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Review

Sand Flies and Their Microbiota

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Abstract: Sand flies are a significant public health concern in many parts of the world where they are known to transmit agents of several zoonotic diseases to humans, such as leishmaniasis. Vector control remains a key component of many anti-leishmaniasis programs and probably will remain so until an effective vaccine becomes available. The sand fly gut microbiota has recently emerged as an encouraging field for the exploration of vector-based disease control. In particular, the gut microbiome was previously reported to either enhance or inhibit parasite activity depending on the species of bacteria and, thus, has the potential to alter vector competence. Here, we describe the technological advances that are currently expanding our understanding of microbiota composition in sand flies. The acquisition and composition of microbiomes are influenced by several abiotic and biotic factors, including host immunity, genetics, and the environment. Therefore, the microbiomes of sand flies can vary substantially between individuals, life stages, species, and over geographical space, and this variation likely contributes to differences in host phenotypes, highlighting opportunities for novel vector control strategies.

Keywords: sand fly; microbiome; *Leishmania*; vector competence; vector control

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1. Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are tiny blood-sucking insects that feed on a wide range of hosts and potentially act as vectors of pathogens responsible for human and animal diseases worldwide. Of the more than 1000 sand fly species that have been described to date, approximately 10% are suspected or proven vectors of various pathogens, including arboviruses and bacteria, but are best known as the principal vectors of *Leishmania*, the etiological agent of leishmaniasis, a neglected tropical disease [1,2]. *Leishmania* have two main lifecycle stages: the motile flagellated promastigote, which is present in the sand fly vector, and the intracellular non-flagellated amastigote, which is present within mammalian host cells. Around 20 *Leishmania* species are known to be pathogenic to humans with clinical symptoms varying from localized, self-limiting cutaneous lesions and severe diffuse and destructive mucocutaneous lesions, to a disseminating visceral infection that is fatal in the absence of treatment [1]. The disease remains a public health problem worldwide, affecting approximately 12 million people in 98 countries, where 350 million people are at risk of infection [3]. An estimated 1.3 million new cases occur each year and cause 20,000 to 30,000 deaths per year [4]. At present, leishmaniasis is undergoing a type of synergy between natural phenomena and man-made conditions that facilitate the spread of the disease, with limited vaccines available and no effective therapeutic treatments against any form of human leishmaniasis [5,6]. Vector control is currently one of the most effective ways to prevent disease transmission. Advances that support evidence-based vector control can be leveraged to further optimize planning and implementation.

Over the last decade, microbial communities associated with sand flies gained relevance, as they have an important impact on the development of the parasite *Leishmania* in

the host's digestive tract [7]. Since the application of molecular methods, novel culture-independent techniques have been developed to study microbial communities in sand flies [8,9], while several studies have used culture-dependent approaches [10,11]. The sand fly microbiota is a dynamic community mostly acquired from the environment, as in other Insecta or Diptera. Sand flies become infected with *Leishmania* when they feed on the blood of an infected host in order to develop eggs and reproduce. The *Leishmania* developmental life cycle within the sand fly vector occurs exclusively in the mid- and hindgut with the presence of symbiotic bacteria; therefore, possible bacteria-parasite interactions occur between the microbial community of the gut and parasite [12,13]. In addition to blood, they take sugar meals from a number of different sources, including the sap of plants or honeydew, through which they may acquire plant bacteria [14].

Although the role of the gut microbiome in the biological cycle of insects is widely recognized, factors modulating the composition of the gut microbiota of sand flies are still poorly defined. Lastly, many studies have aimed to clarify interactions among insect gut microbiota and diet, the local ecosystem and climatic conditions, phylogeny, and life stage. Previous studies reported that variation in the microbiota residing in the insect gut might be mainly explained by the host habitat, diet, developmental stage, and phylogeny all contributing to the structure of insect gut microbiota [15]. Sampling location has been reported to strongly affect the gut microbial community structure of mosquitoes [16], while the composition of the gut microbial community in turtle ants was better explained and influenced by host species [17].

A better understanding of vector-microbiota-pathogen interactions is vital, as it can lead to the discovery of new tools to block disease transmission and provide critical information for the development of intervention strategies for sand fly control. Scientists have discovered a new malaria transmission-blocking microbe in *Anopheles* mosquitoes [18] and *Wolbachia* endosymbionts have already been used successfully when introduced in *Aedes aegypti* to block the transmission of the dengue virus [19–21].

Paratransgenesis is an alternative approach to control disease transmission. In this strategy, bacterial flora native to disease-transmitting vectors are engineered to interfere with pathogen transmission [22,23]. The principal and essential step in paratransgenesis is the identification of suitable bacteria in the vector. These microorganisms should possess the main advantages of being non-pathogenic, easy to cultivate, and easy to genetically manipulate. For example, *Bacillus* (*B.*) *subtilis* and *Brevibacterium linens* isolated from the kala-azar vector *Phlebotomus* (*Ph.*) *argentipes* are currently being considered as possible candidates for paratransgenesis aimed at preventing *Leishmania* (*L.*) *donovani* transmission [24]. The same researchers illustrated the initial proof-of-concept of the paratransgenic approach to *Ph. argentipes* under laboratory conditions. They demonstrated the ability of Green Fluorescent Protein (GFP)- expressing *B. subtilis* to colonize the insect gut [25].

In the following text, we will summarize recent findings that have advanced our knowledge of the complex interactions among sand flies, their microbiota, and the pathogens they transmit. We will open the paper with a general introduction to the sand fly gut bacteria and then turn towards a discussion of particular factors and mechanisms affecting the colonization of the sand fly gut habitat as well as the diverse beneficial roles mediated by the gut microbiota. This review also highlights the technological development that advances our understanding of sand fly microbiota and the use of gut microbiota for novel vector control strategies.

