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Leishmania infantum Infection Modulates the Jak-STAT Pathway in *Lutzomyia longipalpis* LL5 Embryonic Cells and Adult Females, and Affects Parasite Growth in the Sand Fly

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Phlebotomine sand flies (Diptera, Psychodidae) belonging to *the Lutzomyia* genus transmit zoonoses in the New World. *Lutzomyia longipalpis* is the main vector of *Leishmania infantum*, which is the causative agent of visceral leishmaniasis in Brazil. To identify key molecular aspects involved in the interaction between vector and pathogens and contribute to developing disease transmission controls, we investigated the sand fly innate immunity mediated by the Janus kinase/signal transducer and activator of transcription (Jak-STAT) pathway in response to *L. infantum* infection. We used two study models: *L. longipalpis* LL5 embryonic cells co-cultured with *L. infantum* and sand fly females artificially infected with the parasite. We used qPCR to follow the *L. longipalpis* gene expression of molecules involved in the Jak-STAT pathway. Also, we modulated the Jak-STAT mediated immune response to understand its role in *Leishmania* parasite infection. For that, we used RNAi to silence the pathway regulators, protein inhibitor of activated STATs (PIAS) in LL5 cells, and STAT in adult females. In addition, the pathway suppression effect on parasite development within the vector was assessed by light microscopy in late-phase infection. The silencing of the repressor PIAS in LL5 cells led to a moderate increase in a protein tyrosine phosphatase 61F (PTP61F) expression. It suggests a compensatory regulation between these two repressors. *L. infantum* co-culture with LL5 cells upregulated repressors PIAS, suppressor of cytokine signaling (SOCS), and PTP61F. It also downmodulated virus-induced RNA-1 (VIR-1), a pathway effector, indicating that the parasite could repress the Jak-STAT pathway in LL5 cells. In *Leishmania*-infected *L. longipalpis* females, STAT and the antimicrobial peptide attacin were downregulated on the third day post-infection, suggesting a correlation that favors the parasite survival at the end of blood digestion in the sand fly. The antibiotic treatment of infected females showed that the reduction of gut bacteria had little effect on the Jak-STAT pathway regulation. STAT gene silencing mediated by RNAi reduced the expression

of inducible nitric oxide synthase (iNOS) and favored *Leishmania* growth in sand flies on the first day post-infection. These results indicate that STAT participated in the iNOS regulation with subsequent effect on parasite survival.

Keywords: sand fly, innate immunity, RNAi gene silencing, *Leishmania*, microbiota, vector-pathogen interaction

1 INTRODUCTION

Phlebotomine sand flies (Diptera, Psychodidae) are vectors of a group of parasitic diseases classified as cutaneous or visceral leishmaniases, which are transmitted by the bite of an infected female sand fly. These diseases are caused by protozoan parasites belonging to the *Leishmania* genus, with over 20 species identified as infective to humans and endemic to 98 countries and territories distributed from temperate to tropical regions of the globe (1). *Lutzomyia* is the most important genus in the American continent due to its wide distribution and diversity of species (reviewed in 2). This genus includes *Lutzomyia longipalpis*, the main vector of visceral leishmaniasis in Brazil, caused by *Leishmania infantum* (syn. *L. chagasi*) (reviewed in 3).

To prevent the spread of vector-borne diseases, strategies such as genetic manipulation of the vector or paratransgenesis, as well as transmission-blocking vaccines, became promising alternatives to reduce vector competence in transmitting pathogens [reviewed in (4, 5)]. We are interested in understanding how the *L. longipalpis* immune system responds to parasitic challenges, therefore contributing to developing novel molecular-based tools to control *the Leishmania* cycle in the insect.

We have previously identified *L. longipalpis* components of the sand fly innate immunity regulated by the Toll and immune deficiency (IMD) pathways that ultimately produce antimicrobial peptides (AMPs) such as attacin, cecropin, and defensins (6–8). Both pathways were active in *L. longipalpis* LL5 embryonic cells (6, 9). In addition, the activation of the IMD pathway in the female sand fly can reduce the parasite survival in the insect, evidencing the involvement of the insect immunity in parasite control (8). Nevertheless, innate immunity is multi-faceted. Besides AMPs expression mediated by the Toll and IMD pathways, the Janus kinase/signal transducer and activator of transcription (Jak-STAT) pathway regulate the expression of cytokines involved in cell growth, differentiation, and apoptosis. It also regulates other effector molecules that play important roles in the immune response (reviewed in 10–12). Briefly, the pathway is activated by the unpaired (Upd) family of ligands that bind to the transmembrane receptor domeless (Dome), which is followed by the recruitment and transphosphorylation of associated Jak tyrosine kinase Hopscotch (Hop). These events result in the phosphorylation and dimerization of the transcription factor STAT, which is then translocated to the cell nucleus for target genes transcription. This cascade is regulated at different levels by repressor molecules, including the suppressors of cytokine signaling (SOCS) family that bind to Dome receptor and inhibit STAT recruitment. Also, protein tyrosine phosphatases (PTPs) dephosphorylate STAT preventing its translocation to the cell nucleus. In addition, the protein inhibitor of activated STATs (PIAS) inactivates STAT through direct binding [reviewed in (10–12)]. Graphical representations of the Jak-STAT pathway in insects can be found in several reviews (10, 13, 14).

Among STAT-targeted genes, the AMP attacin and the virus-induced RNA-1 (VIR-1) are effector molecules associated with the Jak-STAT pathway in *Drosophila* (12, 15). Nevertheless, attacins in *Drosophila* are mostly regulated by the IMD pathway (16). Attacins are primarily active against Gram-negative bacteria (17) but also have antiparasitic activity (18). VIR-1 is induced by a viral infection such as the *Drosophila* C virus (DCV) (19). In addition, the Jak-STAT pathway can regulate the inducible nitric oxide synthase (iNOS) (reviewed in 20, 21), with a consequent increase of nitric oxide production and deleterious effect on microorganisms (reviewed in 22). Interestingly, upon *Plasmodium vivax* infection, STAT expression was increased in the malaria vector *Anopheles aquasalis*, and the silencing of STAT caused the increase in oocysts numbers in the vector (23). Also, the increased nitric oxide levels in *Anopheles stephensi* hemolymph are important to control the development of *Plasmodium berghei* (24). These findings indicate the potential role of the Jak-STAT pathway and its downstream effects in balancing parasite infection.

