Intestinal Microbiota – A Modulator of the *Trypanosoma cruzi*-Vector-Host Triad

Isabella Teotônio[1], Nayra Dias[1], Luciana Hagström-Bex[1], Nadjar Nitz[1], Amanda Fortes Francisco[2] and Mariana Hecht[1*]

[1] Interdisciplinary Laboratory of Biosciences, Faculty of Medicine, University of Brasilia, Brasilia, Federal District, Brazil.

[2] Department of Pathogen Molecular Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London, United Kingdom.

*Correspondence:
Mrs. Isabella Márcia Soares Nogueira Teotônio
isabella.msn.rocha@gmail.com

Abstract. Chagas disease affects millions of people, and it is a major cause of death in Latin America. Prevention and development of an effective treatment for this infection can be favored by a more thorough understanding of *T. cruzi* interaction with the microbiome of vectors and hosts. Next-generation sequencing technology vastly broadened the knowledge about intestinal bacteria composition, showing that microbiota within each host (triatomines and mammals) is composed by high diversity of species, although few dominant phyla. This fact may represent an ecological balance that was acquired during the evolutionary process of the microbiome-host complex, and that serves to perpetuate this system. In this context, commensal microbiota is also essential to protect hosts, conferring them resistance to pathogens colonization. However, in some situations, the microbiota is not able to prevent infection but only modulate it. Here we will review the role of the microbiota on the parasite-vector-host triad with a focus on the kinetoplastida of medical importance *Trypanosoma cruzi*. Novel strategies to control Chagas disease based on intestinal microbiome will also be discussed.

Keywords: Intestinal microbiota, *Trypanosoma cruzi*, Vector, Host, Chagas disease.

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1. Introduction

The intestinal ecosystem is an environment in which biological and biochemical interactions occur at various hierarchical levels, connecting microbial communities and their hosts. [1, 2] Studies of fecal samples revealed that the microbiota from a wholesome intestine is an intricated ecological community composed of trillions of

microorganisms, from viruses to unicellular eukaryotes.[3] However, in this article, we will use the term microbiota to refer only to the population of bacteria of an organism.

The intestinal microbiota is highly dynamic, it varies over time and is modulated by environmental conditions (use of antibiotics, lifestyle, diet and hygiene preferences, metabolic dysfunction, immunodeficiency and hyper immunity).[4]The application of new high-performance methodologies for analysis of bacterial species, such as the new generation sequencing (NGS) of 16S rRNA, revolutionized the knowledge about the intestinal microbiome. [5] It is now known that about 1000 bacterial species inhabit the human adult intestine; however, the predominant genera are *Lactobacillus, Bifidobacterium, Bacteroides, Eubacterium, Clostridium, Ruminococcus, Peptostreptococcus, and Peptococcus.*[5] Despite the large number of distinct species, they belong to a relatively small number of phyla, especially Bacteroidetes and Firmicutes. [6]

In healthy hosts, the presence of this microbiota contributes to the prevention of pathogen colonization.[7] Additionally, it has an important impact on various aspects of the hosts physiology and metabolism; such as, protection of intestinal epithelium, digestion of host nutrients, production of vitamins and hormones, and regulation of immune responses, modulating the expression of immunological mediators and the recruitment of certain cell populations.[8, 9]

Changes in microbiota composition usually have a direct effect on parasitic infection, in part because parasites and bacteria metabolize substrates interactively and secrete products that affect each other, interfering with the survival and physiology of both. [10]Likewise, the microbial community constitution is an extremely important factor for host immune responses: imbalance between the microbiota and the immune

system may alter the host's homeostasis and lead to greater disease susceptibility, and therefore dictate the success of the intestinal pathogens.