2. Methodological Approaches to Study the Microbiota of Sand Flies

The first paper on sand fly microbiota appeared in 1996 [26]. A culture-dependent method, which involved traditional cultivation on a petri dish containing plate-count agar, was used in that study to isolate bacterial colonies from the guts of field *Ph. papatasi* adults collected from Egypt. Colonies were identified using standard bacteriological methods [27]. Most of the isolated bacteria were *Enterobacteriaceae*, which are ubiquitous, non-fastidious, and fast-growing on the type of medium used. Culture-dependent approaches were increas-

ingly adopted in the subsequent years based on different media and isolation techniques to characterize the bacterial contents in several sand fly species including *Ph. dubosqi*, *Ph. sergenti*, *Ph. kandelakii*, *Ph. perfiliewi*, *Ph. Halepensis*, and *Lutzomyia* (*Lu.*). *longipalpis* [28–31]. In subsequent works, the authors combined both standard bacteriological methods and Sanger-sequencing to identify the taxa (Table 1), and bacterial species considered as potential candidates for paratransgenic or biological approaches for the control of sand fly populations were identified. Recently, researchers became more oriented toward culture-independent approaches because of common problems with bacterial culture including uncultivated bacteria in standard laboratory media. While sequencing information has traditionally been elucidated using a low-throughput technique called Sanger sequencing, high-throughput sequencing (HTS) technologies, now most frequently using Illumina technology, primarily the MiSeq system, are capable of sequencing multiple DNA molecules in parallel, enabling hundreds of millions of DNA molecules to be sequenced at a time. These approaches provide complementary information by producing a more comprehensive view of the bacterial communities that reside in sand flies [32–34]. The hypervariable region(s) of the 16S rRNA gene analysis has become a major tool in the determination of associations among bacteria and it is widely used for identification purposes (Table 1). The variability of 16S rDNA is usually high enough to allow accurate taxonomic characterization but may not always allow unambiguous identification at a lower classification level such as genus or species. On the other hand, different genes beyond the 16S rRNA genes were used to identify gut bacteria, fungi, and viruses [10,31,33,35,36].

Microbial culturomics has recently emerged as a successful tool to isolate high numbers of bacteria and identify yet unknown microbes as a part of the rebirth of culture techniques in microbiology [37–40]. It is a new field that complements molecular techniques and provides another approach to determining the composition of microbial populations, providing exciting new perspectives on host–bacteria associations. Briefly, this approach comprises the combination of multiple culture conditions using different selective and/or enrichment culture conditions followed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) or 16S rDNA amplification and sequencing to identify the growing colonies. This culturomic approach has enabled the culture of 17 new bacterial species that are associated with mosquitoes, suggesting the potential of culturomics to expand our knowledge of the microbiota composition [41]. Despite the public health importance of sand flies, this method has not, to the best of our knowledge, been explored for these insects. Compared with MiSeq which was established to identify unusual, fastidious, or anaerobic bacteria, microbial culturomics has the advantage of providing additional information on the viability of detected symbionts and therefore paratransgenesis candidates. Moreover, culturomics may be more widely used in the future, with advances in automation and when the most effective culture conditions have been defined [42].

Technological advances to examine the microbiota composition in sand flies were described to elucidate the role of bacteria in host phenotypes. The bacterial quantification by both culture-dependent and -independent methods were applied on sand flies fed on an autoclaved regular diet and antibiotics to check their gut bacteria [7,13]. Interestingly, the authors indicated that antibiotics inhibited the growth of a majority of the recoverable sand fly gut bacteria which showed a decrease from 2772 to 10 CFU per gut cultured on LB agar plates [13]. Furthermore, the incorporation of different fluorescent bacteria into the diet and subsequent detection of these fluorescent microorganisms in the gut proved the ability of sand flies' larvae to ingest bacteria [43]. Due to the difficulties associated with obtaining only the midgut of sand flies, whole sand fly bodies were used in the microbiome analyses of several studies [14,32,33]. However, a comparative analysis of microbial communities in whole bodies and midguts from our laboratory-reared *Ph. papatasi* did not reveal any marked difference between samples [33]. It is important to note in this context that a novel bacterial species named *Sphingobacterium phlebotomi* sp. nov., a new member of the family *Sphingobacteriaceae*, was recently isolated from sand fly-rearing substrate [44].

3. Diversity and Composition of the Microbiome in Sand Flies

3.1. Microbiota of Different Sand Fly Developmental Stages

Ingested food and environmental microbes generate larval and adult gut content microbiota. Previous studies on gut microbiota abundance showed that most of these larval-stage bacteria undergo biodegradation during the pupal stage and the bacterial load suddenly and significantly decreases after adult emergence [12,30]. The bacterial composition of larvae, pupae, and newly emerged adults of colony-reared *Ph. duboscqi* was initially investigated using standard bacteriological approaches [30]. In this study, *Ochrobactrum anthropi* was consistently the predominant bacterium at different growth stages, suggesting the presence of bacterial transtadial passage. A more recent study showed the occurrence of *Microbaterium* sp. in immature and adult stages of the same colony-reared sand fly species [12]. On the other hand, many bacterial species were identified in field and laboratory-reared sand flies from the same species [10]. This bacterium is known as a soil microorganism, indicating the influence of external microbial populations on the gut microbiota of immature sand fly stages [45]. This finding suggests that environments play important roles in the colonization of sand flies. The gut composition microbiota across larvae, pupae, and adults of *Lu. evansi* collected from the same locality was recently identified and included a number of soil microorganisms such as *Enterobacter*, *Pseudomonas*, *Bacillus*, and *Lysobacter* genera [46]. The presence of microbial strains in both larvae and adult sand flies will help to implement new efficient biological approaches for the control of sand fly populations in order to prevent *Leishmania* transmission. In a recent study, the genus *Lysinibacillus* was found in the immature (larvae) and adult stages, suggesting that species within this genus could remain transstadially associated with the sand fly [32]. It is important in this context to note that most reports focused on the gut microbial content of field and reared adult sand flies due to the inaccessibility of larvae and pupae in the field.

