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The Fungal Microbiota of the Digestive Tract of the Chagas Disease Vectors *Triatoma infestans* Klug, 1834 and *Panstrongylus megistus* Burmeister, 1835

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AMLM and ACVJ designed the entire experiment. Author ACVJ conducted all fieldwork. Authors ACVJ and LCS processed the triatomines. Authors MFG, CAS and ISS processed all fungal samples. Authors ISS and AMLM identified the fungal strains. Authors AMLM, ACVJ and ISS reviewed and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Chagas is a neglected disease, one of Brazil's main medical and social problems and a serious public health problem in the Americas, with more recent occurrences in non-endemic countries outside of the Americas. Research into the microbiota of triatomines is relevant because of its potential role in vector competence and as a proposed biological control strategy. Stressing a possible insect-fungal interaction in the development of *Trypanosoma cruzi*, and considering the lack of studies on the subject, we analyzed the fungal microbiota of the digestive tract of two species considered important vectors of *Trypanosoma cruzi: Triatoma infestans* and *Panstrongylus megistus*. Specimens were dissected, digestive tracts macerated and contents serially diluted.

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Each aliquot was seeded in three culture media. The plates were incubated in type B.O.D. climate chambers for 21 days, after which isolated colonies were morphological characterized and identified. There have been few published studies on the fungal microbiota of the triatomine digestive tract. Comparing the results found here with existing data reveals that the genera *Aspergillus* and *Penicillium* are commonly found in the digestive tract of the studied triatomines. Among the several genera identified, the species found in the highest percentages were *Aspergillus flavus, Paecilomyces variotii, Penicillium waksmanii, Penicillium raistrickii* and *Penicillium fellutanum*. Quantitative differences in the number of isolated fungal strains were observed according to sex and nymphal stage of the vector. The present findings corroborate those found in the literature, showing that there is a natural fungal microbiota in triatomines. Data revealing quantitative differences in isolated fungal strains found in male, female and nymphs reinforce the idea that their presence is related to physiology and fasting resistance. The secondary metabolite-producing fungi isolated in this work have in their biology great potential to be tested with regard to the establishment of *T. cruzi* in the digestive tract of its vector.

Keywords: Microbiota; fungal; chagas disease; fungus; vector; Trypanosoma cruzi.

ABBREVIATIONS

B.O.D : Biochemical Oxygen Demand
IOC : Oswaldo Cruz Institute
FIOCRUZ : Oswaldo Cruz Foundation
PDA : Potato Dextrose Agar
MEYA : Malt Extract, Yeast Extract and Agar
YEPGA : Yeast Extract, Peptone, Glucose and Agar
BSL-2 : Biosafety Level 2

1. INTRODUCTION

Chagas disease is one of the neglected tropical diseases listed by the World Health Organization [1]. It constitutes a major public health problem in Latin America, with economic and social impacts, while cases have recently been reported outside the Americas [2,1,3,4,5]. About 6–7 million people worldwide, mainly in Latin America, are infected with *T. cruzi* [1], while estimates for Brazil in 2014 ranged from 1.9 to 4.6 million people [6]. Recent data (2012 and 2016) include 19,914 cases of acute. Chagas disease in Brazil, with 1,190 (5.9%) being confirmed [7].

American trypanosomiasis is a parasitic infection caused by the protozoan *Trypanosoma cruzi* Chagas, 1909 [8]. Its vectors are triatomines (Hemiptera: Reduviidae), hematophagous insects in all stages of development, mainly of the genera *Triatoma, Rhodnius* and *Panstrongylus* [9]. *Triatoma infestans* Klug, 1834 is a domiciliary species distributed in Argentina, Bolivia, Brazil, Chile, Ecuador, Paraguay, Peru and Uruguay. *Panstrongylus megistus* Burmeister, 1835 is considered an important vector in Brazil because, in addition to being susceptible to *Trypanosoma cruzi* infection [10], it has a broad geographic distribution and is found in wild, domiciliary and peridomiciliary environments.

The main strategy for vector control has been through the application of insecticides [11,12]. Over the years, however, insecticide resistance has developed among vectors, making control difficult, a fact linked to genetic variability and selective pressure [13]. This resistance occurs with other animal, including human, disease vector insects, such as those of *Culicidae* and *Phlebotominae* (*Sandflies*) [14,15]. This has led to new studies for vector control, among which is biological control through microbiota research.