Since the Jak-STAT pathway regulates the expression of molecules involved in immune response, we investigated the gene expression of Jak-STAT-related molecules in *L. longipalpis* LL5 embryonic cells and adult females challenged by *L. infantum*. Because the gut microbiota can influence the immune response, we used the strategy of depleting the commensal gut bacteria with antibiotics to investigate its impact on the expression of Jak-STAT-related genes in *Leishmania*-infected females. In addition, to study the role of the *L. longipalpis* STAT, a putative pathway transcription factor, we suppressed its expression by using RNAi-mediated gene silencing. We followed the outcome in sand fly Jak-STAT-related gene expression, bacterial and parasite detection, and parasite development in the vector's gut.

2 METHODS

2.1 Jak-STAT Related Gene Sequences

Amino acid sequences of the Jak-STAT pathway-related molecules were selected from available databases of *Drosophila melanogaster* (25), *Anopheles gambiae*, *Aedes aegypti* mosquitoes (26), and NCBI (27). They were used as a query on tblastn and blastp search against the *L. longipalpis* database available in VectorBase portal (26). The query sequences and their corresponding similarity rates with the top *L. longipalpis* blastp hits are shown in **Supplementary Tables 1–7**. A selection of *L.*

longipalpis nucleotide sequence hits with the highest identity rates with mosquitoes sequences were retrieved and used in blastx search against the NCBI database (27) to assess their similarity to previously characterized sequences.

In addition, *L. longipalpis* translated sequences were analyzed using the InterPro Classification of Protein Families 81.0 tool (28) to search for signature domains. Similarities of *L. longipalpis* amino acid sequences with other insects were assessed by MUSCLE multiple sequence alignment (29) built-in Geneious 7.1.9 software (Biomatters, New Zealand). Cladograms were created using MEGA-X software (30), with the Maximum Likelihood method with a bootstrap of 100 replicates to model evolutionary rate differences among sites. Substitution models were defined for each gene using the MEGA-X tool according to the lowest Bayesian Information Criterion (BIC) score. For PIAS, Le and Gascuel's model was used with discrete Gamma distribution, considering part of sites are evolutionarily invariable (LG+G+I). For SOCS, PTP61F, iNOS, and DUOX, we used Le and Gascuel's model with discrete Gamma distribution (LG+G). For STAT, we used Jones-Taylor-Thornton's model with discrete Gamma distribution (JTT+G). Finally, for VIR-1, we used Whelan and Goldman's model with discrete Gamma distribution (WAG+G).

Because the *L. longipalpis* genes identification was based on similarity levels with previously characterized genes in other insect model species, we highlight that their sequences correspond to putative genes.

For relative expression studies, gene-specific oligonucleotides were designed using Primer3 online tool (31) on the open reading frame sequence for further gene expression analysis (**Table 1**).

2.2 Cell Cultures

L. longipalpis embryonic LL5 cells were grown at 29°C in L-15 medium (Sigma, USA) supplemented with 10% fetal bovine serum (Laborclin, Brazil), 10% tryptose phosphate broth, and 1% antibiotics (penicillin 100 U/mL and streptomycin 100 mg/mL, Sigma). For experimental procedures, LL5 cells were seeded in 24 well flat-bottom plates, and after overnight growth, a new supplemented L-15 medium was added and used for subsequent assays.

L. infantum (MHOM/BR/1974/PP75) was cultured in M199 medium (Sigma), adjusted to pH 7.4, and supplemented with 10% fetal bovine serum. For experimental procedures, parasites were harvested at the exponential growth phase and washed with PBS.

2.3 *L. longipalpis* Rearing and Treatment With Antibiotics

L. longipalpis females were obtained from colonized sand flies originally collected in Jacobina, BA, Brazil, and kept at

TABLE 1 | Oligonucleotides.

Reference	Name	Sequence
(6)	Attacin-F	AGGCTGATCCTCTGGGTCCTGT
	Attacin-R	ATGGGCATGGCAGCGTCTCT
(32)	Bac16s-F	TCCTACGGGAGGCAGCAGT
	Bac16s-R	GGAGTACCAGGGTATCTAATCCTGTT
(33)	DUOX-F	GGCAAGACGGAAGACAAG
	DUOX-R	TCAACAAGGGAACGACATC
(34)	Histone-F	GAAAAGCAGGCAAACACTCC
	Histone-R	GAAGGATGGGTGGAAAGAAG
(35)	iNOS-F	TGGCTGTCGCAATTTGTGTG
	iNOS-R	CCGCAATGTTCACCTCAACC
(36)	LeishActin-F	GTCGTCGATAAAGCCGAAGGTGGTT
	LeishActin-R	TTGGGCCAGACTCGTCGTACTCGCT
LLOJ002593	PIAS-F	GCCACAAGGGTTGAGCACAT
	PIAS-R	GACACTGCTTCCCGTTGACTTT
LLOJ008161	PTP61F-F	AATCCCGCGAAATCTTGCAG
	PTP61F-R	TCCAACGTTGTCATCGAGTG
Adapted from (37)	RP49-F	GACCGATATGCCAAGCTAAAGCA
	RP49-R	GGGGAGCATGTGGCGTGTCTT
LLOJ002175	SOCS-F	CCGTGGATGATGGGCTTGT
	SOCS-R	ATCCTTTCGGCTGCTTCG
LLOJ007427	STAT-F	GGCTCCAAAGATTCCGACAA
	STAT-R	AGGAAGAGAAAGAAGCGGGATGTCG
LLOJ005673	VIR-1-F	TAGTCCCGGAATTGACTTGG
	VIR-1-R	GTGTCAGGGGTTCATTCGTT
LLOJ002593	dsPIAS-F*	TGGCGCCCCTAGATGAGGAGGATGCAGACTGCGATAT
	dsPIAS-R*	TGGCGCCCCTAGATGACGTGGGCTTCCGCTCATTCA
LLOJ007428	dsSTAT-F#	CCGtaatacgactcactatagggGGCATCCCGTTCAAGTAG
	dsSTAT-R#	CCGtaatacgactcactatagggTTGGAGCCGTTCCTGTTT
Adapted from (38)	dsLacZ-F#	taatacgactcactatagggagaTATCCGCTCACAATTCCACA
	dsLacZ-R#	taatacgactcactatagggagaGAGTCAGTGAGCGAGGAAGC
(7)	T7+adapter*#	CCGtaatacgactcactatagggTGGCGCCCCTAGATG

