among the three groups: $119.8 \pm$
256 27.7 ng per ant (mean \pm SE) for EXT, 81.1 ± 11.0 for IN \emptyset and 132.3 ± 32.8 for INB
257 (Kruskal-Wallis $H(2, n = 53) = 1.67, P = 0.43$) (See Fig. S1).

258 The hydrocarbon profile was simple (24 peaks, see Fig. S2). The hydrocarbons
1
2 259 observed were mainly methyls (11-+13-+15-MeC29, more than 30%, see table S1; 11-+13-
3
4 260 MeC31 -10%) and the corresponding dimethyls (respectively 11,15-+13,17-DiMeC29, 5%
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7 261 and 11,15-+13,17-DiMeC31, 6%), and the hydrocarbon profile was not changed according to
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10 262 the ant group. In the dendrogram, the samples were mixed in arbitrary groups (see Fig. S3).

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12 263 We found some of the ant hydrocarbons in the bacteria and also in the gelose (see
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14 264 Table S1), but in very small quantities (4.5 and 9.7 ng, respectively). These hydrocarbons
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16
17 265 were all present on the ant's cuticle.

19 266 4. Discussion

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21
22 267 The encapsulation rate of *Acromyrmex subterraneus subterraneus* workers with a
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25 268 visible actinobacteria coating was significantly lower than that of workers without bacteria. It
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27 269 seems that ectosymbionts are not responsible for reducing this immune response because their
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30 270 removal did not increase the encapsulation response. Instead, the results suggest that
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32 271 actinobacteria could give protection to young workers until maturation of their immune
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35 272 system. We affirm that internal workers with bacteria are younger and external workers older;
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37 273 this conclusion is based (i) on our daily observation of laboratory colonies, which included
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39
40 274 several *Acromyrmex* species, and (ii) on the studies conducted by Poulsen et al. (2003a) in
41
42 275 *Acromyrmex octospinosus*. Moreover, temporal polyethism is ubiquitous in social insect
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44
45 276 colonies. Newly emerged workers perform tasks within the nest, such as brood care and nest
46
47 277 maintenance, and progress to tasks outside as they age (Wilson, 1971). Recently, it has been
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49 278 demonstrated that *Actinobacteria* constitute a line of defense against entomopathogenic fungi
50
51
52 279 in Attini ants (Mattoso et al. 2012). These authors verified that experimental removal of the
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54 280 bacterial coating after antibiotic treatment increased the susceptibility of *A. subterraneus*
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56
57 281 *subterraneus* workers to infection by the entomopathogenic fungus *Metarhizium anisopliae*.
58
59 282 This study offered direct evidence for the benefits of actinobacteria ectosymbionts to the
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283 health of the workers. We are also conducting experiments to evaluate the action of an
1
2 284 actinomycete isolate from *A. subterraneus subterraneus* against entomopathogenic fungi
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4
5 285 isolate from the same ant species. Preliminary results have shown inhibitory effects of the
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7 286 actinomycete against the entomopathogenic fungus *Aspergillus ochraceus*.

10 287 The variation of encapsulation rate between the groups is not a function of worker
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12
13 288 location because the encapsulation rate of internal workers without actinobacteria is similar to
14
15 289 that of external workers without actinobacteria. Consistent with our studies, Armitage and
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17
18 290 Boomsma (2010) have found a significant increase in phenoloxydase activity (an enzyme
19
20 291 involved in melanization) in older workers of *A. octospinosus*. Our results, coupled with the
21
22
23 292 studies of Armitage and Boomsma (2010), highlight a pattern of increasing immunity as
24
25 293 *Acromyrmex* workers age.

28 294 Different attine ant species can use different strategies against pathogens. For example,
29
30
31 295 workers of *Atta*, another leaf-cutting ant genus, do not have visible actinobacteria and
32
33 296 completely lost the cuticular structures to rear actinomycetes (Mueller *et al.*, 2008). In *Atta*
34
35
36 297 *sexdens rubropilosa*, workers performing internal activities had a higher encapsulation rate
37
38 298 than those working outside the colony, which is different from what we observed for *A.*
39
40
41 299 *subterraneus subterraneus* (Ribeiro *et al.*, 2011). Comparative studies of immune response
42
43 300 among species, differing in the presence or absence of bacteria coating, could determine
44
45 301 whether ectosymbionts act to modulate innate immune responses in attine ants.

