Reactive Oxygen Species-mediated Immunity against *Leishmania mexicana* and *Serratia marcescens* in the Phlebotomine Sand Fly *Lutzomyia longipalpis* *\(^*\)*

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Background: Reactive oxygen species are part of the sand fly innate immune system.

Results: ROS production in the gut increases in response to a bacterial pathogen but not to *Leishmania*.

Conclusion: Sand flies tolerate the presence of *Leishmania* by differential response of the ROS system.

Significance: The successful use of sand flies as vehicles for *Leishmania* transmission relies partially on the parasite circumventing the ROS immune response.

Phlebotomine sand flies are the vectors of medically important *Leishmania*. The *Leishmania* protozoa reside in the sand fly gut, but the nature of the immune response to the presence of *Leishmania* is unknown. Reactive oxygen species (ROS) are a major component of insect innate immune pathways regulating gut-microbe homeostasis. Here we show that the concentration of ROS increased in sand fly midguts after they fed on the insect pathogen *Serratia marcescens* but not after feeding on the *Leishmania* that uses the sand fly as a vector. Moreover, the *Leishmania* is sensitive to ROS either by oral administration of ROS to the infected fly or by silencing a gene that expresses a sand fly ROS-scavenging enzyme. Finally, the treatment of sand flies with an exogenous ROS scavenger (uric acid) altered the gut microbial homeostasis, led to an increased commensal gut microbiota, and reduced insect survival after oral infection with *S. marcescens*. Our study demonstrates a differential response of the sand fly ROS system to gut microbiota, an insect pathogen, and the *Leishmania* that utilize the sand fly as a vehicle for transmission between mammalian hosts.

Phlebotomine sand flies are the insect vectors responsible for the transmission of leishmaniasis worldwide (1). This group of diseases is caused by parasites of the genus *Leishmania* and threatens ~350 million people in 88 countries around the world (2). Moreover, phlebotomines are also able to transmit other diseases caused by bacteria and viruses (3–5). *Lutzomyia longipalpis* (Lutz and Neiva, 1912) is the main vector species that transmits American visceral leishmaniasis in Brazil (6), American visceral leishmaniasis being one of the most important and dangerous forms of this collection of diseases (7, 8). Although its distribution is restricted to the New World (9, 10), this species has proven to be an excellent model organism given its permissiveness to different species of *Leishmania* (11), wide distribution in urban environments (12), and extensive research regarding laboratory rearing (8, 13).

In recent years, evidence from other dipteran species has highlighted the role of reactive oxygen species (ROS) \(^2\) in insect immunity by regulating potential insect pathogens and determining the profile of the commensal gut microbiota (14–16). ROS are oxygen-derived radical species formed during cell respiration, mainly derived from mitochondrial electron transport. This group includes superoxide anion (\(\text{O}_2^-\)), the hydroxyl radical (\(\cdot\text{OH}\)), and hydrogen peroxide (\(\text{H}_2\text{O}_2\)). Although \(\text{H}_2\text{O}_2\) does not have unpaired electrons, it is usually considered as a ROS because it can be easily transformed into the highly reactive \(\cdot\text{OH}\) via a Fenton-like reaction (17, 18). Moreover, \(\text{H}_2\text{O}_2\) can react with hypochlorous acid, superoxide anion, nitric oxide (\(\text{NO}^-\)), and peroxynitrite, generating singlet molecular oxygen, a strong oxidant (19). ROS production in excess has deleterious effects in the cell, damaging lipids, proteins, and DNA (20). Eukaryotic cells are able to regulate ROS levels through the production of antioxidant enzymes. \(\text{O}_2^-\) is produced by a NADPH oxidase and transformed to \(\text{H}_2\text{O}_2\) by superoxide dismutase, whereas \(\text{H}_2\text{O}_2\) is reduced to \(\text{H}_2\text{O}\) by catalase (17). In *Drosophila melanogaster* ROS are actively produced in the midgut at a basal level in the presence of commensal microbiota (15, 21) and highly generated upon bacterial oral challenge (22). In *Anopheles gambiae*, ROS modulate immunity against bacteria and *Plasmodium* (14, 23). Studies done with *A. gambiae* showed that *Plasmodium* refractory strains were in a constant oxidative stress state exacerbated by blood-feeding and contributed to a higher *Plasmodium* melanization rate in comparison with strains susceptible to the malaria parasite (23). Superoxide anions are secreted into the midgut lumen of the adult *Aedes aegypti* mosquito, and blood meal ingestion decreased ROS levels via blood heme-activated protein kinase C (24). The

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\(^{2}\)The abbreviations used are: ROS, reactive oxygen species; UA, uric acid feed; ABF, after blood-feeding; Contig, group of overlapping clones.
ROS-mediated Immunity in L. longipalpis

complexity of the role of ROS in gut microbe homeostasis was further underlined by the suggestion that gut bacteria-derived ROS may kill Plasmodium in the anopheine mosquito (25).

