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ORIGINAL PAPER

Identification and Semi-quantification of Protozoa from the Digestive System Microbiota of the Lobster Cockroach *Nauphoeta cinerea* Oliver, 1789 (Insecta:Blattaria)

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Abstract

Introduction The lobster cockroach *Nauphoeta cinerea* (*N. cinerea*) is indicated as a promising non-mammalian model, because it presents behavioral and biochemical alterations also observed in conventional models. In this research, we identified and characterized the distribution of protozoa that inhabit the digestive system (DS) of *N. cinerea* cockroaches.

Methods The adult specimens of *N. cinerea* used in this study ($n=32$) were obtained at the Federal University of Santa Maria, dissected and had their visceral contents observed in bright-field microscopy without staining and after application of lugol, Ziehl–Neelsen staining, EA36 trichrome and simulated dark-field microscopy with application of nankin ink. The presence of protozoa in different portions of the DS was semi-quantified by a system of crosses (+).

Results The main taxa observed were: amoebas (Archaemebae:Entamoebida), gregarins (Apicomplexa:Eugregarinide), coccidia (Apicomplexa:Eucoccidiorida), kinetoplastids (Kinetoplastea:Kinetoplastida) and oxymonads (Preaxostyla:Oxymonadida). The highest prevalence of amoebas and gregarines was observed in the medial portion of the DS, while for the other groups, this was seen in the final portion, and in the case of coccidia, such prevalence was specially evidenced by the alcohol-acid coloration. In the present work, the great biological diversity that exists in the microbiota of the digestive system of *Nauphoeta cinerea* was demonstrated, being possible to find several pathogenic species for humans such as *Entamoeba histolytica/dispar/moshkovskii*, *Cryptosporidium* sp. and *Cyclospora cayetanensis*. There is still a lot to know about the interactions between endocommensal protozoa and their respective invertebrate hosts, so the best way to clarify such relationships is through molecular and genetic test.

Keyword *Nauphoeta cinerea* · Protozoa · *Entamoeba* · *Cryptosporidium* · *Cyclospora*

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Introduction

Cockroaches (Insecta:Blattaria) are generally the target of repudiation or aversion for a number of reasons, one of which is their unusual appearance, but the main one is that they have unsanitary ecological habits that expose them to a wide variety of human pathogens, which often can be transmitted to people and cause potentially epidemic outbreaks, with emphasis on *Lophomas blattarum* Stein, 1860, which causes serious nosocomial infections [1, 2]. However, there are many alternative applications for the use of these insects in different areas, such as gastronomy, representing a strong trend in eastern livestock [3] and animal feed [4].

As for the epidemiological aspect, without a doubt, the most studied cockroach species was *Blatella germanica* L., 1757, because it is probably the most widespread species in cities of the modern world [5]. The parasitic profile of *B. germanica* has been documented for over 50 years and is frequently updated, being characterized by the presence of a wide variety of parasitic protozoa, such as: *Entamoeba histolytica* Schaudinn, (1903)/*dispar* Brumpt, (1925)/*moshkovskii* Tshalaia, (1941), *Giardia duodenalis* Filice, (1952) and *Balantidium coli* Claparède and Lachmann, (1858) [6, 7].

Regarding the alternative uses of cockroaches, one of the main uses is for the manufacture of animal feed, which can be cited as the most promising: *Periplaneta americana* L., (1758), *Nauphoeta cinerea*, and *Gromphadorhina portentosa* Schaum, (1853) [4, 8, 9]. Of these, only *P. americana* has been extensively characterized as to its parasitic profile of protozoa, with the main findings being *Entamoeba* spp. Casagrandi & Barbagallo, (1895), and *Nyctoterus ovalis* Leidy, (1850), [10].

The lobster cockroach *Nauphoeta cinerea* is indicated as a promising non-mammalian model in the toxicological evaluation of oxidative markers [11], as it presents behavioral and biochemical alterations observed in conventional models [12]. It is also known that *N. cinerea* presents neurochemical principles that are very similar to those of mammals, so that alterations in neurotransmitters present in mammals such as acetylcholine and octopamine can be analyzed in it [13].

Numerous pathogens transmitted by arthropod vectors are protozoa, such as *Trypanosoma cruzi* Chagas, (1909), transmitted by beetles of the subfamily Triatominae Jeannel, (1919) [14]; the *Leishmania* spp. Laveran & Mesnil, (1903), transmitted by mosquitoes of the subfamily Phlebotominae Kettle, (1984) [15]; and, numerous intestinal parasitic species such as *Entamoeba coli* Grassi, (1879) and *Balantidium coli* that have already been identified in a large number of different species of cockroaches [16].

Several efficient staining methods have already been developed for the visualization of protozoa commonly found in the human digestive system, many of which are applied to the present day. For amoeba staining, the most applied methods are trichrome stains for trophozoites and staining with iodine solutions for cysts [17]. For coccidia staining, the most commonly used methods are those derived from the Ziehl–Neelsen technique [18].

The use of cockroaches as a model for evaluating the neurobehavioral and biochemical effects of various chemical compounds implies the risk of exposure to potentially pathogenic protozoa by those involved in handling the animals, thus it is important to investigate the presence of these infectious agents in relevant species scientific. Thus, the objective of this research was to identify and characterize the distribution of protozoa that inhabit the digestive system of the cockroach *Nauphoeta cinerea* using different staining methods.

Materials and Methods

Insect Rearing and Sex Morphological Determination

The adult specimens of the lobster cockroach *Nauphoeta cinerea* used in this study were obtained from Federal University of Santa Maria (Biochemical Toxicology Laboratory). The animals were reared in plastic boxes with controlled temperature (25–27 °C) and 70% relative humidity, in a 12 h:12 h cycle (light/dark). Cockroaches had free access to water and a basal diet (for the control group), consisting of sterile feed for laboratory mice (PRESENCE®, Brazil), whose nutritional composition is rich in proteins (23%), fibers (5%), vitamins (~ 1%), mineral salts (~ 13%) and fats (in smaller proportions).

The sexes of the adult specimens of *N. cinerea* were morphologically differentiated both by the presence of the anal style in males and by the analysis of the proportion between the size of the wings and the length of the abdomen, so that in males the wings cover at least 2/3 of the abdomen and in females only about 1/2. The differentiation was confirmed during the dissections by the analysis of the reproductive systems, where the observation of the testes in males and immature oothecae in females can be made.

