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Overview of microbial studies in sandflies and their progress toward development of paratransgenic approach for the control of *Leishmania* sp.

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During their whole life insects are exposed to the impact of different microbes from various sources. The role of microbes in the life of insects is of immense importance, and there is an ample of evidence showing that microbes can affect different aspects of insect physiology, behaviors as well as their potential and capacity to transmit pathogens. Sandflies are main vectors of *Leishmania* parasites – causative agent of leishmaniasis. Leishmaniasis is a difficult, disfiguring and if not treated deadly disease ranked among the top 10 neglected tropical diseases. In comparison to other medically and veterinary important insect vectors where microbial investigations already progressed toward field studies and practical application of paratransgenic approach, in the field of sandflies and leishmaniasis control microbial investigations are at their beginning. This mini review provides a summary of the key moments in the overall microbial studies in sandflies and highlights the urgency of detailed microbial investigations to progress toward the development of paratransgenic approach.

KEYWORDS

sandfly, microbiome, paratransgenesis, leishmaniasis, biological control

Introduction

The members of the Diptera order are among the most significant insect disease vectors, and they are involved in transmission of pathogens causing diseases such as malaria, dengue, West Nile fever, Chagas disease, African sleeping sickness, leishmaniasis etc. Among Diptera, mosquitoes alone are responsible for as many as 1 million deaths annually and are of major health care concern on the global level (1). Compared with mosquitos, the mortality rate caused by sandfly borne diseases is significantly lower with approximately 70.000 reported cases per year (2). Regardless of this, sandflies are considered as very dangerous insect disease vectors, as they are the main vectors of *Leishmania* parasites - the causative agent of leishmaniasis. Leishmaniasis is a difficult, disfiguring and if not treated deadly disease, endemic in more than 100 countries all around the world (2). Globally, leishmaniasis is ranked among the top 10 neglected tropical diseases, with over 1 billion of people being in risk of acquiring the disease and more than 12 million people being infected (3). Highest number of leishmaniasis cases have been reported in Brazil, Colombia, Peru, India, Sudan, and Kenya, marking leishmaniasis as a neglected disease of poverty. Due to the severely lacking reporting system, it is suspected that number of cases is significantly higher, as only 20-40% of visceral leishmaniasis gets reported to WHO.

Sandflies are insects that thrive in warm climate, and the effect of global warming, along with the increased international travel/trade and destruction of natural habitats, all served as an incentive to support the spread of sandflies and ultimately diseases they transmit (4). Sandflies are known to transmit bacteria from *Bartonella* genus and several arboviruses with most prominent members belonging to *Phlebovirus* genus; but the biggest health care concern has been the continuous spread and (re)emergence of leishmaniasis in high proportions. The increased number of cases has been observed in correlation with SARS-CoV-2 virus pandemic, as most of the leishmaniasis control strategies have been neglected due the overbearing impact of SARS-CoV-2 on economy and healthcare (5). The great success in leishmaniasis spread is additionally supported by the complex transmission cycle of the *Leishmania* parasites. Among 1000 species, and subspecies of sandflies that are identified, close to 100 are involved in transmission of *Leishmania* parasites. Presence of *Leishmania* parasites have been detected in more than 90 animal species (including humans) with potential to be hosts/reservoirs of the disease, with 20 *Leishmania* species being pathogenic for humans (6).

With this being said, the need for the establishment and continuous implementation of effective sandfly vector/disease control is vital. Currently used control methods generally consider the use of several strategies and are focused either on preventing the sandfly from transmitting the disease, or on treatment of *Leishmania* infected hosts. Successful sandfly vector management proved to be among the most challenging tasks in leishmaniasis control. Vector management that greatly relies on insecticide use faced the major setback when negative effect of these chemical compounds was revealed (7). The implementation of the chemical approach was additionally hampered by fast development of insecticide resistance in many sandfly species (8). These obstacles clearly pointed out toward the great urgency of developing new, biologically friendly control methods. Hence, in recent years exploration of microorganisms, and their potential in *Leishmania* control (re)gained significant interest among the scientific community. This mini review briefly summarizes the key moments of the overall microbial studies in sandflies and highlights the urgency of detailed microbial investigations in order to progress toward development of paratransgenic approach and its future application in the field.