Our previous work has shown that ROS scavenging by means of antioxidant supplementation decreased survival of adult L. longipalpis and led to activation of the phenoloxidase cascade, which was proposed to be due to bacterial proliferation (26). The purpose of the present study was to investigate the ROS activities in the gut of L. longipalpis after feeding Leishmania mexicana and Serratia marcescens, a bacterial insect pathogen also found in wild sand fly populations (27). We manipulated ROS balance in infected flies to analyze its effect during vector-microbe homeostasis. The results suggest that Leishmania infections do not elicit ROS production within the L. longipalpis midgut, whereas Serratia infections increase ROS generation inside the sand fly gut.

EXPERIMENTAL PROCEDURES

Sand Fly Rearing—All experiments were performed using insectary-reared L. longipalpis from a colony first started with individuals caught in Jacobina, Brazil. Insects were kept under standard laboratory conditions (28). Sand flies were fed with 70% w/v sucrose solution in cotton wool (unless stated differently in experiments), kept under a photoperiod of 8 h of light/16 h of darkness, a temperature of 27 °C (±2), and a relative humidity of >80% inside the rearing cages. The females in the colony were fed on rabbit blood via a Hemotek membrane feeder (Discovery Workshops) at 37 °C. All procedures involving animals were performed in accordance with the United Kingdom government (Home Office) and European Commission regulations.

Sand Fly Infections—For parasite infections, L. mexicana MNYC/BZ/62/M379 promastigotes were kindly donated by Prof. P. Bates and kept at 26 °C in M199 medium supplemented with 25 µg/ml gentamicin sulfate (Sigma), 1× Eagle’s Basal Medium vitamins (Invitrogen), and 20% fetal calf serum (PAA Laboratories). Promastigotes were subpassaged into fresh medium when cultures reached late log phase. Axenic amastigotes were obtained from promastigotes as described previously with some modifications (29). Briefly, promastigotes were centrifuged at 2000 rpm for 10 min, resuspended in Grace’s medium supplemented with 25 µg/ml gentamicin sulfate (Sigma), 1× Eagle’s Basal Medium vitamins (Invitrogen), and 20% fetal calf serum (PAA Laboratories) at pH 5.5, and incubated at 32 °C until fully transformed amastigotes were present in the flask. Axenic amastigotes were maintained and subpassaged in supplemented Grace’s medium at 32 °C. For sand fly infections, amastigotes were resuspended in 1 ml of rabbit blood (2 × 10⁶ parasites/ml) and fed to the insects through a chick skin membrane via a Hemotek feeder at 37 °C. Insects were kept under standard laboratory conditions until required for experimental work. For bacterial infections, S. marcescens (NCIMB 1377) was inoculated on LB agar plates and incubated at 26 °C for 20 h. Bacterial suspensions were prepared by transferring a colony of S. marcescens into 5 ml of LB broth and incubating overnight at 37 °C under shaking. The suspension was centrifuged at 13,500 rpm for 5 min, resuspended in PBS, and diluted to a concentration of 5.7 × 10⁶ cfu/ml. Bacteria were then diluted in heat-inactivated blood to a final concentration of 1.14 × 10⁸ cfu/ml and offered to sand flies via a Hemotek feeder as explained above.

RNA Extraction and Gene Relative Expression by RT-PCR—Individual sand flies were homogenized in 50 µl of TRI Reagent® (Ambion, Austin, TX) and kept at −80 °C until needed. RNA was extracted following the manufacturer’s protocol. Total RNA was quantified using a NanoDrop® (NanoDrop Technologies, Wilmington, DE) and normalized to 10 ng/µl. RT-PCR was performed with a SuperScript® III one-step RT-PCR system with a Platinum® Taq DNA polymerase kit (Invitrogen) executing 25 cycles and following the manufacturer’s protocol (primers listed in supplemental Table 1). Relative expression was normalized using a housekeeping gene (GenBank™ AM088777, 60 S ribosomal protein L3). RT-PCR products were analyzed by 1.5% w/v agarose/ethidium bromide gel electrophoresis, and changes in gene expression were determined by densitometric measurement of bands using the GeneSnap/GeneTools software (Syngene).

