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Review

Site specific microbiome of *Leishmania* parasite and its cross-talk with immune milieu

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ABSTRACT

Microbiota consists of commensal, symbiotic and pathogenic microorganisms found in all multicellular organisms. These micro-organisms are found in or on many parts of the body, including the intestinal tract, skin, mouth, and the reproductive tract. This review focuses on interplay of site specific microbiota, vector microbiota along with immune response and severity of Leishmaniasis. Herein, we have reviewed and summarized the counter effect of microbiome post infection with the Leishmania parasite. We have studied skin microbiome along with the gut microbiome of sand-fly which is the vector for transmission of this disease. Our major focus was to understand the skin and gut microbiome during Leishmania infection,their interaction and effect on immunological responses generated during the infection.Moreover, systems biology approach is envisioned to enumerate bacterial species in skin microbiota and Phlebotmus gut microbiota during *Leishmania* infection.

1. Introduction

The microbiota is the collective populations of bacteria, viruses, fungi, protozoa and archaea found in our environment or associated with various tissues and organs throughout our body. It has been estimated that there are from 3 to 10 times more bacterial cells in the body than human cells, and it is evident that the microorganisms associated with our body are important players in our biology. Bacteria are found in or on many parts of the body, including the intestinal tract, skin, mouth, and the reproductive tract. While the exact numbers may vary depending on size and gender of the person, early studies suggested that the intestinal tract harboured the most bacteria with about 10^{14} cells, followed by the skin with about 10^{12} cells, while the rest of the body sites harbor around 10^{12} bacteria combined [1,2]. Many studies have focused on the bacteria in the intestinal tract, but recently studying the commensal bacteria on the skin has become a broader area of interest. Prior to the age of genomics, culture based methods were used to study the bacteria in the environment [3]. However, it became apparent that simply culturing samples was not capturing all the bacteria present [4,5]. The discovery that bacterial phylogeny could be determined based on the well-conserved 16S ribosomal RNA (rRNA) gene [6] set the stage for the present-day microbiota studies. Presently, bacterial communities are identified using high-throughput sequencing. Studies have shown that there is lot of diversity in healthy microbiota and perturbations in this microbiota called as “dysbiosis” are usually associated with inflammation and various diseases such as cancer, infectious diseases, and metabolic disorders [7,8]. While many of these studies show only correlations between dysbiosis and disease, more recent research has focused on determining whether dysbiosis is a cause or consequence of disease. Various studies focusing on intestinal tract of humans during diseased state and normal state have shown that the dysbiosis in intestinal bacterial population leads to drive disease in arthritis, obesity, cancer, and colitis [9–12]. This outcome is mediated through immunomodulatory response. However, some studies have shown a completely opposite data indicating that this dysbiosis can evoke an immune regulatory phenotype for protection against disease [13]. Few studies have been conducted to analyse the co-relation of skin microbiota and diseases. Based on above studies, it is clear that “Site specific microbiota has a defined role in diseases”. This led us to the idea of understanding this in Leishmania disease model where based on species disease has a different pathological site varying from visceral organs to skin viz, *Leishmania major* causing Cutaneous Leishmaniasis and *Leishmania donovani* causing Visceral Leishmaniasis. The present review summarises the site specific microbiota, their role in disease pathology and immunomodulation taking Leishmaniasis as disease model systems.

1.1. Skin

Skin is usually termed as “First Line of Defense”. It serves as a

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Table 1
Distribution of species along diversified microbiome.

S.No.	Bacterial Species	Sample	Site	Laboratory model	Human	Sandfly
1.	*Streptococcus* *Enterococcus* spp. *Staphylococcus aureus*	LCL lesions	Skin		✓ [31]	
2.	*Enterobacter sp* *Proteus sp* *Pseudomonas aeruginosa* *Klebsiellasp*	*single lesions*	*Skin*		✓([32])	
3.	*staphylococcus aureus* coagulase negative *Staphylococcus* *E. coli* *Proteus* sp. *Klebsiella* sp.	CL lesions	Skin		✓ [33]	
4.	*Staphylococcus* *Enterococcus* *Pseudomonas* Serratia *Corynebacterium* Clostridium *Bacillus* Paenibacillus Propionibacterium *Escherichia* Streptococcus Brevibacterium Citrobacter Klebsiella *Lactobacillus* *Finegoldia* *Sarcina* *Anaerococcus* *Bacteroides* *Peptoniphilus* *Prevotella* *Enterobacter* *Salmonella* *Providencia Peptostreptococcus* *Acinetobacter* *Nocardiopsis*	chronic wounds	Skin		✓ [34]	
5.	*Staphylococcus Streptococcus Corynebacterium,* *PeptoniphilusPeptostreptococcus,* *Fusobacterium*	LCL Lesions	Skin		✓ [3]	
6.	Prevotella (2 + 7 + 9) Faecalbacterium Escherichia-Shigella Alloprevotella Bacteroides Ruminococcaceae UCG-002 Bifidobacterium Roseburia Agathobacter Catenibacterium Asteroleplasma Succinivibrio Clostridialesvadin BB60 group Anaerovibrio Dialister Megamonas Megasphaera Mitsuokella Lactobacillus Ruminococcaceae UCG-014 Gastranaerophilales	VL patient and endemic contact faeces	Gut		✓[78]	
7.	*Anoxybacillus* *Flavithermus* *Bacillus clausii* *Bacillus mycoides* *Brevibacteriumcasei* *Geobacillus* *kaustophilus* *Micrococcus tetragenes* *Staphylococcus cohnii* *Staphylococcus nepalensis*	dissected midguts	Gut			√ gut (P. argentipes)