Literature search strategy

An intensive literature search was carried out by screening six online databases (National Center for Biotechnology Information, Web of Science, Science Direct, PubMed, Scopus, and Google Scholar). The search was conducted using a terminology in English language. Commonly accepted terms for sandflies (in singular and plural form), as well as specific names of sandfly genuses and several prominent species were combined with terms “microbiome”, “microorganism”, “bacteria”, “paratransgenesis”, “metagenome”, “culturomics”, and “biological control”. All publications identified during the initial search were further screened based on their content, and studies that didn’t match the topic of this mini review were removed. Reference lists of these remaining articles were screened for other topic matching publications (in English or other languages). In total, more than 100 publications were processed during the final filtering. For this mini review only publications that are most informative and present in full, regardless of the publishing language were chosen. Studies that were too narrow, present only in the form of abstract, or showed duplicate, insufficient, or not relevant data were removed.

Brief overview of microbiome studies in sandflies

Investigations of the sandfly microbiome commenced nearly 40 years ago. First reports on sandfly microbiome date from 1985 and describe microbial composition of colony-reared *Phlebotomus papatasi*, and *P. tobbi* collected from Greece (9). After a decade long gap, research was resumed in 1996 and studies were conducted on *P. papatasi* collected in Egypt (10), *Lutzomyia longipalpis* from Brazil (11, 12) and colony-reared *P. duboscqui* (13). After these publications, slow but steady interest in the topic was noted, and over the past two decades the research of microbial communities associated with sandflies gained significant relevance.

From three sandfly genera - *Phlebotomus*, *Chinius*, and *Sergentomyia*, that constitute the sandfly fauna of the Old World, and approximately 400 identified sandfly species (14), the microbiome has been investigated in only a handful of species. The microbial component among the sandfly vector species present in the Old World is best explored in *P. papatasi*. *Phlebotomus papatasi* is a proven vector of *Leishmania major* (15), and has a wide distribution in the Old World with its presence being reported in more than 25 countries (16). Regardless of *P. papatasi* wide distribution and its immense importance in the transmission of *L. major*, the microbial composition of *P. papatasi* samples collected from the nature has been investigated only in Greece (17), Marocco (18), Egypt (10), Iran (19, 20), India, Turkey and Tunisia (21, 22). From other important vector species present in the Old World, microbiome was investigated in limited number of species including the *P. major*, *P. sergenti*, P. *kandelakii*, *P. perfiliewi*, *P. alexandri*, and *P. halepensis* collected from Iran (19, 23, 24), *P. argentipes* from India and Sri Lanka (25, 26), *P. chinensis* from China (27), *P. perniciosus* from Tunisia (28), *P. neglectus*, *P. tobbi*, and *P. similis* from Greece (17). Besides samples collected from the nature, microbial composition was explored on the colony-reared sandflies of *P. papatasi* (9, 22, 29–31), *P. dubiscqui* (13, 18, 30) and *P. perniciosus* (28).

The sandfly fauna of the New World is even greater, and it is comprised of approximately 600 species. Unfortunately, from the medically and veterinary important sandfly vectors in the New World, the microbial component is best explored in *Lu. longipalpis* collected from Brazil (11, 32–34). Other sparsely investigated species of New World sandflies include *Lu. evansi* (Colombian Caribbean coast (35–37)), *L. cruzi* (Brazil (32)), *Lu. intermedia* (Brazil (38)), *Lu. maranonensis* (Peru (39)), *Lu. ayacuchensis* (Equador and Peru (40)), and *Lu. cayennensis* and *Lu. dubitans* (Colombian Caribbean coast (37)).

The sandfly fauna of the world is accounting roughly 1000 species. Many of them are of medical and veterinary importance, and the control of their populations plays a key role in suppressing leishmaniasis spread and (re)emergence. Despite the great diversity of sandflies and considerable research potential related to the sandfly microbiome investigations, this research topic remains severely geographically restricted and limited to the several key sandfly species.

The microbial role in direct and indirect *Leishmania* control

The role of microbiome in the life of insects is crucial, and there is an ample of evidence that microbial symbionts can supplement insects with essential dietary elements, modulate their signaling pathways, trigger immunity responses, support insects' ability to degrade chemical pesticides etc. Microbial component can also impact insect development, morphogenesis, longevity, fitness, behavior, fecundity etc. (41).