Studies of the microbiota of triatomines began with Duncan [16], who isolated Gram-positive bacteria from the gut of the kissing bug *Rhodnius prolixus*. *Rhodococcus rhodnii* (*Actinobacteria*) has been isolated from *R. prolixus* [17]; this bacterium provides vitamin B, which is necessary for the development of the insect [18,19]. From these studies, others on the intestinal microbiota of triatomines using bacteria have gained relevance. Analyses have shown that microbiota diversity and symbiotic relationships facilitate host nutrition and defense, and that genetically modified bacteria can be used to inhibit insect or parasite development. Other studies with *R. prolixus* have shown that symbiotic bacteria cause damage to *Trypanosoma cruzi* and produce molecules that can inhibit the growth of other microorganisms [20,21,22,23,24,25].

The presence of different fungi in the digestive tract of triatomines has been demonstrated [26,27,28,29]. Nonetheless, there is a lack of

studies on fungal microbiota, with studies in the literature coming from only two research groups: Moraes et al. in Brazil and Marti et al. in Argentina [26,27,30,31,28,29].

Stressing a possible insect-fungal interaction in the development of *Trypanosoma cruzi*, this study aimed to isolate and identify filamentous fungi from the digestive tract of *Triatoma infestans* and *Panstrongylus megistus.* The study also aimed to infer a pattern of fungal species for each vector by comparing with previous studies and observing any changes in this fungal population.

2. MATERIALS AND METHODS

Two species of triatomines —*T. infestans* and *P. megistus*— that are among the main vector species of *T. cruz* were analyzed. They were reared in a colony maintained at Laboratory of Parasitic Diseases of Oswaldo Cruz Institute/ Oswaldo Cruz Foundation -IOC/Fiocruz. Digestive tracts of 19 males, 19 females and 15 nymphs of each developmental stage of the two studied species were used.

At Laboratory of Taxonomy, Biochemistry and Bioprospecting Fungi of Oswaldo Cruz Institute/ Oswaldo Cruz Foundation -IOC/Fiocruz, the adult and nymph specimens were transferred to a laminar flow chamber, sacrificed with chloroform and washed for sterilization of external contaminants.

Wash was performed by emerging specimens in 1% sodium hypochlorite for two minutes and then washing in sterile distilled water, iodized alcohol and finally 70% alcohol, after which they were immediately dried with sterile gauze. After this procedure, the insects were dissected [32]. Digestive tracts were removed with forceps and macerated with a drop of 0.85% saline. From 150 to 200 microliters of this macerate underwent serial dilutions, in which 0.2 ml of each dilution was seeded in Petri dishes. Each dish contained the following culture media plus chloramphenicol: potato dextrose agar (PDA); malt extract, yeast extract and agar (MEYE); and yeast extract, peptone, glucose and agar (YEPGA). All procedures were performed in a BSL-2 biological safety booth. After seeding, the dishes were incubated in type B.O.D. (Biochemical Oxygen Demand) climate chambers at a temperature of 25°C [30,31,28].

To monitor fungal growth, dishes were examined daily up to 10 days from the first day of seeding to count fungal colonies. After 10 days, the plates were examined every three days until 21 days. Colonies were transferred into test tubes containing PDA and malt extract media for 10 for isolation.

Identification to genus followed Barnett and Hunter [33], de Hoog et al. [34,35] and McGinnis [36]. For species identification, microscopic characteristics were evaluated using the microculture technique [37]; the material was stained with lactophenol blue-cotton and observed under a Zeiss Axiophot optical microscope. Macroscopic characterization of the fungal strains was done through the inoculum point technique; after the proposed incubation period for each genus, the diameters of the colonies were measured with a Mitutoyo digital caliper with 0.01mm resolution. Species were identified following the classifications of De Hoog et al. [35], Gerlach and Nirenberg [38], Klich [39], Pitt [40], Raper and Fennell [41] Rifai [42], Samson [43] and Visagie et al. [44]. The isolated strains were preserved under mineral oil.