Cockroach Dissection and Stains Used

The cockroaches were anesthetized by cold, where they were placed in a freezer at temperature of – 10 °C for 20 min [19]. Afterwards, the specimens were placed on a latex rubber accommodated inside a container filled with cockroach saline solution [20], modified by the replacement of anhydrous $CaCl_2$ by $CaCl_2 \cdot 2H_2O$ in an equivalent proportion of Ca^{2+} ions; and their legs amputated at the level of the

thoracic-coxal joint using small scissors. Then, the two pairs of wings were also amputated by cutting in the region of the mesothorax joint and the cockroaches were then attached to the rubber by entomological pins attached to the mesothorax.

Later, two transversal cuts were made in the first segment of the abdomen. Through these openings, cuts were made in an anteroposterior direction in a transverse direction, separating the abdomen into hemispheres, where the upper hemisphere was removed with the aid of entomological tweezers and the posterior externalized viscera. New transversal cuts were made in the opening made by the first lateral cuts and followed in a posteroanterior direction until separating the mesothorax and prothorax into hemispheres, so that the upper hemisphere was again removed and the anterior region of the viscera externalized.

Finally, the head and the last abdominal segment were delicately shredded with entomological forceps and the viscera were placed on a microscope slide. The anterior (foregut), medial (midgut) and posterior (hindgut) regions of the digestive system (DS) were then separated by means of a scalpel, where the anterior one begins in the pharynx and ends at the beginning of the gizzard, the medial one begins at the beginning of the gizzard and ends in the ileum, and the posterior one begins in the ileum and ends in the anus, so that each of the parts it was cross-sectioned and its content analyzed.

Three couples (one male and one female) were used for the manufacture of fresh visualization slides (unstained) and visual enrichment techniques (enhancement of contrast), while for dark-field microscopy simulated with nankin ink, 5 pairs were used and for trichrome staining 2 pairs, due to similar qualitative and quantitative results, totaling 32 specimens analyzed.

Unstained Visualization

The fresh visualization of the visceral contents of the specimens was performed by distending them on a microscope slide with subsequent addition of a drop of modified cockroach saline solution [20], then covered with a glass coverslip, left to rest for 10 min and viewed under a bright-field microscope on 10x, 40 × and 100 × objectives. After the dissection of each region of the DS, the regions that would be dissected then waited immersed in modified cockroach saline solution.

Lugol Stain

In the protocol with concentrated lugol (2%), a drop of potassium iodide (2%) and metalloid iodine (1%) solution with deionized water as solvent (LABORCLIN©, Brazil) was added to the slides with material, and then covered with a cover slip of glass, left to rest for 5 min and then visualized under a bright field microscope on the 10 ×, 40 × and 100 × objectives. After the dissection of each region of the DS, the regions that would be dissected then waited immersed in modified cockroach saline solution.

Ziehl–Neelsen Stain

For acid-alcohol staining (Ziehl–Neelsen), a rapid staining kit (LABORCLIN©, Brazil) was used, following the manufacturer's instructions with some modifications: the entire slide was covered with Ziehl–Neelsen's fenified fuchsin solution, heated until vaporization was observed (taking care that the liquid did not boil or dry), removed from support and quickly washed in running water under low pressure; then, it was decolorized with bleaching solution, dripping it onto the inclined slide until no further dye was removed, washed in running water under low pressure, covered with Loeffler methylene blue for 30 s; finally washed in running water, and subsequently dried in an upright position at room temperature. The slides were viewed under a bright-field microscope on 10x, 40 × and 100 × objectives.

Trichrome EA36

For trichrome staining, EA36 trichrome (eosin azuri 36) (LABORCLIN©, Brazil) was used. The dye is usually applied for Papanicolaou staining [21, 22] for stain cytoplasm, collagen, fibers muscles, and mucus [23]. The composition used was: phosphotungstic acid (0.2%), anhydrous ethyl alcohol (900 ml), yellowish eosin (0.225%), light green (0.225%), brown Bismark Y (0.05%), deionized water (100 ml), saturated solution of lithium carbonate (0.5 ml).

The protocol applied here was that suggested by Fisher Diagnostics [24] with modifications: 70% Ethanol (5 min); 10% ethanol (3 min); Trichrome EA36 staining (10 min); 90% ethanol plus acetic acid (0.5%) (1–3 s); 100% Ethanol (2–3 times dip); 100% ethanol (3 min); 100% ethanol (3 min). The slides were viewed under a bright-field microscope on 10x, 40 × and 100 × objectives.

Dark Field Microscopy Simulated with Nankin Ink

Nankin ink presents itself as a promising source in conducting artificial dark microscopy, since, as a colloid, it hinders light diffusion in the background of the image and increases the visual contrast, in addition to the fact that it is easily obtained. This technique was applied specially to optimize the visualization of gregarine cell structures, since, due to its relatively large size, the visualization is not excessively impaired by the presence of a lot of viscous fluid in the space of the visualized material. This method was performed by adding a drop of insect saline to the material stretched on a slide and a drop of India

ink (Acrilex©, Brazil), which were then covered with a glass cover slip, left to rest for 10 min and then visualized using a bright field microscope on the 10 × objective, where light pressure was applied on the cover slip, facilitating visualization.

Data Analyses

In all slides prepared by the different methods described, a count of the observed protists was made using the 10× (gregarines) and 100× (other) objectives. About 100 fields were analyzed in the slides prepared with the contents of the anterior, medial and posterior regions of the DS of each specimen, similarly to what was done by Goggin & Lester [25], so that a system of "crosses" to semi-quantify the presence of the main observed protozoa, where: the morphotypes observed on average up to 10 times were qualified with a cross (+); those observed on average between 11 and 20 times were qualified with two crosses (++); and the morphotypes observed on average more than 20 times qualified with three crosses (+++). Simple means were in triplicates, and the analyses were performed by a main observer, or in cases of doubt, another two observers were selected, where all consulted the cited literature to corroborate the hypotheses.

Photographs were taken of the specimens observed in all described preparation methods, and the images were analyzed using *ImageJ* software with 64-bit Java 1.8.0_172, so that a measurement of the main structures could be made to corroborate the identifications at the genus or species level, using a 30 µm line for scale calibration. For the identification of the flagellates, videos were later recorded and decelerated using the *VideoFramePlayer* application. The taxa were identified through the observation of synapormophies described by Archibald *et al.* [26] and through photographic comparisons with the CDC image gallery [27].