Underlined nucleotides indicate adapter sequence.

#*Lowercase nucleotides indicate T7 promoter sequence.*

temperatures between 24-28 and 70-80% relative humidity following standard insectary conditions (39). Adult insects were fed on 50-70% sucrose *ad libitum*. Females were blood-fed on anesthetized hamsters or mice once a week for colony maintenance. For bacterial depletion, sand flies were separated and kept with sucrose solution containing a mixture of antibiotics at a final concentration of 100 U/mL penicillin-streptomycin and 10 µg/mL gentamicin immediately after emergence from pupae until needed.

2.4 Double-Stranded RNA Synthesis

DNA templates were amplified from *L. longipalpis* cDNA obtained from insects kept under colony conditions for dsRNA *in vitro* synthesis.

PIAS template was amplified by PCR in two rounds. In the first round, gene-specific primers coupled to an adapter sequence on the 5′ end (dsPIAS-F and dsPIAS-R, **Table 1**) were used to amplify the partial coding sequence of the target gene. This first round product was subsequently used as a template in a second PCR containing primers with the adapter and T7 promoter sequences (T7+adaptor, **Table 1**). On both rounds, PCR conditions were: 95°C for 3 min; 35 cycles of 95°C for 30 sec, 57°C for 45 sec, and 72°C for 45 sec; followed by 72°C for 7 min.

The putative STAT template was amplified by PCR using gene-specific primers directly coupled to T7 promoter sequence (dsSTAT-F and dsSTAT-R, **Table 1**) in a touchdown PCR as follows: 95°C for 3 min; 16 cycles of 95°C for 45 sec, 68 to 50°C (progressively decreasing 1°C per cycle) for 45 sec, and 72°C for 45 sec; 26 cycles of 95°C for 45 sec, 50°C for 45 sec, and 72°C for 45 sec; 72°C for 3 min.

Up to 2 µg of these templates were used in a dsRNA synthesis reaction using MEGAscript RNAi kit (Invitrogen, USA) following the manufacturer's instructions.

2.5 *L. infantum* Challenge and PIAS Gene Silencing in LL5

2.5.1 PIAS Silencing in LL5 Cells

For PIAS silencing, LL5 cells were transfected with a mixture of transfection agents containing 0.25 mL DharmaFECT (Thermo Fisher Scientific, USA), 23.25 mL DCCM medium, and 1.5 mL of PIAS dsRNA for a final concentration of 30 nM. As a control, cells were transfected with a non-related luciferase dsRNA (6). LL5 cells were maintained in this mixture for 16 h and then incubated for 12 h, 24 h, and 48 h at 30°C, before being resuspended in TRIzol reagent (Invitrogen).

2.5.2 *L. infantum* Challenge in LL5 Cells

For the *Leishmania* challenge, parasites were seeded to L-15 medium and added to LL5 cells at a microbe/cell ratio of 10 to 1. Non-challenged LL5 cells were used as control. Samples were collected at 24 h, 48 h, and 72 h post-challenge from three independent experiments by discharging the supernatant medium, washing the cells twice with PBS, adding 1 mL of TRIzol, and storing at -80°C for future RNA extraction.

2.6 STAT Gene Silencing and Artificial Feeding in *L. longipalpis* Females

2.6.1 STAT Gene Silencing in *L. longipalpis* Females

For the putative STAT gene silencing, STAT dsRNA was lyophilized and resuspended in nuclease-free H_2O to 4.5 µg/µL final concentration. Females were microinjected intrathoracically with 32.2 nL of dsRNA using Nanoject II microinjector (Drummond, USA) (40). A non-related LacZ dsRNA was injected in negative control groups (41). For assays involving *Leishmania* infection, sand flies were artificially infected as described above on the following day after dsRNA microinjection and collected on subsequent days according to experimental design. For controlling gene silencing efficiency, injected flies were kept under colony conditions fed on sucrose and collected on three successive days post dsRNA microinjection.

2.6.2 Artificial Feeding in *L. longipalpis* Females

For artificial infection, females (3 to 6 days after emerging from pupae) were fed through chick skin membrane on inactivated New Zealand rabbit blood seeded with *L. infantum* (10^6 parasites/mL of blood), and control groups were fed on blood. Fully engorged females were separated and collected at 24 h, 48 h, 72 h, and 144 h post-feeding for RNA extraction and at 144 h for microscopy analysis. All samples were collected in pools of 10 whole body sand flies, or 15 dissected guts and corresponding carcasses (insect thorax and abdomen without gut) depending on experimental design.