ROS Regulatory Gene Sequence Analysis—Putative gene sequences of L. longipalpis catalase, Cu/Zn superoxide dismutase, and peroxiredoxin and oxidative resistance protein 1 (OXR1) were obtained from a cDNA library constructed from sand fly whole bodies (AM105518, AM095907, AM102380, and AM097733, respectively) (30). Putative gene sequences of L. longipalpis catalase, superoxide dismutase, and peroxiredoxin were obtained from a midgut-specific expressed sequence tag library (ABV60342, ABV60343, and ABV60347, respectively) (31). BLAST was used to compare midgut catalase, superoxide dismutase, and peroxiredoxin and whole body OXR1 sequences with the National Center for Biotechnology Information database (32). Conserved residues in those protein families were retrieved from the Conserved Domains Database (CDD) (33). Multiple alignment, phylogenetic analysis, and Neighbor-joining cladograms were performed with MEGA package (34).

H₂O₂ Profile—Sand flies were infected with either Leishmania or Serratia after 3 days after emergence. Insects were dissected at 24, 48, 72, and 96 h after infection. The control group was blood-fed, noninfected flies. H₂O₂ was also measured in sugar-fed flies, 1 day before infection/blood-feeding. At each time point, two pools of four midguts were homogenized in 60 µl of PBS per pool containing 2 mg/ml of the catalase inhibitor 3-amino-triazole. Samples were flash-frozen in liquid N₂ and kept at −80 °C. Prior to assay, samples were thawed and centrifuged at 16,000 rpm, 5 min at 4 °C. Additional replicates were performed at 48 h after infection using fresh samples (n = 6 pools of four midguts) to validate the use of frozen samples for hydrogen peroxide assays. Five µl of the supernatant were assayed for H₂O₂ using the Amplex Red® hydrogen peroxide/peroxidase assay kit (Invitrogen) following the standard protocol as recommended by the manufacturer. All assays were carried out in triplicate. The experiment was performed twice.

In Vivo Detection of ROS—Sand flies were infected with either Leishmania or Serratia after 3 days after emergence and dissected at 1, 24, 48, 72 h, and 7 days after infection. The control group was blood-fed, noninfected flies. ROS production was also measured in sugar-fed flies, 1 day before infection/
blood-feeding. At each time point, five midguts were dissected for in vivo detection of ROS as described previously (35). Briefly, midguts were dissected in L-15 (Leibovitz) medium (Sigma) and incubated with 30 μmol of dihydroethidium in L-15 medium for 5 min in a dark chamber on a mini orbital shaker (70 rpm) at room temperature. After 3 × 5-min washes under the same conditions to remove dihydroethidium, individual midguts were transferred to 10-well slides. ROS production was monitored via an inverted fluorescence microscope using a U-MWG fluorescence cube (excitation, 530–560 nm; emission, 590 nm). Images were captured using a ×10 objective and analyzed by ImageJ. A semiquantitative approach was used to compare fluorescence between samples. Based on tiff image files saved using the NIS-Elements BR 3.00 imaging software (Nikon), the sand fly midguts were delimited using the paintbrush tool of the ImageJ program, and mean intensity was measured inside the midgut, minimizing interference from background fluorescence. Mean intensity values were then used to compare fluorescence between samples after incubation.

dsRNA-mediated Catalase Knockdown—Sense and antisense catalase-specific primers (supplemental Table 1) flanked by the T7 promoter site amplified by PCR a 1472-bp product obtained from a normalized whole body L. longipalpis cDNA library (30) that was used as template for double-stranded RNA synthesis. Transcription reactions and column purification with the MEGAscript RNAi kit (Ambion®) followed the manufacturer’s protocol. dsRNA purity was assessed by 1.5% w/v agarose/ethidium bromide gel electrophoresis, and dsRNA was quantitated using a NanoDrop ND-1000 spectrophotometer (LabTech). dsRNA was eluted with nuclease-free water at 65 °C, concentrated to 4.5 μg/μL with a Martin Christ® RVC 2–25 rotational vacuum concentrator, and stored at −80 °C. Enhanced green fluorescent protein (eGFP) dsRNA was produced from a 653-bp amplicon of the pEGFP-N1 expression plasmid (Clontech) and used as a “mock-injected” control. RNA interference (RNAi) was achieved by dsRNA injections as described previously (36). After injections, sand flies were transferred to cages, kept under standard rearing conditions, and infected with Leishmania 72 h after injections. Insects were dissected 94 h after infection, 15 midguts were homogenized individually in 50 μl of PBS, and parasites were counted using a hemocytometer. Three whole sand flies were reserved for individual RNA extraction and knockdown evaluation by RT-PCR. Relative expression data were shown as mean ± S.E. from three biological replicates.