(*continued on next page*)

Table 1 (*continued*)

S.No.	Bacterial Species	Sample	Site	Laboratory model	Human	Sandfly
8.	*Alcaligenes faecalis* *Bacillus firmus* *Bacillus flexus* *Bacillus mojavensis* *Bacillus pumilus* *Bacillus vallismortis* *Brevibacillusreuszeri* *Brevibacteriumfrigoritalerans* *Citrobactermurliniae* *Enterococcus gallinarum* *Bacillus altitudinis* *Bacillus amyloliquefaciens* *Bacillus brevis* *Bacillus cereus* *Bacillus circulans* *Bacillus endophyticus* *Escherichia blattae* *Exigobacteriumindicum* *Lysinibacillusboronitolerans* *Bacillus licheniformis* *Microbacteriumimperiale* *Microbacteriumparaoxydans* *Microbacteriumsediminis* *Oceanobacillus species* *Pantoeaananatis* *Planomicrobiumglaciei* *Proteus mirabilis* *Proteus vulgaris* *Providencia rettgeri* *Pseudomonas aeuroginosa* *Pseudomonas geniculate*	dissected midguts	Gut			Sandfly(P papatasi)
9.	*Acinetobacter junii* *Cedecea Sp.* *Citrobacter Sp.* *Diploricketseilla Sp.* *Erwinina sp.* *Escherichia sp.* *Klebsiellaozaenae* *Pantoea sp.* *Pluralibacter sp.* *Pseudomonas marginalis* *Pseudomonas sp.* *Pseudomonas trivialis* *Rickettsia sp.* *Rickettsiella sp.* *Spiroplasma sp.* *Wolbachia sp.*	*dissected midguts*	*Gut*			√ *(P Chinese)*
10.		Dissected midguts	Gut			*P dubosqi*
11.	*Bacillus casamanesis* *Bacillus* *Bacillus galactosidilyticus* *Bacillus olironius* *Bordetella avium* *Brevundimonas terrae* *Burkholderia fungorum* *Ehrlichia sp* *Kocuria polaris* *Lysinibacillus sp* *Microbacterium sp* *Micrococcus sp* *Nocardia ignorata* *Ochrobactrum intermedium* *Rhizobium pusense* *Roseomonas* *ludipueritiae* *Saccharomonaspora sp* *Sporosarcina koreensis* *Wolbachia inokumae*	Dissected midguts	Gut			*P pernicious*
12.	Asaia sp.	Dissected midguts	Gut			*P. sergenti*

primary host for various microbes such as bacteria, fungi and viruses. It has been scientifically proven that these microbes termed as skin microbiome play an important role in healing of wounds, protecting from various infectious agents, initial inflammatory immune response and allergic reactions [14,15]. Various diseases have been co-related to changes in skin microbiota [8,16]. How the changes in these microbiota affect the disease as well as role of these microbes in modulating the dermal cell responses is also not explored a lot. Herein, we are

discussing site specific microbiome in Leishmaniasis.

1.1.1. Skin microbiome and cutaneous leishmaniasis

When it comes to skin, the form of Leishmaniasis which is being manifested at this local site is cutaneous leishmaniasis (CL). The disease is basically divided into three phenotypes based on its clinical manifestation from self-healing to chronic/metastatic lesions [17]:(I) Localized CL(LCL), characterized by painless ulcerative lesion [3] which may vary from a single lesion to many (II) Muco-cutaneous Leishmaniasis characterized by destructive mucosal lesions (III) diffuse CL (DCL), presenting multiple non-ulcerative nodules. The disease spreads by the bite of infected sand-fly [18–20]. The variation of disease from being localized to chronic or metastatic has been explored less, however, few studies says that it is not due to rigorous replication of the parasite but the inflated immune response leading to excessive inflammation [21–26]. LCL which is said to be localised disease and self-healing too recovers very slowly in absence of any treatment. The available drugs have shown good response which includes pentavalent antimonials, amphotericin B and Miltefosine [27]. The long time required for self-healing along with environmental exposure, poor hygienic conditions give the way to growth of microbial population at the site of infection. Many secondary bacterial infections have been identified in the patients and needs antibiotic treatment [28,29]. There have been studies which have shown that disease manifestation in germ-free mice model is different from that of conventional mice but how the skin microbiota is involved in this is still unclear [15,30].

