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ORIGINAL PAPER

Effects of dietary intake of garlic on intestinal trematodes

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Abstract The current strategy for the control of helminth infections relies on chemotherapy. However, resistance appearance is promoting the necessity of developing new drugs against trematodes. Herein, potential trematocidal effects of garlic (*Allium sativum*) are investigated in the context of intestinal foodborne trematodes, employing the *Echinostoma caproni*-mouse model. Daily administration of dietary doses of garlic was conducted in three groups of mice: (i) before infection (prophylaxis), (ii) after infection (therapeutic) and (iii) both, before and after infection (continuous). A fourth group of mice, not exposed to garlic, was used as control. No differences in worm recovery, fecundity and local cytokine expression profiles were found with respect to control infections. However, considerable alterations in tegument structure, including swelling, furrowing, vacuolization and changes in secretory bodies were detected in garlic-exposed parasites using scanning and transmission electron microscopy. Protein secretion was markedly reduced in response to garlic, whereas up-regulation of several proteins, such as major vault protein and tER-ATPase, was observed in treated worms. The results presented herein provide new insights in the anthelminthic activity of bioactive garlic compounds and the manner that parasites respond to toxins.

Keywords *Allium sativum* · *Echinostoma caproni* · Trematoda · Anthelmintic activity · Tegument

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Introduction

Over 100 species of foodborne trematodes are known to cause infection in humans, and more than one billion persons are at risk of infection, mainly in Asia and Latin America (Fürst et al. 2012; WHO 2015). The current global strategy to control foodborne trematode infections, both in humans and livestock, relies on the use of trematocidal drugs, basically praziquantel and triclabendazole. However, it is well known that most of chemical products employed to combat infectious diseases lose their efficacy after several years of use because resistance develops (Mehlhorn et al. 2011). Drug tolerance and resistance are arising against available drugs; hence, there is a need to discover and develop new drugs for the prevention and treatment of these helminthiases (Keiser et al. 2010). In this sense, a strong trend to revisit the pre-chemotherapeutical era has arisen, using natural products as anthelmintic remedies (Abdel-Ghaffar et al. 2011; Klimpel et al. 2011). In 2000, the World Health Organization recommended evaluation of therapeutic uses of plant-derived products. Hence, research into the use of traditional, plant-based medicines is gaining popularity, and the number of studies using plant extracts and other natural products as potential remedies has increased.

Garlic (*Allium sativum*) is one of the earliest documented examples of plants used for maintenance of health and treatment of several diseases with few side effects (Rivlin 2001; Londhe et al. 2011). Medical use of garlic appears to have originated in Central Asia and then spread to China and the

Mediterranean region, before arriving in northern Africa and Mexico (Rivlin 2001). Bioactive compounds of garlic are reported to perform antiparasitic and immunomodulatory effects (Arreola et al. 2015). Particularly, anti-helminthic effects of garlic have been reported against different stages of *Schistosoma mansoni* (Riad et al. 2009; Mantawy et al. 2011, 2012), *Fasciola gigantica* (Singh et al. 2009) and the monogeneans *Gyrodactylus* and *Dactylogyrus* (Fridman et al. 2014). Nematocidal effect against *Haemonchus contortus* larvae has been also described (Palacio-Landín et al. 2015), though it showed no effect against the adult stages of *Trichuris muris* (Klimpel et al. 2011) and *Ascaridia galli* (Velkers et al. 2011).

Sutton and Haik (1999) stated that the antiparasitic activity of garlic is not due to a pharmacological elimination of the parasite, but through the enhancement of the host immune response to the parasite. It has been documented that different garlic preparations significantly reduce the production of inflammatory cytokines and shift the Th1-Th2 balance towards a Th2 response (Liu et al. 2009). Other studies, however, suggest that garlic enhances pro-inflammatory responses against intracellular parasites (Feng et al. 2012). Effects on different immune cells such as macrophages (Shin et al. 2013), dendritic cells (Feng et al. 2012), natural killer cells and γδ lymphocytes (Nantz et al. 2012) have also been described. Other authors have attributed the parasite clearance to the enhancement of antioxidant enzymes in the host's tissues (Mantawy et al. 2012) or to direct effects of garlic on the parasite surface (Riad et al. 2009).

The present work aimed to undertake further studies to evaluate anthelmintic properties of garlic in vivo using the *Echinostoma caproni*-mouse model (Toledo et al. 2009). *E. caproni* is an intestinal trematode with no tissue phase in the definitive host, which allows quicker patent infections in the definitive hosts than other trematode species, providing rapid and cost-effective results (Keiser 2010). Potential anthelminthic effects are addressed from different approaches, including parasitological, immunological and proteomic analyses, together with ultrastructural studies of the tegument by electron microscopy.

Materials and methods

Animals, experimental infections and garlic administration

A total of 20 male CD1 mice (30–35 g) were randomly allocated in four experimental groups, five mice each, according to the schedule of garlic administration (prophylaxis, treatment, continuous and control).

The garlic preparation was made according to Riad et al. (2009). Briefly, garlic cloves were peeled, washed with distilled water and dried before crushing in a blender to obtain a paste of uniform consistency. This paste was diluted in spring water to obtain a stock solution of 1 g/ml, which was aliquoted and stored at −20 °C until use. Individual doses of 50 mg/kg were prepared daily from the stock solution and administered intragastrically. The dose selected is expected to be equivalent to the daily amount of garlic recommend in humans (4 g, approximately) (Riad et al. 2009).