2.7 RNA Extraction, cDNA Synthesis, and Gene Expression Analysis

According to each experimental design, samples were collected at different time points post challenges for total RNA extraction using TRIzol. Extracted RNA was incubated with RNase-free DNase I (Thermo Scientific) at 1 U/µg of total RNA for removing possible traces of DNA. Up to 1 µg of total RNA was used in reverse transcriptase reactions to produce cDNA using SuperScript III Reverse Transcriptase (Invitrogen). Protocols were followed according to each manufacturer's instructions.

Gene expression was assessed by qPCR using cDNA templates, gene-specific primers (**Table 1**), and SYBR Green PCR Master Mix in a 7500 Real-Time PCR System (Applied Biosystems, USA) following manufacturer's standard cycling conditions. The gene expression was calculated relative to a ribosomal protein (RP49) reference gene and expressed in fold change values in comparison to a control group (6) following the $\Delta\Delta C_T$ method (42).

2.8 *Leishmania* Development in Sand Fly Guts

Sand flies were examined at 144 h post-infection by light microscopy for parasite load and localization. Guts were dissected in saline solution (NaCl 0.9%), covered with a thin glass slide, and examined under a 40x magnification objective lens. Parasite loads were estimated and classified as light (below 100 parasites), moderate (between 100 and 1000 parasites), or

heavy infection (above 1000 parasites) (43). In addition, the localization of parasites in the gut (abdominal or thoracic gut, cardia, and colonized stomodeal valve) was recorded to evaluate the progress of *Leishmania* infection, following previously published methods (44).

2.9 Statistical Analysis

We used ordinary two-way ANOVA with Sidak's correction for multiple comparisons test using GraphPad Prism software (version 6.07) (GraphPad Software Inc., USA) to test significant differences between experimental and control groups across various time points. The Sidak's correction for multiple comparisons compute confidence intervals and significance based on adjusted P values. This method was applied to gene expression results obtained by qPCR and in infection estimation and localization results obtained by light microscopy observation.

3 RESULTS

3.1 Gene Identification

We selected key molecules involved in the Jak-STAT pathway, such as regulators (repressors and transcription factor) and downstream effector molecules. We identified sequences similar to repressors PIAS, PTP61F, and SOCS, transcription factor STAT, and downstream related such as VIR-1 in the *L. longipalpis* transcript database available from VectorBase (26). Other related downstream genes such as attacin (6), iNOS (35), and DUOX (9) were identified in previous studies.

There was one PIAS ortholog (LLOJ002593) in the *L. longipalpis* database (**Supplementary Table 1**). The identification of signature domains in the *L. longipalpis* amino acid sequence (*in silico* predicted) showed that the sequence coding for the main pathway repressor contains the N-terminal PINIT (IPR023321) and Zinc finger (IPR004181) domains of the E3 SUMO-protein ligase PIAS1/PIAS3 family (**Supplementary Figure 1A**). The phylogenetic analysis showed that *L. longipalpis* PIAS sequence is distantly related to *Bractrocera*, *Drosophila*, *Musca*, and *Stomoxys* fly species and to *Aedes* and *Anopheles* mosquito species (**Supplementary Figure 1B**).

In the *L. longipalpis* database, there were three SOCS-like sequences (**Supplementary Table 2**). The sequence with the highest similarity with mosquitoes' sequences (LLOJ002175) was selected for our future analyses. This *L. longipalpis* SOCS-like amino acid sequence contains the sarcoma homology 2 (SH2) (IPR000980) and SOCS box (IPR001496) domains (**Supplementary Figure 2A**). This sequence is closely related to *Nyssomyia neivai* and *D. melanogaster* SOCS16D sequences and form a cluster with *A. aegypti* and *A. gambiae* SOCS7. Another putative *L. longipalpis* SOCS sequences form clusters with *D. melanogaster* SOCS44A and SOCS5 from mosquitoes. A third SOCS-like sand fly sequence forms a cluster with *D. melanogaster* SOCS36E and SOCS6 sequences from mosquitoes (**Supplementary Figure 2B**).

The PTP61F amino acid sequence from *L. longipalpis* (LLOJ008161) was the only ortholog identified (**Supplementary Table 3**) and contains the PTP superfamily domain (IPR000242) (**Supplementary Figure 3A**). It formed a cluster with other PTP61F sequences from *Aedes*, *Culex*, and *Anopheles* species while separated from the Drosophila PTP61F cluster in the phylogenetic analysis (**Supplementary Figure 3B**).

Two putative STAT amino acid sequences were found through the blastp search against the *L. longipalpis* database (**Supplementary Table 4**). We chose one STAT-like sequence(LLOJ007428) to proceed with our analysis. This sequence contains STAT (IPR013801) and SH2 (IPR000980) domains forming the STAT family signature (**Supplementary Figure 4A**). In the phylogenetic analysis, the *L. longipalpis* STAT-like sequences form a separate branch from the STAT92E sequences from *D. melanogaster* and clusters containing STAT1 and STAT5 sequences identified in other organisms (**Supplementary Figure 4B**).

Among the Jak-STAT downstream molecules in *L. longipalpis*, the attacin sequence was previously identified. It contains the attacin family signature domain and is similar to other attacin sequences from *N. neivai* and *Phlebotomus papatasi* (41)..

Through the blastp search, we found one putative VIR-1 sequence in the *L. longipalpis* database (**Supplementary Table 5**). To date, VIR-1 has no specific domain associated with it. Nevertheless, the *L. longipalpis* VIR-1-like predicted amino acid sequence shares similarities with the *Drosophila* VIR-1 sequence. In our phylogenetic analysis, the sand fly VIR1-like sequence forms a branch distinct from the *Drosophila* cluster and is closer to the *Culex quinquefasciatus* VIR and other *Aedes* and *Culex* sequences not yet fully characterized (**Supplementary Figure 5**).