1.1.2. Characterization of skin microbiota of cutaneous leishmaniasis

Cutaneous Leishmaniasis (CL) associated microbiome studies have been confined to very little number in LCL lesions in humans, however culture based studies have been performed. It has been shown that *Staphylococcus spp,Streptococcus spp, Enterococcus spp, Pseudomonas spp,* and other opportunistic bacteria are present in LCL lesions [31–33].However, there has been a debate on evaluating the bacterial composition by culture-based technique as it may compromise with the number of species identified. This may be due to low abundance of few species along with the different culture conditions required for growth or may be the species are “unculturable [34,35]. The use of massive molecular methods allows deep insights into the microbiome-composition in general and also in the LCL microbiome because it is more sensitive than culture, as described for other chronic wounds [34].Recently comparative microbiome studies have been reported using high throughput amplicon sequencing approach. Herein, theyhavecompared the skin microbiota between that of laboratorial and LCL lesions with the contralateral healthy skin(HS) microbiome from the same individuals. Restricted biological diversity was observed in LCL lesions when compared with HS. This observation with difference in bacterial colonisation might be due to the fact that LCL lesion are open with a compromised epidermis along with inflammatory responses induced by *Leishmania* infection leading to disturbed microbial composition [35]. The results of this study also showed that LCL lesions gets disturbed due to contamination by commensal bacteria which were adapted to the Leishmania induced inflammation and over-growed less adapted bacteria [35]. They observed *Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria* [36]in HS samples which were comparable to normal skin microbiome along with Cyanobacteria and Fusobacteria. LCL microbiome showed similar profile to non-healing foot ulcers with similar percentage as well viz.,*Firmicutes*(67 %), *Actinobacteria*(14 %),*Proteobacteria*(9.8 %),*Bacteroidetes*(7.3 %), *and Fusobacteria* (1.4 %) at phylum level [37].Aerobic bacteria including *Lactobacillus and Pseudomonas* which play protective role in skin by production of lactic acid and other anti-microbial compounds were found to be decreased in LCL [38]. Few new bacterial species such as (*Fusobacterium, Bacteroides, and Peptoniphilus*), microaerophiles, and facultative anaerobic (*Streptococcus, Staphylococcus, Morganella, Campylobacter, and Arcanobacterium)* bacteria were most frequently detected in LCL. Table 1 enlists all those bacterial species involved in diversified microbiome.

1.2. Immune system and skin microbiome in context of Cutaneous Leishmaniasis

Various studies have established the co-relation between changing skin microbiota and disorders associated with skin for e.g. atopic dermatitis, psoriasis, and chronic diabetic wounds [8,16,36,39]. However, the question that what causes the changes is still unanswered? Reports suggest that alike to gut microbiota, the microbial flora in skin can modulate the immune responses in skin which promote the defense mechanism against the pathogen and alleviate the inflammatory response to maintain the homeostasis in tissue. It has been observed that mice, in which there is no adaptive immunity, are unable to have a control over their skin microbiota and this allows the invasion of pathogens [40]. For e.g., in case of germ free mice when *Staphylococcus epidermidis*is introduced, the mice restores the IL-17A production, thereby indicating as part of skin microbiota, S. *epidermidis* induce Th17 cells along with other T cells that express IL-17A. It has been further observed that Th17 cells present in skin are being modulated by site specific skin microbiota, without having any modulation due to gut microbiota, this suggests that immune responses are controlled in a compartmentalized manner [41,15].

During inflammation, cytokines, chemokines, and antimicrobial peptides are often produced, potentially explaining why there are changes in the microbiota. Table 2 talks about changes in effector immune responses by microbiome.

Bacteria present in the microbiota such as *Salmonella typhimurium* and *E. coli* can use these products of immune response by changing their metabolic processes. This way of adaption to changing conditions post-infection helps the microbiota to survive in inflammatory conditions [42–44]. Above-said has been well established in case of intestine [42], but much is not explored in case of skin. However, it is clear that skin microbiota can modulate the cutaneous immune response.

It has been reported that microbes present in skin microbiota such as *Staphylococcus* can evoke Th1/Th17 immune response in the skin. T-cell response in skin occurs by synchronized activation of skin-resident dendritic cells. This indicates that site specific cells present in particular tissue are tuned to respond in case of changes in microbial population. The skin immune system response can help in protection from the pathogen in some cases, conversely, it can drive the inflammatory response in other pathogens such as *L. major* [15]. The microbial population plays a very important role in immune response, as these may help in developing regulatory responses at an early age which can protect from inflammation at a later stage [45].