The strain of *E. caproni* and the infection procedures were described previously (Fujino and Fried 1993). In short, encysted metacercariae were removed from kidneys and pericardial cavities of experimentally infected *Biomphalaria glabrata* snails. All mice were infected with 50 metacercariae of *E. caproni*, orally administered by gastric gavage. At 2 weeks post-infection (wpi), stool samples were taken and mice were sacrificed to collect the adult worms from the small intestine. Triplicate Kato-Katz thick smears, using standard 41.7 mg templates, were prepared from each stool sample. The Kato-Katz thick smears were examined with a microscope and the number of *E. caproni* eggs per gram of faeces (EPG) was recorded.

Mice in each experimental group were treated as follows: (i) Prophylaxis: mice were given a dose of the garlic preparation daily during the week before infection; (ii) Treatment: garlic was administered from the first day of infection until the end of the experiment at 2 wpi; (iii) Continuous: animals received a daily dose of garlic from 1 week before infection until 2 wpi; and (iv) Control: control mice were not exposed to garlic, but given the same volume of spring water.

Total RNA extraction, RT and real time-PCR

Total RNA was isolated from full-thickness sections of the ileum of mice using Real Total RNA Spin Plus Kit (Durviz), and cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. For quantitative PCR, 9 μl of the product of reverse transcription, diluted 1/10 in sterile water, was added to 10 μl of TaqMan® Gene Expression Master Mix (Applied Biosystem) and 1 μl of the pertinent TaqMan® Gene Expression Assay (Supplementary Table 1). β-Actin was used as a housekeeping gene to normalize for differences in the efficiency of sample extraction and/or cDNA synthesis. Reactions were performed on the StepOnePlus Real Time-PCR System (Applied Biosystems), with the following thermal cycler conditions: an initial step of 10 min at 95 °C followed by 40 cycles of 15 s denaturation at 95 °C and 1 min of anneal/extension at 60 °C each. Samples were analysed in triplicate. The threshold cycle (Ct) was calculated for the genes of interest and the housekeeping in each sample and negative control, and a comparative quantification method ($2^{-\Delta\Delta Ct}$) was applied to evaluate the effect of the infection on gene expression (Livak and Schmittgen 2001).

The method is based on the fact that the difference in threshold cycles (ΔCt) between the gene of interest and the housekeeping is proportional to the relative expression of the gene of interest. The fold change in the target genes was normalized to β-actin and relativized to the expression in control animals (not exposed to garlic) to get a relative quantification of the expression levels (Klein 2002).

Obtaining of ESPs and SDS-PAGE

The excretory/secretory products (ESPs) were obtained by incubation of *E. caproni* adults from each experimental group in pre-heated RMPI 1640 culture medium (Gibco, Life Technologies) and maintained at a concentration of 40 worms/ml for 12 h at 37 °C in RPMI 1640 containing 100 U penicillin, 100 mg/ml streptomycin and complete mini EDTA-free protease inhibitor cocktail (Roche). After incubation, the media were collected and centrifuged at 15,000*g* for 30 min at 4 °C. Then, the supernatant was collected and protein concentration was measured using Bio-Rad protein assay.

To analyse the protein profiles, the ESPs were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. A total amount of 30 μg of each ESP was electrophoresed in either 12 or 8% resolving SDS-PAGE gels, run in Tris-glycine SDS buffer. The electrophoretic profiles were compared visually and differential bands were manually excised from gels for protein identification.

Protein identification by LC-MS/MS and database search

Gel bands were washed twice with double-distilled water and digested with sequencing grade trypsin (Promega). For liquid chromatography and tandem mass spectrometry (LC-MS/MS), digested samples were diluted in 12 μl of 5% formic acid and 6 μl of the resulting suspension was injected onto a 50 mm × 300 μm C18 trap column (Agilent Technologies) using a Shimadzu Prominance Nano HPLC. Samples were desalted on the trap column for 5 min using 0.1% formic acid (aq) at 30 μl/min. Peptides were then eluted onto an analytical nano HPLC column (150 mm × 75 μm 300SBC18, 3.5 μm, Agilent Technologies) at a flow rate of 300 nl/min and separated using a 35 min gradient of 1–40% buffer B followed by a steeper gradient from 40 to 80% buffer B in 5 min. Buffer B contained 90/10 acetonitrile/0.1% formic acid, and buffer A consisted of 0.1% formic acid (aq). The column eluates were subsequently ionized using a 5500 QTRAP system (AB Sciex) operated in an Information Dependent Acquisition, IDA, mode. Full scan TOFMS data was acquired over the mass range 350–1400, and for product ion, MS/MS 80–1400 m/z ions observed in the TOF-MS scan exceeding a threshold of 100 counts and a charge state of +2 to +5 were set to trigger the acquisition of product ion, MS/MS spectra of the resultant 20 most intense ions.

Database search was performed using MASCOT 2.5 (Matrix-Science) search engine on the *E. caproni* genome database, available on-line at http://parasite.wormbase.org/Echinostoma_caproni_prjeb1207/Info/Index/. Searches were done with tryptic specificity, allowing one missed cleavage and a tolerance in mass measurement of 100 ppm in MS mode and 0.6 Da for MS/MS ions. Carbamidomethylation of Cys was used as fixed modification and oxidation of Met and deamidation of Asn and Gln as variable modifications. Only proteins identified with two or more significant peptides were taken into account. BLASTp was performed against NCBInr protein database with taxonomy set in Trematoda.

Electron microscopy: SEM and TEM

A total of 10 adult worms recovered from control mice and 10 from mice exposed to garlic prior to and after infection (continuous group) were analysed. For scanning electron microscopy (SEM), *E. caproni* adults were fixed in Karnovsky's fixative (0.5 M glutaraldehyde, 2.5 M formaldehyde), washed in buffer solution and post-fixed in 2% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.2, for 2 h before dehydration by critical point. Mounted specimens were sputter-coated with gold-palladium and examined in a Hitachi S4100 scanning electron microscope at 5 kV.