We also investigated the presence of signature domains in the sequences associated with the production of oxidative stress identified from previous studies (9, 33, 35, 45). The blast search for the *L. longipalpis* iNOS sequence in the VectorBase database (**Supplementary Table 6**) indicated one ortholog (LLOJ005465) that contains the N-terminal domain of the NOS superfamily (IPR036119). The phylogenetic analysis indicates that the *L. longipalpis* iNOS-like sequence forms a separate branch from the clusters containing *Drosophila*, *Aedes*, and *Anopheles* species (**Supplementary Figure 6**).

The DUOX amino acid sequence used in this study (LLOJ010494) showed high similarity with DUOX sequences from *A. aegypti*, *A. gambiae*, and *D. melanogaster* in our blastp search (**Supplementary Table 7**). This sequence contains the characteristic domains of the NOX-DUOX family including FAD-binding (IPR017927) and NADP binding (IPR013121) domains. The phylogenetic analysis showed that the *L. longipalpis* DUOX amino acid sequence formed a separate branch from the *Drosophila*, *Lucilia*, and *Sarcophaga* flies' cluster. It was also separated from mosquito DUOX sequences (**Supplementary Figure 7**).

Multiple alignments and phylogenetic trees output files generated in the MEGA X software were deposited in a public repository: DOI: 10.6084/m9.figshare.16915891.

L. longipalpis PIAS, PTP61F, SOCS (or SOCS-like), STAT (or STAT-like), VIR-1 (or VIR1-like) genes were identified in this

study by sequence similarity. Therefore, they were considered as putative genes. We use these acronyms only for text simplification purposes.

3.2 Silencing of PIAS in LL5 Cells and Consequent Expression of Jak-STAT-Related Genes

Our first approach was to test the activity of the Jak-STAT pathway in *L. longipalpis* by silencing the pathway repressor PIAS. We hypothesized that PIAS silencing would affect the gene expression of other Jak-STAT-related molecules. We chose LL5 cells, which were shown to be useful models to study *L. longipalpis* innate immunity (6). We transfected them with PIAS dsRNA to follow the expression of selected genes by qPCR.

We observed that PIAS silencing was significantly achieved at 24 h and 48 h post dsRNA transfection compared to the control group transfected with LacZ dsRNA (**Figure 1A**). The expression of the putative repressor SOCS did not alter significantly (**Figure 1B**), while the repressor PTP61F was significantly increased at 24 h (**Figure 1C**). The putative STAT transcription factor, attacin, and VIR-1 showed no significant alteration after the PIAS dsRNA transfection (**Figures 1D–F**).

3.3 Jak-STAT-Mediated Response to *Leishmania* in LL5 Cells

We also hypothesized that LL5 cells' immune response mediated by the Jak-STAT pathway would be affected by the *L. infantum* challenge. Therefore, we co-cultured LL5 cells and *Leishmania* and assessed the expression of Jak-STAT-related genes by qPCR at subsequent times.

The putative PIAS and PTP61F expression significantly increased at 48 h and 72 h post-challenge compared to non-challenged control groups (**Figures 2A, C**), while SOCS expression showed a high variability (**Figure 2B**). The STAT-like transcription factor significantly increased at 24 h, while attacin showed no significant modulation (**Figures 2D, E**). Interestingly, VIR-1 was significantly reduced at 24 h, 48 h, and 72 h post-challenge (**Figure 2F**).

3.4 Expression of Jak-STAT-Related Genes in *L. longipalpis* Females Infected With *Leishmania*

Our following approach investigated the Jak-STAT-related immune response in *L. longipalpis* adult females artificially infected with *L. infantum*. We hypothesized that the parasite infection would alter the pathway expression in the adult female.

The putative pathway regulators PIAS, SOCS, and PTP61F, as well as STAT, did not show significant differences in whole-body samples of sand flies infected with *Leishmania* compared to the blood-fed control group (**Figures 3A–D**). Attacin expression was reduced at 72 h (**Figure 3E**), while VIR-1 and iNOS have not altered post *Leishmania* infection (**Figures 3F, G**). We detected the bacteria load by the 16S ribosomal RNA gene expression, showing no significant differences between infected and non-infected groups (**Figure 3H**). In addition, we assessed the parasite load through the *Leishmania* actin gene expression compared to a control sample collected at 24 h post-infection. The parasite load was increased 48 h post-infection (**Figure 3I**).

It is well known that the gut microbial community plays a considerable role in balancing immune responses (reviewed in

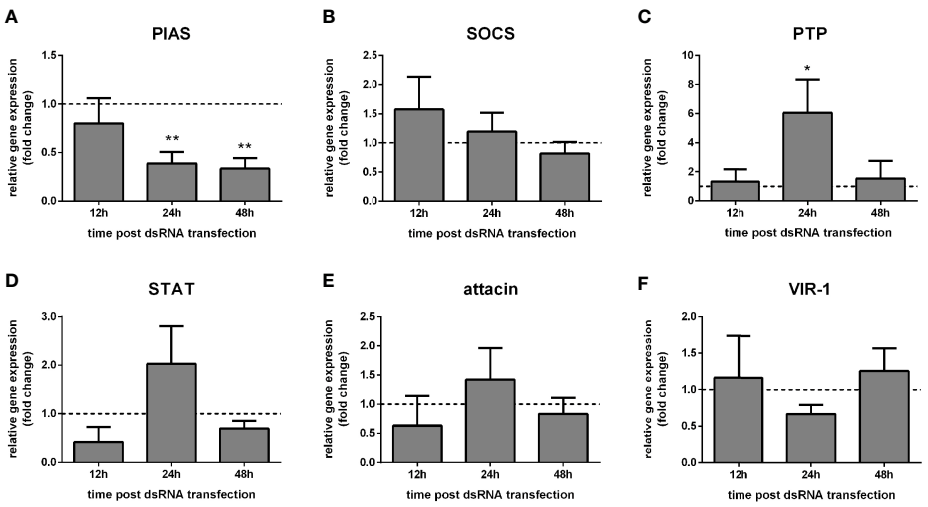

FIGURE 1 | Gene expression of Jak-STAT regulators in LL5 cells after PIAS silencing: *L. longipalpis* relative gene expression was calculated compared to the endogenous reference gene RP49. **(A)** silencing of the PIAS gene; **(B–F)** expression of indicated genes after PIAS silencing. **(A–F)** relative expression of PIAS-dsRNA transfected LL5 cells (y-axis) was expressed as fold change compared to the control group transfected with a non-related dsRNA and collected at each corresponding time point (horizontal dotted line). Samples of experimental and control groups were collected at 12 h, 24 h, and 48 h post PIAS silencing (x-axis). Vertical bars represent the mean with standard error (SEM) of 3 biological replicates. Significant differences were calculated using two-way ANOVA (*$p < 0.05$; **$p < 0.01$).