For the amoeba species, the main criteria used in the identification were the sizes of the cellular structures visualized (cells/cysts, nuclei and karyosomes), the arrangement of the karyosomes and the presence of chromatoid bodies. The morphological criteria of the primate analyzed in the identification of gregarines were: length of the deutomerite; maximum width of the deutomerite; protomerite length; maximum width of the protomerite; width of the protomerite on the equatorial axis; and total length of the satellite. To identify the coccidia, the main morphological criteria applied were the presence and number of oocysts, sporocysts and sporozoites. For the other genera, the criteria were general cell shape and flagella disposition. The rate assumptions were also based on findings, such as case reports, previously documented in the literature, especially in the identification of *Cryptosporidium* sp.

Results

Unstained Visualization

The main taxa observed were: amoebas (Archaemebae:Entamoebida), gregarins (Apicomplexa:Eugregarinide), coccidia (Apicomplexa:Eucoccidiorida), kinetoplastids (Kinetoplastea:Kinetoplastida) and oxymonads (Preaxostyla:Oxymonadida). The visualization of fresh materials allowed identification of all the morphotypes described, where both the cystic (Fig. 1a–d) and mobile (trophozoites) forms of the amoebas were observed, making it possible to distinguish at the species level to the point of characterization of five different species of amoebas: *Entamoeba histolytica/dispar/moshkovskii*, *E. coli*, *E. hatmanni* Prowazek, (1912), *Endolimax nana* Wenyon & FWO'Connor, (1917) and the poorly described *Endamoeba blattae* Bütschli, (1878). The trophozoites of gregarines were extensively observed, both solitary forms (free trophozoites) and associations (syzygy), in all portions of the DS, mainly in the medial portion (gizzard), having been identified five different morphotypes probably belonging to five species (Fig. 1e,f).

The coccidioids visualized were characterized as belonging to the genus *Cryptosporidium* Tyzzer, (1907) and to the species *Cyclospora cyetanensis* Ortega, Gilman & Sterling, (1994). Structures of the oocyst and sporocyst types were observed (Fig. 1g) for the latter and only oocysts for the former. The identification of these structures, mainly of Cryptosporidium, being very difficult in the absence of appropriate coloration. The diplomonads were predominantly observed in the ileum region, and their identification is also quite difficult due to the rapid locomotion of these individuals, belonging to the species *Blattamonas nauphoetae* Treitli *et al.*, (2018) (Fig. 1i). The kinetoplastid species had very similar morphotypes, probably belonging to *Herpetomonas* Kent, (1880), respectively, occurring almost always in the same portions of the DS. (Fig. 1h).

A general analysis demonstrates an apparent predominance of protozoa in the DS of male cockroaches, evidenced by all stained and unstained methods. Visualization without addition of dyes led to the distribution of protozoa specified in Table 1, where it is possible to observe a higher prevalence of amoebae in the terminal portions of the DS, and the same applies for (Kinet.) spp. and *B. nauphoetae*. In the cases of coccidia and gregarines, the highest concentrations of these specimens seem to be located in the median portion of the DS.