Inclusion in LR-white resin for transmission electron microscopy (TEM) was performed by fixing the adult parasites in glutaraldehyde 2.5%, washing in phosphate buffer 0.1 M pH 7.2, and then post-fixing in 2% osmium tetroxide in phosphate buffer. After several washes in distilled water, parasites were sequentially dehydrated in 30, 50, 70 and 96% EtOH. Finally, the worms were sequentially incubated for 2 h in 33% LR-white resin in 96% EtOH, 66% LR-white resin in 96% EtOH, 66% LR-white resin in 100% EtOH and 100% LR-white resin in 100% EtOH. Samples were filtered in resin and polymerized at 60 uC for 48 h. Ultra-thin slices (60 nm) were stained with 2% uranyl acetate prior to visualization by TEM at 60 kV in a microscope Jeol JEM1010. Images were acquired using a digital camera MegaView III with Olympus Image Analysis software.

Statistical analysis

Significant differences among groups in the number of worms recovered, EPG and relative gene expression were analysed by one-way ANOVA. Bonferroni *t* test was performed as a post hoc analysis, and differences between means were considered statistically significant when $p < 0.05$. Prior to analysis, data were log transformed to achieve normality and verified by Shapiro-Wilk test.

Results

Worm recovery and egg production

No significant differences among groups were detected in the number of worms recovered per mouse, nor the EPG (Table 1). These results indicate that garlic consumption at a daily dose of 50 mg/kg is not effective in preventing, nor curing, *E. caproni* infection in CD1 mice and does not affect the egg production of adult worms.

Local cytokine expression

The immunomodulatory effects of garlic were studied by analysing the gene expression of several cytokines at the site of the infection. At 2 wpi, the different garlic-exposed groups showed a local cytokine expression profile very similar to that in non-exposed mice. Significant statistical differences were not detected among groups for any of the cytokines analysed (Supplementary Fig. 1).

Protein secretion and electrophoretic profile of ESPs

The ESPs were obtained by incubation of *E. caproni* adults from each experimental group in RPMI 1640 containing phenol red as pH indicator. A striking fact was that the colour of the culture medium immediately changed from pink to orange when it was added over the worms recovered from all the groups exposed to garlic, indicating a rapid acidification of the medium. Conversely, it did not shift but kept the pink-coloured tone in the control group (Supplementary Fig. 2). After 16 h of incubation, all media showed the same pale-yellow tone and the worms remained alive. The protein content in each ESP decreased progressively as garlic exposure increased. The quantity of protein secreted per worm diminished from 8.24 μg in the control group to a minimum of 1.62 μg in parasites recovered from mice belonging to the continuous group. The amount of protein per worm was 1.9, 3.8 and 5.1 times higher in the control group with respect to prophylactic, therapeutic and continuous groups, respectively (Fig. 1a).

Table 1 *Echinostoma caproni* adults recovery from mice with different exposure to garlic

	Control	Prophylaxis	Treatment	Continuous
Worm recovery[a]	38.0 ± 13.1	39.0 ± 3.5	27.7 ± 7.0	44.7 ± 4.0
EPG ($\times 10^{3*}$)[b]	4.0 ± 0.4	6.2 ± 0.5	3.5 ± 2.0	9.21 ± 3.8

Number of *E. caproni* eggs per gram of faeces in each group

[a] Number of worms recovered per mouse (mean ± standard deviation)

[b] Number of *Echinostoma caproni* eggs per gram of faeces (mean ± standard deviation) counted by Kato-Katz technique

The same amount of protein from each ESP was separated by 1D protein electrophoresis. Figure 1b shows the electrophoretic profile of the ESPs for each experimental group. Protein patterns were very similar among all the groups, though some differences were noticed. Twelve per cent polyacrylamide gels revealed differences in protein amount of one band at around 29 kDa and several bands over 100 KDa. To get a better resolution of the proteins of high molecular weight, the ESPs were separated in 8% polyacrylamide gels. Three bands of approximately 80, 100 and 200 kDa, respectively, showed differences in protein quantity. All the differential proteins showed the same dose-dependent pattern, being more abundant as garlic exposure increased (Fig. 1b).

Protein identification

The results for the identification of differential proteins are compiled in Table 2. Among the proteins identified, two involved in cell signaling, such as 14–3-3 protein and major vault protein (MVP) (bands 4 and 2, respectively), an ATPase from the transitional endoplasmic reticulum (tER-ATPase, band 3) and a secreted proteinase inhibitor, α-2-macroglobulin-like protein 1 (band 1). All the proteins showed very close experimental and theoretical molecular weights, in addition to high values of MASCOT score and emPAI. Furthermore, BLASTp results were also satisfactory, with nearly nil e-values and query coverage over the 90% in all cases (Table 2).

Results of scanning electron microscopy

The potential effects of garlic over the surface of *E. caproni* were analysed on adult specimens recovered from control mice and mice exposed to garlic before and after the experimental infection. Changes on the surface of parasites were compared in different parts of the body, including the cephalic region, ventral sucker, and ventral, lateral and dorsal sides. No substantial differences were observed at the cephalic region. The surface of the collar encircling the oral sucker was smooth, with longitudinal striations from the periphery to the opening of the sucker, both in control and exposed worms. However, there were evident signs of lesion in one specimen from the continuous group, which may be attributable to the effect of garlic. Concretely, areas of desquamation and mild erosion were observed on the surface of the collar (Supplementary Fig. 3a). Furthermore, this specimen displayed eruption and sloughing off of the tegument on the ventral surface (Supplementary Fig. 3b). Alterations in the ventral sucker were not present.

