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1 **Effect of amphotericin B on the infection success of *Schistosoma mansoni* in *Biomphalaria***
2 ***glabrata*.**
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23 ABSTRACT

24 In the present study, we examined the effect of amphotericin B on larval stages (miracidia and
25 primary sporocyst) of the helminth *Schistosoma mansoni*, the causative agent of human
26 schistosomiasis. Amphotericin B (AmB) is a polyene macrolide that disturbs the function of the cell
27 membrane; it is widely used as prophylactic antimycotic agent in *in vitro* culture. We show for the
28 first time that *S. mansoni* miracidia infectivity is considerably reduced after AmB treatment.
29 Moreover we demonstrate that AmB does not affect the development, growth, viability, and
30 behavior of miracidia and primary sporocysts. Our data indicate that AmB effects on *S. mansoni*
31 sporocyst prevalence are linked to the oxidative properties of AmB. These may alter the capacity of
32 sporocysts to respond to the oxidative stress generated by the snail immune defence system.

33

34 Keywords: Amphotericin B, *Schistosoma mansoni*, miracidium, *Biomphalaria glabrata*, reactive
35 oxygen species (ROS).

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37 INTRODUCTION

38

39 Schistosomiasis or bilharzia is a tropical parasitic disease affecting 200 million humans in 74
40 countries, causing 200,000 deaths annually. It is the second most important tropical disease in terms
41 of morbidity after malaria. Schistosomiasis is caused by flatworms of the genus *Schistosoma*, with
42 the most common causative species being *S. haematobium*, *S. japonicum*, and *S. mansoni* (Chitsulo,
43 et al., 2004, Gryseels, et al., 2006).

44 The cycle of the parasite is complex. It requires humans as definitive host and certain freshwater
45 snail species as intermediate hosts. Infection occurs in water by the free-living larval stages
46 (cercaria for the definitive host and miracidia for the intermediate host). Significant attention has
47 been paid to freshwater snails over the past because of their both medical and epidemiological
48 importance as intermediate hosts for schistosome parasites. Moreover, the interaction between
49 *Schistosoma mansoni* and the snail *Biomphalaria glabrata* provides a model of choice in
50 evolutionary biology to investigate the host-parasite coevolutionary dynamics (Beltran and Boissier,
51 2008, Beltran, et al., 2008, Bouchut, et al., 2008, Roger, et al., 2008, Steinauer, 2009). In this
52 context, the *in vitro* maintenance of schistosome intramolluscan stages is a valuable tool to
53 investigate host-parasite interaction, particularly at the molecular level (Coustau and Yoshino,
54 2000). Thus, *in vitro* culture is used often in this model to obtain pure parasites and/or secretion
55 products to identify the molecules involved in host-parasite interplay (Bender, et al., 2002, Guillou,
56 et al., 2007, Roger, et al., 2008, Roger, et al., 2008), to perform pharmacological investigations
57 (Mattos, et al., 2006), or to study parasite development (Azzi, et al., 2009).

58 To avoid the risk of contamination in those *in vitro* culture, antimicrobial substances were widely
59 used, mainly streptomycin, penicillin (as antibiotics) and amphotericin B (as antimycotic) (Smyth,
60 1990). It is known that antimicrobials can have side effects and consequently may affect the
61 cultivated organisms (Kuhlmann, 1995). Moreover, recent work has demonstrated activity of AmB
62 against helminth parasites (Olds, et al., 1981, Reuter, et al., 2003).

63 In this paper we describe the effect of an antimicrobial mix on the miracidium infectivity. This
64 antimicrobial substance is a mixture of two antibiotics, penicillin (PnG) and streptomycin (StrS)
65 and one antimycotic: amphotericin B (AmB). Herein we show that AmB decreases the infectivity of
66 the miracidium, the snail infective larval stage of schistosome parasite. To understand this anti-
67 parasitic effect better, the mode of action of AmB was examined.

68

69 MATERIALS AND METHODS

70

71 *Biological material*

72 The host-parasite association *Biomphalaria glabrata – Schistosoma mansoni* originated from
73 Brazil, and has been maintained in the laboratory for several years. Miracidia from *S. mansoni* were
74 hatched from eggs axenically recovered from infected hamster (*Mesocricetus auratus*) livers after
75 50 days according as described procedures (Roger, et al., 2008). Briefly, livers were collected and
76 kept in sterile 0.85% saline, containing a 1X antibiotic/antimycotic mixture (100 units/ml penicillin
77 G, 0.1mg/ml streptomycin sulfate, 0.25µg/ml amphotericin B). The livers were homogenized and
78 the eggs were filtered, washed and transferred to natural mineral water (Volvic) to allow miracidia
79 to hatch under illumination.