3.2. Microbiota of Wild and Laboratory Sand Flies

3.2.1. New World Sand Flies

Microbiota composition was analyzed in both laboratory strains and wild populations. The first publication on the gut microbiota composition of New World adult sand flies used a reared laboratory colony and different *Lu. longipalpis* populations collected from Brazil [28,29,47]. The composition of the adult midgut microbiota among different *Lu. longipalpis* populations and reared laboratory colonies showed shared opportunistic pathogenic, environmental, and gut-associated bacterial species including *Pantoea agglomerans*, *Stenotrophomonas maltophilia*, *Enterobacter cloacae*, *Pseudomonas* sp., and *Serratia marcescens*. More recent studies on this sand fly species collected from field and laboratory-reared colonies showed that shared bacteria were *Pantoea*, *Serratia*, *Stenotrophomonas*, and *Erwinia* genera [13,14,28,29,36,47,48]. In the same way, *Staphylococcus*, *Clostridium*, and *Bacillus* genera belonging to the *Firmicutes* phylum were identified in *Lu. longipalpis* colony and field-captured insects [49–51]. These bacterial genera are known as pathogenic to several organisms, and the *Bacillus* genus is currently being considered as a possible candidate for paratransgenesis aimed at preventing *Leishmania* infection [52,53]. These findings indicate the capacity of some bacteria to persist in this sand fly species, despite the difference in field and laboratory conditions.

3.2.2. Old World Sand Flies

Microbiota composition was analyzed in both laboratory strains and wild *Ph. perniciosus* populations collected from Tunisia [10]. In this study, *Stenotrophomonas maltophilia*, *Bacillus* sp., and *Lysinibacillus* sp. were identified in both groups of sand flies. These findings indicate that vector control strategies based on modern biotechnological tools in the laboratory might be applicable in the field.

3.3. Microbiota and Sand Fly Species

The microbiota composition of sand flies was largely summarized in a previous meta-analysis study that included all data obtained until 2017 [10]. Here, we highlight some differences and similarities in data on gut bacteria.

3.3.1. New World Sand Fly Species

The bacterial flora shared between different New World sand fly species collected from the field can be compared with the microbiota of field and laboratory-reared sand flies from the same species. Currently, bacterial communities have been investigated in seven New World sand fly species collected in the field (Table 1): *Lu. evansi* [46], *Lu. longipalpis* [14,28,36,47], *Lu. cruzi* [14], *Lu. intermedia* [54], *Nyssomyia* (*Ny.*) *neivai* (synonymous *Lu. neivai*) [55], *Lu. ayacuchensis* [33], and *Pintomyia (Pi.) (Pifanomyia) evansi* [34]. Very few shared bacteria were identified in these sand fly species collected from the field, probably due to diverse habitats and blood host origins. *Staphylococcus agnetis*, potentially pathogenic to poultry [56] and associated with bovine mastitis [57], was shared among *Lu. cruzi*, *Lu. evansi*, and *Lu. longipalpis*. *Pelomonas* sp. was shared between *Ny. neivai* and *Lu. intermedia*, also found in other insects [58]. Three universal bacterial species were identified in three sand fly species: *Lu. evansi*, *Lu. intermedia*, and *Lu. longipalpis: Acinetobacter calcoaceticus*, known for triggering a detectable immune response in tsetse flies [59], *Enterobacter aerogenes* (found in other insects and potential pathogen to humans) [60], and *Pseudomonas putida* (associated with soil and water) [61,62]. On the other hand, *Ralstonia* sp. (a plant-associated species) [14] was shared among *Ny. neivai*, *Lu. intermedia*, and *Pi. evansi*; *Lawsonella* sp. and *Corynebactrium* sp. (found in other insects) [63,64] between *Lu. ayacuchensis* and *Lu. evansi*; *Escherichia* sp. between *Lu. longipalpis* and *Lu. evansi*.