In contrast, the tegumentary surface both at ventral and dorso-lateral sides was damaged in those worms exposed to garlic. The surface of control worms had the typical appearance, with the body surface covered by tegumentary spines,

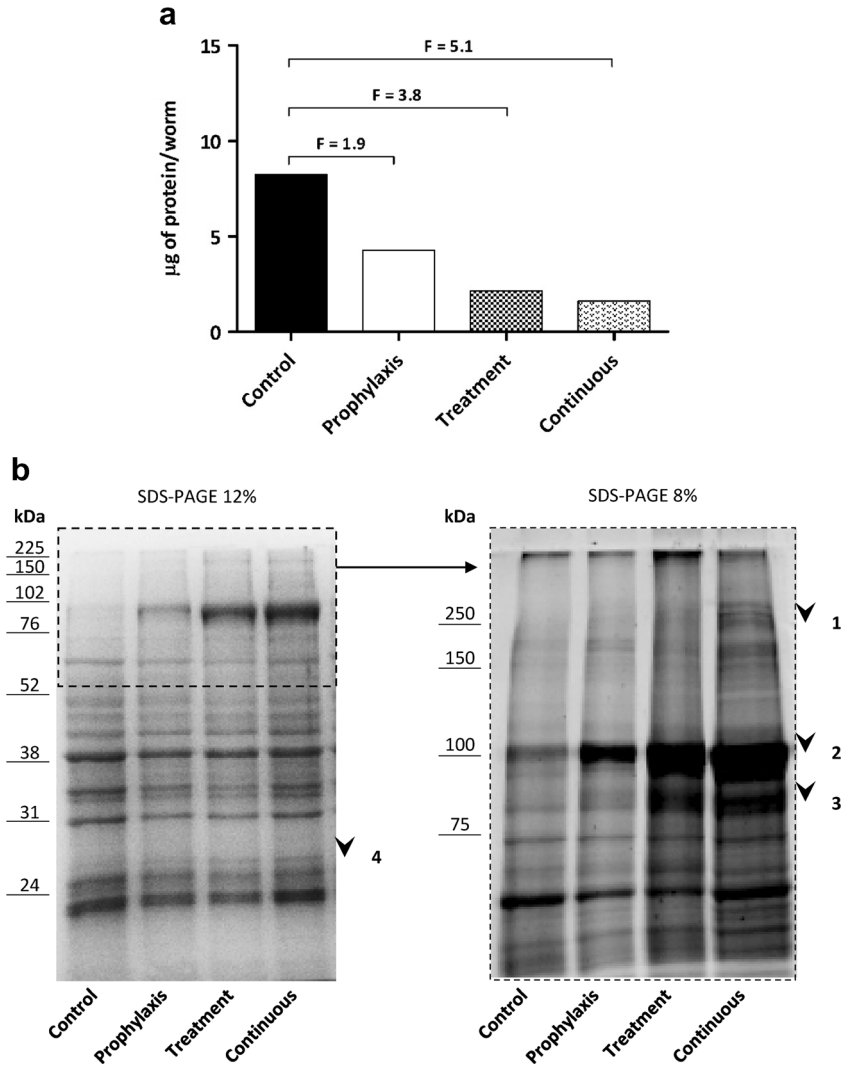

Fig. 1 **a** Quantity of protein excreted/secreted per worm in culture. Fold difference between the protein amount in control and each garlic-exposed groups (*F*). **b** 1D–electrophoretic profile of the excretory/secretory products of *Echinostoma caproni* recovered from mice with different exposure to garlic. *Arrowheads* point at the differential bands that were identified by mass spectrometry and database search (see Table 2)

except in the dorsal part (Fig. 2a, c, e). The ventral side, which shows the highest spine density, appeared smooth and uniform in the control specimens. In garlic-exposed adults, however, both ventral and lateral surfaces were markedly swollen (Fig. 2b, d). Consequently, the actin spines were much less prominent and almost entirely covered by tegument, which gave them a flattened appearance (Fig. 2b, inset). Most of the spines showed shattered tips. Moreover, the tegument surface in exposed worms was not smooth but furrowed (Fig. 2b, d). On the sides of the body, the spines appeared sunken, with their tips protruding from the swollen tegument (Fig. 2d, inset).

The dorsal side of *E. caproni* adults is characterized by the absence of tegumentary spines. Conversely, in this part of the body, the tegument forms grooves (Fig. 2e). Unlike control specimens, a markedly swollen dorsal tegument was observed in garlic-exposed worms. In these parasites, the grooves had disappeared and the tegument displayed a furrowed aspect (Fig. 2f).

Results of transmission electron microscopy

Tegumental and sub-tegumental ultrastructures were studied in worms recovered from mice in control and continuous groups. A normal structure was observed in control specimens (Figs. 3a and 4a). The external surface of the parasite is lined up by a definite, highly folded plasma membrane that forms the microvilli along the body. The syncytial tegument is highly packed with membrane-bound vesicles of different morphologies. Elongated vesicles or T2-like secretory bodies are the most frequently observed in the distal part, showing a characteristic distribution pattern. These are disposed perpendicularly to the external membrane in the microvilli and the apical part of the syncytium but are oriented parallel to the basal lamina further interiorly. Circular vesicles, filled with intermediate electron dense material, appear mainly underneath the microvilli, whereas T1-like secretory bodies are, in general, very scarce. Extracellular vesicles of various sizes are frequently seen outside the tegument. A number of

Table 2 Identification details of garlic-modulated proteins in excretory/secretory products using MASCOT search engine on the *Echinostoma caproni* genome database and BLASTp against NCBInr protein database