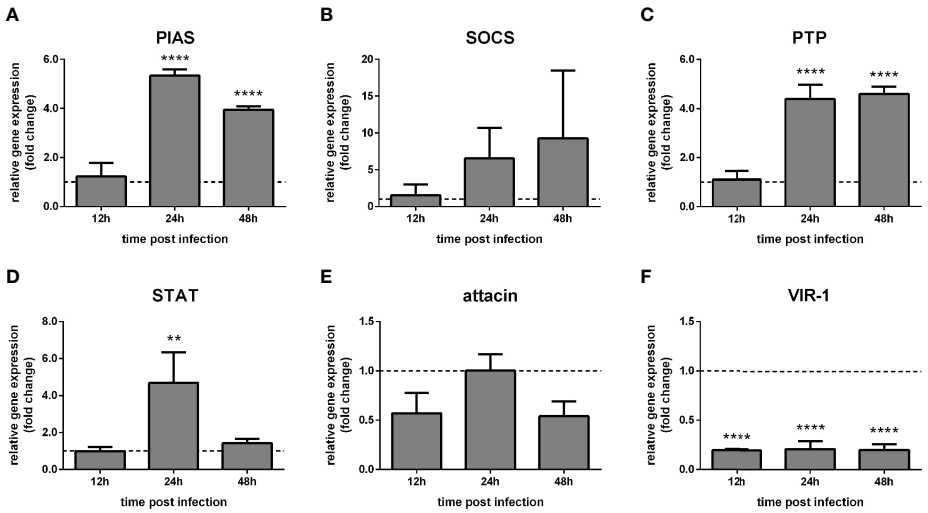

FIGURE 2 | Gene expression of Jak-STAT-related genes in LL5 cells after *Leishmania* challenge: *L. longipalpis* relative gene expression was calculated compared to the endogenous reference gene RP49. **(A–F)** expression of indicated genes after *Leishmania* co-culture. Relative expression in *Leishmania*-challenged LL5 cells (y-axis) was expressed as fold change compared to the non-challenged control group collected at each corresponding time point (horizontal dotted line). Samples of experimental and control groups were collected at 24 h, 48 h, and 72 h post-*Leishmania* challenge (x-axis). Vertical bars represent the mean with standard error (SEM) of 3 biological replicates. Significant differences were calculated using two-way ANOVA (**p < 0.01; ****p < 0.0001).

46). In our study model, both experimental and control groups were infected with *L. infantum*. We chose to deplete the commensal bacteria in the experimental group by feeding with a mixture of antibiotics, while the control group was *Leishmania*-infected but not treated with antibiotics. We tested whether the suppression of gut bacteria would interfere with the expression of Jak-STAT-related molecules in infected sand flies.

The expression of pathway regulators PIAS and STAT-like were not significantly altered in whole sand flies after bacteria depletion (**Supplementary Figures 8A, B**). Attacin expression was also not changed significantly but was highly variable at 144 h post-infection (**Supplementary Figure 8C**). In addition, VIR-1 was also not significantly changed by the antibiotic treatment (**Supplementary Figure 8D**).

3.5 Expression of Jak-STAT-Related Genes in STAT-Silenced *L. longipalpis* Females Fed on Sucrose

To further explore the role of the Jak-STAT pathway, we hypothesized that the suppression of STAT-like transcription factor by RNAi-mediated gene silencing would reduce the gene expression of effector molecules associated with the pathway.

STAT expression was reduced in females at 24 h post STAT dsRNA injection compared to the control group injected with LacZ dsRNA (**Figure 4A**). Upon STAT silencing, attacin expression was unaltered at 24 h and increased significantly at 48 h (**Figure 4B**). VIR-1 expression showed a reduction at 24 h and 72 h and increased at 48 h (**Figure 4C**). iNOS expression was significantly reduced at 24 h and 48 h (**Figure 4D**), while DUOX was increased at 24 h (**Figure 4E**). Bacteria detection by 16S rRNA was not significantly altered (**Figure 4F**).

3.6 Expression of Jak-STAT-Related Genes in STAT-Silenced *L. longipalpis* Females Infected With *Leishmania*

We also investigated the effect of STAT-like silencing in the gene expression of STAT-related downstream molecules in dissected gut and carcasses of *Leishmania*-infected sand flies. In carcasses, STAT expression was significantly reduced at 24 h and 48 h (**Figure 5A**), while attacin was increased at 48 h post-infection (**Figure 5B**). VIR-1 was not significantly altered (**Figure 5C**), while iNOS was reduced at 24 h and 48 h, and DUOX at 24 h (**Figures 5D, E**).

In dissected guts, STAT-like expression was reduced at 24 h and 48 h (**Figure 6A**). Attacin was increased at 48 h post-infection (**Figure 6B**). VIR-1, iNOS, and DUOX were not significantly altered (**Figures 6C–E**). Also, bacteria detection through 16S ribosomal RNA expression was not significantly altered (**Figure 6F**), while *Leishmania* detection through actin expression was significantly increased at 24 h (**Figure 6G**).