Published data demonstrate that the intestinal microbiota usually has a deep influence on the parasite-host relationship.[11] It is well known that intestinal microbiota composition is determinant for some parasites pathogenicity, as described for *Entamoeba histolytica*,[12] *Trichuris* muris,[13] *Schistosoma mansoni*,[14] *Eimeria falciformis*,[15] *Eimeria ovinoidalis*, [16] *Ascaris lumbricoides*,[17] and *Giardia lamblia*. [18]

On the other hand, this microbiota can reduce the damages of other infectious agents, such as *Cryptococcus neoformans*,[19] *Strongyloides venezuelensis*, [20] and almost all enteropathogenic bacteria (*Clostridium difficile, Clostridium perfringens, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Shigella xexneri* and *Vibrio cholerae*). [21, 22, 23] In few reported cases - *Raillietina cesticillus*,[24]]*Isopora* suis,[25]and *Trichuris trichiura* [17]- the microbiota composition appear not to influence the outcome of the disease.

T. cruzi is the etiological agent of Chagas disease, the most important parasitic disease in the Americas, affecting approximately 6 to 8 million people and causing around 12,000 deaths per year. [26]Little is known about the modulation of *T. cruzi* infections by the intestinal microbiota, in insects or vertebrate hosts. Approximately 30% of infected individuals will develop cardiac, digestive or neurological changes during the chronic phase. Chagas disease pathogenesis has not been fully elucidated, and different theories try to explain it, such as parasite persistence and autoimmunity.[27]This fact contributes to the difficulty in developing an effective treatment. In this review, we will summarize the current knowledge on microbiome of

T. cruzi invertebrate and vertebrate hosts, highlighting new approaches and research gaps in this field (Figure 1).

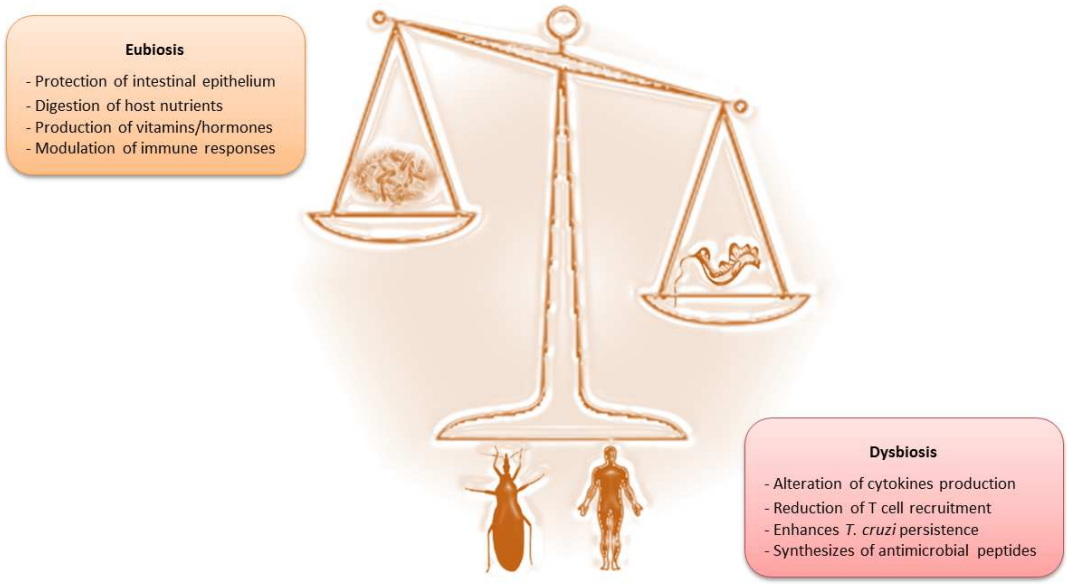

Figure 1. Multi-effects of the intestinal microbiota on the vector-parasite-host triad. In healthy, non-infected, vectors and hosts, the resident microbiota will play an important role in the maintenance of homeostasis (eubiosis). During *T. cruzi* infection, the parasite and bacteria metabolize substrates interactively and secrete products that affect each other and interfere in the survival and physiology of the host (dysbiosis).