3.3.2. Old World Sand Fly Species

The microbial gut content of *Ph. papatasi* females has been largely explored. The first publication identified a species pathogenic to humans, *Enterobacter cloaceae* [65], from Egyptian sand flies [26]. More recently, *Microbacterium* sp., pathogenic to insects [66], was detected in Moroccan sand flies [12]. Several groups of researchers conducted the same kinds of experiments in Tunisia, Turkey, and India, and the *Bacillus* genus was the most dominant among genera [67]. Similar results were obtained in Iran and several bacteria genera and species were identified including *Acinetobacter*, *Enterobacter*, *Microbacterium*, *Staphylococcus*, *Terribacillus*, *B. cereus*, *B. flexus*, *B. licheniformis*, *B. pumilus*, *B. subtilis*, *Pseudomonas aeruginosa*, and *Serratia marcescens*. This last species was previously found associated with wild *Lu. longipalpis* [36] and is also lethal to *Leishmania* in vitro [68]. A recent study showed that *B. subtilis* and *Enterobacter cloacae* were shared among the *Ph. papatasi* habitat, rodent *Rhombomys opimus*, and sand fly gut [69]. Other studies on the gut microbial contents of different sand fly species in Iran and India including *Ph. sergenti*, *Ph. kandelakii*, *Ph. perfiliewi*, *Ph. halepensis*, and *Ph. argentipes* showed that they share the *Bacillus* genus with *Ph. papatasi* [11,24,31,70]. The *Pseudomonas* genus was identified recently in *Ph. chinensis* collected from China and shared with the sand flies cited above, except *Ph. argentipes* [8]. In a recent report, the authors showed the influence of both sand fly species and habitats on the microbial gut content of *Ph. perniciosus* collected from Tunisia [10]. An extensive meta-analysis in this study showed proportions of *Acinetobacter baumanii*, *Escherichia coli*, *Stenotrophamonas maltophila*, *B. subtilis*, *Staphylococcus epidermidis*, *Acinetobacter* sp., *Enterobacter* sp. *Klebsiella ozaenae*, and *Serratia* sp. among at least three phlebotomine insects from the Old and New World. A more recent paper showed that host species determine the composition of the prokaryotic microbiota in *Phlebotomus* sand flies collected from Greece [71]. In this study, *Ph. papatasi* microbiota was the most distinct from *Ph. Neglectus*, *Ph. tobbi*, and *Ph. similis*, dominated by *Spiroplasma*, *Wolbachia*, and *Paenibacillus*.

Table 1. Studies analyzing sand fly microbiota using different methodological approaches.

Sand Fly Species	Sand Fly Origin [Reference]	Source	Developmental Stage	Tissue	Methodological Approach
Ph. duboscqi	Senegal [30]	Colony	Larvae, pupae, and adults	Guts	Culturing
Ph. duboscqi	Senegal [12]	Colony	Pupae and adults	Guts	DNA sequencing V6–V8 of 16S rDNA gene
Ph. papatasi	Egypt [26]	Field	Adults	Guts	Culturing
Ph. papatasi	Morocco [12]	Field	Adults	Guts	DNA sequencing V6–V8 of 16S rDNA gene
Ph. papatasi	Iran [31]	Field	Adults	Guts	Culturing
Ph. papatasi	Egypt, India, Tunisia, and Turkey [67]	Colony and field	Adults	Guts	Culturing and DNA Sequencing V1–V9 of 16S rRNA gene
Ph. papatasi	Iran [69]	Field	Adults	Guts	DNA sequencing V1–V9 of 16S rRNA gene
Ph. papatasi	Iran [11]	Field	Adults	Guts	Culturing and DNA Sequencing V1–V2 and V3–V5 of 16S rRNA gene
Ph. papatasi	Greece [71]	Field	Adults	Guts	Illumina MiSeq V4 of 16S rRNA gene
Ph. argentipes	India [24]	Field	Adults	Guts	Culturing and DNA sequencing 16S rDNA gene
Ph. halepensis	Iran [31]	Field	Adults	Guts	Culturing
Ph. halepensis	Iran [11]	Field	Adults	Guts	Culturing and DNA Sequencing V1–V2 and V3–V5 of 16S rRNA gene
Ph. kandelakii	Iran [31]	Field	Adults	Guts	Culturing
Ph. kandelakii	Iran [11]	Field	Adults	Guts	Culturing and DNA Sequencing V1–V2 and V3–V5 of 16S rRNA gene
Ph. perfiliewi	Iran [31]	Field	Adults	Guts	Culturing
Ph. sergenti	Iran [31]	Field	Adults	Guts	Culturing
Ph. chinensis	China [8]	Field	Adults	Whole body	Culturing and DNA Sequencing V1–V9 of 16S rRNA gene
Ph. perniciosus	Tunisia [10]	Colony and field	Adults	Guts	Culturing and DNA Sequencing V3–V5 of 16S rRNA gene and ITS (16S–23S rRNA)
Ph. argentipes	Sri Lanka [70]	Field	Adults	Guts	Culturing and DNA Sequencing V1–V9 of 16S rRNA gene
Ph. neglectus	Greece [71]	Field	Adults	Guts	Illumina MiSeq V4 of 16S rRNA gene
Ph. tobbi	Greece [71]	Field	Adults	Guts	Illumina MiSeq V4 of 16S rRNA gene
Ph. similis	Greece [71]	Field	Adults	Guts	Illumina MiSeq V4 of 16S rRNA gene
Lu. evansi	Colombia [46]	Field	Larvae, pupae, and adults	Guts	Culturing and DNA Sequencing V1–V9 of 16S rRNA gene and ITS (16S–23S rRNA)
Lu. evansi	Colombia [9]	Field	Adults	Guts	Illumina MiSeq V4 of 16S rRNA gene
Lu. longipalpis	Brazil [28]	Field	Adults	Guts	Culturing
Lu. longipalpis	Brazil [29]	Colony	Adults	Guts	Culturing
Lu. longipalpis	Brazil [47]	Field	Adults	Guts	Culturing and DNA Sequencing 16S rDNA gene
Lu. longipalpis	Argentina and Brazil [36]	Field	Adults	Whole body	High-throughput pyrosequencing Total RNA
Lu. longipalpis	Brazil and Colombia [14]	Field	Adults	Whole body	DNA sequencing V1–V9 of 16S rRNA gene