48 302 Actinomycetes seem to combat primarily *Escovopsis* spp., but inhibitory effects of
49
50
51 303 lower intensity have been demonstrated against other fungi, including entomopathogenic
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53
54 304 fungi (Haeder *et al.*, 2009). Under more vulnerable conditions, where the immune system of
55
56 305 younger workers is less active, actinobacteria may offer protection against pathogens. It has
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58
59 306 been demonstrated that other insects can be protected by symbiotic actinobacteria against

307 pathogens, parasitoids or predators. The actinomycetes' ability to produce a wide range of
308 secondary metabolites, including several with antibiotic properties, partially accounts for this
309 trend in insect-actinomycetes symbioses (Kaltenpoth, 2009).

310 From *Hydra* to humans, we can find examples of epithelia selecting the bacterial
311 community to live on them (Fraune and Bosch, 2010). In Attini ants, actinomycetes live in
312 specialized structures that are elaborate cuticular crypts with associated exocrine glands
313 (Currie et al., 2006). Their abundance is age-dependent, and their dependence on metapleural
314 gland secretion supports the hypothesis of active mechanisms regulating their presence
315 (Poulsen et al., 2003b). Thus, another hypothesis to be tested consists of verifying an increase
316 of ectosymbionts when the workers are immunocompromised.

317 In our study, external workers exhibited a more elevated respiratory rate than did
318 workers with actinobacteria. Although it is not possible to separate the fraction of energy due
319 to the presence actinomycetes, it is at least evident that actinomycetes do not pose a high
320 energy cost to workers. Our data support a pattern of increase of metabolic rate as
321 *Acromyrmex* workers age and their immune system achieves maturation, and at this point,
322 they are able to perform external activities.

323 Actinobacteria do not change the cuticular profile or the hydrocarbon quantities of the
324 host ant; this is in contrast to the fungus symbiont, which is important in colonial recognition
325 (Viana and Lenoir, 1996). This indicates that nestmate recognition is not modified, which was
326 expected because foragers and some internal ants do not have the actinobacteria. Workers
327 with and without ectosymbionts cannot be discriminated based on cuticular odors. Some
328 hydrocarbons found on the actinobacteria culture may be general for all bacteria membranes
329 and may have contaminated the gelose. Hydrocarbon production is very low and most likely
330 is not important compared to ant cuticle production, indicating that the ant cuticular

331 hydrocarbons do not originate from the actinobacteria. Nevertheless, actinobacteria also
1
2 332 produce some hydrocarbons that may be a signal for recognition by ants, as Zhang et al.
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4
5 333 (2007) have recently shown that workers are able to recognize their own bacterial strain.
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8 334 Our studies, along with other independent studies, suggest a possible new role of
9
10 335 actinomycetes for the leaf-cutting ants, thus reinforcing emerging views that integumental
11
12 336 biofilms protect ants primarily against ant diseases (Mueller, 2012). Considering that the
13
14 337 combat of infectious diseases is a major challenge for large insect societies, actinomycetes
15
16 338 may ensure protection to younger attine ants until the maturation of their immune system, and
17
18 339 this protection is achieved with low energetic cost.
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24 25 341 **ACKNOWLEDGMENTS**

26
27 342 The authors would like to thank Ms. Aline Mello and Mr. Alberto Soares Corrêa for technical
28
29 343 assistance. This work was financially supported by CNPq-Brazil, French CNRS and François
30
31 344 Rabelais University of Tours.
32
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35 345

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487 **Figure Captions**

1
2 488 Fig. 1 – Encapsulation rate (darkness value of implant) in three groups of workers of *Acromyrmex*
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4 489 *subterraneus subterraneus*. Superscript letters indicate significant differences among the groups, not
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6 490 among the colonies (Unequal N HSD, $P < 0.05$). The numbers inside each column indicate the n
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8 491 value. (Mean \pm SE)

10 492 Fig. 2 - Encapsulaton rate (darkness value of implant) of workers with bacterial coating after five
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12
13 493 treatments to remove actinomycetes and control. Superscript letters indicate significant differences
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15 494 among the groups (Unequal N HSD, $P < 0.05$). The numbers inside each column indicate the n value.
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17 495 (Mean \pm SE)

20 496 Fig. 3 – Respiration rate ($\mu\text{L CO}_2/\text{h}$) per ant; (median \pm semi-interquartile range; $n = 14$) of workers
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23 497 from three colonies (A, B and C). Superscript letters indicate significant differences among the groups.
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25 498 KKW ANOVA ($H(2, N=42) = 6.94, P = .031$) followed by multiple comparisons test.