80

81 *Effect of antimicrobial substances on* S. mansoni *miracidia infectivity*

82 Different antimicrobial substances were added directly into the dish containing the miracidia,
83 and they were incubated for 3 hours before snail infection. Five groups of miracidia were used: a
84 control group (untreated miracidia), an antibiotic/antimycotic (AM) mixture 1X treated group, a
85 penicillin G (PnG) treated group (100 units/ml), a streptomycin sulfate (StrS) treated group (0.1
86 mg/ml), a PnG (100 units/ml)/StrS (0.1 mg/ml) treated group, and an amphotericin B (AmB) treated
87 group (0.25 µg/ml). For each group, 20 snails (5-7 mm diameter) were exposed individually to 10

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88 miracidia. Following exposure snails were kept in water at a constant temperature of 26°C and fed
89 on lettuce *ad libitum*.
90 The infected or non-infected status of the exposed snails was detected by the observation of the
91 mother sporocysts (Sp1) in the head-foot region. The snails were fixed 15 days post-exposure as
92 described (Theron, et al., 2008). Briefly, the snails were relaxed in water containing an excess of
93 crystalline menthol for 6 hours. The snail shell was removed and the body was fixed in modified
94 Raillet-Henry's solution (930 ml distilled water, 6g sodium chloride, 50ml formol 40%, 20 ml
95 acetic acid). The presence of Sp1 in each snail was determined by dissection of the head-foot zone
96 and visual inspection. The Sp1 were readily observable as translucent white bodies within an
97 opaque yellow tissue background.
98
99 *Effect of antimicrobial substances on S. mansoni miracidia swimming behaviour*
100 Four groups of miracidia were formed and incubated during 3 hours in Volvic water containing
101 different antimicrobial substances AM 1X, PnG (100 units/ml), StrS (0.1 mg/ml), and AmB (0.25
102 µg/ml). An additional control experiment was done with untreated miracidia. The miracidia were
103 filmed, their swimming behavior (sinuosity, rotation) was observed and their linear swimming
104 speed was determined using the video analysis software Kinovea (http://www.kinovea.org).
105
106 *Effect of antimicrobial substances on S. mansoni miracidia* in vitro *transformation to sporocysts*
107 *and sporocyst viability*
108 Miracidia were submitted to *in vitro* transformation to obtain primary sporocysts (Sp1).
109 Miracidia were cultured for 24 h in sterile Chernin's balanced salt solution (CBSS) (Chernin, 1963),
110 containing StrS (0.1 mg/ml) and PnG (100 units/ml) mixture (used as control), AM 1X, or AmB
111 (0.25 µg/ml). The experiments were conducted two times in triplicate on 24 well plates with 20
112 sporocysts per well.

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113 The percentage of transformed miracidia was determined directly by the observation of remaining
114 miracidia not transformed after 24 hours of culture. The viability of the Sp1 was assessed by two
115 different methods: (i) Sp1 were observed with a light microscope and considered alive when their
116 motility and/or the beating of the flame cells' flagella was observed; (ii) the Sp1 were exposed to
117 trypan blue and observed using a light microscope. Sp1 were considered dead when intra-sporocyst
118 trypan blue accumulation could be observed.
119
120 *Effect of AmB on* B. glabrata *hemocyte Reactive Oxygen Species (ROS) production*
121 *Biomphalaria glabrata* hemocytes were recovered after hemolymph puncture as previously
122 described (Bouchut, et al., 2006). Briefly, for each testing condition 500 µl of hemolymph were
123 recovered and centrifuged (600g, 10 min) to pellet the hemocytes. Hemocytes were washed three
124 times in PBS (phosphate buffered saline) to remove hemoglobin. Hemocytes were then incubated in
125 500 µl of PBS (control), or PBS containing (i) 1µM Phorbol 12-Myristate 13-Acetate (PMA), (ii)
126 0.25µM AmB, (iii) 1µM PMA + 0.25µM AmB. After 4 hours 500 µl of Nitroblue tetrazolium
127 (NBT) was added to each sample and incubated at 20°C for 60 min. The four samples were then
128 centrifuged (600g, 10 min) and the hemocyte pellets were resuspended in 70% MeOH. Samples
129 were centrifuged (600g, 10 min), the supernatant was discarded, 200 µl of dimethylsulfoxide
130 (DMSO)/2 M KOH solution was added and samples were mixed vigorously. Absorbance of
131 supernatants was read in a spectrophotometer at 620 nm vs. a DMSO/KOH blank. For each
132 condition, values were expressed as OD for $5x10^4$ hemocytes. The experiments were conducted two
133 times in triplicate for each sample. The ROS-mediated (Reactive Oxygen Species) reduction of
134 NBT results in the precipitation of insoluble blue formazan which can be quantified
135 spectrophotometrically in hemocytes or in hemocytes after treatment with the antimycotic AmB and
136 the respiratory burst stimulator PMA. This protocol was adapted from a published method
137 (Anderson and Brubacher, 1995).
138