From a perspective of microbial role in the life of insects that have medical and veterinary importance, it has been evidenced that microbes can affect insects' competence and potential to transmit pathogens. The microbial role in different pathogen ablation/suppression was extensively investigated in mosquitoes, triatomine bugs and tsetse flies, resulting in a successful development of the paratransgenic approach for the control of various diseases these insects transmit (42).

In the field of sandflies and leishmaniasis, the investigations related to the microbiome and its role in overall life cycle of sandflies and *Leishmania* development/transmission are still at its beginnings. In the recent years colony-reared sandflies have been used for the investigation of mutual interactions of *Leishmania* parasites and microbiome (43–46). The study of Hassan et al. (43) evaluated the effect of microbiome on the development of *L. major* in its natural vector – *P. papatasi*. Obtained results showed that aposymbiotic sandflies were more susceptible to the *L. major* infection rather than sandflies with viable microbiome present in their guts (43). Authors suggested that midgut bacteria could play a significant role in inhibition of *Leishmania* development, thus preventing the parasite transmission. The negative effect of bacteria on *Leishmania* development was also demonstrated within the study of Sant'Anna et al. (46) where *Lutzomyia longipalpis* sandflies were prefeed on a sugar meal containing bacteria and then fed on a bloodmeal containing *Leishmania mexicana* parasites. Authors observed that bacteria prefeed sandflies had reduced number of parasites in the gut (46). Further evidence to support the role of microbiome in *Leishmania* refraction were obtained from the studies of Vivero et al. (45). Authors investigated the role of microbiome in susceptibility to *L. infantum* infection within *Pintomyia evansi*. Results suggested that a fraction of the intestinal microbiota of *P. evansi* may have a protective role and/or prevent the development or establishment of *L. infantum*. Most recently the study of Kykalova et al. (47) showed that the induced variation of the gut microbiota may offer a considerable challenges for the *Leishmania* parasites, and speculated that the outcome of this balance reveals the *Leishmania* adaptability potential. On the contrary to these studies, the Louradour et al. (44) demonstrated the positive correlation between the population size of the midgut microbiome and the number of *L. major* metacyclic promastigotes (44). Although results of these studies are slightly contradictory, there are irrefutable evidence that microbiome plays a role in *Leishmania* development directly in the sandfly vectors, and that possibility of utilizing microbiome in the control of leishmaniasis shouldn't be disregarded. In addition to these studies that explored the interactions of microbiota with *Leishmania* parasites in the sandfly vectors, several studies have been conducted under *in vitro* conditions supporting the ablative potential of bacteria toward *Leishmania* (43).

Besides above-mentioned studies where direct impact of microbiome on *Leishmania* parasite was demonstrated, the microbiome can have an indirect impact on the insect vector by controlling insect populations via entomopathogenic microorganisms such as bacteria, fungi, nematodes etc. (48), or by modulating insects longevity, fecundity etc. which subsequently affects its transmission potential. The results of the Peterkova-Koci et al. (49) study suggested that presence/absence of the microbial component in the nutrition of sandflies impacts the sandfly development as well as their fecundity potential. Although microbiome-insect host interactions were vastly explored in other insects, the effect of the microbiome on aspects of sandfly physiology was evaluated only superficially. It is necessary to firstly understand the impact of the microbiome on the sandfly host, to proceed toward interpretations of their interactions with *Leishmania*. Therefore, it would be advisable to perform various studies on the microbiome-sandfly model and determine their interactions before paratransgenic studies are instigated.

Current focus and future perspectives of microbiome studies in sandflies

Several approaches are currently being used in order to identify and describe the sandfly microbiome. The oldest approach – culturing, is based on growing and isolating bacterial colonies.

The early published papers in the field of microbial analysis in sandflies, starting from 1985 all up to 2002, were mainly based on the use of culture-dependent techniques. In the subsequent works along with the development of modern techniques such as NGS, the authors combined both culture-dependent and culture-independent methods. In contrast to the culturing, culture-independent methods are focused on retrieving and investigating genetic material under the scope of metagenomic. The fast advancements of modern techniques and their accessibility rapidly revived the studies of the sandfly microbiota. Hence, in recent years the scientific community almost completely refocused their efforts on metagenomic approach, and it has been silently accepted that culture dependent techniques won't be needed any more.