28 **Appendix**

31 500 Table S1 – Relative hydrocarbon quantities (mean % \pm SE) of the three worker categories (INB, INØ
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33 501 and EXT), all workers, actinobacteria and gelose used for actinobacteria culture.

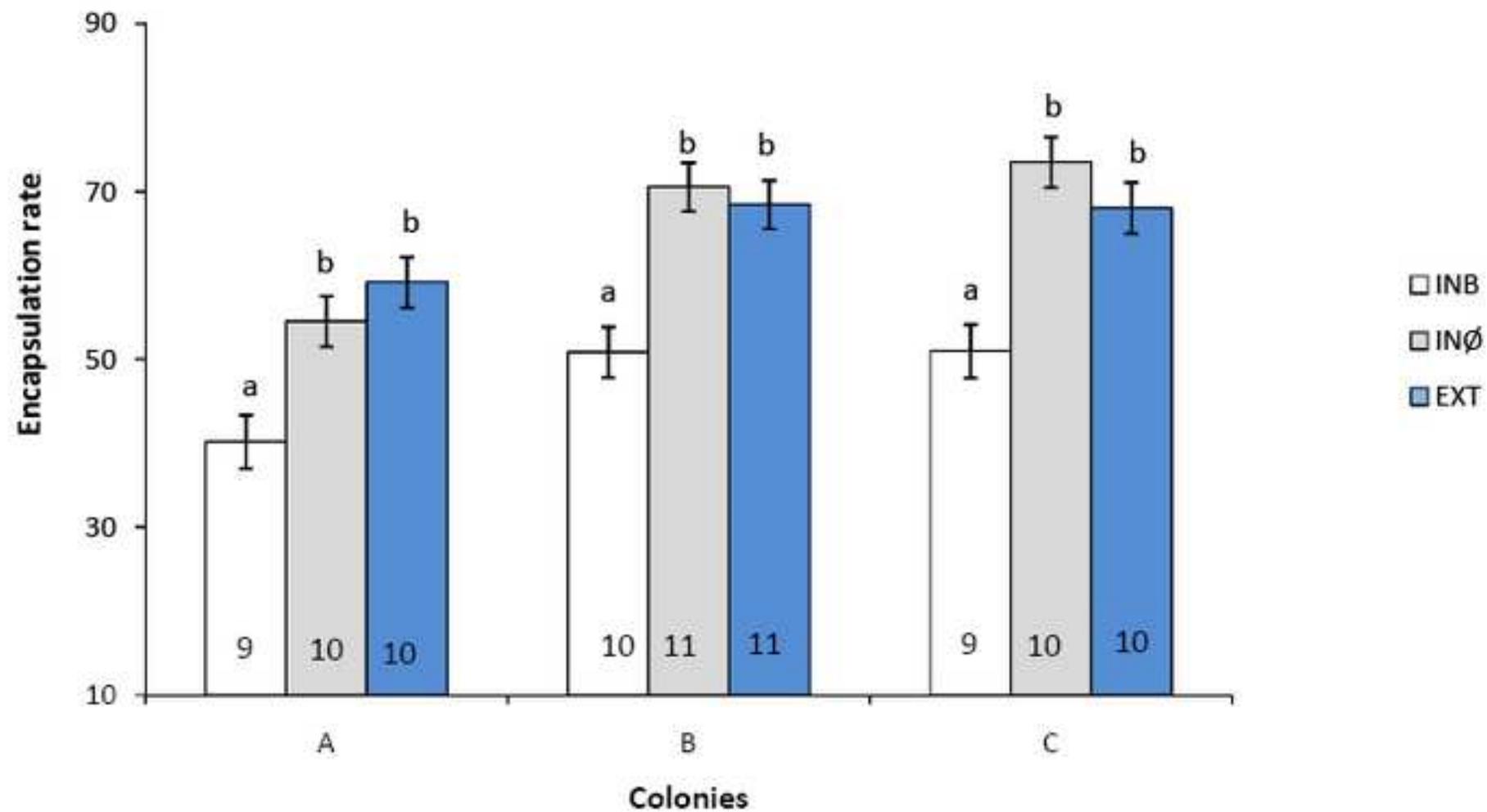
36 502 Fig. S1. Cuticular hydrocarbon quantities (head + thorax + legs) mean per worker \pm SE.