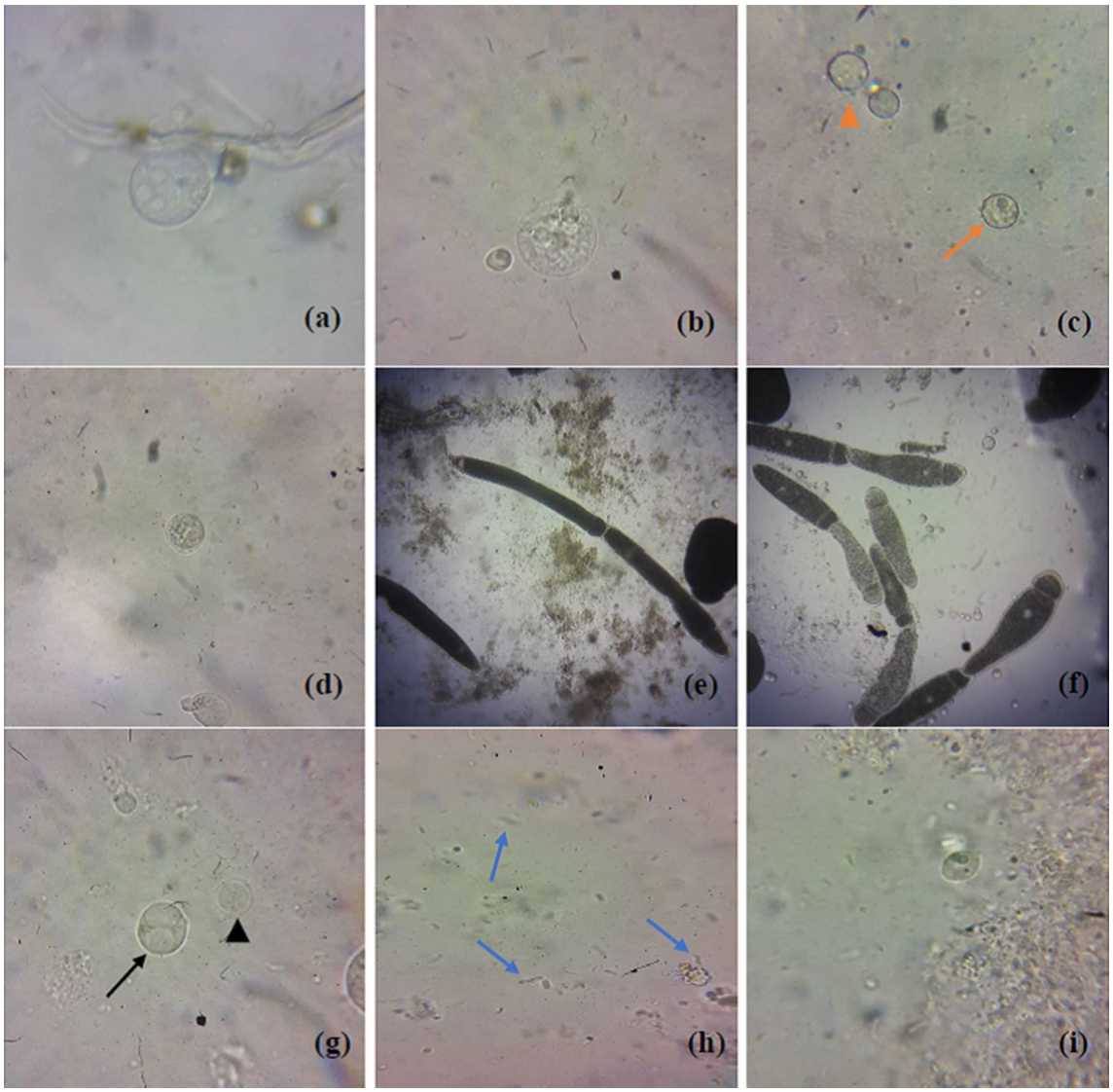

Fig. 1 Unstained visualization of protists from DS microbiota of *N. cinerea*: **a** *Entamoeba histolytica/disparity/moshkovskii* cyst; **b** *E. coli* cyst; **c** *E. hartmanni* (arrow) and *Endolimax* nana (arrowhead) cysts; **d** Probable *Endamoeba blattae* cyst; **e**, **f** Different morphotypes of gregarins; **g** Sporulated (arrow) and non-sporulated (arrowhead) gametocysts of *Cyclospora cayetanensis*; **h** Cylindrical (*Herpetomonas*) shaped kinetoplastids (arrows); **i** *B. nauphoetae* in the material from ileum region. In **a**–**d** and **g**–**i**: 1000 × magnification; in **e**, **f**: 100 × magnification

Table 1 Semi-quantification of protists from SD microbiota of *N. cinerea* without staining

UNSTEIN	*Amoeba* spp.	*Eugregarinida* spp.	*Eucoccidiorida* spp.	*Herpetomonas* sp.	*B. nauphoetae*
Male (foregut)	+	+ +	+	0	0
Male (mindgut)	+ + +	+ + +	+ +	+	0
Male (hindgut)	+ + +	+	+	+ + +	+ + +
Female (foregut)	+	+	+	0	0
Female (mindgut)	+ +	+ + +	+	0	0
Female (hindgut)	+ +	+	+	+ + +	+ + +

Lugol stain

After applying the concentrated lugol solution, it was possible to observe more easily some species and structures such as amoebas (Fig. 2a–f), where their nuclei, karyosomes and vacuoles were better observed. As well as *C. cayetanensis* oocysts and sporocysts (Fig. 2g,h) and kinetoplastids (Fig. 2i), while the visualization of gregarine trophozoites and *B. nauphoetae* was hampered, or even rendered unfeasible in the case of the latter. Another difficulty brought about by the use of this staining method is the fact that it stains pseudo-encysted structures, making it difficult to differentiate between those and cellular structures.

Table 2 below shows the distribution of protozoa obtained after application of lugol, showing a higher concentration of amoebas in the midgut of the animals, while confirming the higher prevalence of gregarines, coccidia, kinetoplastids and diplomonadides in the same places described above (Table 1), which would be midgut for the first two and hindgut for the last two.

Ziehl–Neelsen Stain

Using the classic acid-alcohol staining technique, it was possible to observe numerous oocysts of *Cryptosporidium* sp. (in red) (Fig. 3a–c), all having the same morphotype,

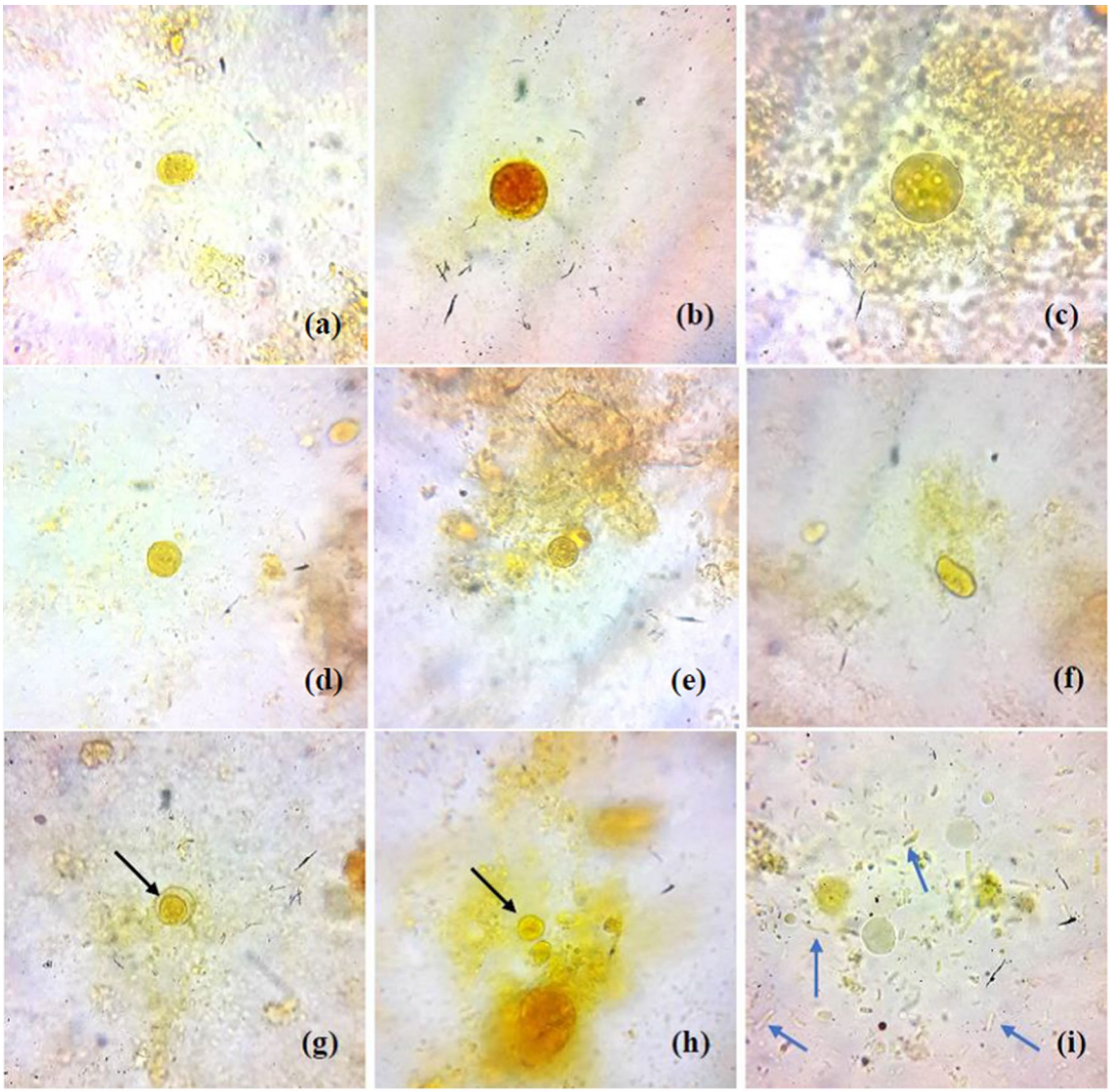

Fig. 2 Visualization of protists from DS microbiota of *N. cinerea* after staining with concentrated lugol solution: **a** *Endolimax nana* cyst; **b** *Entamoeba histolytica/dispar/moshkovskii* cyst; **c** *E. coli* cyst; **d** Probable juvenile *Endamoeba blattae* cyst; **e** *Entamoeba hartmanni* Cyst; **f** Probable *E. hartmanni* trophozoite; **g** *Cyclospora cayetanensis* non-sporulated oocyst (arrow); **h** *C. cayetanensis* free sporocyst (arrow); **i** Cylindrical *(Herpetomonas)* shaped kinetoplastids (arrows). In **a**–**i**: 1000 × magnification

