Tripartite interactions: *Leishmania*, microbiota and *Lutzomyia longipalpis*

Thais Bonifácio Campolina, Luis Eduardo Martinez Villegas, Carolina Cunha Monteiro, Paulo Filemon Paolucci Pimenta, Nagila Francinete Costa Secundino*

Laboratory of Medical Entomology, René Rachou Institute–FIOCRUZ, Minas Gerais, Brazil

*secundinon@gmail.com

Abstract

The microbial consortium associated with sandflies has gained relevance, with its composition shifting throughout distinct developmental stages, being strongly influenced by the surroundings and food sources. The bacterial components of the microbiota can interfere with *Leishmania* development inside the sandfly vector. Microbiota diversity and host-microbiota-pathogen interactions regarding New World sandfly species have yet to be thoroughly studied, particularly in *Lutzomyia longipalpis*, the primary vector of visceral leishmaniasis in Brazil. The native microbiota of different developmental stages and physiological conditions of *L. longipalpis* (Lapinha Cave), was described by culturing and 16s rRNA gene sequencing. The 16s rRNA sequencing of culture-dependent revealed 13 distinct bacterial genera (*Bacillus*, *Enterococcus*, *Erwinia*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Lysinibacillus*, *Pseudocitrobacter*, *Providencia*, *Pseudomonas*, *Serratia*, *Staphylococcus* and *Solibacillus*). The *in vitro* and *in vivo* effects of each one of the 13 native bacteria from the *L. longipalpis* were analyzed by co-cultivation with promastigotes of *L. infantum chagasi*, *L. major*, *L. amazonensis* and *L. braziliensis*. After 24 h of co-cultivation, a growth reduction observed in all parasite species. When the parasites were co-cultivated with *Lysinibacillus*, all parasites of *L. infantum chagasi* and *L. amazonensis* died within 24 hours. In the *in vivo* co-infection of *L. chagasi*, *L. major* and *L. amazonensis* with the genera *Lysinibacillus*, *Pseudocitrobacter* and *Serratia* it was possible to observe a significant difference between the groups co-infected with the bacterial genera and the control group. These findings suggest that symbiotic bacteria (*Lysinibacillus*, *Serratia*, and *Pseudocitrobacter*) are potential candidates for paratransgenic or biological control. Further studies are needed to identify the nature of the effector molecules involved in reducing the vector competence for *Leishmania*.

Author summary

According to the World Health Organization Leishmaniasis is the second parasitic disease that kills the most in the world; the first is malaria. Despite this, knowledge about the *Leishmania* parasite and its interaction with vertebrate hosts concerning the transmitting insect is still relatively fewer and fragmented. Studies on insects microbiota have great potential to control Leishmania.
importance to obtain basic information. How a vector responds to the presence of different microorganisms and how they interact with various pathogens and may lead to the development of new strategies or tools that can be used to prevent or hinder the transmission of the protozoan by the vector insect. Considering the knowledge about the intestinal microbiota of sandflies, we aim to study the effect of bacterial isolates on *Lu. longipalpis* infection by different species of *Leishmania*, and it believed that these bacteria might influence the development of *Leishmania*, preventing, and hindering transmission, contributing to Leishmaniasis control strategies.

**Introduction**

In the last decade, microbial communities associated with sandflies gained relevance, as it has observed to play an essential role in *Leishmania* development within the host’s digestive tract [1]. The sandfly microbiota is a dynamic community mostly acquired from their environment as in other Insecta or Diptera. Its composition shifts throughout its distinct developmental stages, being strongly influenced by the surroundings and food sources crossed during their life cycle [2,3,4]. For example, sandflies acquire bacterial symbiont as immature stages by feeding upon the organic matter from the humid soil in which they develop. From this larval food, some symbionts remain prevalent as members of the adult’s microbiota, suggesting a transstadiual transmission after the pupation process [2,3]. In addition to niche-acquired symbionts, feeding sources may modify adult microbiota diversity. Both females and males feed on plant sap and honeydew aphids.

However, only females feed on blood, which can acquire from a variety of vertebrate hosts, and which required for egg development [4]. Microorganisms like bacteria, fungi, and protozoan parasites ingested during blood feeding can become established within the autochthonous community and modulate insect vectorial capacity [4–7].

The ingested blood meal is digested and processed inside the midgut, where distinct parasites, like *Leishmania*, develop to be transmitted by the vector. However, after the infective blood-meal, the parasite has to survive multiple host barriers. These may impact a mature infection since it affects the resistance to digestive enzymes, colonization of midgut, parasite differentiation, including metacyclogenesis [8,9]. The host barriers involving interactions between the microbial communities and *Leishmania* during its life cycle within the insect remain unclear.

Some studies have demonstrated the microbiota bacterial components interfering with *Leishmania* development inside the sandfly vector. For instance, *Serratia marcescens*, a well-known pathogenic bacteria for many insects [10], negatively interacted with *Leishmania infantum chagasi* and *Leishmania braziliensis*, inducing lysis of the parasite cell membrane [11,12]. An in vivo study revealed reduced infection rates of *Leishmania mexicana* in *Lutzomyia longipalpis* sandflies previously fed on a microbial suspension of *Pseudozyma* sp., *Asaia* sp. or *Ochrobactrum* *intermedium* [5]. The same study showed a pre-infected sandfly challenged with a high dose (5x10⁷ CFU/ml) of *S. marcescens* survived longer compared to control (uninfected but challenged with bacteria). These results suggest that *Leishmania* directly protects the sandfly from the bacterial infection, or modulates its effect by priming the host immune response, as observed in other models like *Anopheles gambiae* infected with *Plasmodium* [13].

Also, Hanssan et al. [3] showed that *Phlebotomus papatasi*, after antibiotic treatment became more susceptible to *Leishmania major* infection compared with the untreated control, suggesting that resistance to *Leishmania* infection was due to the presence of symbionts.
By contrast to the studies showing an adverse effect of the microbiota on *Leishmania* development, recent results suggest that the native microbiota is essential for *Leishmania* development and survival, with antibiotic treatment of sandflies inhibiting parasite growth and differentiation into the infectious metacyclic form [1,14]. In-depth knowledge of the underlying dynamics between bacteria and a host is needed to identify candidate bacteria that can be used in paratransgenic studies, or other biological approaches, aiming to control sandfly populations and *Leishmania* transmission [15].

In this context, the microbiota diversity and the host-microbiota-pathogen interactions regarding New World sandfly species have yet to be thoroughly studied, with only a few reported studies from Brazil, one from Argentina and one from Colombia [2,14,16–21].

In this report, we describe the culture-dependent native microbiota associated with *Lu. longipalpis*, a vector of visceral leishmaniasis. We assessed whether co-culturing members of associated microbiota and *Leishmania* would inhibit parasite growth rates *in vitro* and *in vivo*.

**Materials and methods**

**Ethics statement**

This study was conducted by the Oswaldo Cruz Foundation’s Handbook for Animal Use (Ministry of Health of Brazil. It approved by The Animal Use Ethics Committee, Oswaldo Cruz Foundation (number LW-17/15).