3. RESULTS AND DISCUSSION

A total of 177 fungal strains were isolated from the digestive tracts of males and females and from the different nymphal stages of *T. infestans* and *P. megistus.* For *T. infestans,* 97 strains were isolated, 13 from female, 53 from males and 31 from nymphs, distributed among five genera: *Penicillium* (43), *Aspergillus* (40)*, Paecilomyces* (12), *Trichoderma* (1) and *Purpureocillium* (1). For *P. megistus,* 80 strains were isolated*,* 11 in females, 25 in males and 44 in nymphs, distributed among five genera: *Penicillium* (48), *Aspergillus* (22)*, Paecilomyces* (5), *Fusarium* (4) and *Purpureocillium* (1).

Comparative analysis of the diversity of identified fungal species from different stages of *T. infestans* and *P. megistus* revealed that *Penicillium waksmanii* Zalessky, 1927 was predominant in females and males of *P. megistus* and in females of *T. infestans,* together with *Penicillium raistrickii* Smith, 1933*. Penicillium raistrickii* was the dominant species for nymphs of *P. megistus,* while *Aspergillus flavus* Link, 1809 was the most isolated fungus species for males and nymphs of *T. infestans.* Comparing the two species of triatomines, *T. infestans* had a higher number of isolated fungal strains and one less species (16) identified than *P. megitus* (17), which was the most diverse triatomine species even though it had the lowest number of isolates (Fig. 1).