	Identification details						BLASTp details[f]			
Band[a]	Accession number[b]	Protein	Species (Acc.[c])	MASCOT Score	Matches[d]	Peptides[e]	*E*-value	Total score	Query Cov (%)	Ident (%)
1	ECPE_0000476901-mRN-A-1	Alpha-2-macroglobulin-like protein 1	*Clonorchis sinensis* (GAA52061)	2222	52	33	0	927	99	39
2	ECPE_0001276401-mRN-A-1	Putative major vault protein	*Schistosoma mansoni* (CCD75353)	2019	44	31	0	952	99	65
3	ECPE_0001043101-mRN-A-1	Transitional endoplasmic reticulum ATPase	*C. sinensis* (GAA28937)	2855	63	42	0	1417	97	95
4	ECPE_0000984501-mRN-A-1	14-3-3	*C. sinensis* (GAA36880)	5504	75	21	$8{\cdot}10^{-134}$	380	93	77

[a] Band reference number (see Fig. 1)

[b] Accession number in the *Echinostoma caproni* genome database

[c] Accession number in the protein database of NCBI

[d] Number of significant matches

[e] Number of significant peptides

[f] Details of protein blast of translated genome annotations against the NCBInr protein database

mitochondria are present in the syncytium, except in the apex (Fig. 3a). Beneath the basal lamina, there is the muscular layer, which consists of densely packed longitudinal and transversal bundles of muscle fibres. The tegument-forming cells are located further inside and are fused to the syncytium through cytoplasmic connections (Fig. 4a).

Alterations in the normal ultrastructure of the tegument were observed in worms exposed to garlic in vivo (Figs. 3b, 4b, c and 5). The tegumental syncytium was abnormally thick in exposed specimens (Fig. 3b). Anomalous T2-like secretory bodies, which were larger and often filled with an unusually electron dense material, were common (Fig. 5a). Moreover, the organized disposition of these bodies in the syncytium was lost beneath the microvilli. Circular vesicles were highly abundant in the apex, whereas a fewer mitochondrion was present. In contrast, the formation and release of extracellular vesicles seemed not to be affected (Fig. 5a). Occasionally, vacuolization of the syncytium was observed (Fig. 5b), while swelling of the basal lamina and basal infolds was more common (Fig. 3b). Several alterations were also seen in the subtegument (Fig. 4b, c). Disruption of muscle bundles was seen in some cases. Below the basal lamina, small spaces appeared between muscle bundles, both longitudinal and transversal, and the parenchymal tissue was loosely packed and disorganized (Fig. 4b). Sings of injury were observed sometimes in tegumental cell bodies. These consist in alterations in the morphology of the nucleus, presence of swollen mitochondria and autophagic vesicles, and a disorganized, loosely packed cytoplasm (Fig. 4c).

Discussion

Potential health benefits of garlic have been recognized from ancient times to date (Rivlin 2001). Although in vivo anthelminthic effects have been previously reported against *S. mansoni* (Riad et al. 2009; Mantawy et al. 2011, 2012) and monogeneans (Fridman et al. 2014), the results presented herein indicate that daily consumption of garlic at dietary doses is not effective in preventing nor curing *E. caproni* infections in mice. However, garlic-induced changes in parasite tegument and protein secretion reveal the potential usefulness of bioactive compounds in garlic for the development of new trematocidal drugs.

The immunomodulatory activity of garlic is well documented (Arreola et al. 2015). Herein, key cytokines in the regulation of *E. caproni* infections (Sotillo et al. 2011; Trelis et al. 2011), together with cytokines that are known to be modulated by garlic (Kang et al. 2001; Keiss et al. 2003; Makris et al. 2005), were studied. No statistical differences in the local cytokine expression profile were observed among experimental groups, indicating that garlic-induced changes are not mediated by the host immune response.