**Sandflies**

Wild-caught *Lu. longipalpis* were collected with CDC traps [22] at Lapinha Cave located in the city of Lagoa Santa, 60 km from Belo Horizonte (longitude 43˚57’W; latitude 19˚03’S), state of Minas Gerais, Brazil, a non-endemic region for transmission of Visceral Leishmaniasis. The adults collected with a CDC trap [22]. These traps placed in the early afternoon and removed in the following day morning, over four years during the spring to the summer season, and an average of 800 sandflies per collection. Immediately after the field trip, the insects collected kept at the insectary of the Laboratory of Medical Entomology of the Rene ´ Rachou Institute (IRR) with constant temperature and humidity (25˚C and 75% humidity). The flies were morphologically identified, according to Young and Duncan [23], and processed following midgut contents [16]. A sub-sample of *Lu. longipalpis* females scored as blood-fed were allowed to lay eggs. Larva from hatched eggs was maintained as described by Secundino & Pimenta, [24]. Immature stages (parental line), larvae, and pupae used for posterior analyses.

For *in vivo* experiments, flies were collected in the field separated under a scope, and the non-fed females maintained with a filtered 10% sucrose *ad libitum* at the insectary.

**Specimen preparation**

Samples previously immobilized at -20 for few seconds and their surfaces sterilized by one-minute submersion in 1% hypochlorite, 15 to 30 seconds in 70% ethanol, and then rinsed three times with PBS [25]. After that, immature forms (larvae and pupae) and adults (midguts) with specific midgut contents: i) UF (fed on sucrose, non-blood-fed); ii) BF (Uninfected blood-fed); iii) GR (Gravid, empty midguts without blood and with developed ovaries), iv) P/S treated (sandflies pre-treated with Pen Strep for three days) and vi) PI (post coinfection with *L. i. chagasi* and bacterial isolates) identified and processed. Also, the internal controls added (insect carcass imprint, media alone). All the samples were pooled in groups of 15 (individuals) for culture-dependent bacterial profiling, in a minimum of 3 replicates each group.
Bacterial diversity profiling using a culture-dependent technique

Bacterial members of the microbiota within each pool were isolated as follows: samples were homogenized following [26], in tubes containing 200 μL of brain-heart infusions broth (BHI) (Sigma, Missouri, United States), a non-selective medium, to promote the growth of an ample range of bacteria. The homogenates (100 μL) were then pour-plated in the BHI agar medium at 27˚C for 48 hours. The observed colonies, differentiated by color and morphologic characteristics, were subjected to three passages in agar medium. The pure colonies expanded in the liquid medium, and each of the isolates differentiated by Gram staining and taxonomical profile. Finally, after the in vitro assays, the bacteria were re-plated and re-sequenced to ensure that the bacterial genera remained the same.

16S rRNA oriented profiling

The bacterial gDNA extraction was performed with DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The genomic material was quantified, and its purity assessed using the NanoDrop Spectrophotometer (Thermo Fisher Scientific). Bacterial gDNA from each sample served as a template for a PCR using Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, United Kingdom) and primers 16S ribosomal RNA 27 sense 5'-AGAGTTTGATCCTGGCTCAG-3', and 1492 antisense 5'-TACGGTCTACCTTTGTACGACTT-3'.

Amplification conditions were a 96˚C hold for 2 min, 30 cycles of 95˚C for 1 min, 50˚C for 1 min, and 72˚C for 3 min, followed by 5 min on 72˚C. Amplified products were visualized on a 1% agarose (Fisher Bioreagents, New Hampshire, United States) gel and cleaned using Wizard SV Gel and PCR Clean-up System (Promega, Wisconsin, United States). Twenty ng of the purified PCR product was sequenced using the above-described PCR primers and the Dye-Namic ET Terminator on a DNA Sequencer—ABI 3730 (Life Technologies, California, United States). Sequences were aligned, merged into contigs, and trimmed using Sequencer software (version 5.4.6). Resulting sequences were analyzed searching for similarity, using the sequence analysis tool RDP (Ribosomal Database Project—Update 5) and against the NR database (Non-redundant, NCBI database) using BLASTN algorithm with default parameters [27]. The best BLAST hit was selected, considering a 97% identity threshold. Taxonomic profiling of bacterial communities using 16S rRNA sequences as a target has some well-known limitations, including database bias [e.g., due to a small amount of sandfly associated curated sequences deposited]. The differences in each 16S rRNA variable regions exhibit when resolving taxonomic levels [28,29], and even cross-kingdom amplification [30] amongst other issues [31,32]. We cautiously chose to report taxonomic identifications at the genus level only.

Parasites

*L. i. chagasi* (MHOM/ BR/1970/BH46), *L. amazonensis* (IFLA/BR/67/PH8), *L. braziliensis* (MHOM/BR/75/M2903) and *L. major* (MHOM/IL/80/FN). Promastigotes were cultured in Medium 199 (Sigma, Missouri, United States) supplemented with 10% fetal bovine serum (Cultilab, São Paulo, Brazil), and other components: penicillin (100 U/ml) (Gibco, California, United States), streptomycin (50 mg/ml) (Gibco, California, United States), Hepes (40 mM) (Sigma, Missouri, United States), adenine (0.1 mM) (Sigma, Missouri, United States) and hemin (2.5 mg/ml) (Sigma, Missouri, United States) at 26˚C [33].

In vitro Leishmania co-culture

Parasites were washed twice in sterile Phosphate-buffered saline (PBS), counted, and re-suspended in fresh DMEM (Dulbecco’s Modified Eagle Medium) (Sigma, Missouri, United
States). Afterward, mixed with native bacteria previously isolated from sandflies, (as described below) and both (parasites and bacteria) were co-cultured in DMEM. The number of bacteria was estimated following the colony-forming units (CFU) counting technique, using serial dilutions in Petri dish with BHI medium. The $10^{-8}$ dilution it was possible to count 300 CFU that used in all experiments. i) Isolate assays performed using $4 \times 10^6$ parasites and $10^8$ bacteria / mL. Each parasite strain (listed above) and a single bacterial isolate (Tables 1 and 2) were incubated at 25°C, 0hr to 120 hours. ii) Supernatant assay, the bacterium culture was grown for 16 hours in the BHI medium; after that, centrifuged for 10 minutes at 15000xg, and only the supernatant (100μl) used for the co-culture experiments. The samples were co-cultured (parasites and supernatant) at 25°C through 0hr to 120 hours [11,12]. In the end, the number of alive promastigotes estimated by counting in a hemocytometer. After in vitro assays, the bacteria were re-plated and re-sequenced to ensure that the bacterial genera remained the same.