H₂O₂ Feeding—Sand flies were kept under standard rearing conditions and allowed to feed ad libitum on cotton wool soaked in 5 mmol of H₂O₂ in 70% w/v sucrose solution since emergence and until the end of the experiment. Hydrogen peroxide solutions were freshly prepared from an H₂O₂ 30% w/w stock solution (Sigma). Insects were infected with Leishmania 3 days after emergence. The control group was fed on plain 70% w/v sucrose solution. Flies were dissected 94 h after infection, 15 midguts were homogenized individually in 50 μl of PBS, and parasites were counted in a hemocytometer. Experiments were repeated twice.

Uric Acid Feed (UA), Insect Survival, and Bacteria Counts—Sand flies were kept under standard rearing conditions and allowed to feed ad libitum on cotton wool soaked in 10 mmol of uric acid in 7% w/v sucrose solution (pH = 8.9) since emergence and until the end of the experiment. Uric acid solution was freshly prepared every day. The control group was fed on sucrose 7% w/v, no UA (pH = 8.9). Insects were infected with Serratia 3 days after emergence as explained above. Control flies were fed on noninfected blood. Survival was recorded every day. Twelve flies were collected at 48 h after infection and dissected, and four pools of three midguts were homogenized in 50 μl of PBS per pool. Serial dilutions were inoculated onto LB agar plates, and colony-forming units (cfu) were counted after incubation at 26 °C for 24 h. Experiments were performed three times.

Statistical Analysis—Survival analyses were performed using the Kaplan-Meier Log Rank χ² test. Comparisons between means of two independent groups were done with a pairwise t test, and multiple comparisons were done with one-way analysis of variance. For nonparametric data, multiple comparisons were done with a Kruskal-Wallis test, and pairwise comparisons were done with a Mann-Whitney test. Results are expressed as the group mean ± S.E. Significance was considered when p < 0.05. All data were analyzed with the SPSS Data Editor software (version 17.0, SPSS Inc.).

RESULTS

ROS Regulatory Gene Sequences from cDNA Libraries Constructed from Whole-body versus Midgut Exhibit High Similarity and Identity—Sequences corresponding to catalase, Cu/Zn superoxide dismutase, and peroxiredoxin retrieved from a sand fly whole body cDNA library showed a high level of identity and similarity against sequences obtained from a midgut-specific library (Table 1) (GenBank ABV60342, ABV60343, and ABV60347) (31), suggesting that these genes are also expressed in the midgut of L. longipalpis. Putative OXR1 could not be found in the midgut-specific database, and the best match to the L. longipalpis midgut expressed sequence tag library was a 14.5-kDa midgut protein (GenBank ABV60314, Table 1). All sequences obtained showed high levels of identity with homologous proteins from other organisms (supplemental Fig. 1, A–E), with conserved catalytic residues (catalase, superoxide dismutase, and peroxiredoxin) or domains (OXR1), which suggests that they are functional midgut proteins. Phylogenetic comparisons (supplemental Fig. 3) with sequences from these protein families showed a close proximity with corresponding proteins from other dipteran species, suggesting that these genes are orthologues to genes from other dipteran fly species including Drosophila and Anopheles.