Table 1. *Cont.*

Sand Fly Species	Sand Fly Origin [Reference]	Source	Developmental Stage	Tissue	Methodological Approach
Lu. longipalpis	Brazil [13]	Colony	Adults	Guts	Illumina MiSeq V4 of 16S rRNA gene and 18S rDNA
Lu. longipalpis	Brazil [48]	Field	Adults	Guts	Illumina MiSeq V3–V4 of 16S rRNA gene
Lu. longipalpis	Brazil [32]	Field	Larvae, pupae, and adults	Whole body and guts	Culturing and DNA Sequencing V1–V9 of 16S rRNA gene
Lu. cruzi	Brazil [14]	Field	Adults	Whole body	DNA sequencing V1–V9 of 16S rRNA gene
Lu. intermedia	Brazil [54]	Field	Adults	Guts	Illumina MiSeq V1–V3 16S rDNA
Nyssomyia neivai (syn. Lu. neivai)	Brazil [55]	Field	Adults	Whole body	DNA sequencing 16S rDNA
Lu. ayacuchensis	Ecuador and Peru [33]	Field	Adults	Whole body	Illumina MiSeq V3–V4 16S rDNA gene
Pintomyia evansi	Colombia [34]	Field	Adults	Guts	Illumina MiSeq V4 of 16S rDNA

4. Gut Microbiota Alterations and Their Impact on Flies' Life Traits and *Leishmania* Infection

4.1. Gut Microbiota Alterations and Their Impact on Flies' Life Traits

A previous study reported that the sand fly gut microbiota influences different aspects of flies' life traits [72]. According to this report, the process of laying eggs was more efficient in *Lu. longipalpis* flies fed on rabbit feces than those fed on sterilized feces, which eliminates all rabbit intestinal track-supplied bacteria. The larvae from this last habitat showed delayed hatching and lower survival rates. When different bacteria were reintroduced into sterile feces, there were wide differences in hatching time and survival. On the other hand, it has been demonstrated that all L1-larvae were hatched from homogeneous disinfected eggs and developed on sterilized material [73]. It is important to mention that although bacterial diversity decreases after a blood meal, bacterial numbers actually increase [13,30]. These findings provide new data on microbial dynamics in the sand fly gut which may be used for the development of novel control strategies. The larval nutrition associated with the putative breeding sites of the sand fly *Lu. longipalpis* might affect their oviposition, development, microbiome, and susceptibility to *Leishmania* which plays an important role in the epidemiology of leishmaniasis [73].

4.2. Gut Microbiota Alterations and Their Impact on Leishmania Infection

The influence of the microbial contents on *Leishmania* development in sand flies was investigated in several previous studies. *Serratia marcescens*, which are considered to be pathogenic bacteria for many insects [74], negatively affect *L. infantum chagasi* and *L. braziliensis* by inducing lysis in vitro of the parasite cell membrane [68,75]. Furthermore, it has been demonstrated in vivo that the infection rate of *L. mexicana* in *Lu. longipalpis* sand flies was reduced when fed on *Pseudozyma* sp., *Asaia* sp., or *Ochrobactrum intermedium* [76]. The same experiments, in which *L. mexicana* colonized the sand fly gut prior to being fed *Serratia marcescens*, showed that the survival of flies with a *Leishmania* infection was significantly higher compared with those without *Leishmania* infection. This might be due to the protection offered by *Leishmania* to the sand fly from the bacterial infection or to modulation of the host immunity response by this parasite, as reported in other models such as *Anopheles gambiae* infected with *Plasmodium* [77]. In the same context, *Ph. papatasi* was treated with *an* antibiotic cocktail to deplete gut bacteria and was experimentally infected by *Leishmania*. The bacterial composition of the gut was previously reported to either enhance or inhibit *Leishmania* activity. Previous studies showed that treatment with antibiotics reduces the

richness and diversity of microbiota, but *Leishmania* infection increases, indicating that the microbiota can be a barrier to the establishment and development of promastigotes in *Ph. papatasi* and *Pi. evansi* [34,78]. These findings strengthened the theory that any manipulation that reduces the size and/or diversity of the natural microbiota should enhance the ability of *Leishmania* to establish infections in sand flies or other pathogens in mosquitoes [79]. It has been demonstrated that endosymbionts, such as Microsporidia infections, were more frequently associated with guts without *Leishmania* infection, whereas *Arsenophonus* was only found in guts with a high load of *Leishmania* infection and treated with antibiotics [34]. It has been shown that Microsporodia impairs *Plasmodium falciparum* transmission in *Anopheles arabiensis* mosquitoes [80]. This finding is in agreement with the previous study of the potential influence of this endosymbiont on *Leishmania*. On the other hand, in *Ph. dubosqui* and *Lu. longipalpis*, treatment with antibiotics results in females being highly refractory to the development of transmissible infections [7,13]. It has been demonstrated, for example, that sucrose utilization by the microbiota is essential to promote the appropriate osmotic conditions required for the survival of infective stage promastigotes in vivo [7]. Together, these diverse data suggest that the sand fly midgut microbiome is a critical factor for *Leishmania* growth and differentiation to its infective state prior to disease transmission. As part of a paratransgenic approach, further studies are needed to identify candidate bacteria that can be used, or other biological approaches, to control sand fly populations and *Leishmania* transmission [10]. A more recent study showed that *Lysinibacillus*, *Pseudocitrobacter*, and *Serratia*, which are potential candidates for paratransgenic or biological control, strongly inhibited *Leishmania* growth and survival in vitro and co-infected *Lu. longipalpis* [32].