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139 *Effect of AmB on* S. mansoni *sporocysts resistance to oxidative stress*

140 Miracidia were cultured 24 h in sterile Chernin's balanced salt solution (CBSS), containing 141 StrS (0.1 mg/ml) and PnG (100 units/ml) mixture (used as control), or AmB (0.25 µg/ml). After 24 142 hours of culture, increasing amounts of H_2O_2 were added to the culture medium of primary 143 sporocysts (Sp1). Five concentrations of H_2O_2 were tested: 0, 200, 400, 800 and 1600 µM. After 4 144 hours, the viability of Sp1 was assessed as described above

145 The experiments were conducted two times in triplicate on 24 well plates with 20 sporocysts per 146 well.

147

148 *Effect of AmB on* S. mansoni *sporocysts total antioxidant capacity*

149 Quantitative measurement of the cumulative antioxidant capacity of sporocystes was determined 150 following exposition to different AmB concentrations. Total antioxidant activity was measured 151 using the Antioxidant Assay Kit (Sigma). For each test, 2000 sporocystes were *in-vitro* transformed 152 and cultured in sterile Chernin's balanced salt solution (CBSS), containing 0; 0.25; 0.5; or 1.25 153 µg/ml of AmB. After 24h fully transformed sporocystes were recovered by gentle centrifugation 154 (800g, 5 minutes, 4°C), sonicated (3 pulses of 20 seconds), centrifuged to pellet cell debris (12000g, 155 15 minutes, 4°C) and antioxidant capacity of supernatant was determined following manufacturer's 156 recommendations. Protein amount in the supernatant was determined using Bradford protein 157 quantification assay and used as a correcting factor. The experiment was done six times for each 158 AmB concentration tested.

159

160 *Statistical analysis*

161 Results of miracidia infection, miracidia transformation and sporocyst viability were analyzed 162 using a two-tailed Fisher's Exact test. Results of miracidia linear swimming speed and hemocyte 163 ROS production were analyzed using the Kruskal-Wallis test. Results of AmB effects on sporocysts

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164 resistance to oxidative stress and antioxidant capacity were analyzed using the Student T test.
165 Levels of confidence lower than 0.05 were considered to be statistically significant.
166
167 RESULTS
168 *Effect of antimicrobial substances on* S. mansoni *miracidia infectivity*
169 We assayed the effect of miracidia treatment with antimicrobial substances on infectivity (
170 Fig. 1). Miracidia treated with 1X AM and AmB display significant prevalence decrease (P<0.006
171 and P<0.005 respectively) compared to the control. We observed a reduction of more than 50% of
172 *S. mansoni* miracidia infectivity after those two treatments. Prevalence for the control was 65% and
173 only 32.5% for 1X AM and 25% for AmB (Fig. 1). There is no significant difference between AM
174 1X and AmB treatments, and neither StrS nor PnG had effects on miracidia infectivity compared to
175 the control (Fig. 1). These results permit us to conclude that AmB is reducing *S. mansoni* miracidia
176 infectivity and that no synergistic or antagonistic effects could be observed with the other
177 antimicrobial substances tested here
178 *Effect of antimicrobial substances on* S. mansoni *miracidia swimming behavior*
179 Swimming behavior of miracidia was investigated after treatment with antimicrobial
180 substances. Linear swimming speed (Fig. 2A), sinuosity and miracidia swimming rotation (data not
181 show) did not differ from that of the control. The linear swimming speed was of 1.35, 1.18, 1.17,
182 1.21, and 1.16 mm/s for the control, 1X AM, AmB, PnG, and StrS respectively. No significant
183 differences were observed between those values (Kruskal-Wallis ; P=0.63). Thus, none of the
184 molecules tested have an effect on swimming behavior of miracidia
185
186 *Effect of antimicrobial substances on* S. mansoni *miracidia* in vitro *transformation to sporocysts*
187 *and sporocysts viability*
188 In this experiment the use of antimicrobial molecules in the culture medium was necessary
189 to avoid microbial contamination. Thus, a PnG/StrS mixture was used as control. Miracidia