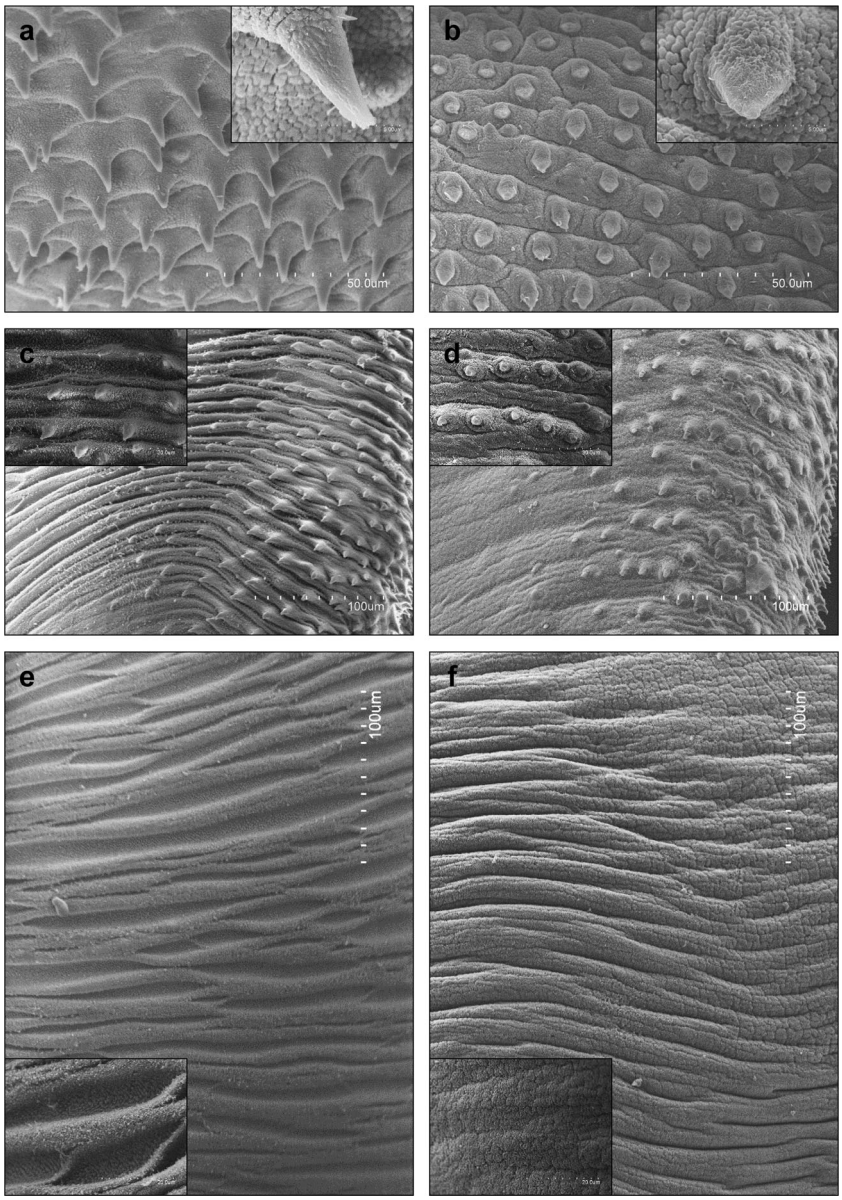

Fig. 2 Representative micrographs of scanning electron microscopy of control (**a**, **c**, **e**) and garlic-exposed *Echinostoma caproni* adults (**b**, **d**, **f**). Ventral (**a**, **b**), lateral (**c**, **d**) and dorsal (**e**, **f**) sides are shown. *Insets* show detailed regions of each full image. *Dots in scale bars* delimitate a tenth part of the indicated length

However, remarkable alterations were noticed in several phenotypic features of adult worms in response to garlic. Changes in tegumental structure were evident. Tegument ultrastructure of control *E. caproni* observed by SEM and TEM coincided with previous descriptions (Sotillo et al. 2010; Andresen et al. 1989; Simonsen et al. 1990). It is noteworthy that garlic-induced alterations are similar to those induced by trematocidal drugs in other foodborne trematodes. Swelling and furrowing of the external surface, together with sunken spines, have been described in other trematode species in response to clorsulon (Meaney et al. 2005), triclabendazole (Halferty et al. 2009), artesunate (O'Neill et al. 2015) and praziquantel (Goncalves et al. 2013). However, severe lesions including bebbling, peeling and erosion of the tegument or loss of tegumentary spines were not observed herein. The lack of changes in oral and ventral suckers, which are involved in vital processes such as feeding and mucosal attachment, respectively, may explain the high worm recoveries obtained in garlic-exposed mice.

Alterations in the ultrastructure of tegument and subtegument were also evident in exposed worms. The large increase in the thickness of the tegumental syncytium and basal lamina may account for the swelling seen externally. Loosely packing of muscle bundles, parenchymal tissue and tegument-forming cells were observed in some areas and can contribute to this result. Similar changes have been described in *F. hepatica* following triclabendazole and artesunate administration and were associated with disruption of the osmoregulatory capacity of the tegument (Halferty et al. 2009; O'Neill et al. 2015). Likewise, appearance of vacuoles in the

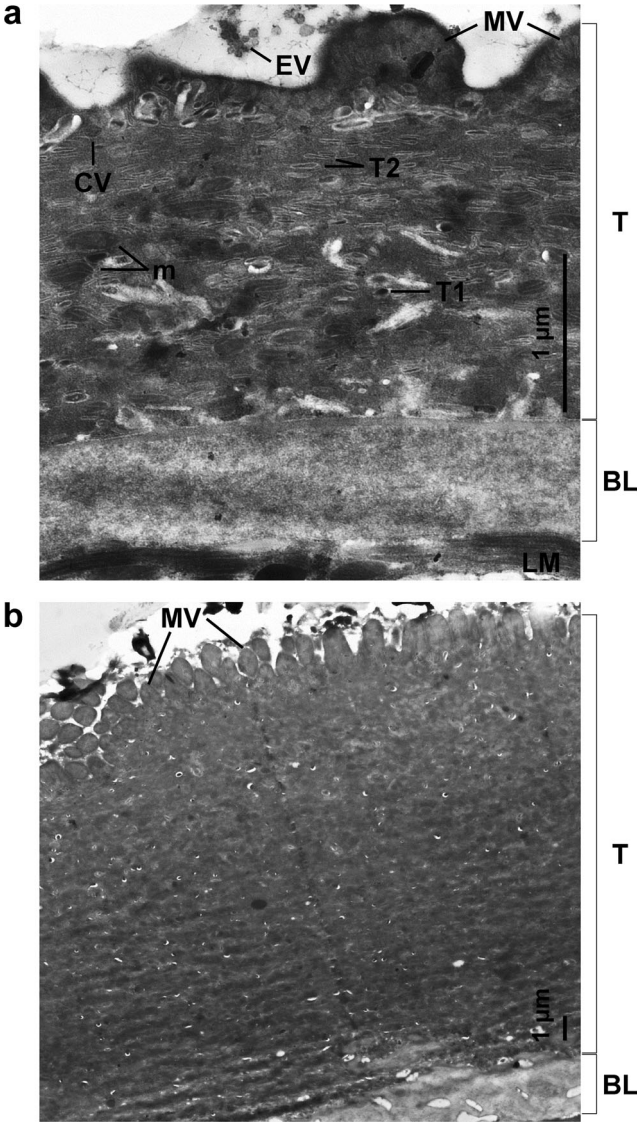

Fig. 3 Representative micrographs of transmission electron microscopy of the tegument of **a** control and **b** garlic-exposed *Echinostoma caproni* adults. Note the enlarged tegumental syncytium (*T*) and basal lamina (*BL*) in exposed worms. *MV* microvilli, *EV* extracellular vesicles, *CV* circular vesicle, *T1* T1-like secretory bodies, *T2* T2-like secretory bodies, *m* mitochondria, *LM* longitudinal muscle