Despite the enormous potential of sequence screening and metagenomic approach, it has become evident that these new methods have multiple drawbacks, starting with the most common problem of incomplete and outdated genomic databases (50). These databases do not follow the current taxonomical developments, which are ever changing, especially in the field of microbiology. This is resulting in significant difficulties in assigning a precise taxonomic rank to a sizeable number of sequences. On the other hand, for the practical application of these findings (as it is the case with paratransgenesis), modern methods do not provide a proof-of-concept sample, i.e. the pure culture of the microorganism which is crucial for further investigations (51). Based on the well-established criteria that defines the paratransgenic microbiota validity (42), for bacteria to be considered as paratransgenic candidate, it must be cultured, amenable for genetic modification etc. (Criteria are given in "Paratransgenesis for leishmaniasis control" section of this article). The metagenomic approach unfortunately doesn't provide information on none of these criteria. Having this in mind, in recent years a handful of scientists redirected their approach back toward culturomics. Microbial culturomics approach has been originally introduced in order to optimize the culture conditions, and disprove the general belief that most of the microorganisms are uncultivable (52). Within microbial culturomics, for the full microbial identification in addition to the practical approach of culturing under different conditions, culture techniques are coupled with MALDI-TOF MS and/or 16S rRNA gene sequencing. The complementarity between culture-dependent and culture-independent studies was well proven (53), and culturomics has played an important role even in the description of the human microbiota (53, 54). These two approaches complement one another and should be used together in order to fully describe the sandfly microbiota with more efficiency, as well as to provide a biologically viable sample for further investigations.

Paratransgenesis for leishmaniasis control

Paratransgenesis is a promising new strategy currently being developed for the control of various vector-transmitted diseases including leishmaniasis. Paratransgenesis utilizes the native microbiome of the insect vector that has been genetically manipulated, to inhibit or kill the disease pathogen (42). Generally, the native symbionts or commensals are isolated from the vector and genetically transformed *in vitro* conditions to produce antipathogen factors. These transformed microbes are then reintroduced into the insect where they interrupt the life cycle of the pathogen. Most used organisms for paratransgenic approach are mutualistic symbiotic and commensal bacteria, fungi, and occasionally viruses (42).

When compared to the traditional approach in sandfly/leishmaniasis control that relies on massive use of insecticides, the application of paratransgenesis has multiple benefits. Besides being eco-friendly and not causing massive negative effect on environment and non-targeted organisms, paratransgenic approach might be even beneficial from economic point of view, as large numbers of transformed microbes can be produced under laboratory conditions. These transformed microorganisms usually undergo a massive multiplication in the insect vector and may be passed horizontally/vertically from one generation to another. Furthermore, they can have the ability to colonize a wide range of different insect vector species, which is especially important in leishmaniasis control since different sandfly species can transmit the same *Leishmania* species.

There are several criteria for microorganisms to be considered as a viable candidates for paratransgenesis. Among the most important criteria is the fact that microorganisms must be a culturable symbionts or commensals susceptible to genetic manipulation. This microorganism should occupy the same body tissue as the pathogen in the insect host, with a potential to colonize a range of insect species/strains. Additionally, the microorganism shouldn't be pathogenic to humans/animals, and should colonize all developmental stages of the insect i.e. demonstrate the transstadial potential (42).

Current status and possibilities for further progress in developing paratransgenic approach in sandflies

With the recent revival of the interest in microbiome studies in sandflies, quite a substantial number of publications were generated. Most of the studies were focused on the sole identification of microbial composition within the sandfly gut, or the whole insect. Regardless of this immense effort, the identification of suitable bacteria (maintainable in culture, has transstadial potential, is non-pathogenic to humans, can be modified, widespread among sandflies etc.) still remains the biggest obstacle in developing the paratransgenic approach in sandflies. In the recent years several review articles were published summarizing the list of identified bacteria (55, 56). Gram-negative bacteria of the Proteobacteria phylum, and Gram-positive bacteria from the phylum Firmicutes and Actinobacteria are among the most commonly found bacteria within sandflies. Several bacterial species, including *Serratia marcescens*, *Pantoea agglomeratus*, *Enterobacter cloacae* and

Escherichia coli, that have been identified in sandflies of different species, have been previously tested in mosquitoes or triatomines. As some of these species are pathogenic for humans (which directly contradicts to the above stated criteria for paratransgenic candidate incrimination) or are entomopathogenic (which may cause problems with bacteria reintroduction to the insect), further studies on detection and identification of alternative candidate species should be intensified.