Table 2 Semi-quantification of protists from SD microbiota of *N. cinerea* after staining with lugol

LUGOL	*Amoeba* spp.	*Eugregarinida* spp.	*Eucoccidiorida* spp.	*Herpetomonas* sp.	*B. nauphoetae*
Male (foregut)	+	+	0	0	0
Male (mindgut)	+ + +	+ + +	+ +	+	0
Male (hindgut)	+	+	+	+ + +	0
Female (foregut)	+	0	+	0	+
Female (mindgut)	+ + +	+ +	+	0	0
Female (hindgut)	+	+	+	+ + +	+ +

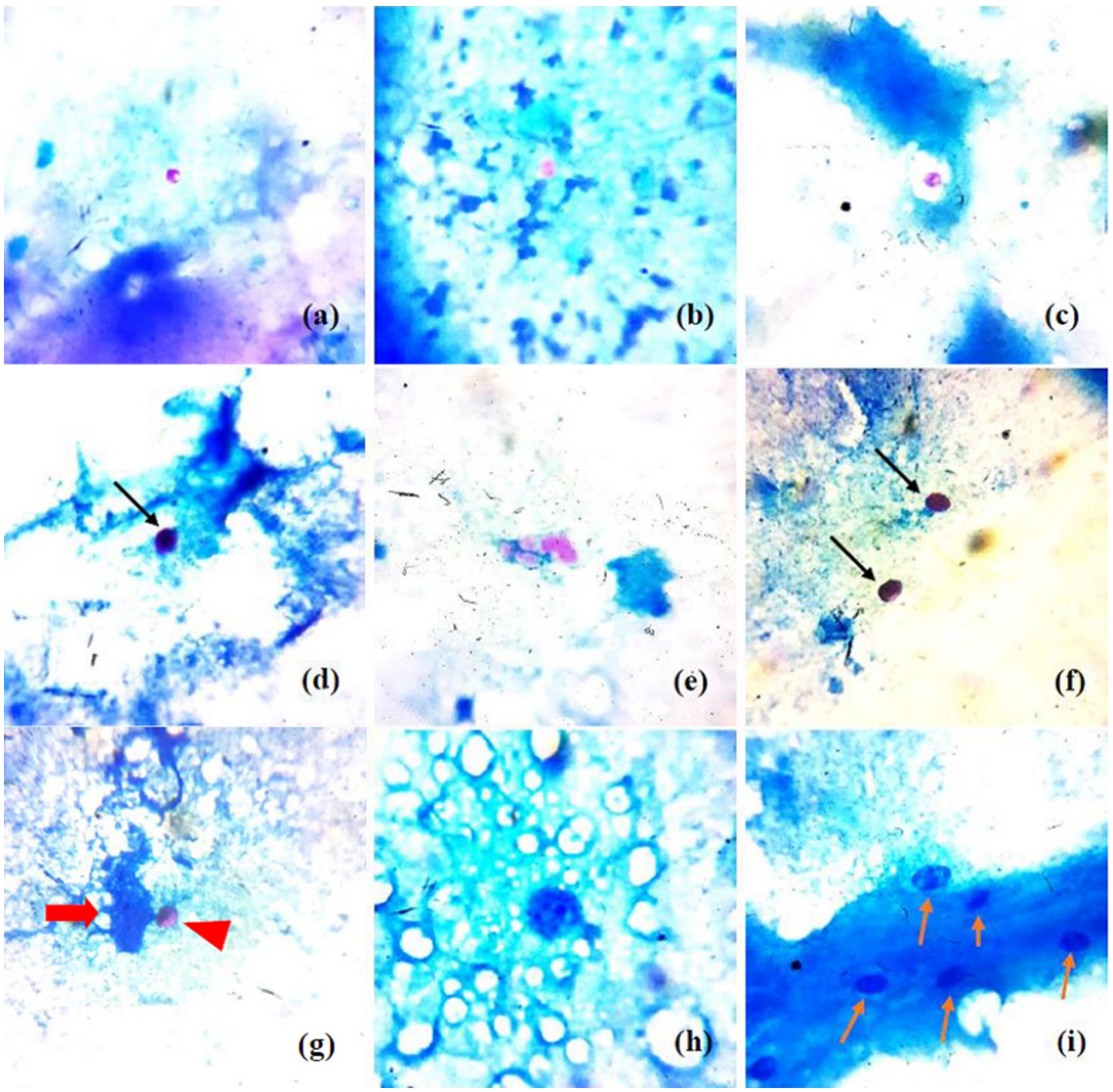

Fig. 3 Visualization of protists from SD microbiota of *N. cinerea* after acid-alcohol staining: **a** *Cryptosporidium* sp. oocyst; **b**, **c** Oocysts of *Cryptosporidium* sp. with colored sporozoites; **d** *Cyclospora cayetanensis* non-sporulated oocyst (arrow); **e** Sporulated oocyst of *C. cayetanensis*; **f** *C. cayetanensis* free sporocysts with two intensely stained sporozoites each (arrows); **g** *Entamoeba coli* trophozoite (arrow) and *C. cayetanensis* free sporocyst (arrowhead); **h** Probable *E. histolytica/dispar/moshkovskii* cyst; **i** Numerous *B. nauphoetae* (arrows). In **a**–**i**: 1000 × magnification

so they probably belong to the same species. Such structures were not observed in any of the other visualization methods applied despite their relatively high concentration. *Cyclospora cayetanensis* structures were also more easily visualized using the alcohol-acid coloration (in dark red), even allowing its identification at the species level through the morphological analysis of the sporozoites located inside the sporocysts (Fig. 3d–f).