Various studies have suggested that the microbial population in the skin can influence the skin immunity, but it has been less explored that the imbalance or “Dysbiosis” in these bacteria can affect disease progression, if any. In case of atopic dermatitis, it has been shown that the dysbiosis can promote the disease progression [46]. As discussed above, the immune system/microbiota interaction can help in disease progression or control, depending on the circumstances [13,15,46]. Post-infection changes in skin microbiota were observed in both humans and mice when infected with *L. major*. In mice, *Staphylococcus spp*. was found dominantly in case of moderate lesions and higher percentage of *Streptococcus spp*. in severe lesions. Gimblet et al., have further shown that in humans dominance of both these species was found. One interesting observation was that in skin during infection, innate immune response, molecules such as antimicrobial peptides (AMPs) can target some bacterial species and these may be involved for imbalance in skin microbiota [38,47–49]. This area is of interest and needs to be explored more in case of Leishmania infection.

It was observed that the expression of AMP was changed post-infection and mice which were deficient in cathelicidin-type antimicrobial peptide (CAMP) were more susceptible to infection. There is a great possibility that these AMPs might cause changes in skin

Table 2
Changes in effector immune responses by microbiome.

Sno.	Bacterial species/ severity of infection	Immunological response	Disease model	Microbiome
1.	*Staphylococcus* spp. dominant in moderate lesions and *Streptococcus* spp. increasing in more severe lesions in group A	more neutrophils and pro-IL-1β production in the skin in group A	A). *L. major* infected mice B). Control mice (Gimblet et al.)	Skin
2.	Relative to SPF mice, GF mice infected intradermally with *L. major* manifested smaller lesions with reduced edema and necrosis GF+ *S. epidermis*	Impaired immune response in GF mice, with reduced- *Leishmania*-specific IFN-γ & TNF-α by cutaneous T cells Mono-associated GF mice with *S. epidermidis* rescued protective immunity in these animals	A) Germ free mice B) Special pathogen free mice (Naik et al.)	Skin
3.	Germ-free mice failed to heal lesions and presented a higher number of parasites at the site of infection than their conventional counterparts.	Germ-free mice produced elevated levels of IFN-γ and lower levels of IL-4 but no controlled infection Isolated macrophages from Germ-free mice, exposed to IFN-γ and infected with amastigotes in vitro were not as efficient at killing parasites as macrophages from conventional animals ** Indicates importance of microbiota in macrophage activation	A) Swiss/NIH germ-free mice B) Conventional (microbiota-bearing) mice **Both infected with *Leishmania major*	Skin
4.	*Clostridia (phylum Firmicutes)* and *Gammaproteobacteria (phylum Proteobacteria)* *Actinobacteria* and *Bacteroidia* classes	IL-1β positively co-related with most of the microbial classes in the self-healing mice but only with *Bacilli* and *Gammaproteobacteria* in non-healing mice IL-12 and Il-10 have the most immune-microbial correlations out of all the cytokines in the non-healing mice Strong positively correlation with IL-10 levels which suggested that these bacteria may be responsible for exerting an IL-10 dependent anti-inflammatory effects on the host	C57BL/6 (resistant) BALB/c (susceptible) mice **both infected with *L. major*	Gut of mice(faeces sample)
5.	Gut microbes from the sand fly are egested into host skin alongside Leishmania parasites	Egested microbes triggered inflammasome and produced IL-1β, which sustains neutrophil infiltration. Reducing midgut microbiota by pretreatment of Leishmania-infected sand flies with antibiotics, or neutralizing the effect of IL-1β, in bitten mice abrogates neutrophil recruitment.	*L. donovani*-infected sand flies harboring transmissible infections reproducibly transmit about 10^3–10^4 parasites to mice ears (Dey et al.)	Gut (Sand-fly)

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Table 2 (*continued*)

Sno.	Bacterial species/ severity of infection	Immunological response	Disease model	Microbiome

microbiota but the mechanism still needs to be elucidated. It is well established that virulent factors can help in resistance for AMPs in case of bacteria and both the species *Staphylococcus spp*. and *Streptococcus* spp. found in abundance in skin site express the genes required for protecting them from AMP [50–52]. This might have helped in survival of both bacterial species during Leishmania infection. It was also proven using mouse with dysbiotic skin microbiota, that naturally acquired dysbiosis can cause change in inflammatory responses and disease progression in *Leishmania*. There are evidences suggesting that environmental conditions affect skin microbiota [53]. Data suggested that *Leishmania* infection disturbs the first defense system i.e., skin and also causes dysbiosis in skin microbiota. This concluded a hypothesis which was of great attention that this dysbiosis caused due to Leishmania infection leads to recruitment of neutrophils and IL-1β recruiting cells to the skin, and causes increased lesion severity [23,25,54,55].