Demonstrating a transstadial potential i.e. ability to survive the full metamorphosis and be found among all developmental stages of sandflies is one of the main prerequisites in incriminating bacteria as a suitable paratransgenic candidate. Despite the fact that many bacteria have been recorded among the wild and colony reared sandflies (55, 56), only several were evaluated for their transstadial potential. Findings of Malta et al. (57) showed that some midgut bacteria are able to survive the metamorphosis despite being exposed to the gut histolysis and remodeling. Morphological changes in the sandfly midgut commence during the 4th larval instar, and epithelial degeneration is followed by the remodeling with the differentiation of regenerative cells in pre-pupa and pupa (57). First bacteria incriminated with transstadial potential belonged to *Ochrobactrum* genus and they were isolated from the guts of larvae, pupa and freshly emerged females of the colony reared *Phlebotomus duboscqi* (13). *Lysinibacillus fusiformis* and *Bacillus cereus* were found among all investigated developmental stages of *P. argentipes*, revealing transstadial passage of these endogenous microbes (58). Most recently Vaselek et al. (31) demonstrated a full transstadial transmission of *Ochrobactrum intermedium*. This bacterium was found in the gut of all immature stages including larvae, pre-pupa and pupa. The presence of *O. intermedium* in adult stage was demonstrated in newly emerged adults of both genders, where bacterial recovery and increase in bacterial abundance was noted (31). The suitability of this bacteria was further evaluated in engorged females showing their ability to remain in the gut even after blood defecation (31). Up to this point, within the field of sandflies only several studies have been conducted that are leading toward the design of the paratransgenic approach. The study of Abassi et al. (59) investigated the colonization of *P. papatasi* with *Enterobacter cloacae* subsp. *dissolvens* that was modified to express a defensin gene. Modified bacteria were transformed with a red fluorescent protein in addition to the defensin plasmid and offered to a 1st instar sandfly larva. Examination of the microbial composition of the immature stages revealed that transformed bacteria plasmid remained in the sandfly gut for up to 36 days post-feeding (59). Unlike *Bacillus* (58) or *Ochrobactrum* (31), this bacterium unfortunately didn't demonstrate the transstadial transmission as its presence has not been detected in guts of adult flies (59).

In order to progress with the development of microbial/paratransgenic studies in sandflies, among others it will be necessary to identify microbial component (bacteria, fungi, or even virus) that is shared between wide range of sandfly species. It would be recommended that sandfly microbiome studies be expanded to cover other sandfly species in addition to the few species that are currently investigated. Identified microbes should be cultivated and evaluated directly in the sandfly vectors for their ability to ablate *Leishmania*, as well as assessed for their potential to survive a full metamorphosis (demonstrate the transstadial potential). In addition, the mechanism of interaction on the level microbes-*Leishmania*-sandfly need to be elucidated. More intense and in-depth studies need to be conducted in this area to successfully target appropriate genes responsible for *Leishmania* ablation and further use them for bacteria modification.

Conclusions

Leishmania parasites develop exclusively in the sandfly gut and as such they are exposed to the impact of diverse microbial complex present in the gut. These microbes may be transmitted from immature stages to the adult sandfly (inherited microbiome), or they can be ingested with different meals taken by the sandfly (acquired microbiome). As seen from the above-mentioned text, microbiome can impact *Leishmania* development and transmission directly or indirectly. Therefore, it is of immense importance to conduct more targeted investigations of the sandfly microbiome, especially in the field of their correlation and impact on *Leishmania* parasites. Within these studies it would be recommended to apply both culture dependent and culture independent approaches in order to isolate microbes for further paratransgenic experiments. It is of immense importance to devote attention toward in-depth evaluation of microbes that have been previously incriminated with paratransgenic properties. Additionally, research on the impact of microbiota on the aspects of sandfly physiology and life are severely lacking. It is essential to accumulate the data from all these areas in order to move forward with proper designing of the paratransgenic approach in sandflies. In conclusion, much additional work is required within the field of sandflies before paratransgenic approach can be fully instigated for field trials.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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