2. Gut microbiota in parasite-vector interface

Hemiptera insects began to inhabit our planet about 400 million years ago, being favored by the emergence of vascular plants, whose phloem served as their food source. Throughout the evolutionary process, adaptations of the oral apparatus of these arthropods allowed the acquisition of new feeding habits, such as hematophagy. [28, 29]Triatomines (Hemiptera: Reduviidae), popularly known as kissing bugs, are life-long obligatorily hematophagous arthropods which feed on various animals, mainly mammals. During hematophagy, several microorganisms can reach triatomines alimentary tract and begin its colonization.

In recent years, triatomines microbiota has been evaluated by NGS, showing that the ecological diversity of its microbiome is low but dynamic, changing according to genera and gender, development stages origin, and blood sources.[30, 31, 32] The assessment of *T. brasiliensis* and *T. pseudomaculata* microbiome by denaturing gradient gel bands sequencing revealed their microbiota was mostly composed by Proteo- and Actinobacteria; being *Serratia* the predominant genus. [33]Analyzes of the 16S rRNA gene of the intestinal microbiota of *Triatoma maculata* and *Rhodnius pallescens* captured in the same locality of Colombia, showed the distinct composition of bacteria community. In *R. pallescens*, *Williamsia* and *Kocuria* (orders Corynebacteriales and Actinomycetales, respectively) were the most prevalent genera, while in *T. maculate*, *Dietzia*, *Aeromonas* and *Pelomonas* (orders Actinomycetales, Aeromonadales and Burkholderiales, respectively) were predominant.[30]Another study confirmed that 70% of *Triatoma diminiata* microbiome was composed by bacteria from orders Bacillales, Actinomycetales, Enterobacteriales and Burkholderiales. However, the predominating bacteria in bugs fed on dogs was Burkholderiales, in those fed on humans was Bacillales, and for those fed on porcupine was Enterobacteriales. [31]Interestingly, Rodríguez-Ruano et al., [34] showed that the microbiome composition is particularly determined by host species, receiving less influence of locality and environment.

Following a blood meal, kissing bugs can also ingest the protozoa *T. cruzi*. Once inside the insect gut, *T. cruzi* have to invade surrounding tissues of the vector and transform to epimastigote forms and later, in infective metacyclic forms, which are eliminated with excreta and can achieve the host bloodstream through the bite site. During this journey, *T. cruzi* and the resident microbiota maintain an intimate interaction looking for a balance for the establishment of both.

Independently of gut microbiota composition, most of *T. cruzi* is destructed in the first hours of vector infection. [35]After that, parasite-microbiota interaction is essential to control *T. cruzi* amount. *In vitro* experiments showed that bacterial clusters can adhere to *T. cruzi* surface through D-mannose recognizing fimbriae and lead to parasite lysis. [35]Furthermore, a control of parasite replication is also orchestrated by the local bacteria.[36]Thus, to provide continuity to its life cycle in the digestive tract of triatomines and increase their chances of reaching a new host, *T. cruzi* needs to overcome the microbiota trypanolytic activity. The interaction between parasite and microbiota could vary among different vectors. As an example, *T. cruzi* Dm28c strain when stimulated induce antibacterial activities in *Rhodnius prolixus*, resulting in fewer bacteria and higher parasitemia. However, the *T. cruzi* Y strain is not able to produce the same effects, being inefficient in the establishment of the infection in the vector.[37]

Vectors infected with *T. cruzi* synthesize antimicrobial peptides, such as defensins and prolixicin, to control the expansion of the new invader, in a strain-dependent manner. [38]These bioactive molecules may also affect the resident microbiota richness,[34]and consequently, benefit or impair parasite survival. For example, the use of a selective inhibitor of NF-κb in *R. prolixus* modulated the gene expression of defensins, increasing the microbiota and reducing *T. cruzi* population. [39]Furthermore, the knockdown of the antimicrobial product from *Triatoma infestans* midgut (TiAP) increased by 600 times the amount of gut bacteria and, consequently, reduced the number of *T. cruzi* epimastigotes.[40]So, TiAP controls microbiota growth, contributing to *T.* cruzi establishment in the vector. Similarly, a Kazal-type inhibitor from the midgut of *R. prolixus* (RpTI) is involved in microbiota regulation and its silencing with RNA interference technology resulted in higher bacterial loads.[41]In contrast, Díaz et al.[42]reported that triatomines challenged with *T. cruzi* have their

microbiome altered in a species-specific manner; harboring a more diverse bacterial community than the negative controls. The significance of this increase in diversity must be better investigated.