In the case of *C. cayetanensis*, the observation of sporulated oocysts was much less frequent than that of other structures, with more free sporocysts being observed, indicating that this staining technique can damage mature oocysts. Cysts of amoebas and trophozoites of gregarines and amoebae were easily observed (Fig. 3 g, h), as well as cells of *B. nauphoetae* (in blue) (Fig. 3i).

With the Ziehl-Nielsen staining technique, the distribution status of the amoebae (Table 3) was again confirmed in the terminal portions of the DS, especially in the mindgut, and the same applies to the gregarines, where it was possible to observe a decrease in the frequency of visualization of these groups after staining. The highest prevalence of coccidia was indicated as being in hindgut by this method, differing from previous results (Tables 1 and 2). The distribution of *B. nauphoetae* and kinetoplastids remained characterized by the highest prevalence of specimens in the last portion of the DS.

Trichrome EA36

Staining with EA36 trichrome by the proposed method generated slides with a much cleaner visual background, which facilitated the identification of cellular structures, especially amoebas and gregarines, despite a noticeable loss of material (Fig. 4). Cytoplasm was stained dark pink/red, while chromatin and nuclei were stained light pink, where the former became lighter (Fig. 4a–h). The lipophilic regions, such as cell membranes, showed a greenish color, as can be seen in the *B. nauphoetae* of Fig. 4i. The nuclear size of *Entamoeba* spp. seems to have been reduced in the process (Fig. 4e, f). This method also allowed a better visualization of the coccidia, as can be seen in Fig. 4a, g.

In Table 4, the distribution of amoebas in the DS is indicated as being predominant in the final portion of the DS (hindgut), as well as that of the gregarines, which differs a little from the results obtained by the other stains. While the presence of kinetoplastids and diplomonadides is again confirmed as predominant in hindgut. It is also possible to observe a large reduction of specimens observed for all identified taxa caused by the staining process.

Dark Field Microscopy Simulated with Nankin Ink

With the application of nankin ink, the contrast of the images of Eugregarinida spp. was naturally increasing, facilitating the visualization of its internal and external morphological elements, so that it was possible to distinguish the probable presence of five species of gregarines: *Blabericola migrator* (Clopton, 1995) Clopton, 2009, *B. haasi* (Geus, 1969) Clopton, (2009), *Protomagalhaensia wolfi* (Geus, 1969) Hays and Clopton, 2006, *P. cerastes* Clopton, (2010) and *Gregarina steini* Berndt, (1902). The decreasing order of prevalence of these species in SD *N. cinerea* observed during the tests mentioned was: *Protomagalhaensia wolfi* < *P. cerastes* < *Gregarina steini* < *Blabericola haasi* < *B. migrator* (Fig. 5).

Discussion

As already reported in several other cockroach species, the different parts of the digestive system of *Nauphoeta cinerea* are inhabited by a surprising variety of taxa of endocommensal protozoa. Regarding the observation of different species of amoeba, surprisingly, the most efficient methods were unstained visualization and the Ziehl–Neelsen method (Figs. 1, 2, 3, 4 and Tables 1, 2, 3, 4). The findings described here are in agreement with results obtained by different prospecting methods carried out on different species of cockroaches. In the case of *Periplaneta amercana*, a deep phylogenetic evaluation of the populations of *Entamoeba* present in its DS has already been carried out, revealing the presence of numerous genetic variants of non-pathogenic species for humans, such as *Entamoeba coli* and *E. hartamanni*, as well

Table 3 Semi-quantification of protists from SD microbiota of *N. cinerea* after alcohol-acid staining

Ziehl-Nielsen	*Amoeba* spp.	*Eugregarinida* spp.	*Eucoccidiorida* spp.	*Herpetomonas* sp.	*B. nauphoetae*
Male (foregut)	+ +	+	+	+	0
Male (mindgut)	+ + +	+ +	+	+	+
Male (hindgut)	+ +	+ +	+ +	+ +	+ + +
Female (foregut)	+	0	+	0	0
Female (mindgut)	+ +	+ +	+ +	0	0
Female (hindgut)	+ +	+	+ + +	+ +	+ + +