Table 1. Bacterial genera isolated from different developmental stages and physiological conditions of *Lu. Longipalpis*.

| Genus /Gram | *Lu. longiplapis* | UF | BF | GR |
|-------------|-------------------|----|----|----|
| *Bacillus* (+) | Larvae | X | | |
| *Enterobacter* (-) | Pupae | X | | |
| *Enterococcus* (+) | | X | | |
| *Escherichia* (-) | | | | |
| *Providencea* (-) | | | | |
| *Lysinibacillus* (+) | | X | | |
| *Pseudomonas* (-) | | X | | |
| *Serratia* (-) | | | | |
| *Staphylococcus* (+) | | | | |

(+ ) Gram-positive and (-) Gram-negative; UF = Non-blood-fed; BF = Uninfected blood-fed; GR = Gravid.

https://doi.org/10.1371/journal.pntd.0008666.t001

Table 2. Bacterial genera isolated from *Lu. Longipalpis* after treatment with PenStrep and post-infection with *L.i. chagasi*.

| Genus /Gram | *Lu. longiplapis* | UF | PS TREATED | PI |
|-------------|-------------------|----|------------|----|
| *Bacillus* (+) | | X | | |
| *Enterobacter* (-) | | X | X | X |
| *Enterococcus* (+) | | X | | |
| *Erwinia* (-) | | | X | |
| *Escherichia* (-) | | | | X |
| *Klebsiella* (-) | | | | |
| *Lysinibacillus* (+) | | | X | |
| *Providencia* (-) | | | | |
| *Pseudocitrobacter* (-) | | | | |
| *Serratia* (-) | | | X | |
| *Solbacillus* (+) | | | | |
| *Staphylococcus* (+) | | | | X |

(+ ) Gram-positive and (-) Gram-negative; UF = Non-blood-fed; PS TREATED = Sandflies pre-treated with Pen Strep; PI = Post infection.

https://doi.org/10.1371/journal.pntd.0008666.t002
In vivo—sandfly infection

Groups of 150 flies previously treated with 50 U/ml penicillin plus 50μg/ml streptomycin (daily changed) in the sugar meal three days before the infective meal [1, 34]. After that, flies fed through a chick skin membrane apparatus filled with heparinized mouse blood reconstituted with heat-inactivated serum, plus 4 × 10^6 mL logarithmic phase promastigotes and bacterial isolates at 1 × 10^8 bacteria/ mL.

As a control, one group fed only with blood and parasites, and another group only pre-treated with the antibiotic (Pen Strep) were analyzed. After the infective blood meal, engorged flies were separated and maintained at 27˚C and 75% humidity and provided 10% sucrose ad libitum. The sandflies were analyzed three and six days post-infection (d.p.i).

Ten flies were frozen (-20˚C) and killed in a 5% soap solution, and then midguts were dissected and transferred individually into a tube containing 30 μl PBS. The midguts, individually homogenized using a plastic pestle, then 10μl of the supernatant (containing the released promastigotes) counted under a hemocytometer, each experiment was performed three times.

Bacteria add back

The bacteria added after the experimental infection with L.i.chagasi carried out following the previously described protocol (in vivo—sandfly infection). Wild-caught sandflies previously treated for three days with Pen Strep and then experimentally infected with L.i.chagasi (4 × 10^6 mL logarithmic phase promastigotes). Three days after the infective blood meal, at the end of the digestive process, a sterile solution of sucrose with 1x10^8 bacteria/ mL of Lysinibacillus or Serratia was offered to the flies. The flies were analyzed three days after the addition of bacteria, corresponding to six d.p.i.

Statistical analysis

The results were analyzed using GraphPad Prism 7. The non-parametric Kruskal-Wallis test (ANOVA) used for statistical analysis. Values of p < 0.05 were considered significant.

*p* < 0.05, **p** < 0.001, ***p*** < 0.0001 and ****p*** < 0.00001.

Results

Sandfly native microbiota diversity revealed by a culture-dependent method

The 16s rRNA gene sequencing of cultivated bacteria from the native microbiota of Lu.longipalpis showed shifts in the community composition (in terms of genus richness) across the tested life stages, as well as in the distinct physiological conditions evaluated. A total of 92 CFUs of all groups were isolated and sequenced, revealing ten bacterial genera (Bacillus, Enterococcus, Erwinia, Enterobacter, Escherichia, Lysinibacillus, Providencia, Pseudomonas, Serratia, and Staphylococcus). General aspects, 54% had a whitish color, and all colonies were circular, the predominant elevation and margin type were umbonate and entire (56%), respectively; six were Gram-negatives (60%), and four were Gram-positives (40%) (Table 1).

Immature forms

Regarding larval and pupal stages, there were respectively three genera (Bacillus, Lysinibacillus, and Pseudomonas) and two families (Bacillaceae and Pseudomonadaceae) present in the isolated colonies, with only Pseudomonas present in both groups.
Adults

The UF group showed the highest bacterial diversity, with eight different genera. Being four Gram-positive (Bacillus, Enterococcus, Lysinibacillus and Staphylococcus), and four Gram-negative (Erwinia, Enterobacter, Escherichia and Providencia). Except for Lysinibacillus, Bacillus (Bacillaceae), and Staphylococcus (Staphylococcaceae), the other isolates were Enterobacteriaceae family members. The only genus identified associated with the BF group was Serratia, a Gram-negative Enterobacteriaceae. Two distinct genera identified in the GR group, (Erwinia and Providencia), both Gram-negative Enterobacteriaceae.

Finally, genera shared between immature and adults groups were Bacillus and Lysinibacillus, Gram-positive taxa, identified in pupae and UF, and larvae and UF group, respectively.

In vitro assay: short and long evaluation

The effect of Lu. longipalpis native microbiota on the survival of Leishmania species.  

i) Short time frame evaluation: The activity of each one of the ten native bacteria isolated from Lu. longipalpis were analyzed by co-cultivation with promastigotes of L. i. chagasi, L. major, L. amazonensis, and L. braziliensis. After 30 minutes of co-cultivation of the Leishmania promastigotes with the native bacteria, we observed a reduction in parasite numbers for all tested species. When compared to the 0 hr time point, the co-culture with Lysinibacillus and Serratia killed 13.25% and 8.75% of L. chagasi, respectively, and 12.25% and 8.5% of L. amazonensis, respectively. When these same bacteria were co-cultured with L. braziliensis or L. major, they killed 10.5% and 7%, and 13.25% and 9.5%, respectively (Fig 1).

At 60 minutes, the killing persisted for the majority of co-cultures. At the 120 minutes time point, when comparing to the 0 hr mark, the respective killing of L. chagasi, L. amazonensis, L. braziliensis and L. major following co-culture with Lysinibacillus or Serratia was 25.5% & 15%, 26 & 16.75%, 23% & 14.5%, and 28% & 23.25%, respectively.

Statistically significant differences were observed for most groups when comparing parasite reduction numbers against the control (only the Leishmania) (α = 0.05). In particular, no significant reductions found when any parasite expose to Enterobacter and Pseudomonas and when L. i. chagasi (A) and L. amazonensis (B) were co-cultivated with Escherichia (Fig 1).

ii) Long time frame evaluation: After 24hrs of co-culture with associated sandfly bacteria, the Leishmania promastigote numbers decreased for all parasite species. Co-culture with Serratia killed 41.3% of L. major, and 36.25% of L.i chagasi, or L. amazonensis or L. braziliensis promastigotes (Fig 2). Serratia produced additional mortality in all species at 48 hrs, and after 72 hrs, all the parasites co-cultured with Serratia were dead. But, when the parasites were exposed to Lysinibacillus for 24 hrs, L. i. chagasi and L. amazonensis were totally killed, whereas the mortalities for L. braziliensis and L. major were 88.75% and 81.25%, respectively. After 48 hrs, all of the parasite species were completely dead (Fig 2).

At 72 hrs, all of the other co-cultured groups (Bacillus, Enterobacter, Enterococcus, Erwinia, Escherichia, Staphylococcus, Providencia and Pseudomonas) showed an inhibitory effect on bacterial growth or survival. Between 96 to 120 hrs, and in all co-cultured groups, the Leishmania parasites were failing or dead.