3. Gastrointestinal microbiota in the parasite-mammal interface

Novel bioluminescence imaging systems have evidenced the persistence of *T. cruzi* infection in the GIT (gastrointestinal tract) during the acute and chronic Chagas disease.[43, 44]The persistence of *T. cruzi* in the gut could contribute to the development of GIT disorders, notably megacolon and/or megaesophagus, resulting in altered peristaltic movements, dysphagia and pain. It is believed that the chronic gastrointestinal symptoms of Chagas disease are a consequence of the destruction of the myenteric neurons by the parasite. [45]Furthermore, continuous migration of *T. cruzi* from the GIT to other organs such as the heart has been suggested, indicating that the intermittent traffic of parasites can be involved in chronic Chagas cardiomyopathy.[44, 46]

In the gut, *T. cruzi* may interact with thousands of commensal bacteria, but little is known about this ecological relationship. Apparently, an indirect contact should occur between parasite-bacteria, since *T. cruzi* is preferentially found in the muscularis externae of GIT.[47]The impact of this protozoa on microbiota profile and metabolome were characterized in an immunocompetent murine model, [48]in which *T. cruzi* disrupted fecal microbiome and caused biochemical alterations in a synchronized manner. For example, variations in linoleic acid metabolism could be observed. [48] Linoleic acid metabolism has been associated with an important immune-modulating response, affecting T cell recruitment and cytokines production in the colon, [49]which could favor *T. cruzi* persistence.

Researches on germ-free mice infected with T. *cruzi* have been performed to characterize immunoregulation and clinical evolution of Chagas disease in this experimental model. Silva et al., [50]showed that the lack of the natural microbiome negatively influenced parasitemia intensity, mortality rate, spleen size, and cardiac damage. However, the same findings were not obtained by Duarte et al.,[51]in whose study the infection outcome did not alter significantly between control and germ-free mice, despite a higher production of inflammatory cytokines in the first group.

The role of specific species of bacteria on Chagas disease immunomodulation was also evaluated in germ-free mice.[52]Mono-association of *T. cruzi* with *E. coli, E. faecalis, B. vulgatus* or *Peptostreptococcus* sp produced a Th1 immune response, higher levels of IgGs and increased survival rate. Interestingly, these tested bacteria are predominant in the indigenous microbiota, but there is no evidence that this population group is more resistant to the development of Chagas disease clinical manifestations.[53]In this respect, characterization of the microbiome in coprolites and colon of a chagasic pre-Columbian Andean mummy revealed that paleofeces were constituted predominantly by Firmicutes, with *Clostridium* spp. and *Turicibacter* spp. representing the most abundant bacterial genera. [54]

Since gut microbiome depends on intestinal health, it is expected its impairment during Chagas disease, regardless of ancestry. Quantitative and qualitative analysis of the microbiota in chagasic megaesophagus and health esophagus showed a more elevated bacterial concentration and variability in chagasic patients, with a predominance of the aerobic gram-positive bacteria *Streptococcus* sp and the anaerobic *Veillonella*. [55]In the proximal jejunum of patients with megacolon, it was observed an overgrowth of facultative and strict anaerobes microorganisms, which returns to normality after surgical treatment.[56]

Dysbiosis in Chagas disease may also be associated with the emergence of secondary diseases. The proliferation of certain bacteria in the esophageal lumen can cause pulmonary infections, dysplasia of the esophageal mucosa and cancer.[55]Individuals with a more advanced stage of esophageal dilation have elevated concentrations of *Staphylococcus* sp, *Corynebacterium* sp, *Peptostreptococcus* sp and *Veillonella* sp, bacteria that are capable of reducing nitrate into nitrites, which have been associated with the formation of proven esophageal carcinogens nitrosamines. [57, 58]