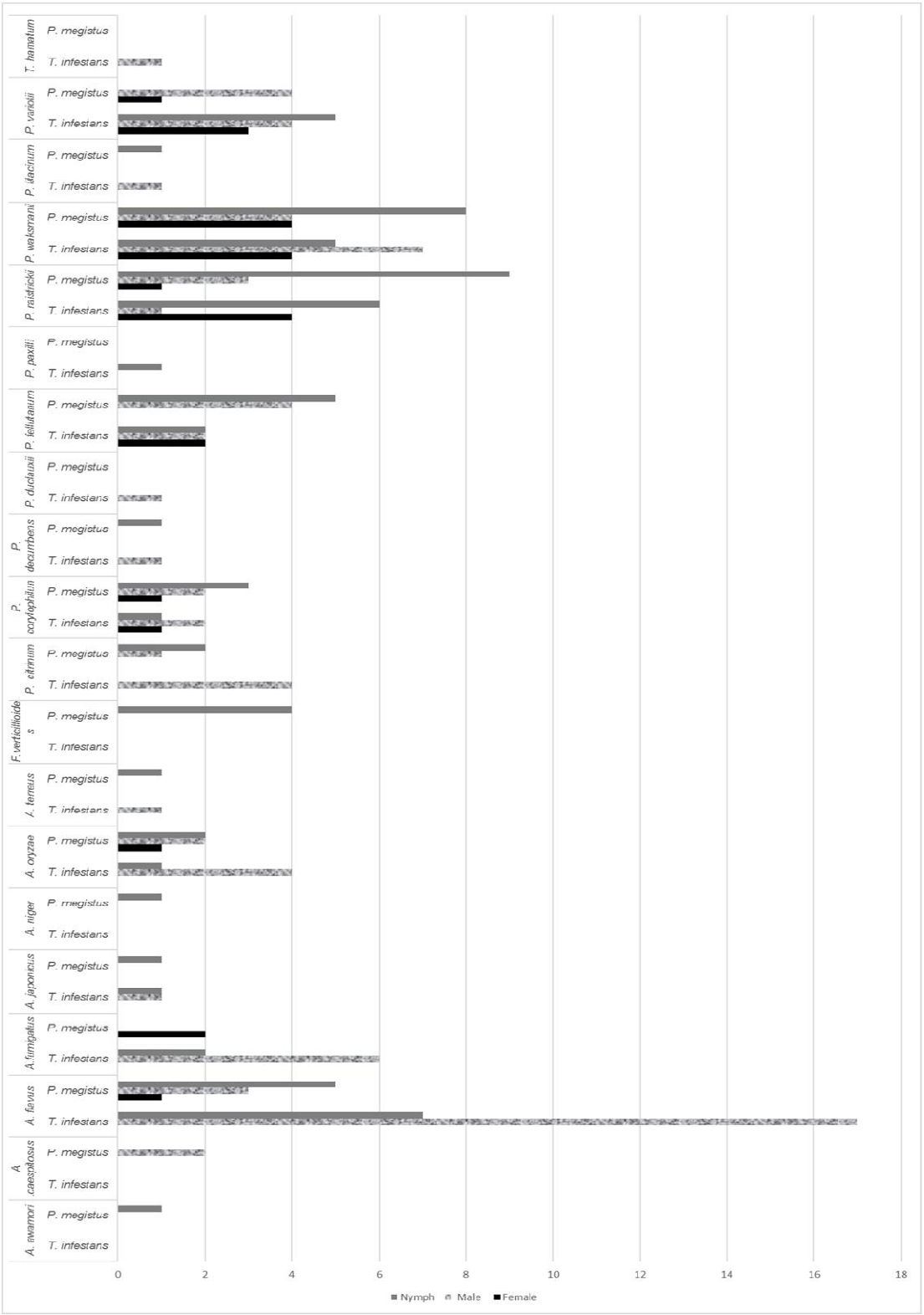

Fig. 1. Comparison of the diversity of fungal microbiota isolated from different stages of *T. infestans* and *P. megistus*, according to sex and nymphal stage of the vector

Different species of filamentous fungi were isolated from the digestive tract of adults and nymphs of *T. infestans* and *P. megistus*, all widely found in nature on different substrates. The genera *Aspergillus* and *Penicillium* were more prevalent, regardless of the genus or growth stage of the triatomine species evaluated. Studies have related these genera of fungi to some arthropods, such as Coleoptera: Scolytidae [45,46,47]; Hymenoptera: Apidae [48]; Diptera: Culicidae [49]; and Diptera: Muscidae [50]. Although there is very little work on the intestinal microbiota of triatomines, the presence of these genera has been previously reported, as well as that of others isolated in the present work, such as *Fusarium, Trichoderma* and *Paecilomyces* found in the digestive tract microbiota of the order Coleoptera [46,26,27,30,31,29].

Among the species isolated in the present study, A. flavus, *P. waksmanii, Penicillium corylophilum* Dierckx, 1901*, P. raistrickii, Penicillium fellutanum* Biourge, 1923 and *Paecilomyces variotii* Bainier, 1907 stand out because they were isolated in practically all developmental stages of both triatomines, and were related to the microbiota of triatomines in the literature, confirming the present findings [30,31,28,29]. Based on this, these fungal species can be confirmed to be part of the natural microbiota of *P. megistus* and *T. infestans*, raised in a colony. Among the most represented genera are the strains *Aspergillus niger* and *P. corylophilum*, which Moraes et al. [28] reported as being part of the natural microbiota of *P. megitus, Rhodnius prolixus, R. neglectus* and *Diptelagaster maximus*. Another study by Moraes et al. [31] showed that *A. niger, P. corylophilum* and a species of the genus Acremonium were absent from the digestive tract of nymphs of *P. megistus* infected with *T. cruzi*. Based on this observation, it was proposed that *A. niger* and *P. corylophilum* could be used as a barrier to protozoan colonization.

Marti et al. [29] studied the microbiota of female and male *T. infestans* raised in the laboratory and identified seven species: *Alternaria alternata, Aspergillus fumigatus, Cladosporium cladosporioides, Cladosporium sphaerospemum, Mucor hiemalls, Penicillium* sp. and *Phoma glomerate*. This differed from the present study, which showed greater fungal diversity with 15 identified fungal species. The only species found by both Marti et al. [29] and the present study were *A. fumigatus* and *Penicillium* sp., but the genera *Cladosporium* and *Alternaria* have also been isolated from triatomines by a previous study [31], reinforcing the idea that these fungal species are part of the microbiota of triatomines.

On the influence of the microbiota present in vectors, studies have been developed involving bacteria, such as Azambuja et al. [21], for example, who evidenced the presumed inhibitory action of microbiota on the life cycle of *T. cruzi*. Studies with *R. prolixus* have shown that bacteria can influence the development of *T. cruzi* by causing damage such as protozoan lysis [20,21,51,23,22]. There is also an inverse relationship in which *T. cruzi* may reduce the quantity of bacteria in the digestive tract of triatomines [52], which has also even been shown with the fungal microbiota of triatomines [30].