5. Microbiota-Driven Mechanisms Affecting Vector Competence

5.1. Genetic Determinants of Sand Fly Competence for Leishmania Parasites

Previous studies highlighted, among other factors, the role of host phylogeny in the composition of sand flies and other insect gut microbial communities [17,71,81,82] which may influence susceptibility to *Leishmania* infection. In addition to the species differences, intraspecific population divergence caused by multiple environmental factors such as climate, distance, altitude, and geographic barriers has been suggested to influence vector competence [83–85]. A previous study clearly demonstrated apparent genetic divergence among populations of *Lu. ayacuchensis* with different vector competence [86]. In a subsequent study, the role of the existing midgut bacterial microbiota in the observed phenotypic variability in the susceptibility phenotype was investigated [33]. The findings suggest that the ecological diversity of sand flies may contribute to shaping the structure of associated microbiota, which may influence susceptibility to *Leishmania* infection; however, it was not possible to elucidate their role or conclude clear outcomes regarding potential pathogenic effects or interactions with *Leishmania*. Previous studies focused on natural vector-parasite interactions and confirmed that mosquito genetics play a major role in determining vector competence [87,88]. Moreover, a primary evolutionary consequence of environmental variations is the maintenance of genetic diversity in both host and parasite populations, provided some genotypes are favored in one environment, whereas other genotypes perform better in other environments [89,90].

5.2. Non-Genetic Determinants of Sand Fly Competence for Leishmania Parasites

The ability of symbiotic bacteria to inhibit pathogen colonization is mediated via several mechanisms. The microbiota promotes direct colonization resistance through (1) killing: it has already been suggested that killing or growth suppression may play a dominant role in colonization resistance against some pathogens [91,92]; (2) Inhibitory metabolites: metabolic byproducts produced by bacteria can also have an inhibitory effect on other bacteria, which may influence pathogen development [92–94]; (3) Competition for nutrients and space: previous experiments reported that substrate limitation was an important determinant of successful colonization of the bacterial gut [92,95,96]; and

(4) Physical niches: it has been shown that, in addition to functional nutrient-based niches, bacteria must compete for physical space. Indeed, some species prefer living on the food matter in the lumen, in the outer mucus layer, or more rarely at the epithelial surface. Close physical contact with the epithelium is an essential part of some pathogens' lifestyles, so physical competition for adhesion sites (often glycan structures) could prevent infection or pathology [92,97] (Figure 1). Interestingly, microbes can also change the presence of host adhesion sites indirectly. Aside from direct competition, microbes can compete with pathogens indirectly by acting on the host. This usually involves stimulation of the innate or adaptive immune system (Figure 1), but other non-immune defenses can also take part. For example, a recent study suggested that sucrose utilization by the microbiota is essential to promote the appropriate osmotic conditions required for the survival of infective stage promastigotes in vivo [7].

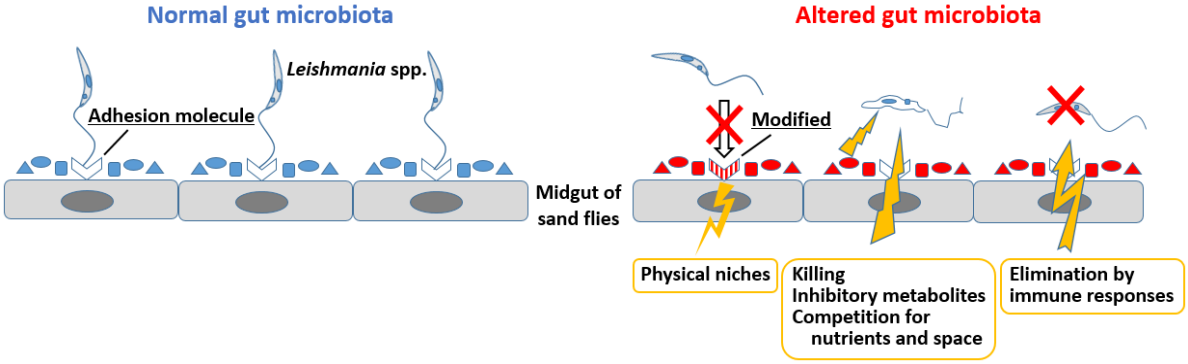

Figure 1. Factors interacting with microbial symbionts and determining vector competence of sand flies.

The sand fly immune response is constantly regulated to balance and respond to different microbial challenges [98]. Simultaneously, *Leishmania* parasites can adapt to these changes by expressing a plethora of genes during their cycle [99]. Previous studies reported that the Toll and immune deficiency (IMD) pathways and antimicrobial peptides (AMPs) could affect both *Leishmania* and gut bacteria [100–104]. On the other hand, the first report on putative sand fly genes involved in the Janus-kinase/signal transducers and activators of transcription (Jak-STAT) pathway has just been published and it provides additional information on the complex interaction between the *Lu. longipalpis* immune response to *L. infantum* [105]. The authors used the strategy of depleting the commensal gut bacteria with antibiotics to investigate its impact on the expression of Jak-STAT-related genes in *Leishmania*-infected females. Suppression of the JAK-STAT pathway favored *Leishmania* growth in sand flies on the first day post-infection, although it was insufficient to yield a higher rate of infection on late-phase infection.