Statistically significant differences were detected for the majority of groups when comparing parasite reduction numbers between the control and the endpoints (α = 0.05). The exception was L. major with Enterobacter against the control group (only Leishmania) (Fig 2).

In vivo assay

Evaluation of experimental coinfection in vivo—Lu. longipalpis, L. i. chagasi and bacterial isolates.  

Our in vitro results have shown that some bacterial genera have a powerful effect
of inhibiting the survival of different *Leishmania* species, causing the death of promastigotes in a few hours or days. To test the effects of bacteria in vivo, all ten isolates obtained in this study were used to challenge *L. i. chagasi* infected *Lu. longipalpis* sandflies, a natural parasite—vector pair. Three days post-infective blood-meal, the number of parasites per midgut was detected, and the groups co-infected with *Serratia* and *Lysinibacillus* had 90% fewer parasites compared to the treated control group. At six d.p.i. for all isolates, the number of parasites per infected fly was lower than controls. However, the co-infection with *Serratia* and *Lysinibacillus* again showed the lowest numbers of parasites per infected fly (Fig 3).

In summary the infection rate was 100% in all groups and the median of parasite per group was 7500 parasites/ per sandfly for *Bacillus* after 3 d.p.i and 408 parasites/ per sandfly after 6 d.p.i. (**`). For *Enterobacter* the median was 5500 parasites/ per sandfly after 3 d.p.i (`) and 581 parasites/ per sandfly after 6 d.p.i. For *Enterococcus* the median was 4900 parasites/ per sandfly after 3 d.p.i. and 790 parasites/ per sandfly after 6 d.p.i. (`). For *Erwinia* the median was 5382 parasites/ per sandfly after 3 d.p.i and 592 parasites/ per sandfly after 6 d.p.i. (```). For *Escherichia* the median was 6000 parasites/ per sandfly after 3 d.p.i and 1100 parasites/ per sandfly after 6 d.p.i. For *Lysinibacillus* the median was 226 parasites/ per sandfly after 3 d.p.i (**`**) and 30 parasites/ per sandfly after 6 d.p.i. (```). For *Providencia* the median was 5000 parasites/ per sandfly after 3 d.p.i and 910 parasites/ per sandfly after 6 d.p.i. For *Pseudomonas* the median was 2727 parasites/ per sandfly after 3 d.p.i and 497 parasites/ per sandfly after 6 d.p.i. (```). For *Serratia* the median was 174

Fig 1. The short effect, in the parasite, when growth co-cultured with native bacteria. A short temporal evaluation (0 to 120 minutes) showing the activity of each one of the ten native bacteria isolated from *Lu. longipalpis* when co-culture with four *Leishmania* species: (A) *L. i. chagasi*, (B) *L. amazones*, (C) *L. braziliensis*, and (D) *L. major*.

https://doi.org/10.1371/journal.pntd.0008666.g001
Composition of the bacterial community associated with *Lu. Longipalpis* after co-infection

The cultivable bacterial methodology showed the bacterial communities associated with *Lu. longipalpis* post-co infection composed by eight bacterial genera with the following composition: i) UF group—*Bacillus, Lysinibacillus* and *Solibacillus*, all Gram-positive; ii) P/S treated group—*Enterobacter, Klebsiella*, and *Pseudocitrobacter*, all Gram-negative; iii) PI group—*Enterobacter, Escherichia*, and *Serratia*, all Gram-negative (Table 2).

Our results showed a shift in the composition of the bacterial community associated with *Lu. longipalpis* before and after infection (unfed and blood-fed), as follows: *Klebsiella, Pseudocitrobacter*, and *Solibacillus*. Also, those bacterial isolates effects were in vitro tested with *L. chagasi, L. amazonensis, L. braziliensis*, and *L. major* up to 120 hours (S1 Fig).

Co-infection of *Lu. longipalpis* with native isolates of bacteria and *Leishmania* spp

After analysis of all the in vitro (Figs 1 and 2 and S1 Fig) and in vivo results (Fig 3), *Lysinibacillus, Pseudocitrobacter*, and *Serratia* showed to be the most competitive or aggressive bacteria.

![Fig 2. The long term mortality of *Leishmania* species when co-cultured with native bacteria.](https://doi.org/10.1371/journal.pntd.0008666.g002)
Fig 3. The effect of co-infection of *L. i. chagasi* and native bacteria. *In vivo* co-infection showing the result of the co-infection of *L. i. chagasi* (initial concentration 4x10^6 parasites/mL) with all ten bacterial taxa (initial concentration of 10^8 CFU/mL) isolated from *Lu. longipalpis*. The midguts individually macerated, and the number of parasites alive counted under a hemocytometer. The groups co-infected with *Serratia* and *Lysinibacillus* showed the lowest infection rate (statistically significant differences observed. α = 0.05). Six days post-infection, for all isolates, the number of parasites per midgut was lower than the pre-treated control.

https://doi.org/10.1371/journal.pntd.0008666.g003
against the parasites. These three isolates used for co-infection assays with *L. i. chagasi*, *L. amazonensis*, and *L. major*.

Again, a decrease in the number of parasites per midgut observed for all *Leishmania* species tested (Fig 4). Live parasite counts showed significant reductions in all the coinfected groups at both day three and six day d.p.i., and compared with the control group, similar to the *in vitro* results (Fig 4 and S1 Fig). The exception was *Pseudocitrobacter*, which was able to cause a significant decrease in the number of parasites in *L. i. chagasi* (median 396 parasites/per sandfly) and *L. major* (median 671 parasites/per sandfly), but not *L. amazonensis* (median 291 parasites/per sandfly).

In all the groups, co-infected tested had significant differences detected for bacterial effectors when compared with the control group, and the number of parasites per gut dropped similarly to the *in vitro* results ($\alpha = 0.05$).

**Bacteria add back**

To evaluate the effect of *Lysinibacillus* or *Serratia* after the establishment of *L. i. chagasi* infection in *Lu. longipalpis*, these two bacterial genera were offered to sandflies three days of post-blood infection. The experimental infection with *L. i. chagasi* carried out following the previous protocol. As a control, only infected sandflies, no bacterial genera offered. On the third day after bacterial addition, there was a significant decrease in the number of parasites per midgut for both *Lysinibacillus* (***') and *Serratia* (****') (S2 Fig).

**Discussion**

In Diptera, the native microbiota plays many distinctive roles in the life cycle, such as development, immune response, reproductive biology, and vector competence. The intestinal diversity of microbiota associated with insect larvae and adults is variable and influenced by where they live and by their feeding behavior. Additionally, transstadial transmission is a mechanism that has been described in other arthropods [35].
In sandflies, the larval stages acquire different organisms because they feed in fertile soil where fungi and bacteria thrive [36]. Members of the microbiota acquired during the larval stage may remain autochthonously associated with the adults. Such a process implies that bacterial OTUs (Operational Taxonomic Units) would persist in the symbiotic assemblage throughout the whole insect metamorphosis [4, 37–39]. In anopheline mosquitoes, also observed that bacterial genera Acinetobacter, Bacillus, Enterobacter, Staphylococcus, Pseudomonas, Cryseobacterium, and Serratia persist as members of the insect microbiota throughout its life stages [40]. Here, we used the method of cultivable bacteria to address the native microbiota of Lu. longipalpis in immature stages and adults at distinct developmental stages and physiologic conditions.