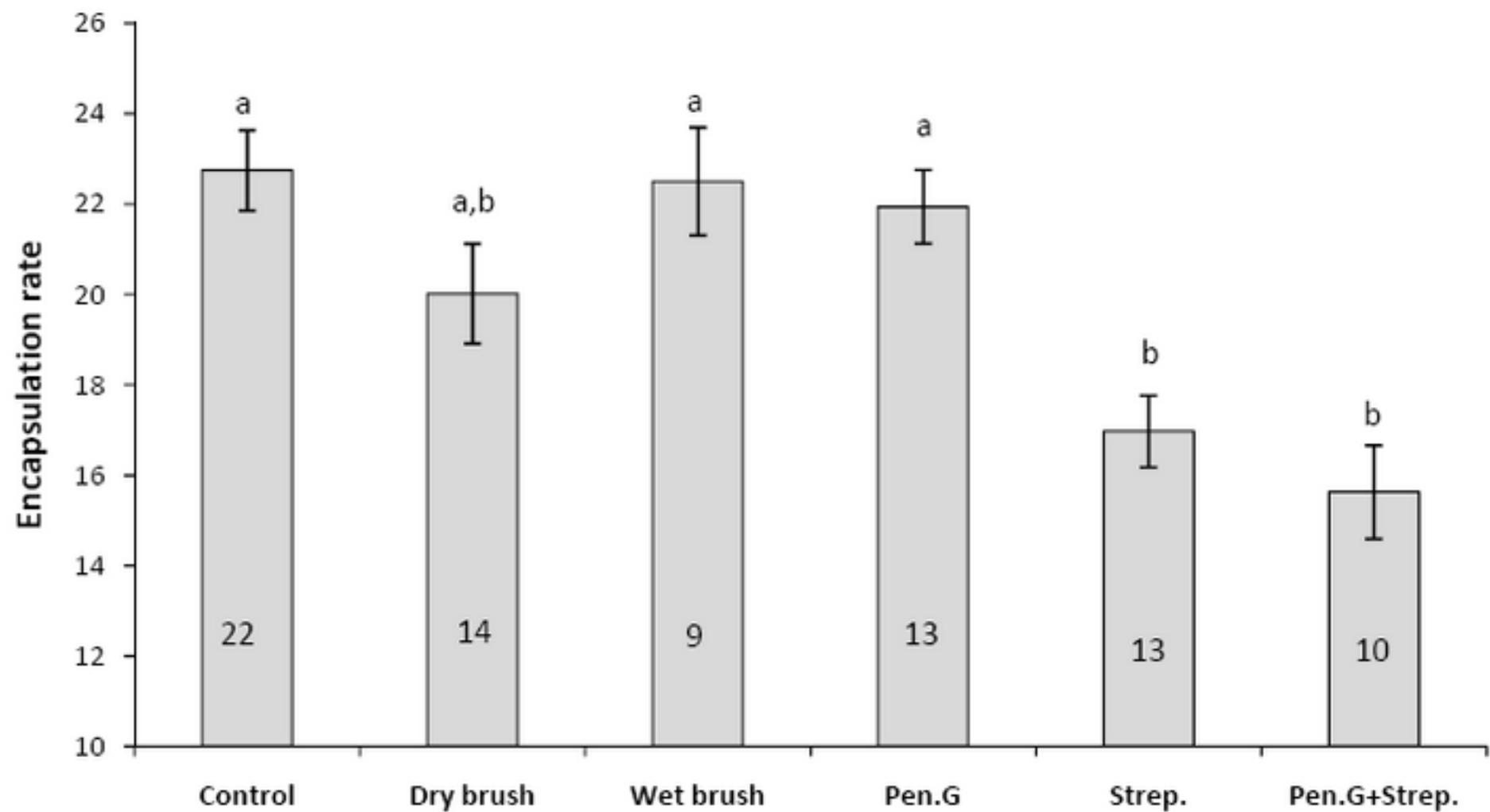
40 503 Fig. S2. – Chromatogram of *Acromyrmex subterraneus subterraneus* workers. Numbers refer to Table
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42 504 S1.