Although the above studies offered some basic knowledge on the genetic and molecular mechanisms underlying sand fly suitability for *Leishmania* parasites, they mostly involved unnatural laboratory host-parasite associations, which can be poor approximations of what occurs in natural ecosystems. Previous studies suggested that both extrinsic and intrinsic factors can also play critical roles in modulating host-parasite interactions [33,89,90] (Figure 2). It has been demonstrated in mosquitoes that non-genetic factors influence vector competence for malaria parasites. The authors reported that factors such as temperature, mosquito larval and adult diets, and the maternal environment can affect microbial gut flora and play a major role, comparable to host and parasite genotypes, in shaping mosquito competence (direct effects on parasite versus indirect effects via mosquito immune defenses) [106] therefore affecting the epidemiological conditions. In addition to immunological defenses, insect hosts can use behavioral defenses including behavioral fever and self-medication to better resist or tolerate their parasites [107–110]. In sand flies, it has been suggested that the richness of the gut microbiota may be related to seasonal

activity [10]. It has demonstrated that a high bacterial load and diversity decreased competence in mosquitoes (i.e., aseptic mosquitoes harbored about eight times more oocysts than their septic counterparts) [79,111,112]. Under field conditions, insect populations are made up of individuals that differ not only in their genetic background but also in regard to factors affecting their gut microbiota such as age and reproductive status. This heterogeneity can have important consequences for vector competence. For example, the host immune system may weaken with age, resulting in increased susceptibility to pathogens [106].

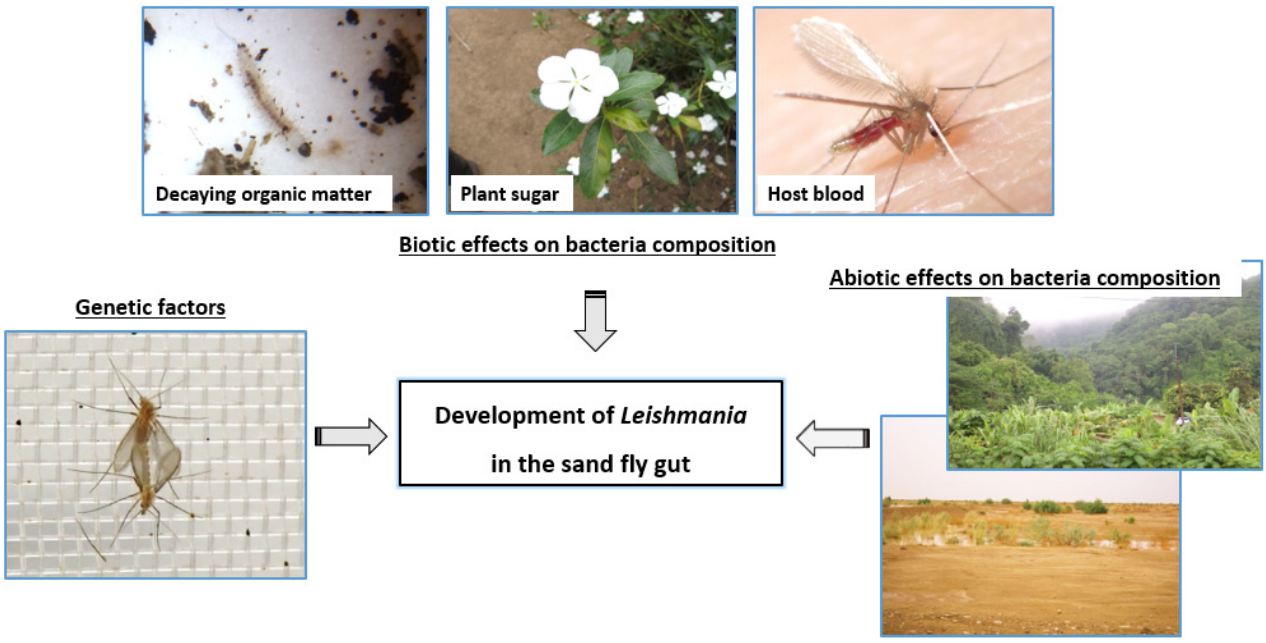

Figure 2. Extrinsic and intrinsic factors that may influence microbial symbionts which affect sand fly competence for *Leishmania* parasites.

6. Fungi Associated with the Midgut of Sand Flies

Although the bacterial component of sand fly microbiota has been investigated in several studies, few papers reported on the fungal diversity in sand flies [31,36,113]. A comparative analysis of fungal communities revealed the absence of fungi in *Lu. longipalpis* guts collected from an endemic area, whereas fungi were found in a non-endemic area, including *Cunninghamella bertholletiae*, *Peronospora conglomerata*, *Mortierella verticillata*, and *Toxicocladosporium irritans* [36], suggesting that fungi are excluded in the presence of *Leishmania*. However, contradictory findings identified fungal genera in sand flies collected from endemic areas of northern Iran and southern Peru, including *Ph. papatasi*, *Ph. sergenti*, *Ph. kandelakii*, *Ph. perfiliewi*, *Ph. halepensis*, and *Lu. ayachensis* [31,33]. In these areas, species belonging to *Penicillium*, *Aspergillus*, *Acremonium*, *Fusarium*, *Geotrichum*, *Candida*, and *Malassezia* genera were identified [31,33,113]. However, it was not possible to elucidate their role or conclude any outcomes regarding potential pathogenic effects or interactions with *Leishmania*.

7. Microbiota as a Target for Novel Vector Control Strategies

The lack of an effective vaccine against leishmaniasis, narrow ranges of effectiveness and unfavorable side effects of available drugs, and development of drug resistance in the parasite highlight the need for novel approaches to control vector transmission of *Leishmania* [11]. Furthermore, emerging knowledge of vector-microbiota-pathogen interactions can lead to the discovery of new tools to block disease transmission and provide critical information for the development of intervention strategies for sand fly control. Two main strategies may be applied in vector control using microbiota: the first strategy consists of using microbiota taxa that were shown to have physiological impacts on the host or display

anti-vector and anti-pathogen effects. The second alternative strategy, the paratrangenic strategy, involves transforming symbiotic or commensal microbes of host insects to express gene products that interfere with pathogen transmission.