Serratia and Not Leishmania Induces Changes in H₂O₂ Concentration in the Midgut—Catalase degrades toxic H₂O₂ into water and oxygen. To understand whether these changes in catalase expression have an effect on hydrogen peroxide levels, midgut-specific H₂O₂ concentration was measured in L. mexicana- and S. marcescens-infected L. longipalpis. Sand flies were infected, and midguts were assayed for H₂O₂ at 24, 48, 72, and 94 h ABF. Time 0 was considered as H₂O₂ concentration of non-blood-fed flies before infection. Additional replicates
ROS-mediated Immunity in L. longipalpis

TABLE 1

| Accession | Contig | Protein Database (NRPD) excluding | Best match to Non-redundant NRPD | Best match to L. longipalpis midgut EST library | Putative ROS-regulatory genes |
|-----------|--------|-----------------------------------|---------------------------------|-----------------------------------------------|-----------------------------|
| ABY63132  | H11002 | Putative catalase | ABV60342 | 11 | Putative catalase |
| ABY63135  | H11002 | Putative Cu/Zn superoxide dismutase | ABV60343 | 10 | Putative Cu/Zn superoxide dismutase |
| ABY63136  | H11002 | Putative peroxiredoxin | ABV60347 | 7 | Putative peroxiredoxin |
| ABY63137  | H11002 | 10 | 14.5-kDa midgut protein | ABY60314 | 1.1  |

* represents best match with a putative oxidation resistance protein function.

FIGURE 1. Hydrogen peroxide concentration in the midgut of Leishmania- and Serratia-colonized sand flies. Female flies were fed with a noninfected blood meal (Control), an L. mexicana-infected blood meal, or an S. marcescens-infected blood meal. Midguts of sucrose-fed sand flies were dissected 24 h before blood feeding and included in the analysis. Samples were homogenized and flash-frozen. Midguts were pooled (n = 2 pools of four midguts) and assayed for H2O2 concentration. Bar chart represents mean ± S.E. of combined samples from two independent experiments. An extra set of replicates was performed at 48 h after infection using fresh samples (n = 6 pools of four midguts). Data from both frozen and fresh samples were pooled for statistical analysis. Asterisk indicates statistical difference at p < 0.01 between Serratia versus Leishmania or control samples.

within the 48-h group were assayed using fresh, not flash-frozen, samples, and there was no significant difference between all fresh and frozen samples (analysis of variance, p > 0.39, n = 6). Only Serratia-infected flies exhibited a significant increase of H2O2 concentration at 48 h ABF when compared with the Leishmania-infected group (p < 0.01, Fig. 1). These results show that L. mexicana infection does not induce changes in hydrogen peroxide concentration in the midgut of L. longipalpis from 24 to 96 h ABF, in contrast to S. marcescens, which induced a significant increase in H2O2 concentration at 48 h after inoculation.

Serratia and Not Leishmania Induces Changes in Midgut ROS Production in Vivo—To further investigate ROS generation within L. longipalpis midguts, ROS levels were monitored in vivo in Leishmania- and Serratia-infected sand flies. Serratia-infected midguts showed a significant increase in ROS production at 24, 48, and 72 h ABF when compared with both Leishmania-infected and negative control midguts (p < 0.02, Fig. 2A). Leishmania-infected midguts did not show significant differences in ROS levels when compared with blood-fed negative controls (Fig. 2A). All Serratia-infected flies were dead at 7 days after infection. ROS significantly decreased at 24 and 48 h after blood feeding when compared with sugar-fed flies (Fig. 2A, p < 0.05), returning to similar values from sugar-fed flies after 72 h and 7 days after blood feeding (Fig. 2A). These results show that Serratia inoculation dramatically increases ROS production within L. longipalpis midgut, whereas ROS production in Leishmania infections did not increase in comparison with blood-fed control insects up to 7 days after infection.

Continuous H2O2 Feeding to Sand Flies Negatively Affects Leishmania Survival in Vivo—To analyze whether H2O2 has a negative effect in Leishmania survival inside the midgut, sand flies were allowed to feed ad libitum throughout the experiment on a 5-mmol H2O2-supplemented 70% w/v sucrose solu-
tion followed by Leishmania infections to determine the effect of chronic H₂O₂ feeding on Leishmania survival. Midgut homogenates of 96 h ABF sand flies fed on H₂O₂ had significantly fewer parasites when compared with negative controls fed on plain sucrose (p < 0.05, Fig. 3). These results show that H₂O₂ exposure in vivo decreases L. mexicana survival within the L. longipalpis midgut.

dsRNA-mediated Knockdown of Catalase Negatively Affects Leishmania Survival in the Midgut—Expression of four ROS regulatory genes was assessed by RT-PCR in the midgut of L. longipalpis infected with either S. marcescens or L. mexicana. (supplemental Fig. 4). Noninfected blood-fed flies were used as negative control. Catalase exhibited the highest variation in expression among all ROS-detoxifying gene sequences analyzed. To further understand the deleterious effects of ROS on the development of L. mexicana within the sand fly midgut, L. longipalpis catalase was knocked down by RNAi. A dsRNA-mediated catalase knockdown of ≥ 50% was achieved at 96 h after injection (Fig. 4A). Catalase gene depletion was detrimental to L. mexicana survival within L. longipalpis midgut at 4 days after infection (Fig. 4B), suggesting that changes in catalase activity within the sand fly gut had a negative outcome on Leishmania survival and development.