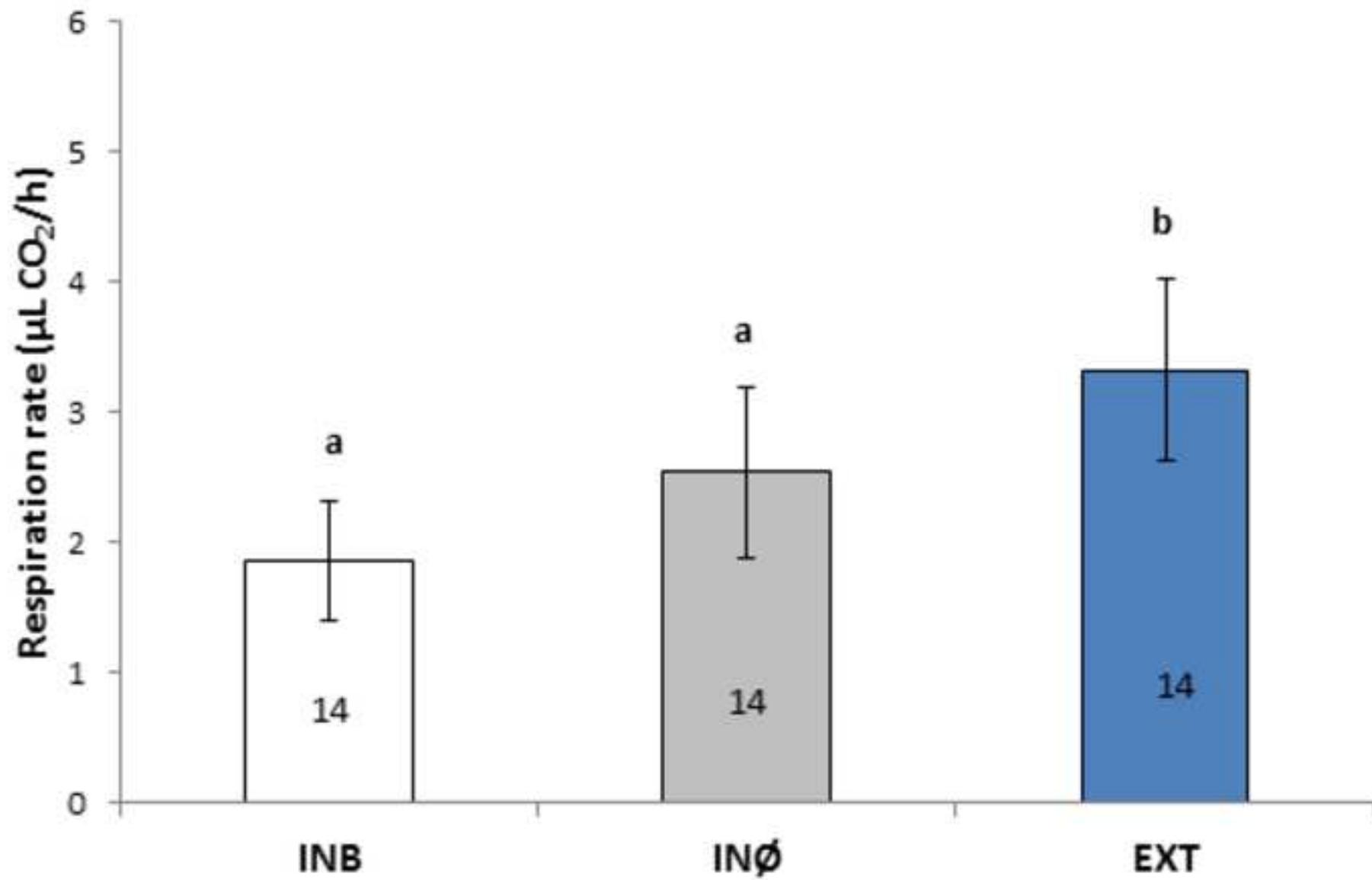
45 505 Fig. S3. Dendrogram of the cuticular hydrocarbon profiles of workers (Ward method, Euclidian
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48 506 distance). The first letter refers to colony origin (A, B or C), and the second refers to the group of
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50 507 workers (B=internal worker with actinobacteria, S = internal worker without actinobacteria, and E =
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52 508 external workers).