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190 transformation and sporocyst viability was compared between this control and 1X AM or AmB (see
191 Fig. 2B). Miracidia transformation did not differ from that of the control. The percentage of
192 transformed miracidia to sporocysts was 97, 96 and 98% for the control, 1X AM, and AmB,
193 respectively. The sporocysts viability after 24 h in culture was very good with 93.2, 93.8, and
194 91.9% of live sporocysts for the control, 1X AM, and AmB (Fig. 2B.1). Miracidia and sporocysts
195 did not show any tegumental lysis or shape modifications when comparing the control and AmB
196 (Fig. 2B.2). Motility, beating of the flame cells' flagella or trypan blue efflux were the same for all
197 samples. To conclude, none of the molecules tested seem to have an effect on miracidia
198 transformation and sporocyst viability.
199
200 *Effect of AmB on* B. glabrata *hemocyte Reactive Oxygen Species (ROS) production*
201 Here we tried to quantify the ROS production of *B. glabrata* hemocytes after treatment with
202 AmB in the presence or absence of PMA, a respiratory burst stimulator. The ROS produced by the
203 hemocytes reduce NBT and the optical density (OD) of reduced NBT was quantified. Intracellular
204 ROS production by AmB-treated and PMA-stimulated cells did not differ from that of
205 untreated/unstimulated control cells (Fig. 2C). The OD of reduced NBT for $5x10^4$ hemocytes was
206 0.084, 0.083, 0.088 and 0.095 for the control, PMA, AmB and AmB/PMA respectively (Fig. 2C).
207 No significant differences were observed between those values (Kruskal-Wallis ; P=0.84). It seems
208 that AmB did not affect *B. glabrata* hemocyte ROS production, with or without burst stimulation.
209
210 *Effect of AmB on* S. mansoni *sporocysts resistance to oxidative stress*
211 Resistance of Sp1 to oxidative stress was investigated after treatment with AmB. Here we
212 used exposure to H_2O_2 for 4 hours as oxidative stress. Different concentrations of H_2O_2 were used.
213 No significant differences between AmB-treated and untreated sporocysts (control) were observed
214 for 0, 200, 400 and 800 µM of H_2O_2 (Fig. 3). However, for 1600 µM a significant difference was
215 noted (Student T-test; P=0.048) (Fig. 3). Viability values were 95.5 and 99.3% for AmB-treated and

216 untreated, respectively. This suggests that Sp1 exposed to AmB would be less able to respond to
217 oxidative stress.
218
219 *Effect of AmB on* S. mansoni *sporocysts total antioxidant capacity*
220 Total antioxidant capacity of sporocysts was determined after exposition to increase concentrations
221 of AmB (Fig. 4). Sporocysts antioxidant activity decreases when AmB concentrations increase.
222 Antioxidant concentration was 214.02µM when no AmB was present in the culture medium and
223 significantly decreased to 105.08µM when 1.25µg/ml of AmB was added to the culture medium
224 (Student T-test; P=0.00107) (Fig. 4). To conclude it seems that AmB affect directly the total
225 antioxidant capacity of sporocysts.
226
227 DISCUSSION
228 AmB is known to be an antifungal agent that binds to sterols (cholesterol and ergosterol) in
229 the fungal cell wall, forming transmembrane channels resulting in osmotic lysis and death of the
230 organism (HsuChen and Feingold, 1973, HsuChen and Feingold, 1973, Reuter, et al., 2003). On the
231 other hand, several studies suggest that AmB has an effect on prokaryotes and eukaryotes
232 (protozoan and metazoan) (Cruz, et al., 1980, Lachaud, et al., 2009, Olds, et al., 1981, Reuter, et al.,
233 2003, Thomas, et al., 1973). The protective effect against those pathogens was linked mainly to
234 AmB immunomodulatory effects and induction of macrophage activation (Beccari, et al., 1991,
235 Kumar and Chakrabarti, 2000, Little, et al., 1978, Olds, et al., 1981, Wilson, et al., 1991, Wolf and
236 Massof, 1990). However on helminths AmB was shown to act by two different ways. Alveolar
237 echinococcosis caused by the parasitic cestode *Echinococcus multilocularis* was significantly
238 reduced by AmB treatment (Reuter, et al., 2003). AmB seems to act directly on the parasite cell
239 membrane. This destructive effect suppressed parasite growth and development (Reuter, et al.,
240 2003). With the helminth *S. mansoni,* AmB treatment increases resistance in mice to a challenge
241 with *S. mansoni* cercariae (Olds, et al., 1981). AmB treatment was effective in protecting mice by

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242 killing schistosomula, the migrating larval stage of the parasite. This study demonstrated activation
243 of murine macrophages by AmB, which contributes to enhanced mice resistance to infection (Olds,
244 et al., 1981).