Chronic Feeding of a Potent ROS Scavenger Reduces Sand Fly Survival in Serratia-infected Flies and Increases Naturally Occurring Microbiota—To test whether ROS depletion by chronic feeding of an antioxidant would have an effect on L. longipalpis survival after Serratia inoculation, insects were fed from emergence and throughout the experiment on a uric acid-supplemented sucrose solution in cotton wool and then infected with S. marcescens in rabbit blood. Serratia-infected sand flies fed with uric acid-supplemented sucrose exhibited a significant decrease in survival when compared with Serratia-infected flies fed on plain sucrose solution (p < 0.001, Fig. 5A). Uric acid supplementation had no effect on survival as no significant reduction could be observed in blood-fed control flies chronically fed with uric acid in comparison with blood-fed control flies (Fig. 5A). To analyze whether reduction in insect

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**FIGURE 2. In vivo detection of ROS in Leishmania- and Serratia-infected sand flies.** Female flies were fed with a noninfected blood meal, an L. mexicana-infected blood meal, or an S. marcescens-infected blood meal. Individual midguts were dihydroethidium-stained and photographed to analyze ROS production. Midguts of sucrose-fed sand flies were dissected 24 h before blood feeding and included in the analysis. A, bar charts represent mean values of net color intensity ± S.E. of at least five individual midguts. Asterisk indicates statistical difference at p < 0.05 when compared with the sucrose (~24 h) group. Dagger represents no survivors (Serratia 7 days). B–D, selected representative images of noninfected and Leishmania- and Serratia-infected midguts, respectively. Scale bar represents 200 μm.

**FIGURE 3. Leishmania infection after continuous feeding of female L. longipalpis with a hydrogen peroxide-supplemented sucrose meal.** Flies were fed ad libitum from emergence on either 70% sucrose (Sucrose) or a 5-mmol H₂O₂-supplemented 70% sucrose solution (Sucrose + H₂O₂) and infected 3 days after emergence. Individual midguts were dissected and sampled for L. mexicana at 96 h after infection. Circles represent the number of parasites per individual midgut. Horizontal line represents mean ± S.E. of combined samples from two independent experiments. Groups were compared using the Mann-Whitney U test.
survival was due to an increase in *Serratia* bacterial cells within the sand fly gut, midguts of sand flies inoculated with *Serratia* with or without UA were dissected at 48 h ABF, and homogenates were diluted in PBS and inoculated onto LB agar plates. *Serratia* population in flies fed on UA-supplemented sucrose were significantly lower when compared with controls (*p* ≤ 0.012, Fig. 5B). However, control flies (noninfected) fed on UA-supplemented sucrose exhibited a larger population of resident *Serratia* bacterial cells within the sand fly gut, midguts of sand flies inoculated with *Serratia* with or without UA were dissected at 48 h ABF, and homogenates were diluted in PBS and inoculated onto LB agar plates.
microbiota ($p \leq 0.009$, Fig. 5C). To investigate whether naturally occurring microbial growth displayed a similar behavior in Serratia-infected samples, colony counts of resident microbes were performed. UA supplementation significantly increased resident microbiota numbers in Serratia-infected flies ($p \leq 0.037$, Fig. 5B).

**DISCUSSION**

The present study shows that ROS regulation influences the sand fly host-Leishmania parasite-gut microbiota interaction and that changes in midgut ROS levels by feeding uric acid (an exogenous ROS scavenger) can alter the dynamics of sand fly midgut homeostasis, favoring the growth of commensal sand fly gut bacteria.