A total of thirteen genera were identified, eight Gram-negative (61.5%), (Erwinia, Enterobacter, Escherichia, Klebsiella, Pseudocitrobacter, Providencia, Pseudomonas and Serratia) and five Gram-positive (38.5%) (Bacillus, Enterococcus, Lysinibacillus, Staphylococcus, and Solibacillus) with the majority identified as Enterobacteriaceae. These findings are in agreement with Oliveira et al. [20,21], who characterized the intestinal microbiota as prevalently Gram-negative in Lu. longipalpis, and with Kelly et al. [14], who defined Enterobacteriaceae as a dominant taxon under both sucrose-fed and blood-fed conditions.

Using a high throughput and NGS metagenomic analysis, [16,19,34], also reported the predominance of Gram-negative bacteria in blood feed and infected groups. The first study McCarthy et al. [19], analyzed males and females of Lu. longipalpis and Pires et al. [16] only females (from the same study location explored here), while the last study Monteiro et al. [34] targeted females of Lu. intermedia from a different geographical region and thus ecological niche.

In our studies, the genera Bacillus and Lysinibacillus were found in the immature stage (pupa) and adult, suggesting that species within these genera could remain transstadially associated to the sandfly. Volf et al. [4] showed that P. dubosqui featured a bacterial consortium associated with the gut immediately after adult emergence. Dillon et al. [6] had hinted the same in a study with P. papatasi, where the bacterial diversity profile within the sandfly gut was proposed to be dynamic, fluctuating during its lifespan. For example, in Lutzomyia evansi from field sites in Central America, the potential symbionts identified were Enterobacter, Pseudomonas, Bacillus, and Lysobacter, these taxa present across larvae, pupae and adults [41].

Regarding the immature stages, the genera we identified were Bacillus, Lysinibacillus, and Pseudomonas. In other studies involving laboratory colonized sandfly larvae were found, Enterobacter and Bacilli [1,42].

Lysinibacillus, Gram-positive, also found in the adults (UF) along with Staphylococcus and Solibacillus (UF). Although less prevalent (38.5%), Gram-positive bacteria appear to be part of the shared microbiota of sandfly of the Old and New World. In the Old World, Maleki-Ravasan et al. [7,42] reported the Gram-positive Bacillus, Staphylococcus, and Pseudomonas. In contrast, Hillesland et al. [43] reported Staphylococcus and Bacillus in their studies with Phlebotomus argentipes, and the genus Solibacillus was described in P. papatasi [36]. In the New World, Oliveira et al. [20] and Pires et al. [16] found Bacillus, Staphylococcus, and Pseudomonas and Dey et al. [44] characterized the microbiota of Lu. longipalpis infected with L. donovani, identifying Bacillus and Lysinibacillus.

Also, the genus Lysinibacillus formally nominated as Bacillus spp. and well-known founded in soil and aquatic environments. Members of this genus can produce spores when submitted to adverse conditions [45]. A member of this taxon, Lysinibacillus sphaericus, named initially as Bacillus sphaericus, is used in commercial larvicides as part of effective insect control strategies. It has a well-documented toxic action on flies, especially for Culicidae [46–48].

In adult insects, bacterial isolates from the midgut, using a culture-dependent method, revealed predominantly bacteria belonging to the family Enterobacteriaceae (Gram-negative);
Erwinia, Enterobacter, Escherichia, Klebsiella, Serratia, Pseudocitrobacter and Providencia. Enterobacteriaceae appears to be the most abundant family identified by other investigators [4,6,14,16,49,50]. That might be explained by the fast growth of these taxa in culture, which may outcompete the growth of other taxa [6].

Among the genera mentioned above, Erwinia and Enterobacter have been identified in plants [51,52]. Therefore, their presence could linked to adult feeding behavior on plant surfaces. Members of the Escherichia genus have also described in sandflies of the New and Old World [16,18,42,53,54]. Providencia is usually found in aquatic and terrestrial environments [55], is present in the hemolymph of Drosophila melanogaster [56] and the midgut of Culex quinquefasciatus [57] and Lu. longipalpis [16]. Serratia is a genera often identified in sandflies [4,6,16–18,21,34,42,54,58]. Pseudocitrobacter and Klebsiella were associated with Lu. longipalpis post-infection. Klebsiella was already associated with Lu. longipalpis sandflies sampled from field sites (Alagoas, Brazil) [17].

During this study, we aimed to assess in vitro and in vivo if bacterial isolates from immature stages and different physiological states of Lu. Longipalpis, a permissive laboratory vector, had the potential to inhibit the growth and survival of Leishmania spp. In all co-cultures, in vitro, and in vivo tests, the presence of the metacyclic forms was observed a small number (1%). A possible explanation should be the decrease in pH caused by the presence of the bacteria, the medium composition, temperature, culture phase, and density of the parasite, and all these factors significantly influence the development of metacyclic promastigotes [59,60,61,62].

We reported an inhibitory effect of bacterial symbionts upon promastigotes of L. infantum chagasi, L. major, L. amazonensis, and L. braziliensis. In the in vitro co-cultures, Lysinibacillus and Serratia registered the most harmful effect for all species tested shows (Figs 1 and 2). Although in vitro studies showed a similar impact of co-cultured microbiota upon parasite survival has been reported for Serratia, Bacillus, and Haemophilus parainfluenzae, all of which induced lysis of promastigotes [11,12,3]. Also, we observe a reduction in the number of live parasites when the Leishmania spp. was co-cultivated with Pseudocitrobacter (S1 Fig).

A possible explanation for the parasite mortality is the formation of a structured bacterial community recognized as biofilms [63–64, 65]. Moraes et al. [11,12] observed by scanning electron microscopy that the bacteria S. marcescens (strain SM365) when incubated with L. chagasi and L. braziliensis, adhered to the entire cell body and flagellum of the parasite. Filamentous structures identified as biofilms formed, which could have induced the lysis of the cell membrane of the parasites. We do not rule out the hypothesis that other probable explanations may be due to the saturation of nutrients present in the culture medium, which leads to a faster death of the parasites due to the high multiplication of bacteria.

Several studies have already demonstrated the interference of bacteria upon insect infection by human pathogens [35]. It accepted that naturally occurring microorganisms in the midgut of insects might play an essential modulating role in the development of pathogens within their arthropod vectors.

In our in vivo studies, we tested all 13 isolates. It was showing that the genera Lysinibacillus, Serratia, and Pseudocitrobacter caused the death of the parasites within the midgut of the insect vector, the same as observed in the in vitro experiments. An in vivo study by Sant’Anna et al. [5] assessed how re-colonizing the intestines of females of Lu. longipalpis (Jacobina, Bahia) with bacteria and yeasts extracted from their midgut interfered in the capacity of the L. mexicana to colonize the insect host. A reduction was observed in the number of infected sandflies that had been pre-fed with Pseudozyma sp., Asaia sp. and Ochrobactrum intermedium, as well as a reduction in parasitic load.