1.3. Gut microbiome

1.3.1. Gut microbiota and leishmaniasis

Various studies have been carried out to understand the effect of bacteria in intestinal tract causing dysbiosis in disease model systems such as arthritis, obesity and cancer [9,10,12].It has been found that the effect of microbiota on the disease outcome is through modulation of immune response. There are reports suggesting both way through, i.e., the immune system may go along with the intestinal microbiota to either enhance the disease, protect against the disease or help in protecting host from inflammatory responses [13]. It has been reported that gut microbiome have the ability to modify the immune responses by various mechanisms including activating macrophages and effecting the differentiation of T-cells. For animal model studies, usually germ free mice are used for study of microbiota because of the fact that in mice susceptible to systemic infection, there is a strong possibility that the parasite can modulate the host-microbiota [56]. The germ free mice would either be more susceptible or resistant to the disease [57]. Very few studies have been conducted on this aspect in Leishmaniasis. Oliveira et al. [30], has conducted the study using Swiss/NIH germ-free mice and microbiota bearing normal mice to understand the effect of host microbiota on T cell differentiation post infection with *Leishmania major*. They have shown that there was no lesion healing in germ free mice along with a higher parasitic load at the site of infection than their conventional counterparts. The initial levels of cytokine IL-2, IL-12 and IFN-γ was nearly equal to their control group i.e, conventional mice. During the course of infection germ free mice produced high levels of IFN-γ and lower levels of IL-4. The data suggested that Th-1 response was induced in the germ free mice as well. However, it was found that macrophages isolated from germ free mice and stimulated with IFN-γ, were not able to kill the Leishmania parasite once infected with them concluding the fact that microbiota effected the activation of macrophages without having any effect on Th1 response. This indicates the correlation between gut microbiota and modulation of the immune response during *Leishmania* infection, which may finally change the disease outcome.

Recently, a study used meta-taxonomic analysis to analyse the faecal samples of individuals from endemic areas for visceral leishmaniasis (VL) in India to determine the composition of gut prokaryotic and eukaryotic microflora and find a co-correlation with diseased and non-diseased state by comparing the difference between VL cases and non-VL endemic controls. High abundance of *Escherichia-Shigella* (9.1 % aggregate abundance) was obtained from bacterial microbiota in a subset of individuals that could define a different enterotype or sub-enterotype in the population (North-East India endemic) for VL compared to other Indian studies. It was inferred that high *Escherichia-Shigella* was associated with an overall dysbiosis of the gut bacterial flora [78].

A study was conducted to verify the fact that does the diverse bacterial population in the gut effects colonization of pathogens negatively. *Phlebotomus duboscqi* was treated with antibiotic with an aim of improving their vector competency, however it resulted into flies which were refractory to the development of transmissible infection and it happened because in the treated group parasites were not able to differentiate into the infective, metacyclic stage. Once these flies were fed with different symbiont bacteria, the defect in parasite development was overcome. It was also observed that when the antibiotic treated flies were given low sucrose concentration meals, the inhibitory effect of antibiotic treatment was moderate. These observations suggested that competing with microbiota for sucrose utilization, produced appropriate nutrient stress along with osmotic conditions for stage differentiation and survival of infectious metacyclic promastigotes in vivo.

Most of the studies in gut microbiota and Leishmaniasis have been focused on gut microbiota of the disease vector that are sandflies. In this section, we are focusing on various aspects of gut microbiota of sandfly.

1.4. Gut microbiome of Sand-fly and Leishmania infection

It is a well-known fact that during Leishmania infection, parasite resides in two forms in two hosts, one is the promastigote form which resides in the gut lumen of the sand-fly and other is the amastigote form within macrophages of infected human host [58]. There are only few species of sand-fly which combined with Leishmania species transmit metacyclic parasites to human host which include *L. donovani—Phlebotomusargentipes, L. major—P. papatasi,L. tropica—P. sergenti* [59]. *Leishmania* parasites which have been taken along with the blood meal escape through peritrophic matrix and get attached to mid gut epithelium [60]. It has been well proven that gut of insects are rich in commensal bacteria [61]and in case of mosquitoes and tsetse flies these microbiota affects the ability of insects as vector for the disease [62,63].

2. Characterization of gut microbiome of the sand-fly

The symbiotic microorganism present in vector causing disease affects various aspects of vector which may include reproduction, nutrition and homeostasis of immune system. This microbiota present in vector along with affecting the vector can also hamper its ability for pathogen transmission by inducing various factors such as innate defence molecules, enzymes and toxins [63]. Sand-fly phlebotomine which is a vector for transmission of Leishmaniasis might acquire the microbiota from soil, plants and since the life cycle of *Leishmania* parasite in the invertebrate host occurs in digestive tract, there is a strong possibility of interaction of various stages of Leishmania parasite with the gut microbiota.

A study based on this hypothesis for Leishmania has addressed the question that “Does Sand-fly helps in developing infectious Leishmania parasites for transmission to host?” They used comprehensive 16SrDNA gene high-throughput sequencing of DNA obtained from the dissected midguts of infected or uninfected *Lutzomyia longipalpis*, which transmits *Leishmania infantum*. Nearly 2091 *Lu. Longipalpis* were used to isolate midgut and varied array of bacterial species were identified. These species varied based on the source of the insect’s meal and infection status. It was found that there was a drastic and regular loss in bacterial community post *L. infantum* infection as the infection progresses [64]. It was observed that bacteria of phylum differed significantly in microbiome of infected sand-flies from that of uninfected controls fed on either blood or sucrose along with bacteria from the family *Phyllobacteraceae* and the genus *Trabulsiella* in both the groups. They also observed prominent presence of *Enterobacteriacae* under both sucrose-fed and blood-fed conditions which were surpassed by *Acetobacteraceae*, 12 days post infection. Other studies which are based on different identification methods including denaturing gradient gel electrophoresis (DGGE), bacterial culture have also identified various bacterial species [65]. The denaturing gel electrophoresis performed with DNA of *Lu. longipalpis* or *Lu. Cruzi*t aken from regions of rural orsylvatic Brazil