3.7 *Leishmania* Infection Development in STAT-Silenced *L. longipalpis* Females

In addition, we hypothesized that the downstream effects of STAT-like silencing could cause an alteration on *L. infantum* colonization in the sand fly gut since we observed an increase in *Leishmania* detection after STAT silencing. Therefore, we investigated the intensity and localization of parasites on six days post-infection in sand fly dissected guts. *Leishmania* infection estimation showed that proportions of STAT-silenced sand flies carrying heavy, moderate, or light infections, as well as non-infected, were not significantly different from the control group injected with LacZ dsRNA (**Figure 7A**). The parasite localization in the stomodeal valve, thoracic gut, and

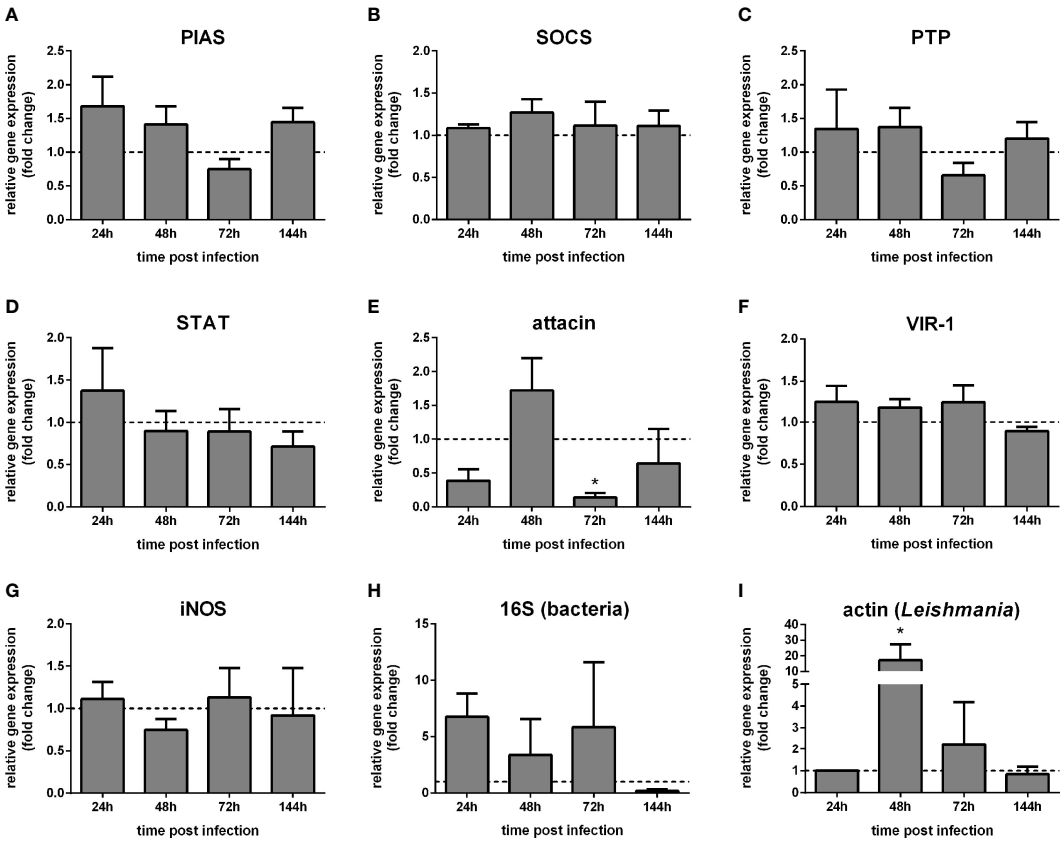

FIGURE 3 | Gene expression of Jak-STAT-related genes in *L. longipalpis* infected with *Leishmania*: *L. longipalpis* relative gene expression was calculated compared to the endogenous reference gene RP49. **(A–G)** expression of indicated genes after *Leishmania* infection; quantification of bacteria **(H)** and *Leishmania* **(I)**. Relative gene expression in *Leishmania*-infected females (y-axis) was expressed as fold change compared to the non-infected female control group collected at each corresponding time point (horizontal dotted line) **(A–H)**. *Leishmania* actin expression was expressed as fold change compared to a control sample collected at 24 h post-infection **(I)**. Samples of experimental and control groups were collected in pools of 10 whole body sand flies at 24 h, 48 h, 72 h, and 144 h post-infection (x-axis). Vertical bars represent the mean with standard error (SEM) of 3 biological replicates. Significant differences were calculated using two-way ANOVA (*$p < 0.05$).

abdominal gut did not differ significantly between STAT-silenced and control groups (**Figure 7B**).

A summarized workflow of the experimental approach and outcomes of the manuscript is shown in **Supplementary Figure 9**.

4 DISCUSSION

Sand flies trigger a repertoire of molecular mechanisms to fight potential microbial harm (reviewed in 47). In *L. longipalpis*, these mechanisms include AMPs production regulated by the Toll and IMD pathways (6, 8, 41, 48). Nevertheless, the complexity of the immune response in this insect is not fully understood. We were interested in investigating the role of the Jak-STAT pathway in the context of *L. longipalpis* interaction with *L. infantum*.

4.1 Jak-STAT Pathway

We identified *L. longipalpis* transcripts involved in the Jak-STAT pathway based on their moderate similarity with genes identified in *Drosophila*, *Aedes*, and *Anopheles*, which are well-characterized models. These *L. longipalpis* sequences indicate that the pathways genes are transcribed and have moderate similarity with other dipterans, including vectors of zoonotic diseases. Such similarities are shared across invertebrates and vertebrate species (reviewed in 49).

In insects, this pathway is activated upon microbial infections. In *Drosophila*, it is triggered upon viral infections by DCV, *Drosophila* X virus (DXV), invertebrate iridescent virus 6 (IIV6) (50). In mosquitoes, it is triggered by the West Nile virus (WNV) in *C. quinquefasciatus* (51), dengue virus (DENV) (52, 53), and Zika virus (54) infection in *Aedes aegypti*, to cite a few. However, only a reduced number of studies showed the role of this pathway against parasitic infection. In *Anopheles aquasalis* and *A. gambiae*, Jak-STAT was activated toward *P. vivax* and *P. berghei*, respectively (23, 55), indicating the pathway role in the insect response against the parasitic infection.