4. Novel approaches based on intestinal microbiota to control chagas disease

In triatomines, obligate bacterial symbionts are essential to obtain some nutrients from the blood-diet, without which several aspects of insect physiology would be compromised, notably its development. [59, 60]It is noteworthy to note that the availability of nutrients affects the vector, the *T. cruzi* population density and the number of metacyclic tripomastigotes in the rectum. [61] Therefore, bacterial communities in the insect gut are essential for *T. cruzi* survival.[62]Interestingly, new methodologies are being developed to facilitate the characterization of triatomines gut ecosystem: RADseq-based analysis was used to disclose mixed DNA from vectors abdomens, enabling the determination of *T. cruzi* DTUs, microbial diversity, and blood meal source. [63]

In this sense, it is quite plausible to think about novel strategies of *T. cruzi* transmission blocking and vector control based on its microbiota (Figure 2), since the traditional strategies seem to be ineffective, such as the use of insecticides. [64]Studies employing antibiotic treatment, specific antibodies or rearing gnotobiotic lines has brought important information about the role of intestinal bacteria on parasites.[65]Triatomine engineering aiming antimicrobial peptides reduction results in

increased bacterial load in the midgut and decreased *T. cruzi* parasitemia, influencing vector competence. [40] It is noted that production of genetically-modified vectors that interferes in microbial colonization is an advantageous strategy because it can be applied to all species of triatomines and impairs *T. cruzi* survival. Intestinal microbiota can also be modified by RNA interference-based technologies to control vectors: *E. coli* expressing specific dsRNAs for *Rhodnius prolixus* heme-binding protein and catalase affected mortality, molting and oviposition rates.[66]Other examples of promising alternatives to control vector infection are the use of bacteria with trypanocidal activity, such as *Serratia*, a commensal of triatomine guts that deregulates *T. cruzi* mitochondrial activity.[67] and the treatment of *R. prolixus* with physalin B, a natural secsteroid that promotes an increase in gut bacterial microbiota and significantly decreases the number of *T. cruzi*. [68]

In mammals, commensal microbes interact with parasites that cohabitate and change the progression of the infection. Recent discoveries, [44; 43]show the intestine as a preferential site of *T. cruzi*, where local bacteria can act directly on the parasite and determine its infectivity. Furthermore, infection can also be modulated at distance. In both cases, the mutualism developed between parasites and microbiota seems to be associated with subclinical manifestations. [69]Therefore, the administration of prebiotics and probiotics to replace the resident microbiota can be promising, since the newly introduced bacteria will compete with the parasites for nutrients and space as well as stimulate the host's immune system to react against infection.[70]Identification of which prebiotics/probiotics can boost protective immune responses can contribute to the success of future treatments. In this respect, oral and intraperitoneal inoculation of *L. casei* in NIH mice resulted in reduction of circulating parasites.[71]

Associated to this, specific diets may contribute to the growth of the microbiota species of interest that diminish the virulence and survival of the parasites.[69]High fat diet and protein deficiency seems to increase parasitemia and leucocyte infiltration in cardiac tissue. [72, 73] Such aspects become more evident when analyzed in germ-free mice. Cintra et al. [74]showed that protein deficiency resulted in a more severe Chagas disease in germ-free mice than the controls. Santos et al. [75]observed that the effect of a deficient fatty acid diet on a germ-free *T. cruzi*-infected model resulted in a larger amount of tripomastogotes per ml of blood and a lower survival rate.

New insights about which mechanisms are involved in parasite-microbiota interaction are also needed. For example, the role of inflammasome should be better elucidated, since its activation controls microbial dysbiosis, protecting the organisms from autoinflammatory responses. However, parasites can reduce inflammasome activation, promoting dysbiosis.[76]