In the study of Louradour et al. [1], the addition of Enterobacter and Serratia genera to the infective blood-meal promoted significant recovery of the number of metacyclic that
developed in *P. duboscqi* treated with Pen Strep post-blood-meal excretion. Here, when both *Lysinibacillus* and *Serratia* were added after the establishment of the infection, a significant decrease in parasites number occurred (S2 Fig).

In summary, we showed that *Lysinibacillus, Pseudocitrobacter*, and *Serratia* strongly inhibited *Leishmania* growth and survival *in vitro* and co-infected *Lu. longipalpis*, the primary vector of American visceral leishmaniasis. After 24 h of *in vitro* co-cultivation of the *Leishmania* with the native bacteria, it was possible to observed growth reduction in all parasite species. When the parasites were co-cultivated with the bacterial genus *Lysinibacillus*, all parasites of *L. infantum chagasi* and *L. amazonensis* died within 24 hours. In the *in vivo* co-infection of *L. chagasi, L. major*, and *L. amazonensis* with the genera *Lysinibacillus, Pseudocitrobacter* and *Serratia*, it was possible to observe a significant difference between the groups co-infected with this bacterial genus and the control group pre-treated. These findings suggest that these three symbiont bacteria are potential candidates for paratransgenic or biological control. Further studies are needed to identify the nature of the effector molecules involved in reducing the vector competence for *Leishmania*.

Supporting information

**S1 Fig. Leishmania species co-cultivated with bacteria isolated after an infective blood meal.** Mortality observed over 120 hours with the four *Leishmania* species: (A) *L.i. chagasi*, (B) *L. amazonensis*, (C) *L. braziliensis*, and (D) *L. major*, co-cultivated with three distinct genera of bacteria (*Pseudocitrobacter, Klebsiella, and Solibacillus*) isolated from the midgut of *Lu. longipalpis* after infection blood meal with *L.i. chagasi*.

(TIFF)

**S2 Fig. In vivo assay showing the effect of addition bacteria after infection of the sandfly.** To evaluate the effect of *Lysinibacillus* or *Serratia* after the establishment of *L.i. chagasi* (initial concentration 4x10^6 parasites/mL) infection in *Lu.longipalpis*, these two bacterial genera were offered to sandflies (pre-treated with pen-strep) three days post the infectious (3 d.p.i) blood infection. Were provided a sterile solution of sucrose with 1x10^8 CFU / mL of *Lysinibacillus* or *Serratia* and the flies were analyses three days after the bacterial feeding. It observed that on the third day after bacterial addition, there was a significant decrease in the number of parasites per midgut for both *Lysinibacillus* and *Serratia*. Statistical significance at p = 0.05.

(TIFF)

Acknowledgments

We would like to thank the Oswaldo Cruz Foundation (FIOCRUZ), Brazilian Council for Scientific and Technological Development (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior–Brasil (CAPES).

Author Contributions

**Conceptualization:** Nagila Francinete Costa Secundino.

**Data curation:** Thais Boniﬁácio Campolina, Luis Eduardo Martinez Villegas.

**Formal analysis:** Thais Boniﬁácio Campolina, Luis Eduardo Martinez Villegas, Carolina Cunha Monteiro, Paulo Filemon Paolucci Pimenta, Nagila Francinete Costa Secundino.

**Funding acquisition:** Paulo Filemon Paolucci Pimenta, Nagila Francinete Costa Secundino.
Methodology: Thais Bonifácio Campolina, Luis Eduardo Martinez Villegas, Carolina Cunha Monteiro.

Resources: Thais Bonifácio Campolina, Luis Eduardo Martinez Villegas, Carolina Cunha Monteiro, Paulo Filemon Paolucci Pimenta, Nagila Francinete Costa Secundino.

Supervision: Nagila Francinete Costa Secundino.

Writing – original draft: Thais Bonifácio Campolina, Paulo Filemon Paolucci Pimenta, Nagila Francinete Costa Secundino.

Writing – review & editing: Thais Bonifácio Campolina, Paulo Filemon Paolucci Pimenta, Nagila Francinete Costa Secundino.

References

1. Louradour I, Monteiro CC, Inbar E, Ghosh K, Merkhofer R, Lawyer P, et al. The midgut microbiota plays an essential role in sandfly vector competence for Leishmania major. Cell Microbiology. 2017;e12755, https://doi.org/10.1111/cmi.12755 PMID: 28580630

2. Peterkova-Koci K, Robles-Murguia M, Ramalho-Ortigao M, Zurek L. Significance of bacteria in oviposition and larval development of the sandfly Lutzomyia longipalpis. Parasite & Vectors. 2012; 5:145. Epub.

3. Hassan MI, Al-Sawaf BM, Fouda MA, Hammad KM. A Recent Evaluation of the Sandfly, Phlebotomus Papatasi Midgut Symbiotic Bacteria Effect on the Survivorship of Leishmania Major. J Anc Dis Prev Rem. 2014; 2:110.

4. Voil P, Kiewegova A, Nemec A. Bacterial colonization in the gut of Phlebotomus duboscqi (Diptera:Psychodidae): transtadial passage and the role of female diet. Folia Parasitologica. 2002; 49:73–77.

5. Sant’anna MRv, Aguiar-Martins K, Sales ASW, Cavalcante RR, Dillon MV, et al. Colonization resistance in the sandfly gut: Leishmania protects Lutzomyia longipalpis from bacterial infection. Parasites & Vectors. 2014; v. 7, n. 1, p.329–339.

6. Dillon RJ, el Kordy E, Shehata M, Lane RP. The prevalence of a microbiota in the digestive tract of Phlebotomus papatasi. Ann Trop Med Parasitol. 1996;Dec; 90(6):669–73. https://doi.org/10.1080/00034983.1996.11813102 PMID: 9039284

7. Maleki-Ravasan N, Oshaghi MA, Afshar D, Arandian MH, Hajikhani S, Akhavan AA, et al. Aerobic bacterial flora of biotic and abiotic compartments of a hyperendemic Zoonotic Cutaneous Leishmaniasis (ZCL) focus. Parasit Vectors. 2015; Jan 29; 8:63. https://doi.org/10.1186/s13071-014-0517-5 PMID: 25630498

8. Sacks DL, Kamhawi S. Molecular aspects of parasite-vector and vector-host interaction in Leishmania. Annu Rev Microbiol. 2001; 55:453–83. https://doi.org/10.1146/annurev.micro.55.1.453 PMID: 11544384

9. Serafim T., Coutinho-Abreu IV, Oliveira F, Meneses C, Kamhawi S, Valenzuela JG. Sequential blood meals promote Leishmania replication and reverse metacyclogenesis augmenting vector infectivity. Nat Microbiol. 2018; https://doi.org/10.1038/s41564-018-0125-7 PMID: 29556108

10. Grimont PA, Grimont F, Le Minor S, Davis B, Pigache F. Compatible results obtained from biotyping and serotyping in Serratia marcescens. J Clin Microbiol. 1979; 10:425–32; https://doi.org/10.1128/JCM.10.4.425-432.1979 PMID: 393712.

11. Moraes CS, Seabra SH Castro DP, Genta FA, Brazil RP, de Souza W, et al. Leishmania (Leishmania) chagasi/interactions with Serratia marcescens: Ultrastructural studies, lysis and carbohydrate effects. Experimental Parasitology. 2008; 118:561–568.