245 In our study we investigated the effect of AmB on the *S. mansoni* larval stage interacting
246 with the invertebrate host *B. glabrata*. To our knowledge, we demonstrate for the first time a
247 reduction of *S. mansoni* miracidia infectivity after AmB treatment (Fig. 1). We observed a reduction
248 of Sp1 prevalence of more than 50% after treatment of miracidia with AmB (Fig. 1).

249 Based on previous work conducted on the AmB effect on helminthes, we tried to determine the
250 mechanisms by which AmB reduces *S. mansoni* miracidia infectivity. A direct deleterious effect on
251 miracidia was considered. We investigated the effect of AmB on parasite and host behavior and
252 physiological traits but no significant differences were noted (Fig.2).

253 Thus AmB would not be able to impair (i) the capacity of *S. mansoni* miracidia to find and
254 penetrate into snail host in aquatic environment, (ii) the capacity of *S. mansoni* miracidia to
255 transform into sporocysts, (iii) the sporocysts growth, development and viability. The present
256 results showed that AmB did not have any direct deleterious effect on miracidia and sporocysts.

257 To go further we looked at the immunomodulatory effect of AmB. In vertebrates AmB was known
258 to activate macrophage oxidative burst by the production of reactive oxygen metabolites (Wilson, et
259 al., 1991, Wolf and Massof, 1990). In invertebrate host of *S. mansoni, Biomphalaria glabrata,* the
260 internal defense system is mediated by either humoral and/or cell components (Ataev and Coustau,
261 1999, Bayne, et al., 2001). The most important effectors of immunity in molluscs are the circulating
262 hemocytes. These phagocytic cells are able to distinguish foreign, non-self material, and
263 encapsulate, kill and eliminate invaders such as parasite sporocysts (de Jong-Brink, et al., 2001,
264 Hahn, et al., 2001). Several studies have indicated that reactive oxygen species (ROS), like
265 hydrogen peroxide, produced by hemocytes play a crucial role in the killing of the parasite (Adema,
266 et al., 1994, Bayne, et al., 2001, Dikkeboom, et al., 1988, Hahn, et al., 2001). In the present study
267 we investigated the *in vitro* effect of AmB on *B. glabrata* hemocyte ROS production. ROS were

268 quantified by NBT reduction after hemocytes treatment with AmB alone or in combination with the
269 respiratory burst stimulator PMA. Intracellular ROS production by AmB treated and PMA
270 stimulated hemocytes did not differ from that of untreated/unstimulated control cells (Fig. 2C). It
271 seems that AmB did not affect *B. glabrata* immune effectors. Unlike vertebrates it seems that AmB
272 did not induce immunomodulatory effect in the invertebrate's phagocytic cells and did not activate
273 hemocyte oxidative burst.
274 Decreased prevalence of *S. mansoni* sporocysts after AmB treatment observed in the present paper
275 would thus neither be related to a direct deleterious effect on the parasite's membrane nor to
276 activation of invertebrate host immunity. However, AmB may induce oxidative damage in
277 conjunction with other oxidizing agents (Brajtburg, et al., 1985). Thus we investigated the
278 resistance of Sp1 to H_2O_2 oxidative stress after treatment with AmB. A significant difference was
279 noted between AmB-treated and untreated sporocysts (Fig. 3). This suggests that Sp1 exposed to
280 AmB are less able to respond to oxidative stress. To go further and clarify the influence of AmB on
281 parasite antioxidant activity, we measure the total antioxidant capacity of the sporocysts after
282 treatment with AmB. The results show that AmB treatment causes a decrease of the sporocyst
283 antioxidant capacity in a dose-dependant manner. Living organisms have a large number of
284 antioxidants, including macro and micro molecules, and enzymes that play a central role in
285 preventing oxidative stress. AmB affects the global antioxidant capacity of the parasite and thus
286 directly reduces its ability to respond to oxidative stress.
287 It is known that miracidia and sporocysts use antioxidant molecules and ROS scavengers to protect
288 themselves against hemocyte-mediated cytotoxicity (Bernal, et al., 2006, Connors, et al., 1991,
289 Guillou, et al., 2007, Knudsen, et al., 2005, Perez-Sanchez, et al., 2006, Roger, et al., 2008). If
290 parasite antioxidant molecules were used to overcome AmB oxidant effects, those molecules would
291 not be available and effective against ROS snail host activities. Thus sporocysts would not be able
292 to protect themselves from a second oxidative stress that occurred when sporocysts are confronted
293 by snail hemocytes ROS products (Bayne, 2009, Bayne, et al., 2001, Bender, et al., 2005).