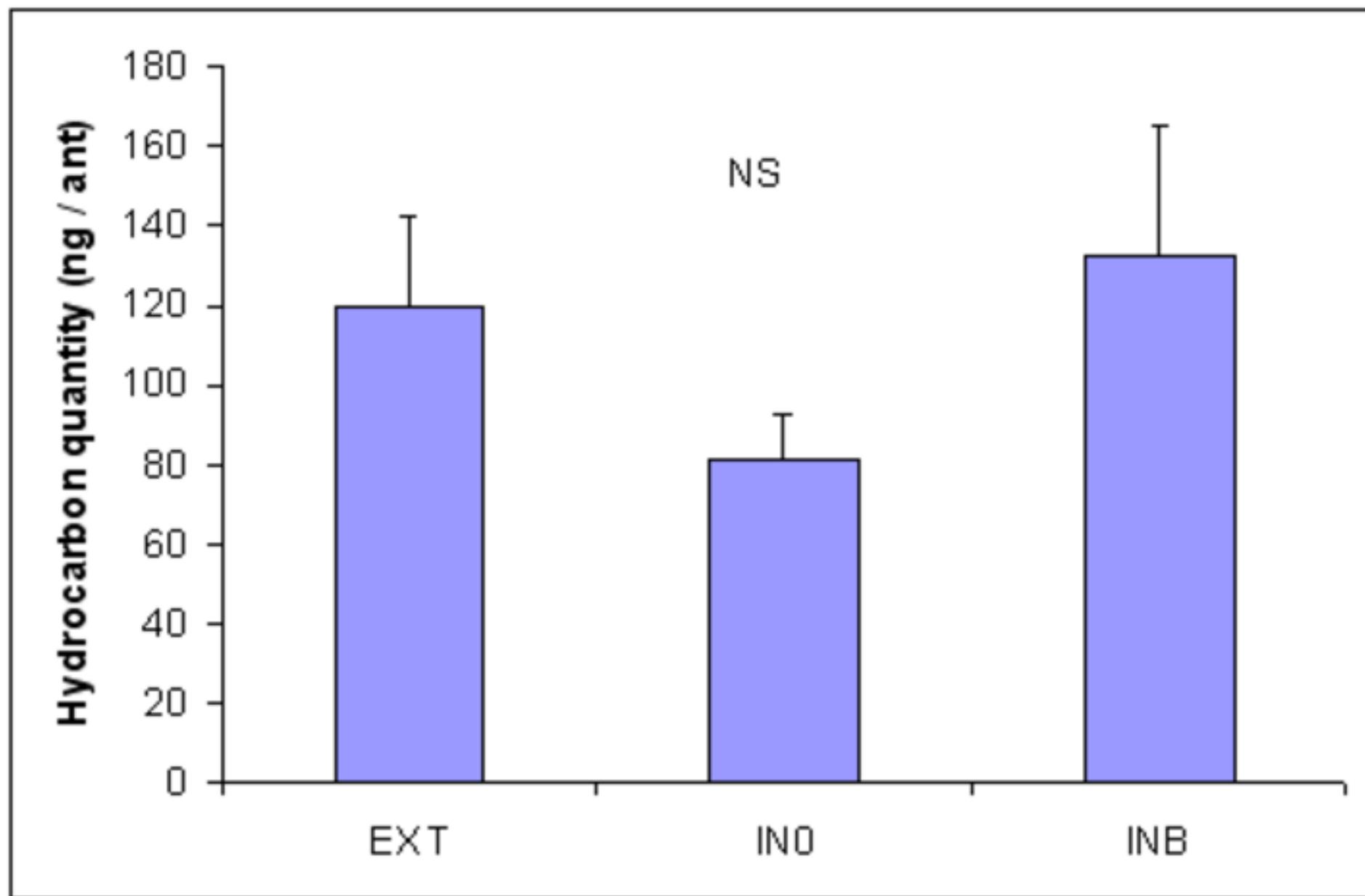
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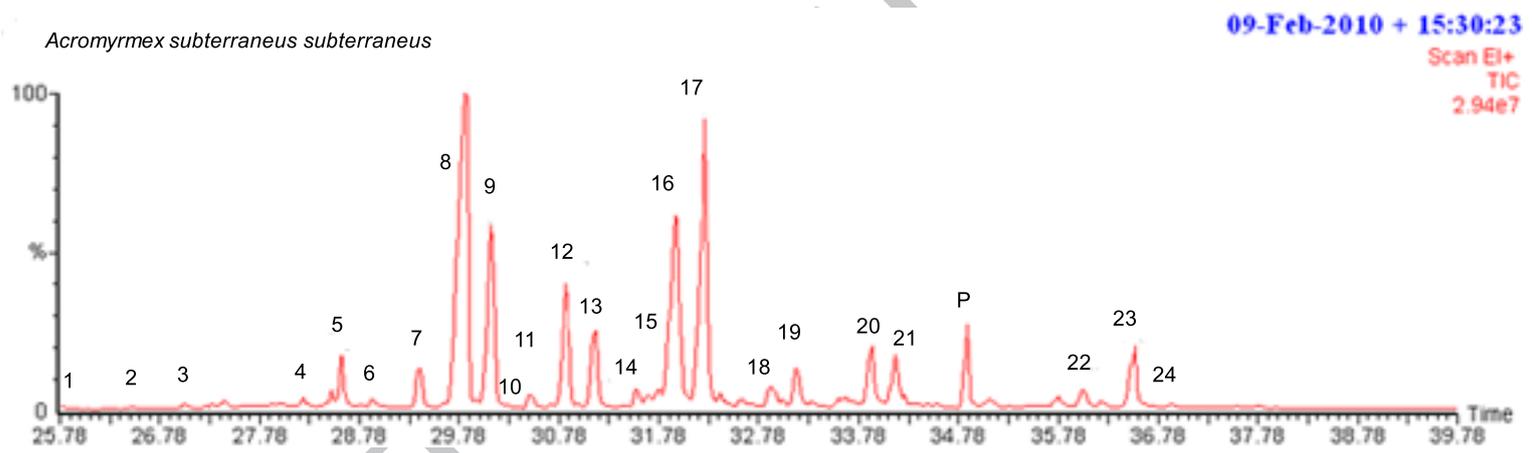
N°	name	EXT		INB		INO		All ants		Bacteria	Gelose
		mean	SE	mean	SE	mean	SE	mean	SE		
1	C27	0.60	0.05	0.99	0.34	0.85	0.08	0.82	0.12	10.11	5.57
2	11-+13-MeC27	1.00	0.09	1.35	0.22	1.62	0.13	1.33	0.09	4.03	2.61
3	3-MeC27	0.67	0.07	0.66	0.12	0.96	0.14	0.77	0.07	6.24	3.69
4	C28	0.72	0.08	1.23	0.14	2.13	0.25	1.37	0.13	13.87	11.25
5	12-meC28	3.96	0.22	4.39	0.32	4.13	0.28	4.17	0.16	6.52	2.21
6	10,14-DiMeC28	0.86	0.12	1.00	0.18	0.70	0.08	0.85	0.08	3.73	0.47
7	C29	3.58	0.51	2.65	0.33	2.47	0.31	2.89	0.23	6.44	5.26
8	11-+13-+15-C29	32.33	1.63	30.89	1.17	30.90	0.72	31.36	0.69	12.44	24.23
9	11,15-+13,17-DiMeC29	12.03	0.59	12.85	0.78	12.82	0.69	12.57	0.40	5.22	11.03
10	5-MeC29	0.12	0.05	0.17	0.06	0.08	0.03	0.13	0.03	0.00	0.00
11	C30	0.99	0.06	0.90	0.05	0.92	0.07	0.94	0.04	3.70	2.29