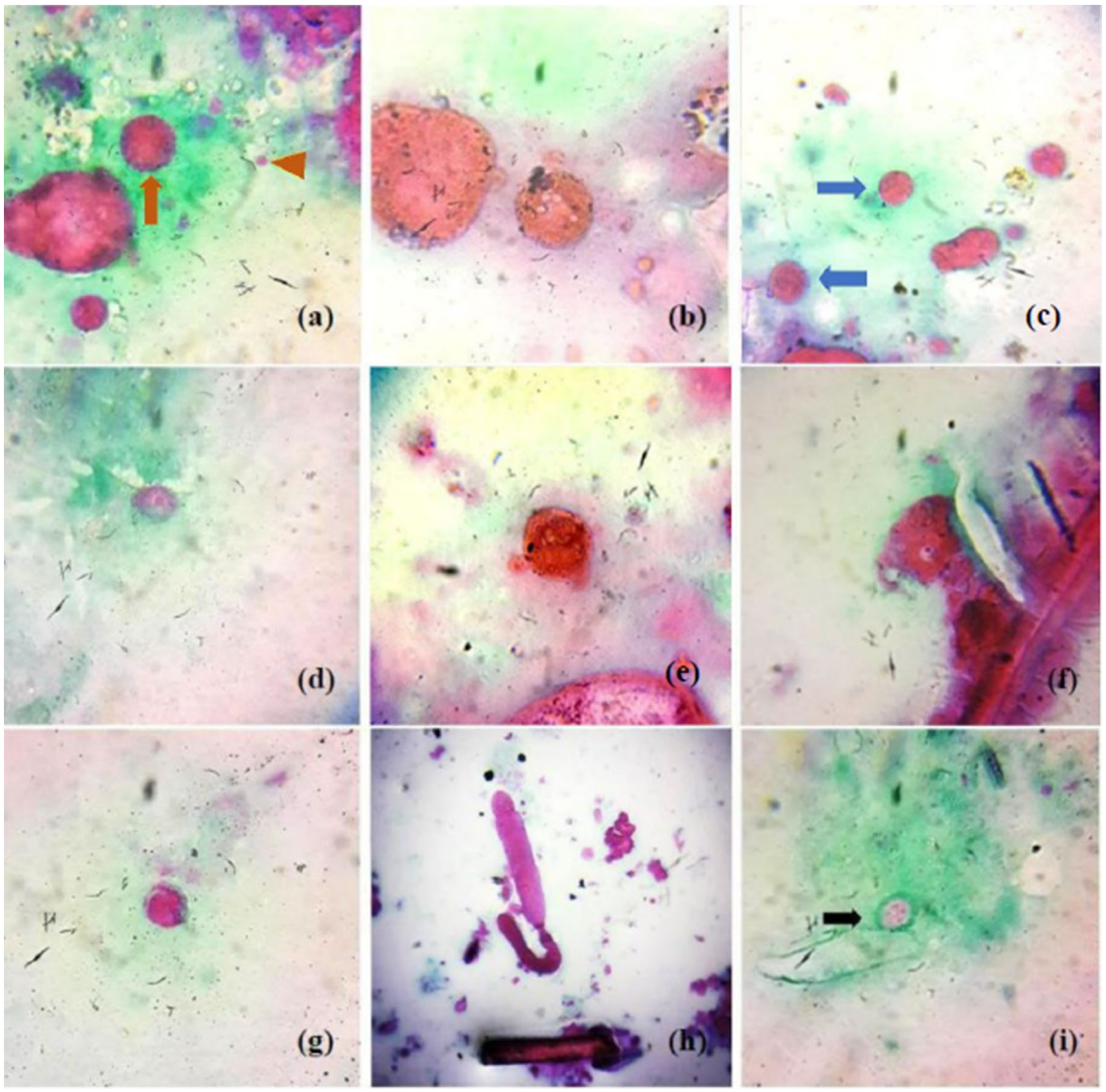

Fig. 4 Visualization of protists from SD microbiota of *N. cinerea* after staining with EA36 trichrome: **a** Immature cyst of *Entamoeba histolytica/dispar/moshkovskii* (arrow) and probable oocyst of *Cryptosporidium* sp. (arrowhead); **b** *E. coli* cyst; **c** *E. hartmanni* cysts (arrows); **d** Probable *Endamoeba blattae* precyst during encystment; **e** *E. histolytica/dispar/moshkovskii* trophozoite; **f** *E. coli* trophozoite; **g** *Cyclospora cayetanensis* sporulated oocyst; **h** Small caudal–frontal association (syzygy) morphotype of gregarine; **i** *B. nauphoetae* (arrow). In **a**–**g**, **i**: 1000 × magnification; in **h**: 100 × magnification

Table 4 Semi-quantification of protists from SD microbiota of *N. cinerea* after staining with EA36 trichrome

TRICHROME EA36	*Amoeba* spp.	*Eugregarinida* spp.	*Eucoccidiorida* spp.	*Herpetomonas* sp.	*B. nauphoetae*
Male (foregut)	0	+	+	0	0
Male (mindgut)	+	+ +	+ +	0	0
Male (hindgut)	+ +	+ + +	0	+ + +	+
Female (foregut)	0	+	0	0	0
Female (mindgut)	+	+ +	+	0	0
Female (hindgut)	+	+ +	+	+ +	+