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302

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543 **Legends to figures:**

544 **Figure 1:**

545 Prevalence and abundance of *S. mansoni* sporocysts in *B. glabrata* exposed to miracidia
546 treated with different antimicrobial substances. The snails were individually exposed to 10
547 miracidia treated either by 1X AM, 100U/ml PnG, 10mg/ml StrS, or 0.25µg/ml AmB. The control
548 was untreated miracidia. The asterisks indicate treatment for which the decrease of prevalence is
549 significant ($p<0.05$).

550

551 **Figure 2**

552 Influence of the antimicrobial substances on *S. mansoni* and *B. glabrata* behavioral and
553 physiological traits. (A) Linear swimming speed of miracidia exposed to 1X AM, 100U/ml PnG,
554 10mg/ml StrS, or 0.25µg/ml AmB. (B.1) Viability % of sporocysts after 24 hours in CBSS medium
555 containing a mixture of 100U/ml PnG and 10mg/ml StrS used as control or1X AM or 0.25µg/ml
556 AmB. (B.2) *S. mansoni* miracidia and cultivated sporocysts in presence or absence of 0.25µg/ml
557 AmB. Miracidia and sporocysts were exposed to AmB for 3 hours and 24 hours respectively. The
558 detached ciliated plates in the sporocysts culture were indicated with black arrows. Scale bars are 35
559 µm. (C) AmB (0.25µg/ml) effect on ROS production by *B. glabrata* hemocytes stimulated or not by
560 PMA. The ROS production was assessed by the reduction of nitroblue tetrazolium measured
561 spectrophotometrically at 620 nm.

562

563 **Figure 3**

564 Sporocyst viability 4 hours after exposure to different H_2O_2 concentrations. The sporocysts
565 were treated with AmB (0.25µg/ml) for 24 hours in CBSS medium or untreated. The asterisk
566 indicate significant difference ($p<0.05$) of viability between AmB treated and untreated sporocysts.

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568 **Figure 4:**

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569 Total antioxidant capacity of sporocysts exposed to different concentrations of AmB. The
570 sporocysts were treated with AmB (0.25; 0.5; 1.25μg/ml) for 24 hours in CBSS medium or
571 untreated. The asterisk indicate significant difference ($p<0.05$) of antioxidant concentration between
572 AmB treated and untreated sporocysts.
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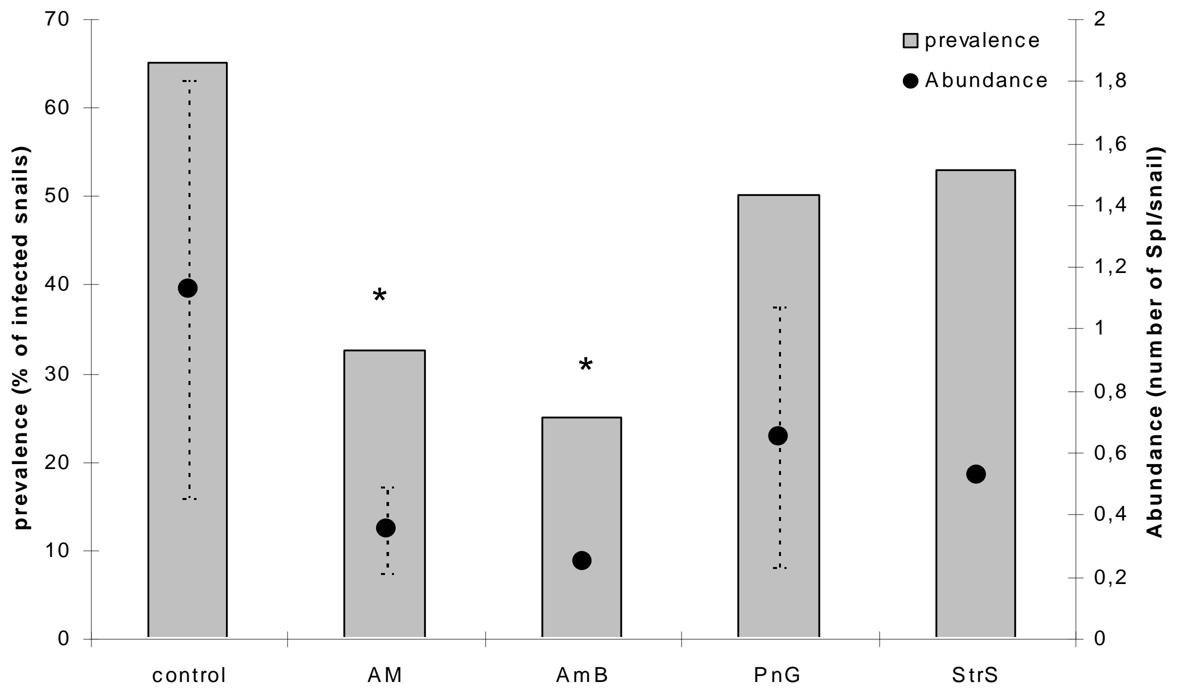
Figure 1