7.1. Introduction of Symbionts to Manipulate Host Life Traits

According to a previous report, bacteria have an important impact on different aspects of life-history traits, starting with early development stages [72]. It was demonstrated in this study that *Lu. longipalpis* flies fed a diet containing raw rabbit feces were much more likely to lay eggs than flies fed sterilized feces. A delayed hatching time and altered survival were noted when different bacteria were reintroduced into sterile feces, showing the importance of bacteria presence for insect development [72]. These findings may reveal additional candidates to be explored as tools for sand fly population control. In mosquitoes, bacterial endosymbionts, such as *Wolbachia*, *Spiroplasma*, and *Arsenophonus*, are capable of manipulating host reproduction [114–117]. Some entomopathogenic fungi shorten the mosquito lifespan or reduce blood feeding success [118].

7.2. Exploitation of Endosymbionts with Antipathogen Effects

A number of microbiota members were shown to have antipathogen activities. For example, a new study that has just been published showed that *Lysinibacillus*, *Pseudocitrobacter*, and *Serratia*, which are potential candidates for paratransgenic or biological control, strongly inhibited *Leishmania* growth and survival in vitro and in co-infected *Lu. longipalpis* [32]. Furthermore, there is special interest in the *Bacillus* species for potential sand fly biological control. *B. subtilis* isolated from *Ph. argentipes* is currently being considered as a possible candidate for paratransgenesis aimed at preventing *L. donovani* transmission [24,25]. Bacteria belonging to the *Bacillus* genus display a host-specific distribution, with only *B. subtilis* being isolated in more than one sand fly species, leading to the implementation of action control strategies to block disease transmission [10,11]. On the other hand, *Wolbachia* has been detected in sand flies of the genera *Phlebotomus* and *Lutzomyia* [8,33,71], but the impact of *Wolbachia* on the *Leishmania* infection load has not been reported. *Wolbachia* endosymbionts have already been used successfully when introduced in *Aedes aegypti* to block the transmission of the dengue virus [19,21].

7.3. Paratransgenesis Approaches

In the paratransgenic strategy, symbiotic microbes of host insects need to be identified and transformed to express gene products that interfere with pathogen transmission. These genetically altered microbes, which are engineered to interfere with pathogen transmission, are re-introduced back into the insect. To date, there are no known reports of any paratransgenic platform to control the transmission of leishmaniasis. Below, we describe how this approach should be utilized to render sand flies resistant to *Leishmania* infections, already attempted by Hurwitz et al. [25]. This approach has been successfully utilized to reduce the carriage rates of *Trypanosoma cruzi*, the causative agent of Chagas disease, in the triatomine bug *Rhodnius prolixus* [119,120].

There are several requirements for a paratransgenic approach. The identification of suitable symbionts that are non-pathogenic to humans or animals among the commensal bacteria that insects harbor is paramount for the success of a paratransgenic system [24]. These bacterial symbionts should be amenable to culture and genetic manipulations. Identification and characterization of sand fly breeding sites will be of marked importance to introducing transformed commensal bacteria. Sand flies, unlike mosquitoes, do not breed in water and there is relatively little information on their breeding sites [121]. Although small numbers of larvae have been recovered from diverse habitats [121,122], more productive sites have been identified in Sardinia and Panama where several hundred *Phlebotomus* (Larrousius) spp. and over two thousand *Lutzomyia* spp. larvae were found, respectively [123–126]. Moreover, modified microbes should show vertical transmission to the progeny, allowing their self-sustenance in the field [127]. Indeed, although trans-stadial

bacterial colonization of the adult gut is known to occur in sand flies, most microorganisms are eliminated due to physiological changes during metamorphosis [72,128]. The modified bacteria should be adapted not only to sand fly guts but also be able to compete with other bacteria in the digestive tract as well as have a life cycle compatible with the adult sand fly vector so that they can readily colonize the midgut to produce the effectors in the necessary amounts [129]. The chosen bacteria should be capable of colonizing a wide variety of insect species, increasing the range of sand flies that can be controlled without affecting the life history traits of the insect vector. Furthermore, the number of bacteria increases markedly after ingestion of blood [13] which increases the number of the modified bacteria leading to various unknown outcomes, such as those reported in mosquitoes [22,130–132]. Producing recombinant bacteria in sufficient numbers is much simpler than creating transgenic sand flies.

8. Conclusions

Data on the microbial consortium associated with sand fly microbiota have highlighted that microorganisms influence many aspects of host biology including their survival, development, and vector competence. This means that these insects can no longer be considered isolated entities and instead should be considered inseparable from the microbiota with which they interact and form a holobiont. Interestingly, sand fly microbiota has been reported to enhance or inhibit *Leishmania* development and transmission. Moreover, the sand fly gut-associated microbiota continues to accompany the pathogens deposited during the *Leishmania*-infected sand fly bite, potentially affecting certain clinical outcomes. Achieving a deeper understanding of the physiological, metabolic, and molecular mechanisms underlying the interaction between microbiota and pathogens is essential to promoting the development of new vector control strategies. Since environmental factors have been reported to affect parasite development and insects' physiological state and immune response, which can all interact with microbial symbionts, sand fly microbiota should be described in light of its close connections with the environment to explore how environmental parameters interact with these potential biocontrol agents.

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