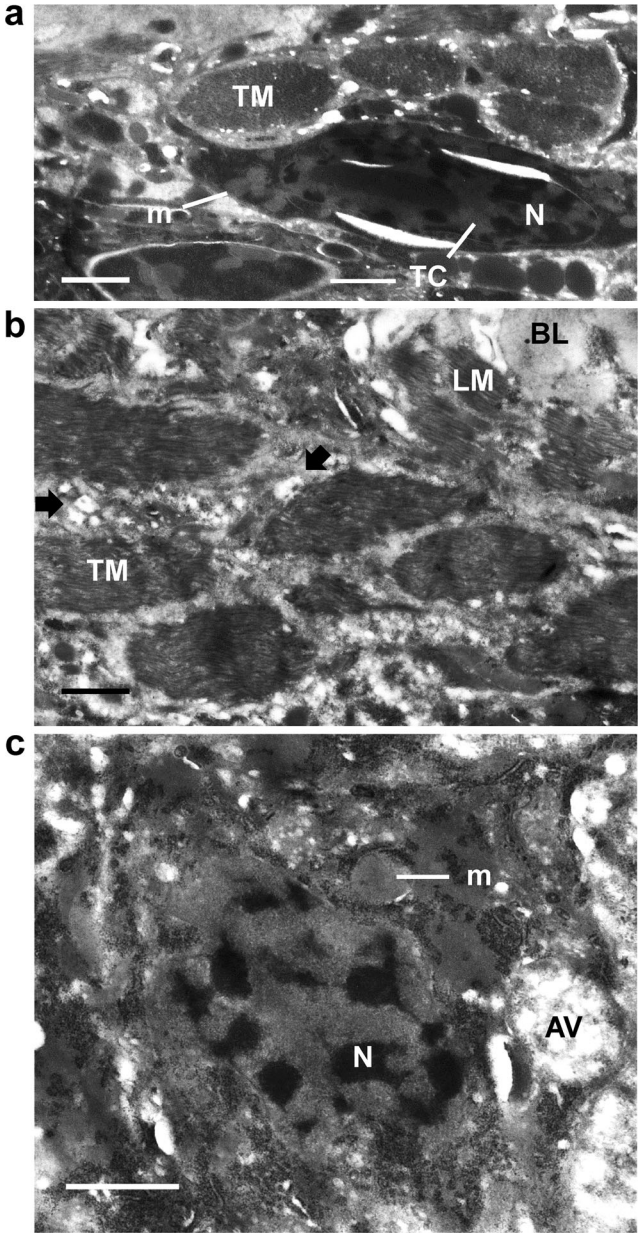

Fig. 4 Representative micrographs of transmission electron microscopy of the sub-tegument of **a** control and **b**, **c** garlic-exposed *Echinostoma caproni* adults. Note the disruption of the connective tissue between muscle bundles (*arrows*) and parenchymal tissue (**b**) and damaged tegument-forming cell showing anomalous nucleus, swollen mitochondria and autophagic vacuole (**c**). *BL* basal lamina, *TM* transversal muscle, *LM* longitudinal muscle, *TC* tegument-forming cell, *N* nucleus, *m* mitochondria, *AV* autophagic vacuole. *Scale bar* = 1 μm

syncytium has been reported in *E. paraensei* (Goncalves et al. 2013), *F. hepatica* (O'Neill et al. 2015) and *S. mansoni* (Xiao et al. 2002) after exposure to different trematocidal drugs. Edema, vacuolization and disruption of tegument-forming cells were also observed in *S. mansoni* adults recovered from mice daily exposed to the same dose of garlic employed in this paper (Riad et al. 2009).

Reduction in the number of secretory bodies is also a common fact after drug exposure (Goncalves et al. 2013; O'Neill et al. 2015) and may precede the loss of the tegumental syncytium, as they are crucial to maintain the syncytial layer (Fairweather et al. 1999). Although a substantial reduction in the number of secretory bodies was not noticed herein, circular vesicles became highly abundant. The increase of circular vesicles at the apex of the tegument may indicate a stress response to guarantee the integrity of the external plasma membrane (O'Neill et al. 2015). A dominance of these vesicles and the accumulation of anomalous T2-like secretory bodies below the microvilli may be the cause of decreased protein secretion, which became dramatically reduced as garlic exposure increased.