12. Moraes CS, Seabra SH, Albuquerque-Cunha JM, Castro DP, Genta FA, de Souza W, et al. Prodigiosin is not a determinant factor in lysis of Leishmania (Viannia) braziliensis after interaction with Serratia marcescens D-mannose sensitive fimbiae. Experimental Parasitology. 2009; 122 84–90.

13. Simões ML, Dimopoulos G. A mosquito mediator of parasite-induced immune priming. Trends Parasitol. 2015; 31(9):402–404. https://doi.org/10.1016/j.pt.2015.07.004 PMID: 26254960

14. Kelly PH, Bahr SM, Serafim TD, Ajami NJ, Petrosino JF, Meneses C, et al. The gut microbiome of the vector Lutzomyia longipalpis is essential for survival of Leishmania infantum. mBio. 2017; 8:e01121–16. https://doi.org/10.1128/mBio.01121-16 PMID: 28996493

15. Fraihí W, Fares W, Perrin P, Dorkeld F, Sereno D, Barhoumi W, et al. An integrated overview of the midgut bacterial flora composition of Phlebotomus perniciosus, a vector of zoonotic visceral leishmaniasis.
in the Western Mediterranean Basin. PLoS Negl Trop Dis. 2017; 11(3):e0005484. https://doi.org/10.1371/journal.pntd.0005484 PMID: 28355207

16. Pires A, Villegas LEM, Campolina TB, Orfano AS, Pimenta PFP and Secundino NFC. Bacterial diversity of wild-caught *Lutzomyia longipalpis* (a vector of zoonotic visceral leishmaniasis in Brazil) under distinct physiological conditions by metagenomics analysis. Parasites & Vectors. 2017; 10, 627. https://doi.org/10.1186/s13071-017-2593-7 PMID: 29284535

17. Sant’Anna MRV, Darby AC, Brazil RP, Montoya-Lerma J, Dillon VM, Bates PA, et al. Investigation of the Bacterial Communities Associated with Females of *Lutzomyia* SandFly Species from South America. PLoS ONE. 2012; 7(8):e42531. https://doi.org/10.1371/journal.pone.0042531 PMID: 22880020

18. Gouveia C, Asensi MD, Zahner V, Rangel EF, Oliveira SMP. Study on the Bacterial Midgut Microbiota Associated to Different Brazilian Populations of *Lutzomyia longipalpis* (Lutz & Neiva) (Diptera: Psychodidae). Neotrop Entomol. 2008; 37:597–601. https://doi.org/10.1590/s1519-566x2008000500016 PMID: 19061048

19. McCarthy CB, Diambra LA, Pomar RVR. Metagenomic Analysis of Taxa Associated with *Lutzomyia longipalpis*, Vector of Visceral Leishmaniasis, using an unbiased High-Throughput Approach. PLoS Negl Trop Dis. 2011; 5(9):e1304. https://doi.org/10.1371/journal.pntd.0001304 PMID: 21909446

20. Oliveira SMP, Moraes BA, Gonçalves CA, Giordano-Dias CM, d’Almeida JM; Asensi MD, et al. Prevalence of the microbiota in the digestive tract of wild caught females of *Lutzomyia longipalpis* (Lutz & Neiva) Diptera: Psychodioide. Revista da Sociedade brasileira de Medicina tropical. 2000; 33(3):319–322. https://doi.org/10.1590/s0037-86222000003000012 PMID: 10967602

21. Oliveira SMP, Moraes BA, Gonçalves CA, Giordano-Dias CM, Vilela ML, d’Almeida JM, et al. Digestive tract microbiota in female *Lutzomyia longipalpis* (Lutz & Neiva) Diptera: Psychodiade feeding in blood meal and sacarose plus blood meal. Cadernos de Saúde Publica do Rio de Janeiro. 2001; 17(1) 229–232.

22. Sudia WD; Chamberlain RW. Battery-operated light trap, an improved model. Mosq News. 1962; 22:126–9

23. Young DG, Duncan MA. Guide to the identification and geographic distribution of Lutzomyia sandflies in Mexico, the West Indies, Central and South America (Diptera: Psychodidae). Mem Amer Entomol Inst. 1994; 54:1–881.

24. Secundino NF, Pimenta PFP. Scanning electron microscopy study of the egg and immature stages of the sandflies *Lutzomyia longipalpis*. Acta Microscopica. 1999:v. 8, p. 33–38

25. Lacey LA, Brooks WM. Initial handling and diagnosis of diseased insects. In: Lacey LA, editor. Manual of Techniques in Insect Pathology. 1st ed. San Diego: Academic Press Press. 1997; P. 409.

26. Gaio ADO, Gusmão DS, Santos AV, Berbert-Molina MA, Pimenta PFP, Lemos FJA. Contribution of midgut bacteria to blood digestion and egg production in *Aedes aegypti* (diptera: culicidae) Parasites & vectors. 2011; Jan; 4(1):105.

27. Altschul et al., Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J. Basic local alignment search tool. J. Mol. Biol. 1990; 5, pp. 403–410, https://doi.org/10.1016/S0022-2836(05)80360-2

28. Wang Y., Qian P. Y. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. PLoS ONE. 2009; 4:e7401. https://doi.org/10.1371/journal.pone.0007401 PMID: 19816594

29. Yang B, Wang Y, Qian PY. Sensitivity and correlation of hypervariable regions in 16S rRNA genes in the Bacteria l Communities Associated with Females of *Lutzomyia intermedia* (dipitera: culicidae) Parasites & Vectors. 2016; 480, n. 9, p.1–6.
35. Minard MM, Minard G, Mavingui P, Moro CV. Diversity and function of bacterial microbiota in the mosquito holobiont. Parasites & Vectors. 2013; 6:146. https://doi.org/10.1186/1756-3305-6-146 PMID: 23688194

36. Mukhopadhyay J, Braig HR, Rowton ED, Ghosh K. Naturally occurring culturable aerobic gut flora of adult Phlebotomus papatasi, Vector of Leishmania major in the old world. PLoS ONE. 2012; 7(5): e35748. https://doi.org/10.1371/journal.pone.0035748 PMID: 22629302

37. Moll RM, Romoser WS, Modrzakowski MC, Moncayo AC, Lerdthusnee K. Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. J Med Entomol. 2001; 38:29–32. https://doi.org/10.1603/0022-2585-38.1.29 PMID: 11268687

38. Wang Y, Gilbreath III TM, Kukutla P, Yan G, Xu J. Dynamic gut microbiome across life history of the malaria mosquito Anopheles gambiae in Kenya. PLoS one. 2011; 6(9):e24767. https://doi.org/10.1371/journal.pone.0024767 PMID: 21957459

39. Abraham M, Massebo F, Lindtjorn B. High entomological inoculation rate of malaria vectors in the area of high coverage of interventions in southwest Ethiopia: implication for residual malaria transmission. Parasite Epidemiol Control. 2017; 2:61–69. https://doi.org/10.1016/j.parepi.2017.04.003 PMID: 29774282

40. Rani A, Sharma A, Rajagopa R, Adak T, Bhatnagar RK. Bacterial diversity analysis of larvae and adult midgut microbiota using culture-dependent and culture-independent methods in lab-reared and field-collected Anopheles stephensi—an Asian malarial vector. BCM Microbiol. 2009; 9:96–118.