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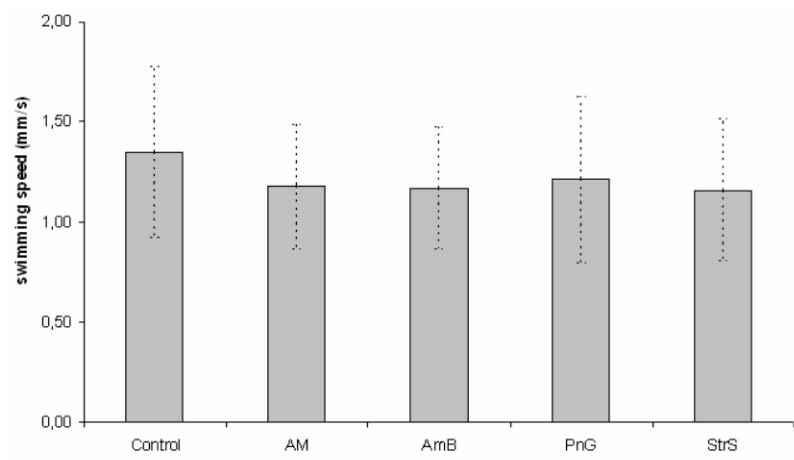

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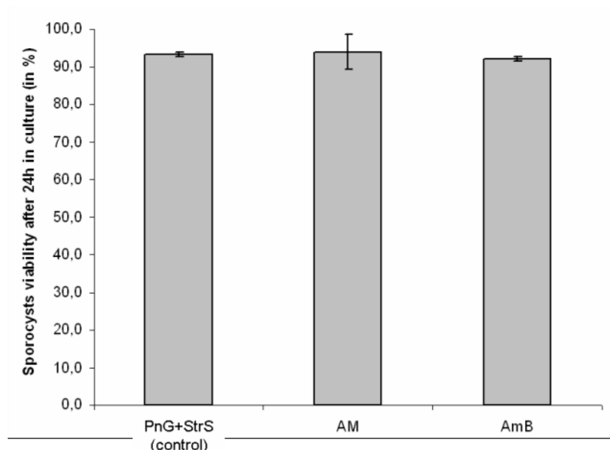

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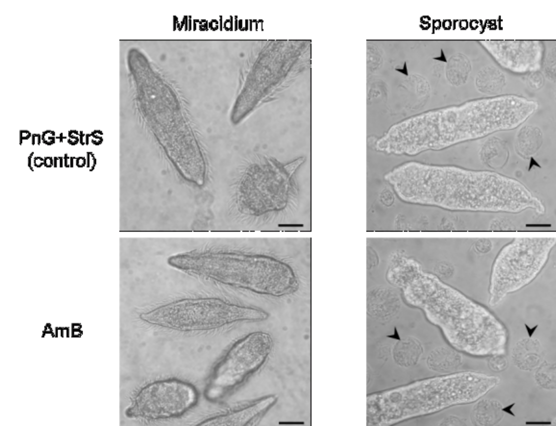

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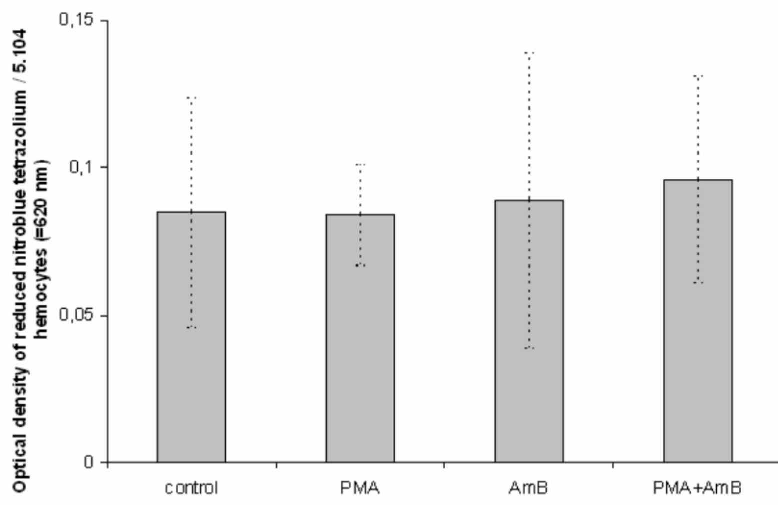