or Colombia identified *Proteobacteria Erwinia and Ralstonia spp*. [66]. Sequencing of midgut metagenome of *Lu. intermedia* which is the vector for *L. braziliense*s was performed by Monteiro et al. They identified *Enterobacteriacae* in both the uninfected and gravid groups but represented only 4.2 %of the bacterial families of blood-fed groups. Rickettsiaceae was found in most abundance in *Lu. intermedia* blood-fed fly pool of which genus Wolbachia comprised nearly 46.7 % of the sequences. Both the *Lu. intermedia* and *Lu. Longipalpis* showed the presence of Proteobacteria and Brucellaceae along with Pseudomonas (phylum Proteobacteria) [67]. Other species of sand-fly vector were also characterized. The microbiota composition in the midgut of *P. perniciosus* revealed that lab-reared *P. perniciosus*had less rich bacterial microbiome in midgut than in field-collected sand –flies which might be due to difference in food intake. *P. perniciosus* midgut, contained bacteria belonging to *Burkholderia* genus and *Stenotrophomonas maltophilia*. They also identified few bacterial species which are not characterized in sand-fly midgut but are present in human or other mammalian midgut like *Veillonella* sp.in addition to *Sporosarcinakoreensis, Rhizobium pusense* and *Nocardia*. The difference in midgut microbiota of *Lutzomyia* sp. and *P. perniciosus* was attributed to enormous factors which included long divergence of evolution between the two subgenera [68].

3. Sand-fly microbiota and Leishmania

We have discussed that Leishmania infection in midgut of sand-fly is accompanied by gut microbiota which has predominance of bacterial species. It has been suggested that microbiota has an important effect on the physiology of any disease transmitting vector and also influence the innate immune system [63]. There are various reports suggesting that microbiota can activate the innate immune pathway, thereby affecting the parasitic infection and induce some effector molecules which can control the infection. It has been shown that the suppression of ROS in midgut of *L. longipalpis* facilitates the leishmania infection proving the importance of microbiota [69]. In line with observation in other vector parasite disease models, the competency of sand-fly is influenced by microbiota in Leishmania infection as well [70,71]. It was found in experiments conducted with colony-raised *L. longipalpis*, that if the insects are feeded with bacteria *Asaia* sp., *Ochrobactrumintermedium* and a yeast-like fungus *Pseudozyma* sp. which were isolated from midgut of wild and laboratory reared female *L. longipalpis* before leishmania infection, the disease does not get established [66]. It was verified in the same study that once infected with *L. mexicana, L. longipalpis* was resistant to *Serratia* infection. Many studies have further shown that the development of promastigote stage of Leishmania parasite in the vector sand-fly is dependent on the microbiota of the vector's midgut. Study carried out by Kelly et al. [64] first characterized the phylogenies of bacteria present within the midgut of three categories of sand-fly *L. longipalpis* i.e, sugar-fed, blood-fed and *L.infantum*-infected. This observation was also accompanied by observing the effect of treatment by antibiotic against Leishmania infection. The results showed that once infected with Leishmania parasite, the diversity of bacterial population in midgut of *L. longipalpis* was lost gradually; moreover, the treatment with antibiotic affected the replication of *L. infantum* and its metacyclic form. More or less similar observation was found Louradour et al. [72], where they have shown that development of *L. major* in *P. duboscqi*is is hampered by antibiotic treatment. The fact that microbiota of sand-fly has a role in Leishmania development and replication in midgut was further proved by using engineered antibiotic-resistant bacteria isolated from natural *P. duboscqi* and in this condition antibiotic treatment did not affect the Leishmania infection in the sand-fly midgut [66]. The importance of exploring the area of sand-fly microbiota in Leishmaniasis was strengthened again by the work carried out in *Leishmania donovani* infected mammals by Dey et.,al. which would be subsequently discussed in the next section [73].

3.1. Gut microbiota and immune system interplay

An extensive study carried out by Lamour SD et al. [74] on mouse microbiota has co-related the microbiota with the cytokines produced. They have investigated the effect of *L. major* infection on microbiota of C57BL/6 (resistant) and BALB/c (susceptible) mice. They found that faeces of both the mice models contained bacteria mainly from two classes i.e. *Clostridia (phylum Firmicutes)* and *Gammaproteobacteria (phylum Proteobacteria)*. It was found that *Clostridium* increased significantly post infection in BALB/c mice than in C57BL/6 mice which was initially similar. Initially post 2 weeks of infection in both animal models, *Gammaproteobacteria* decreased but at the time of termination of experiment it was found that this bacterium was significantly higher in the resistant mouse strain. This higher presence of *Gammaproteobacteria* class was correlated with resistance in C57BL/6 to infection with *L. major* based on the observation that although levels of *Gammaproteobacteria* decreased in both the mice models at the end of the study but the levels of this bacteria in C57BL/6 mice remained significantly higher than in BALB/c mice.