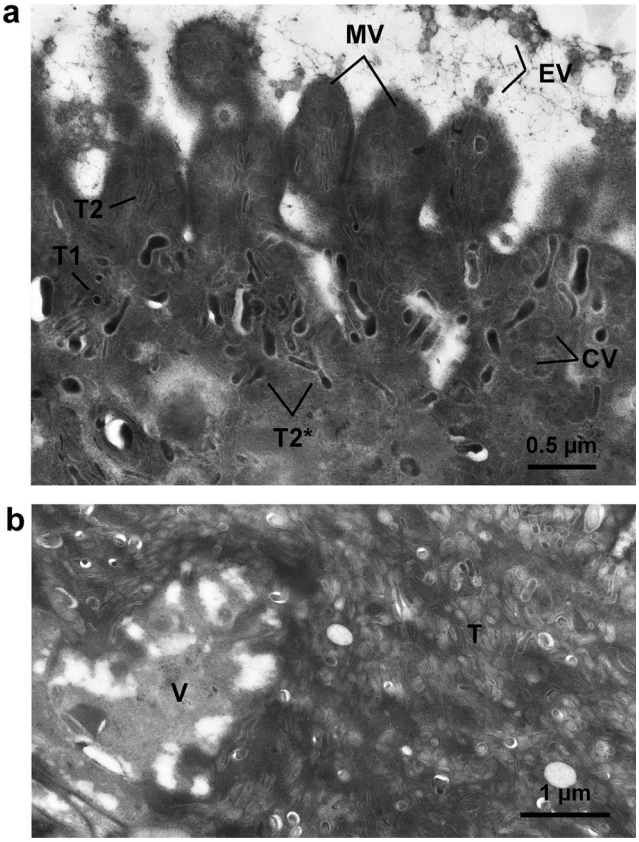

Fig. 5 Representative micrographs of transmission electron microscopy of garlic-induced alterations in the tegumental syncytium of *Echinostoma caproni* adults. **a** Accumulation of anomalous T2-like secretory bodies beneath the microvilli. **b** Vacuolization of tegumental syncytium. *MV* microvilli, *EV* extracellular vesicle, *CV* circular vesicle, *T1* T1-like secretory bodies, *T2* T2-like secretory bodies, *T2** anomalous T2-like secretory bodies, *V* vacuole, *T* tegumental syncytium. Normal appearance of the tegumental syncytium in control worms (not exposed to garlic) is shown in Fig. 3a

Analysis of the secretome showed that several proteins were up-regulated in response to garlic in a dose-dependent manner. Four differentially secreted proteins were identified. The 14-3-3 proteins are key signaling molecules that participate in the regulation of several processes, including cell response to stress (Siles-Lucas and Gottstein 2003). In parasitic trematodes, 14-3-3 proteins are found in the tegument, subtegument, muscle, parenchyma and ESP (Schechtman et al. 2001a; Wang et al. 2012) and have been proposed as vaccine candidates (Wang et al. 2012; Schechtman et al. 2001b). tER-ATPase is involved in endosomal trafficking, autophagy and mitochondrial quality control (Yamanaka et al. 2012). Thus, the up-regulation of this protein could be linked to changes in the tegument ultrastructure observed by TEM, such as alterations in vesicle and mitochondrial numbers, mitochondrial swelling and vacuolization. Enhanced production of a 14-3-3 and tER-ATPase in *E. caproni* exposed to garlic suggests that they may be important in the stress response generated against toxic compounds and can be considered as potential drug targets.

MVP, another protein involved in cell signaling, was up-regulated in response to garlic. MVP is the main component of vaults, a sort of ribonucleoprotein extremely conserved across multiple species. The cellular function of vaults is not fully understood. However, several studies suggest a role of these complexes in multidrug resistance, signal transmission and immune response (Berger et al. 2009). Recently, up-regulation of MVP has been reported in different stages of *S. mansoni* resistant to praziquantel (Reis et al. 2014). Up-regulation of MVP in garlic-exposed *E. caproni* adults suggests a role of this complex in the response to toxic compounds and may be indicative of the activation of resistance mechanisms to promote parasite survival. An alpha-2-macroglobulin-like protein was also up-regulated in garlic-exposed worms. Alpha-2-macroglobulins are broad spectrum protease inhibitors that operate through the entrapment of the target proteases, which are considered to be part of the innate immune system of metazoans (Armstrong 2006). Although sequence identity in key functional domains does exist, there is a lack of functional data on alpha-2-macroglobulins in helminths. Up-regulation of this protein in the context of a toxic environment suggests that it may be involved in the defence response of the parasite and/or its interaction with the host.

A striking result was the rapid acidification of the culture medium in garlic-exposed worms with respect to controls. Medium acidification in other trematodes is due to the release of lactic acid, which needs to be eliminated to avoid poisoning metabolic pathways and maintain high rates of glycolysis. In *S. mansoni*, lactate is exocytosed through the tegumental protein aquaporin (Githui et al. 2006; Faghiri et al. 2010). Functional aquaporins are needed to worm swelling in response to changes in the tonicity of the medium and to maintain parasite viability (Faghiri and Skelly 2009). In garlic-exposed *E. caproni*, rapid acidification of the culture medium coincided with marked swelling of the tegument, suggesting that both alterations may share a common cause.

Overall, the results presented herein demonstrate that, although dietary consumption of garlic appeared not to be effective as an anthelmintic, dose-dependent detrimental changes have been observed in exposed parasites. Injurious effects on the tegumental surface, which resemble those inflicted by commercially available trematocidal drugs, were evident. Since tegument integrity is essential for vital functions such as nutrition, immunoprotection and osmoregulation, lethal effects can be expected at higher doses. Study of the secretome in response to garlic exposure provides a novel approach that may be valuable for the identification of new molecular drug targets. Moreover, this throws light on the molecular mechanisms through which the parasite responds and that may lead to development of resistance. The present study has the advantage that patent effects of garlic on intestinal helminths

have been identified in vivo. Indeed, one of the most attractive results is that some effects, such as quantitative and qualitative changes in protein secretion or rapid acidification of the culture medium, occur also in worms that were not exposed to garlic directly, but when it was given as prophylaxis. This fact opens the possibility that garlic compounds may also act indirectly, inducing more or less permanent changes in the intestine of the host that may affect the parasites established later on.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures involving animals were approved by Ethical Committee of Animal Welfare and Experimentation of the University of Valencia (Ref#A18348501775). Protocols adhered to Spanish (Real Decreto 53/2013) and European (2010/63/UE) regulations.

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