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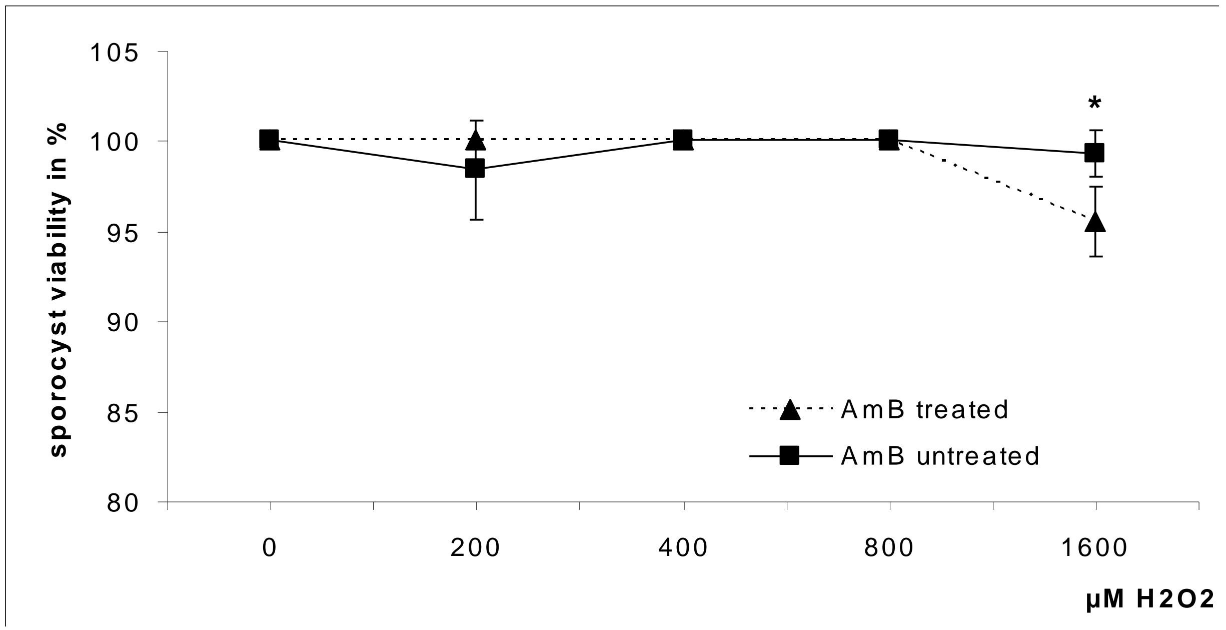

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642 Figure 4

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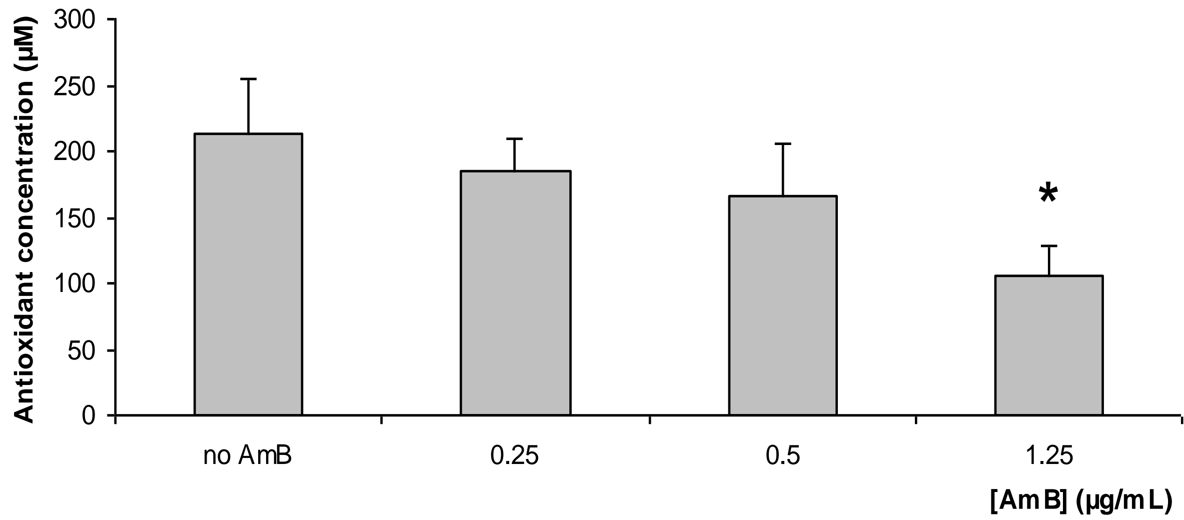

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