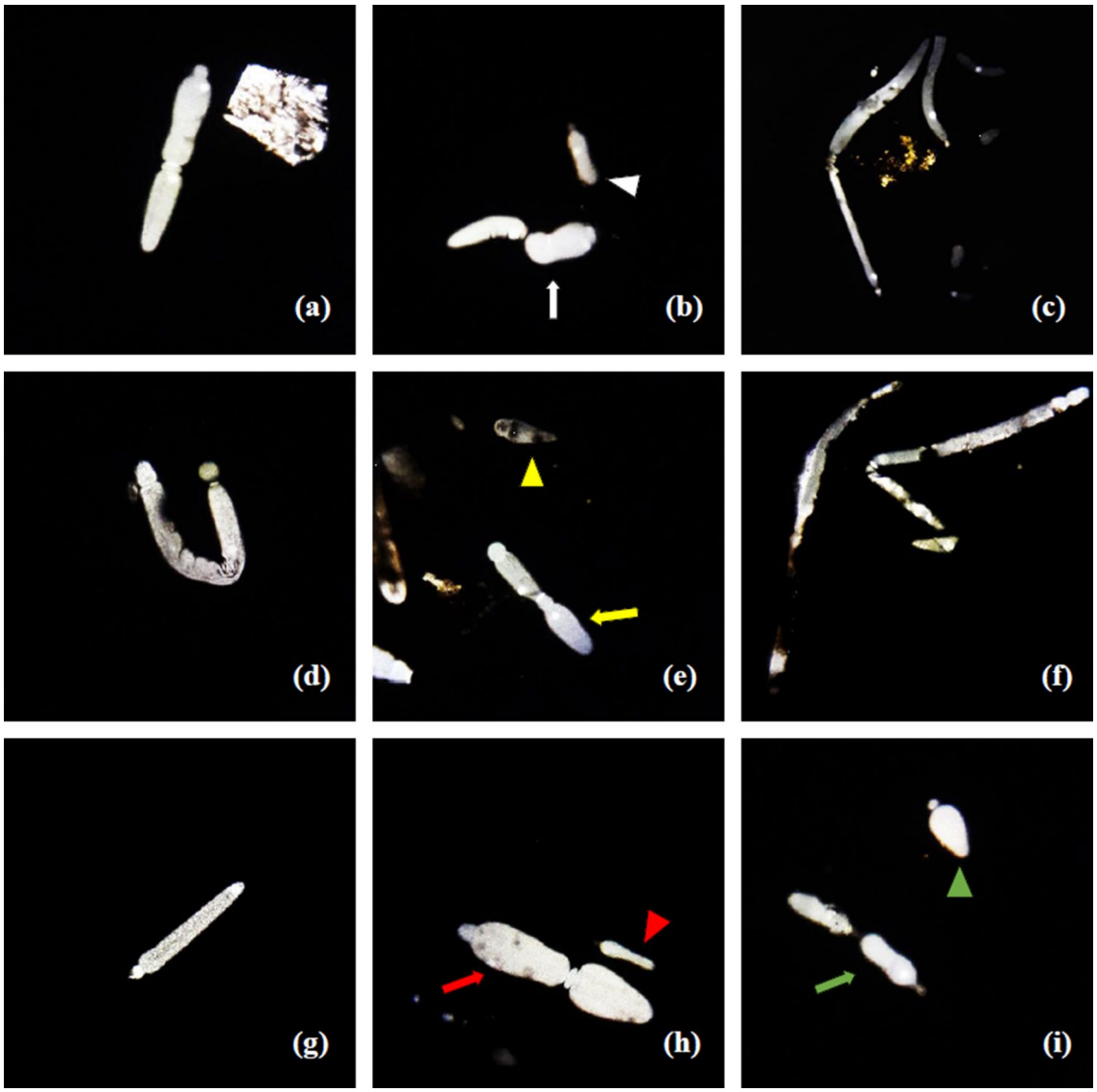

Fig. 5 Visualization of protists from SD microbiota of *N. cinerea* after nankin ink application: **a** *Blabericola migrator* tail–frontal association (syzygy); **b** Syzygy from *B. haasi* (arrow) and free trophozoite from *Protomagalhaensia cerastes* (arrowhead); **c** *P. wolfi* small syzygy; **d** *P. cerastes* syzygy primite; **e** Small syzygy morphotype of *Gregarina steini* (arrow) and free trophozoite of the same species (arrowhead); **f** Syzygy from *P. wolfi*; **g** *P. wolfi* free trophozoite; **h** Syzygy from *B. migrator* (arrow) and young free trophozoite from *P. cerastes* (arrowhead); **i** Syzygy from *B. haasi* (arrow) and free trophozoite from *B. migrator* (arrowhead). In **a**–**i**: 100 × magnification

as pathogenic species such as *E. moshkovskii* and *E. histolytica* [28]. Previous studies have already corroborated the presence of *E. coli* and *E. histolytica* along the DS of *P. americana* in different parts of the world [16, 29].

The species *Entamoeba histolytica* is known to be the main cause of intestinal amoebiasis in humans, often causing diarrhea and lesions in the intestinal epithelium, which can also infect primates and occurs mainly in developing countries, such as Tanzania, where it was recently responsible for infecting thousands of individuals and hundreds of non-human primates [30, 31]. In asymptomatic cases of *E. histolytica* infection, visible ulcer lesions and/or erosions are limited around the cecum, whereas, conversely, in symptomatic invasive patients, lesions may be identified at multiple sites along the colon by endoscopy [32].

Regarding the different species of gregarins efficiently identified with nankin ink in the SD of *Nauphoeta cinerea* here (Figs. 1e,f, 4 and Tables 1, 2, 3, 4), these are similar to what was observed by Clopton and Hays [33], who described *Protomagalhensis wolfi* in specimens from *N. cinerea*, found extensively here in the last two portions of the DS. Likewise, *Blabericola haasi* was described from the DS content of *N. cinerea* specimens. The other gregarine species identified were only previously found in different species of

cockroaches such as: *P. cerastes*, found in *Phoetalia pallida* Brunner von Wattenwyl, (1865) and *Blabericola migrator* found in *Gromphadorhina portentosa* [34, 35]. No group of gregarins described so far has been characterized as a vertebrate parasite.

The presence of *Cryptosporidium* sp. in the DS of *N. cinerea* (Figs. 3, 4 and Tables 1, 2, 3, 4) evidenced exceptionally well by the Ziehl–Neelsen stain, is a finding of relative epidemiological importance, as this parasite is responsible for large-scale epidemic outbreaks, where it has been shown that around 52% of the outbreaks in England are caused by *C. parvum*, with the predominance of the IIaA15G2R1 subtype, which presents a morphotype very similar to the specimens observed here: spherical and less than 5 µm in length [36, 37]. Furthermore, findings of *C. parvum* in specimens of *P. americana* in a house where there was a serious case of infection by this protozoan, so that other possible sources of contamination (water and other domestic insects) were ruled out, also support our identification [38]. *Cryptosporidium* spp. usually occur in rural areas and are likely to have an underestimated prevalence, with watery diarrhea as the main symptom [39].

Similar to the case of *Cryptosporidium* sp., the presence of *Cyclospora cayetanensis* (Figs. 1, 2, 3, 4 and Tables 1, 2, 3, 4) also well demonstrated by the Ziehl–Neelsen stain, is a significant finding from an epidemiological perspective, as it is known that they are responsible for rapidly progressing outbreaks, especially among populations of immunocompromised individuals, whose most recurrent symptom is diarrhea [40, 41]. Recently, multiple cases of *C. cayetanensis* infection have been documented in the United States, characterized by the symptoms: watery diarrhea, loss of appetite, weight loss, cramps, abdominal distension, increased gas, nausea and fatigue [42].

In non-human primates, it has been shown that infection by *C. cayetanensis* can cause serious histopathological changes in the stomach, intestines, lymph nodes and liver, where the latter usually presents congestion of blood vessels, reactive mitotic activity in crypts, parasites in enterocytes and intense lymphocytic infiltration [43]. Similarly, a case study demonstrated that in humans there are also areas of erythema over the descending colon with normal terminal ileum and in other parts of the colon, as well as numerous intracellular protozoa in the apical half of the enterocytes of the terminal ileum and duodenum, followed by blunting of the villi with infiltration of nonspecific inflammatory cells from the terminal ileum is also being observed [44].

Endocommensals (non-pathogenic) of the genus *Herpetomonas* were well identified here especially by lugol staining (Fig. 2 and Table 2). They are predominantly associated with Diptera species such as *Herpetomonas isaaci* Teixeira & Camargo, (2013) and *Herpetomonas puellarum* Teixeira & Camargo, (2013), however, *Herpetomonas tarakana* Kostygov *et al.*, (2015) has been described from different species of cockroaches [45, 46]. Specimens of the genus *B. nauphoetae* are commonly found parasitizing the intestines of fish and even as planktonic forms in phytotelmata [47, 48]. Here, specimens of *Blattamonas nauphoetae* were well evidenced by the Ziehl–Neelsen stain (Fig. 3 and Table 3). B. nauphoetae was recently described from *N. cinerea* isolates, being the only metamonad found in this organism and living as a commensal [49].

Conclusion

In the present work, the great biological diversity that exists in the microbiota of the digestive system of *Nauphoeta cinerea* was demonstrated. It was possible to find several species of amoebas, coccidia, gregarines, kinetoplastids and diplomonadides, where many of them have the potential to cause diseases in humans, such as is the case for *Entamoeba histolytica/dispar/moshkovskii*, *Cryptosporidium* sp. and *Cyclospora cayetanensis*, therefore, requiring some care in the handling of specimens of *N. cinerea*. There is still a lot to know about the interactions between endocommensal protozoa and their respective invertebrate hosts, so that the best way to clarify such relationships is through molecular and genetic tests, enabling a better phylogenetic identification of the protist species.

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Declarations

Conflict of Interest The authors have nothing to declare.

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