The influence of the microbiota present in vectors was analyzed by searching out studies on fungal influence on protozoan colonization. Schlein et al. [53] studied *Phlebotomus* and observed that insects with fungi in the digestive tract did not contain any type of protozoan. Moraes et al. [30] used xenodiagnoses with *P. megistus* and observed a reduction in the fungal population of digestive tracts that were positive for *T. cruzi*. Based on the studies of Schlein, Moraes *et al.* hypothesized that the presence of these fungi in the intestinal tract of triatomines has an influence on *T. cruzi*, since there were qualitative and quantitative differences in the species of the digestive tract of *T. cruzi* negative nymphs, which led to considering the possibility that fungi may be inhibitors of parasite growth.

Investigations with fungi have also shown action against protozoans, such as *T. cruzi* and *Leishamnia infatuam,* through their secondary metabolites. The first report of the action of the fungus genus *Pleurotus* was from Ramos-Ligonio et al. [54], who demonstrated the interaction of ergosterol peroxide in the membrane of *T. cruzi.* Other studies with the species *Pleurotus salmoneostramineus* extracted ergosterol, which had action against amastigote forms of *L. infatum* and trypomastigote forms of *T. cruzi.* This action was through permeabilization of the plasma membrane as well as depolarization of the mitochondrial membrane, leading to parasite death [55].

Similarly, the effect was verified with *Bipolaris sp.* by testing a crude extract against parasites and demonstrating biological activity on trypanothione reductase. This enzyme is involved in the

maintenance of intracellular homeostasis in trypanosomatids (*Leishmania* and *Trypanosoma*), and is essential for their survival [56].

From the above reports found in the literature, the antiparasitic potential of fungi against *T. cruzi* should be highlighted, be it by metabolites or crude extracts. The genera *Aspergillus* and *Penicillium*, which were the most isolated genera in the present study, also have species that produce secondary metabolites, among which are antibiotics, enzymes and mycotoxins [57]. Studies have shown the action of secondary metabolites, such as *Penicillium* sp., which can produce dictyosphaeric acids A and B that have action against *Staphylococcus aureus,, S. faecium* and *Candida albicans* [58]; A*spergillus* sp., which has species that produce phenylamides B−D and the cyclic pentapeptide asperpeptide, which have antimicrobial action [59]; *Fusarium sp.*, which produces fusaranthraquinone, fusarnaphthoquinone (A, B and C) and fusarone, which have antibacterial and antimalarial action [60]; and *Alternaria sp.* which produces altenusin that acts against *Trypanosoma, Leishmania* and *Paracoccidioides brasiliensi* [61]. This led us to think that metabolites secreted by the species identified in the present study may have antiparasitic action against this parasite since several isolated species are metabolite producers.

The physiology of triatomines may favor fungal colonization, which may be directly associate with the species richness and diversity found in the different phases and stages of development of triatomines. Triatomine digestion is slow, a period called fasting resistance, which varies from species to species and among individuals within the same species [62]. This long digestion period facilitates the adherence of conidia, and during this process the wall of the stomach undergoes plastinization, causing hydration of the area and making the environment conducive to the development of fungi [63,62]. Males and 5^{th}-stage nymphs have greater fasting resistance, whereas females have more sensitivity to lack of food, which may be related to higher nutrient requirements for egg maturation and fecundity [62,64,65,32]. This contrast was observed in the present study with females having fewer isolated fungal strains, which leads to the presumption that this may be related to their lesser fasting resistance. Males had a greater number of isolates in both triatomine species, probably due to their higher resistance to fasting. The number of isolates in nymphs varied, which was expected between the species, but there were more identified fungal strains than for females.

4. CONCLUSION

The present study confirmed some species as belonging to the natural triatomine fungal microbiota and showed that the number of isolated fungal strains varies among females, males and nymphs, which may be related to fasting resistance. Studies show that metabolites produced by fungi influence protozoans, so the secondary metabolite-producing fungi isolated in this work have in their biology great potential to be tested, with direct implications for biological control.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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