When cytokine profile was co-related with the microbial population it was found that IL-1β has shown positive co-relation with most of the microbial classes in the self-healing mice but co-related only with *Bacilli* and *Gammaproteobacteria* in non-healing mice. Similarly, it was found that IL-12 and Il-10 have the most immune-microbial correlations out of all the cytokines in the non-healing mice. Two bacterial classes namely, *Actinobacteria* and *Bacteroidia* classes showed a very strong positively correlation with IL-10 levels which suggested that these bacteria may be responsible for exerting an IL-10 dependent anti-inflammatory effects on the host. This profile shows an extensive and strong co-relation between immune response and microbiota in self-healing mice which was different from non-healing mice which might explain the difference in two animal models for Leishmania infectivity. Since above section has shown the importance of sand-fly microbiota in Leishmania infection, we focused on this and found that recently a group has given a fundamental role of sand-fly microbiota in Leishmania infection in mammalian host [73]. They have shown that when *L. longipalpis* sucks the blood meal for infection than along with the parasite, vector microbiota is also egested with the parasite inoculum. The microbe population of sand-fly leads to activation of the neutrophil inflammasome of mouse and further results in a rapid production of interleukin-1β (IL-1β), which sustains neutrophil infiltration. Due to these neutrophils *Leishmania donovani* parasites are shielded and this helps in promoting infection of macrophages post transmission. Impairing the sand-fly microbiota by antibiotic treatment affects the infection of *Leishmania donovani*. This data gave a new insight that sand-fly midgut microbiota can not only affect the Leishmania within it, but these micro-organisms when present modulate the host immune response in favor of the parasite transmission and survival.

Another work based on the fact that LACK-specific Tcells accumulate IL-4 mRNA very rapidly in mice infected with *L.major*, characterized the phenotype of cells before infection. They demonstrated a very interesting fact that the lymphoid organs of naive BALB/c mice were found to have microbial Ag-specific T cells which had the ability to cross-react with LACK and that express a memory/effector phenotype. This could have been the reason for secretion of IL-4 shortly after infection by LACK-specificT cells.The group incubated five different LACK-specific Tcell hybridomas with crude extracts from various aerobic and anaerobic bacteria extracts and it was found that *Escherichia coli* and *Enterococcus faecalis,* but not *Proteus mirabilis* and *Clostridium perfringens* extracts induced secretion IL-2 by T-cell hybridomas. Overall, data suggested that the IL-4 burst induced by parasite on priming of LACK-specificT cells is due to microbial Ags, Theseare among very few studies which has shown the effect on systemic Ag-specific immune responses mediated by intestinal flora and suggests that the immune response generated against the cutaneous parasite may be due to cross-priming of T-cells by microbial Ags from