12	14-+15-MeC30	6.46	0.19	6.50	0.13	6.59	0.09	6.52	0.08	4.96	5.86
13	12,16-DiMeC30	4.41	0.42	4.65	0.28	4.59	0.29	4.55	0.19	0.00	5.23
14	C31	1.16	0.10	1.05	0.06	1.10	0.09	1.10	0.05	0.00	1.21
15	Alcohol	0.91	0.12	0.91	0.08	0.88	0.13	0.90	0.06	0.00	0.00
16	11-+13-MeC31	10.84	0.46	10.69	0.92	10.46	0.60	10.66	0.40	8.50	9.27
17	11,15-+13,1-DiMe7C31	10.97	0.85	10.87	0.44	10.84	0.44	10.89	0.34	5.89	9.81
18	C32	0.50	0.06	0.53	0.03	0.38	0.06	0.47	0.03	0.00	0.00
19	10-+11-+12-MeC32	0.34	0.06	0.32	0.04	0.39	0.18	0.35	0.06	4.47	0.00
20	11-+13-MeC33	0.98	0.15	1.13	0.14	0.97	0.17	1.03	0.09	3.87	0.00
21	x,y-DiMeC33	1.81	0.27	1.81	0.16	1.44	0.16	1.68	0.11	0.00	0.00
22	11-+13-MeC35	2.37	0.09	2.39	0.18	2.61	0.13	2.46	0.08	0.00	0.00
23	11,15-DiMeC35	2.25	0.24	1.99	0.19	2.06	0.19	2.09	0.12	0.00	0.00
24	x,y-DiMeC35	0.12	0.03	0.09	0.03	0.11	0.04	0.11	0.02	0.00	0.00
	Total	100.00		100.00		100.00		100.00		100.00	100.00
		n=	17		18		18		53	1	1