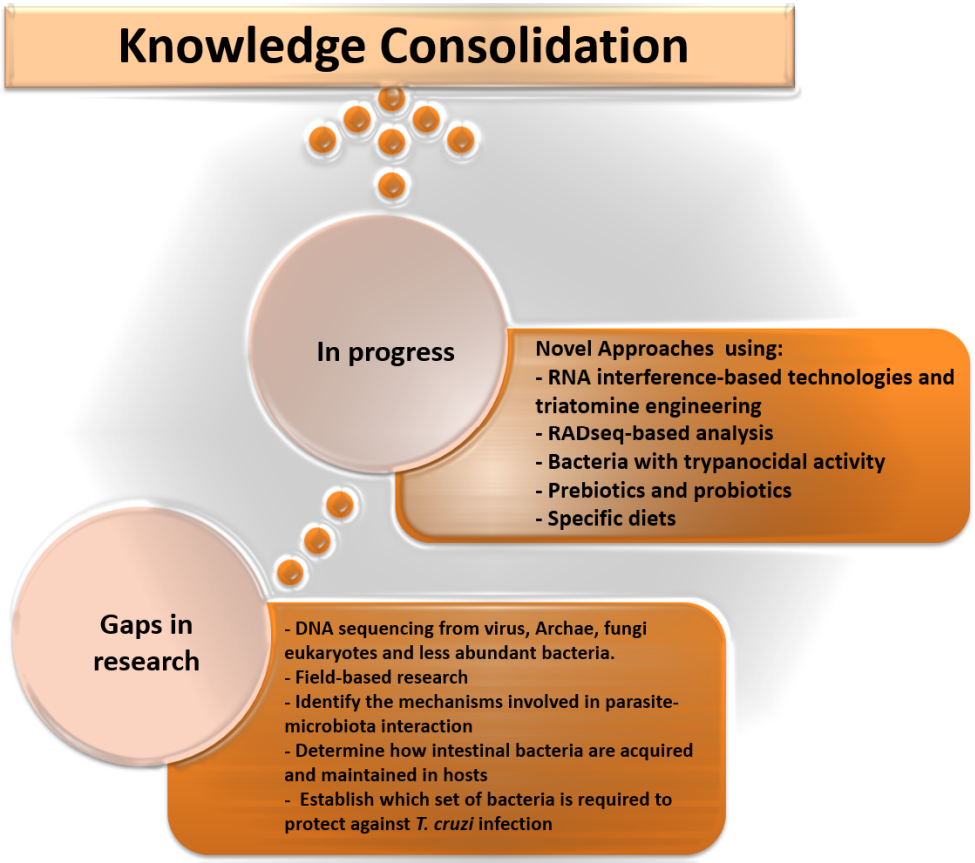

Figure 2. Challenges to consolidate the knowledge about the role of the intestinal microbiota on the vector-parasite-host triad. Gaps in research need to be fulfilled to determine the real importance of the intestinal microbiota on *T. cruzi* infection. Novel approaches are essential to elucidate crucial issues.

5. Conclusions

Reports on parasites and microbiota interaction have become extremely common because of next-generation sequencing technology. However, a bias may have been created because of the possibility of lack of DNA sequencing from less abundant, but of pathological importance, bacteria populations. Furthermore, expanding knowledge about Archae diversity [77]and its interaction with the microbiota can evidence new aspects of the complex GIT ecosystem. Also, the inclusion of virus, fungi and, eukaryotes should be considered in the next studies.

Importantly, some results may be valid for certain ecological conditions, but not to others. So, field-based research can bring to light information that could not be

obtained in controlled lab-models. Another research line that should be further explored in order to address how intestinal bacteria are acquired and maintained in hosts and which combination of bacteria could be required to protect against *T. cruzi* infection. [78]Understanding the mechanisms that interfere in infection progression is essential. Experiments with *T.* cruzi infected animals treated with antibiotics and recolonized with specific bacteria can provide important information of how these microorganisms modulate the infection. Gene exchange among microbiome-parasite-hosts is a possibility that should be considered in this intimate relationship.

Disclosure

There is no conflict of interest to be discussed.

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Highlights

- Intestinal microbiota has a deep influence on the parasite-host relationship
- In triatomines, gut microbiota can benefit or impair *T. cruzi* survival
- In mammals, *T.* cruzi-associated dysbiosis affects immune responses
- Novel approaches based on gut microbiota can be proposed to control Chagas disease