41. Vivero RJ, Jaramillo NG, Cadavid-Restrepo G, Soto SI and Herrera CX. Structural differences in gut bacterial communities in developmental stages of natural populations of Lutzomyia evansi from Colombia’s Caribbean coast. Parasites and Vectors. 2016; 9. 496. https://doi.org/10.1186/s13071-016-1766-0 PMID: 27618991

42. Maleki-Ravasan N, Oshaghi MA, Hajikhani S, Saeidi Z, Akhavan AA, Gerami-Shoar M, et al. Aerobic Microbial Community of Insectary Population of Phlebotomus papatasi. J Arthropod Borne Dis. 2014; Dec 18; 8(1):69–81. PMID: 25629067

43. Hillesland H, Read A, Subhadra B, Hurwitz I, McKelvey R, Ghosh K, Das P, Durvasula R. Identification of aerobic gut bacteria from the kala azar vector, Phlebotomus argentipes: a platform for potential para-transgenic manipulation of sandflies. American Journal of Tropical Medicine and Hygiene. 2008; 79, 881–886. PMID: 19052297

44. Dey R, Joshi AB, Oliveira F, Pereira L, Guimaraes-Costa AB, Serafim TD, et al. Gut microbes egested during bites of infected sandflies augment severity of leishmaniasis via inflammasome-derived IL-1beta. Cell Host & Microbe. 2018; 23, 134–143 e136.

45. Crickmore N, Baum J, Bravo A, Lereclus D, Narva K, Sampson K, et al. “Bacillus thuringiensis toxin nomenclature”. 2016. Available from: http://www.btlnomenclature.info/.

46. Mittal PK. Biolarvicides in vector control: challenges and prospects. Journal of Vector Borne Diseases. 2003; Delhi, v. 40, n. 1–2, p. 20–32. PMID: 15119068

47. Regis L, Silva-Filha MH, Nielsen-LeRoux C, Charles J-F. Bacteriological larvicides of dipteran disease vectors. Trends in Parasitology, Oxford. 2001; v. 17, n. 8, p. 377–380.

48. Thiery I, Back C, Barbazan P, Sinegre G. Applications of Bacillus thuringiensis and de B. sphaericus dans la démoustication et la lutte contre les vecteurs de maladies tropicales. Annales de l’Institut Pasteur/Actualités, Paris. 1996; v. 7, p. 247–260.

49. DeMaio J, Pumpuni CB, Kent M, Beier JC. The midgut bacterial flora of wild Aedes triseriatus, Culex pipiens and Psorophora columbiae mosquitoes. Am J Trop Med Hyg. 1996; 54:219–223. https://doi.org/10.4269/ajtmh.1996.54.219 PMID: 8619452

50. Pumpuni CB, Demajo I, Kent M, Davis JR, Beier JC. Bacterial population dynamics in three anopheline species: the impact on Plasmodium sporogonic development. Am J Trop Med Hyg. 1996; 54:214–218. https://doi.org/10.4269/ajtmh.1996.54.214 PMID: 8619451

51. Zhang Z, NIAN Z. Erwinia persicina, a possible new necrosis and wilt threat to forage or grain legumes production. European Journal of Plant Pathology. 2014; Lanzhou, v. 139, n. 2, p. 343–352, jul.

52. Hoffmann H, Stindl S, Stumpf A, Mehlner A, Monget D, Heesemann J, Schleifer KH, et al. Description of Enterobacter ludwigii sp. nov., a novel Enterobacter species of clinical relevance. Syst Appl Microbiol. 2008; Gauting, Germany, v. 82131, n. 2, p.206–212, 28 abr. https://doi.org/10.1016/j.syapm.2004.12.009 PMID: 15900967.

53. Schlein Y, Polacheck I, Yuval B. Mycoses, bacterial infections and antibacterial activity in sandflies (Psychodidae) and their possible role in the transmission of leishmaniasis. Parasitology. 1985;(1) 57–66. https://doi.org/10.1017/S0031182000049015.

54. Akhoundi M, Bahktiari R, Guillard T, Baghaei A, Tolouei R, Sereno D, et al. Diversity of the Bacterial and Fungal Microflora from the Midgut and Cuticle of Phlebotomine SandFlies Collected in North-
Western Iran. Chaturvedi S, ed. PLoS ONE. 2012; 7(11):e50259. https://doi.org/10.1371/journal.pone.0050259 PMID: 23226255

55. Yoh M, Matsuyama J, Ohnishi M, Takagi K, Miyagi H, Mori K, et al. Importance of Providencia species as a major cause of travellers' diarrhoea. J. Med. Microbiol. 2005; 54:1077–1082. https://doi.org/10.1099/jmm.0.45846-0 PMID: 16192440

56. Ryan KJ, Ray CG. Sherris Medical Microbiology: an introduction to infectious diseases. 4th ed. McGraw-Hill Medical; 2003.

57. Chandel K, Mendki MJ, Parikh RY, Kulikarni G, Tikar SN, Sukumaran D, et al. Midgut Microbial Community of Culex quinquefasciatus Mosquito Populations from India. PLoS ONE. 2013; 8(11):e80453. https://doi.org/10.1371/journal.pone.0080453 PMID: 24312223

58. Warburg A. Entomopathogens of Phlebotomine sandflies: Laboratory experiments and natural infections. Journal of invertebrate pathology. 1991; 58, 189–202. https://doi.org/10.1016/0022-2011(91)90063-v PMID: 1783777

59. Sacks DL, Perkins PV Identification of an infective stage of Leishmania promastigotes. Science 223. 1984:1417–1419. https://doi.org/10.1126/science.6701528 PMID: 6701528

60. Bates PA, Tetley L. Leishmania mexicana: induction of metacyclogenesis by cultivation of promastigotes at acidic pH. Exp Parasitol. 1993; 76:412–423. 4. https://doi.org/10.1006/expr.1993.1050 PMID: 8513879

61. Bates PA. Leishmania sand fly interaction: progress and challenges. Curr Opin Microbiol 2008; 11:340–344. https://doi.org/10.1016/j.mib.2008.06.003 PMID: 18625337

62. Cunningham ML, Titus RG, Turco SJ, Beverley SM. Regulation of differentiation to the infective stage of the protozoan parasite Leishmania major by tetrahydrobiopterin. Science. 2001; 292:285–287. https://doi.org/10.1126/science.1057740 PMID: 11303103

63. Watnick P, Kolter R. Biofilm, city of microbes. Journal of Bacteriology. 2000; 182, pp. 2675–2679. https://doi.org/10.1128/jb.182.10.2675-2679.2000 PMID: 10781532

64. Weitere M, Bergfeld T, Rice SA, Matz C, Kjelleberg S. Grazing resistance of Pseudomonas aeruginosa biofilms depends on type of protective mechanism, developmental stage and protozoan feeding mode. Environmental Microbiology. 2005; 7, pp. 1593–1601. https://doi.org/10.1111/j.1462-2920.2005.00851.x PMID: 16156732

65. Queck SY, Weitere M, Moreno AM, Rice SA, Kjelleberg S. The role of quorum sensing mediated developmental traits in the resistance of Serratia marcescens biofilms against protozoan grazing. Environmental Microbiology. 2006; 8, pp. 1017–1025. https://doi.org/10.1111/j.1462-2920.2006.00993.x PMID: 16689722