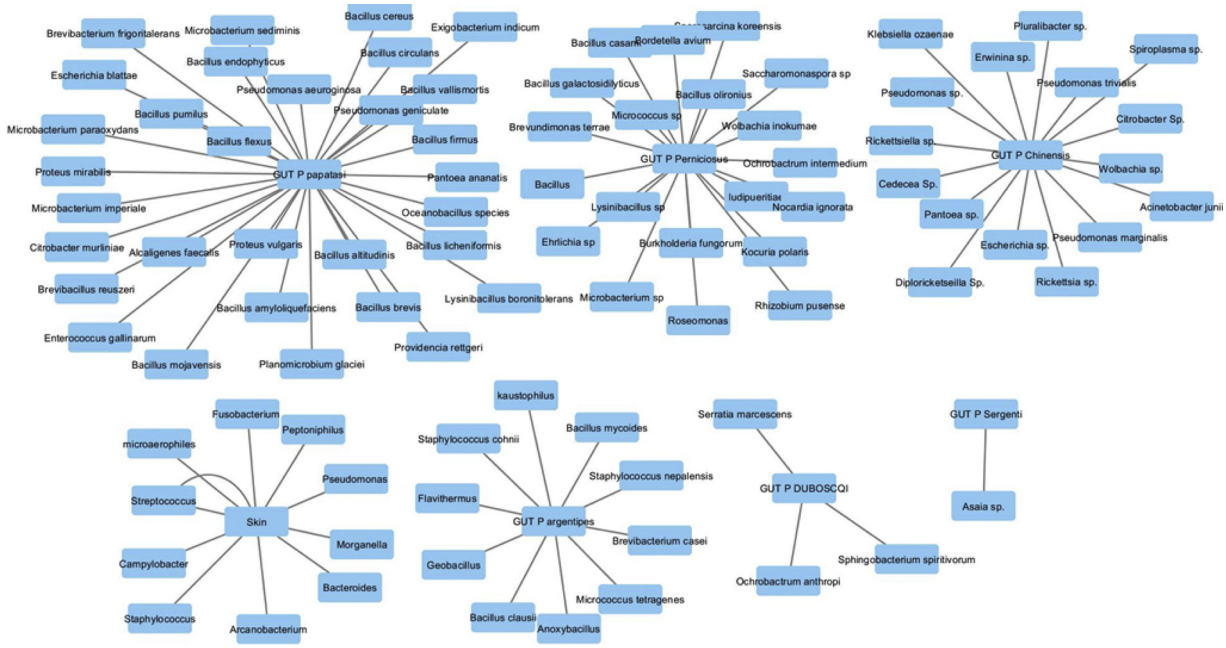

Fig. 1. Connectome of the Microbiome.

the indigenous intestinal flora [75].

4. Metabolites and microbiota interplay in Leishmaniasis

Metabolic profiling reveals an insight into the altered host microbiome across diverse sets of parasite-rodent models evident through a set of microbiota-associated metabolites in urine and plasma. To evaluate the importance of the host microbiome on the immune response specifically differentiation of T cell subsets during the course of infection, assessment of various parameters such as lesion development, parasite loads, and cytokine production was carried out in Swiss/NIH germ-free mice and conventional (which has microbiota) mice.Despite showing strong Th1 immune response in *L.major* infection models, results are indicative that germ-free mice failed to heal lesions in comparison to their conventional counterparts [30]. Having cited that, the study demonstrates intermittent role of microbiota in mounting a successful host response to the parasite.

In general, when any parasite gets introduced in the mammalian system, it might disrupt the microbiota present and this may lead to changes in metabolism. In various studies, when the parasite and rodent model was characterized, a strong co-relation was observed between the metabolites, altered host microbiome and infection. These data are based on the metabolite profiling in conjunction to associated microbiota either in urine or plasma [76]. Metabonomics approach was used in case of *Schistosoma mansoni* infection in mice for characterizing the intergenome interaction between the gut microflora and infection. They found many microbial population related metabolites also such as trimethylamine, phenylacetyl glycine, acetate, p-cresol glucuronide, butyrate suggesting that post infection there is disturbance in gut microbiota. One more study conducted by Dumas et al. [77] has shown a complex interaction between the gut microbiota and host co-metabolites in impaired glucose homeostasis induced by diet, and non-alcoholic fatty liver disease (NAFLD), in the strain of susceptible mouse. The samples of plasma and urine were collected for the study using ^{1}H NMR. Their data indicated that host metabolism is altered due to the alteration in metabolism of gut microbiome. NAFLD is associated with the disturbances in choline mechanism, wherein, there are low levels of plasma phosphatidylcholine in circulation and urine consists of high levels of methylamines which are co-processed by gut microbiota and mammalian enzyme systems. This reduction in choline which was mimicked by choline-deficient diet caused NAFLD. Having said that, it strongly indicates the prominent role of gut microbiome in insulin resistance [77]. In case of *Leishmania major*, it has been observed that inspite of generating a strong Th1 type protective response, the germ free mice were not able to heal the lesions when compared to their normal counterparts [30].The data suggests strong role of microbiota in elucidating a strong host response to the infection. Another study was performed to evaluate the response to infection in a self-healing C57BL/6 and a non-healing BALB/c mice model for cutaneous leishmaniasis. They combined three important aspects to evaluate the outcome viz., immune response, metabolic outcomes and gut microbiota response in the host. Urine, plasma and faeces were included for metabolic profiling, peripheral cytokines and faecal bacterial constituents were analysed [74]. The study identified a strong co-relation between immunological response, metabolic profile and microbiota in *L. major*. Direct statistical interaction was observed after correlation network analyses using metabolome of host, cytokines and the microbial composition of faeces. It was found that self-healing strain has more number of co-relations among the above-said parameters whereas non-healing mice did not have.

4.1. Skin microbiome and gut microbiome interaction

Very few studies have been done to understand the role of the indigenous microbiota during Leishmania infection. It would be interesting to study the dynamic interaction between the host, disease site and the microbiota. We envisioned through systems biology approach to churn some bacterial species in skin microbiota and *Phlebotmus* gut microbiota during *Leishmania infection*, but surprisingly we did not find any common species inspite of the fact that both the sites are involved in disease manifestation. Supplementary Table mentions the connectome of the microbiome constructed with their corresponding Pubmed ID. This indicates that identifying a single bacterium that can be applied to control strategies targeted to a majority of sand-fly vectors, skin manifestation of disease will be quite challenging (Fig. 1). Community level modularity in different microbiomes may be associated with decreased level of variability in the gut environment or with the lack of temporal regularities. Integrated computational-microbiome model may ultimately help devise a predictive framework for targeted community manipulation in disease model systems and even for

informing clinical interventions. To determine which aspects of gut microbiome may contribute to disease and decipher the mechanism linking to host pathophysiology and immunity may shed some important scientific questions to ponder upon in future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.10.004.

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