**ROS Production with Bacteria**—The H$_2$O$_2$ concentration was significantly higher in Serratia-infected flies 2 days after blood feeding (Fig. 1). Additionally, in vivo detection of ROS confirmed high levels from 1 to 72 h after bacterial challenge (Fig. 2A). It is possible that the increase in ROS was due to epithelial cell death caused by pathogen proliferation (37). Alternatively, this increase could be part of the oxidative burst against pathogenic bacteria that has been observed in Drosophila (21). A similar inverse correlation between bacterial growth and ROS levels has been shown recently in A. aegypti (24). In a previous work, we showed that feeding sand flies with the ROS scavenger ascorbic acid was detrimental to survival (26) and suggested that mortality could be caused by bacterial infections due to ROS reduction in the midgut. In the present study, oral administration of a ROS scavenger also decreased survival in Serratia-infected flies continuously fed on uric acid when compared with Serratia-infected flies fed on sucrose solution. It is very unlikely that differences in mortality were caused by uric acid toxicity because flies fed on this antioxidant did not show any differences in survival when compared with control.

The addition of dietary uric acid increased resident gut microbiota in both Serratia-infected and noninfected flies but had an opposite effect on the Serratia population. In similar experiments performed in Aedes with Enterobacter asburiae, the addition of dietary ROS inhibitors increased both endogenous microbiota and Enterobacter (24). In our work, midguts were dissected 48 h after infection, when mortality reached ~80%. It is possible that survivors sampled at that time point were more resistant to infection and harbored a lower Serratia load. Another possibility is that the increase in the Serratia population was impeded by resident microbes, which were significantly higher in Serratia-infected flies supplemented with uric acid.

**ROS Production with Leishmania**—We have shown that hydrogen peroxide can kill Leishmania in vitro (supplemental Fig. 2) and that oral administration of H$_2$O$_2$ to infected flies is detrimental to Leishmania survival within the sand fly gut. RNAi-mediated gene silencing of the H$_2$O$_2$-detoxifying enzyme catalase led to lower Leishmania population in the gut, providing further confirmation that Leishmania parasites are sensitive to ROS generation. An estimated reduction of ~50% in catalase expression was achieved in catalase dsRNA-injected sand flies in comparison with mock-injected controls. Catalase knockdown sand flies exhibited a reduction in parasite numbers when compared with dsGFP-injected insects. Because only fully engorged females were selected for the experiments, it is unlikely that the size of the blood meal could account for the difference in parasite numbers. It was quite intriguing to find a lack of ROS activity in Leishmania-infected sand flies in comparison with Anopheles and Plasmodium (14). The fact that two different ROS (O$_2^-$ and H$_2$O$_2$) did not exhibit induction in biochemical assays in Leishmania-colonized sand fly midguts raises the possibility that Leishmania could “evade” the oxidative burst by an unknown mechanism or avoid eliciting a ROS-based response to ensure survival within the gut. One potential scenario would be detoxification of ROS by the Leishmania during blood meal digestion using the antioxidant enzymes of the protozoan. Antioxidant enzymes are crucial for Leishmania parasites during infections inside the macrophage. It has been shown that virulence in Leishmania correlates with antioxidant enzyme expression in the parasite (38, 39), as well as with its resistance to hydrogen peroxide toxicity (40–42). Moreover, in an interspecies microarray performed in L. mexicana, one of the most up-regulated genes in promastigotes when compared with lesion amastigotes was peroxidoxin (43), which when expressed as a protein was able to break down ROS in protozoa (44–46). The hypothesis of a Leishmania-mediated ROS detoxification during blood meal digestion is currently being studied in our laboratory.

An increasing body of sand fly sequence data (30, 31, 47, 48) allows us to infer that the sand fly gut immune response will be similar in overall organization to that of the other, more extensively studied dipterans: mosquitoes and Drosophila. In these insects, there is a reliance on the innate immune response mainly via the two types of effectors: ROS and antimicrobial peptides. Manipulating the sand fly ROS system revealed the potential complexity underlying immune homeostasis in the gut. The challenge for the fly is to regulate ROS production within the gut to attempt suppression of potential pathogens while allowing development of potentially beneficial microorganisms. Our results suggest that ROS are harmful to the Leishmania and that experimental activation of the ROS system in the sand fly results in a reduced Leishmania population. However, there is an apparent tolerance of the Leishmania by its sand fly host, allowing the development of large populations. The addition of antioxidant to the gut and subsequent effects on cohabitating bacterial species provide us with a glimpse into the “fine tuning” between ROS levels, bacterial communities, and the sand fly vector of Leishmania.

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