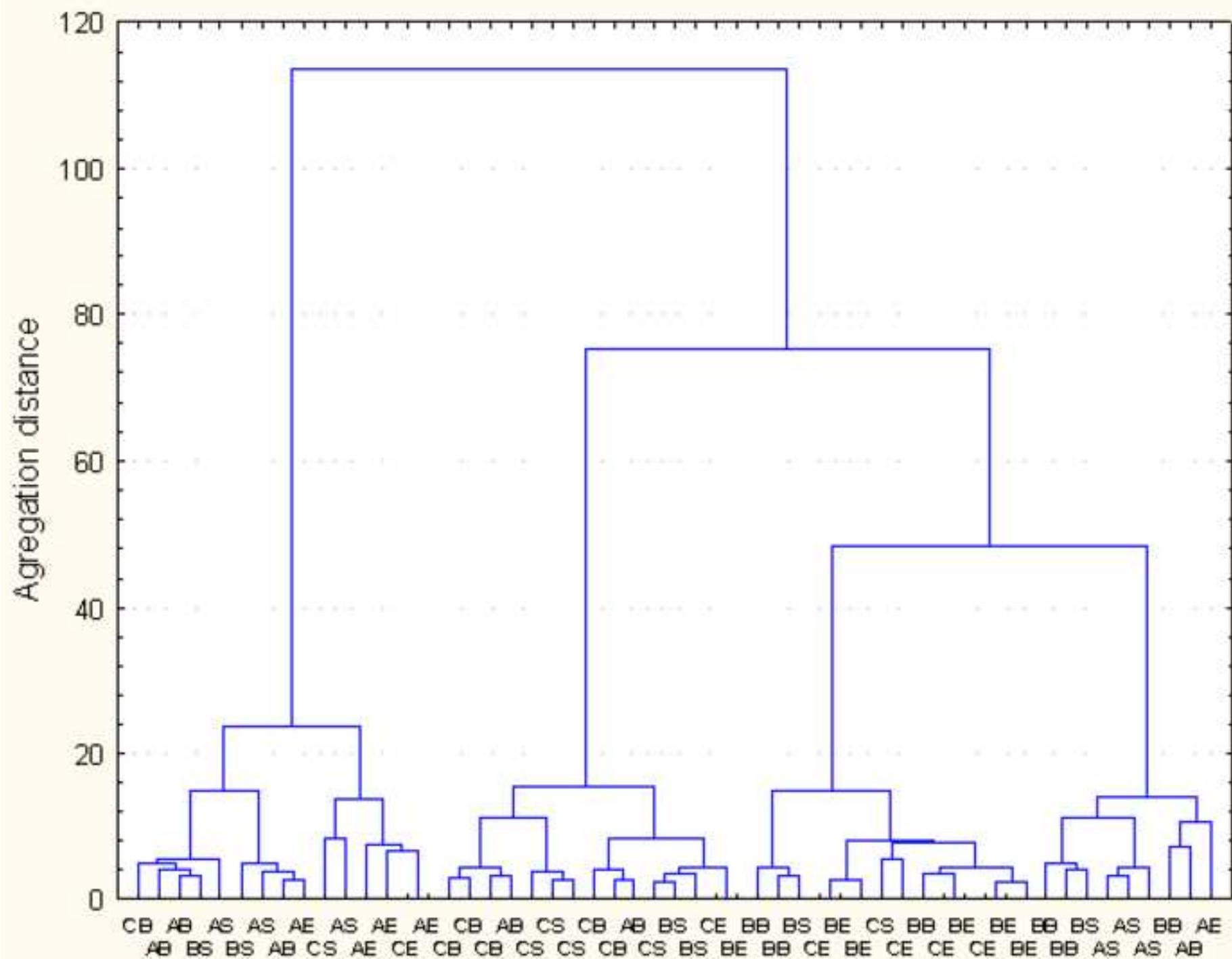












Highlight :

<*Acromyrmex subterraneus subterraneus* workers covered with actinobacteria have lower encapsulation response<

ACCEPTED MANUSCRIPT