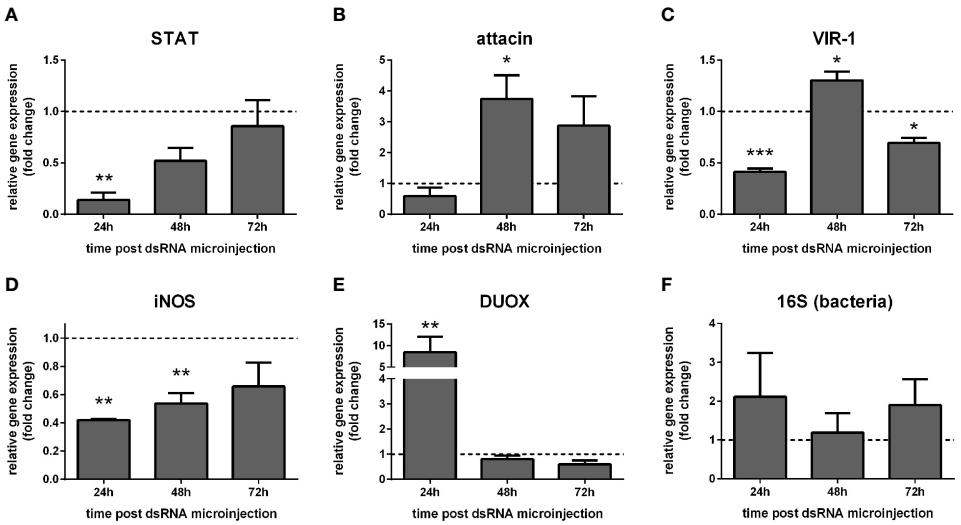

FIGURE 4 | Gene expression of Jak-STAT-related genes in STAT-silenced sucrose fed *L. longipalpis*: *L. longipalpis* relative gene expression was calculated compared to the endogenous reference gene RP49. **(A)**: silencing of the STAT-like gene; **(B–E)**: expression of indicated genes after STAT-like gene silencing. **(F)** quantification of bacteria. **(A–F)** relative expression of STAT-like dsRNA injected females (y-axis) was expressed as fold change compared to the control group of LacZ dsRNA injected females. Both group samples were collected at each corresponding time point (horizontal dotted line). Samples of experimental and control groups were collected in pools of 10 whole body sand flies at 24 h, 48 h, and 72 h post-STAT silencing (x-axis). Vertical bars represent the mean with standard error (SEM) of 3 biological replicates. Significant differences were calculated using two-way ANOVA (*p < 0.05; **p< 0.01; ***p < 0.0001).

4.2 PIAS Knockdown in LL5 Cells and effect on Jak-STAT-Related Genes

To test this pathway activity in *L. longipalpis* LL5 cells, we knocked down PIAS, a major pathway repressor, and followed the consequent effect on other Jak-STAT related genes. The LL5 is a suitable model for studying immune pathway modulation since experimental variables are easier to control in these cells than in insects. These cells were used to investigate arboviral infections (56, 57) and have an antiviral response (7, 57, 58). Moreover, they were used in studies investigating the interaction with *Leishmania* (59)

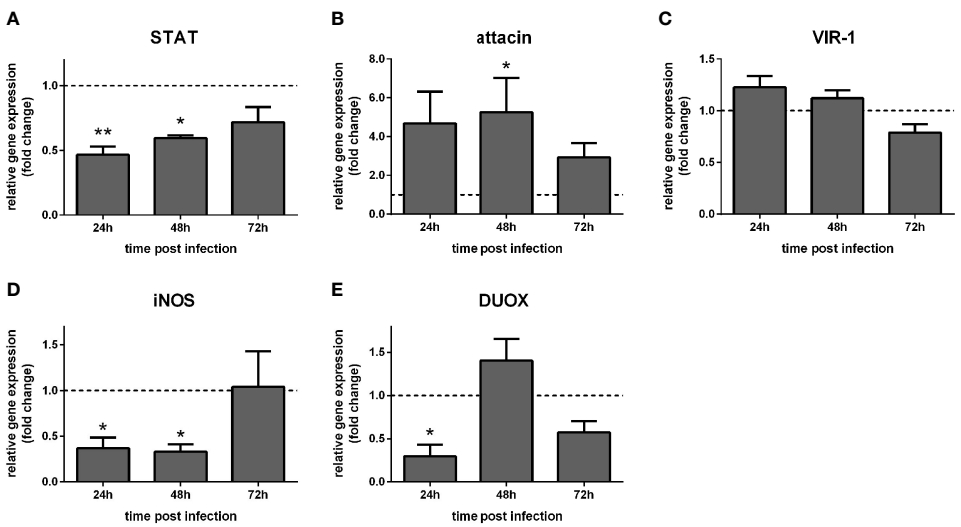

FIGURE 5 | Gene expression of Jak-STAT-related genes in carcasses of STAT-silenced *L. longipalpis* and infected with *L. infantum*: *L. longipalpis* relative gene expression was calculated compared to the endogenous reference gene RP49. **(A)** silencing of the STAT-like gene; **(B–E)**: expression of indicated genes after STAT-like gene silencing. **(A–E)** relative gene expression in STAT-like dsRNA injected females (y-axis) was expressed as fold change compared to the control group of LacZ dsRNA injected females. Samples were collected at 24 h, 48 h, and 72 h post-infection (x-axis). Vertical bars represent the mean with standard error (SEM) of 3 biological replicates. Significant differences were calculated using two-way ANOVA (*p < 0.05; **p< 0.01).

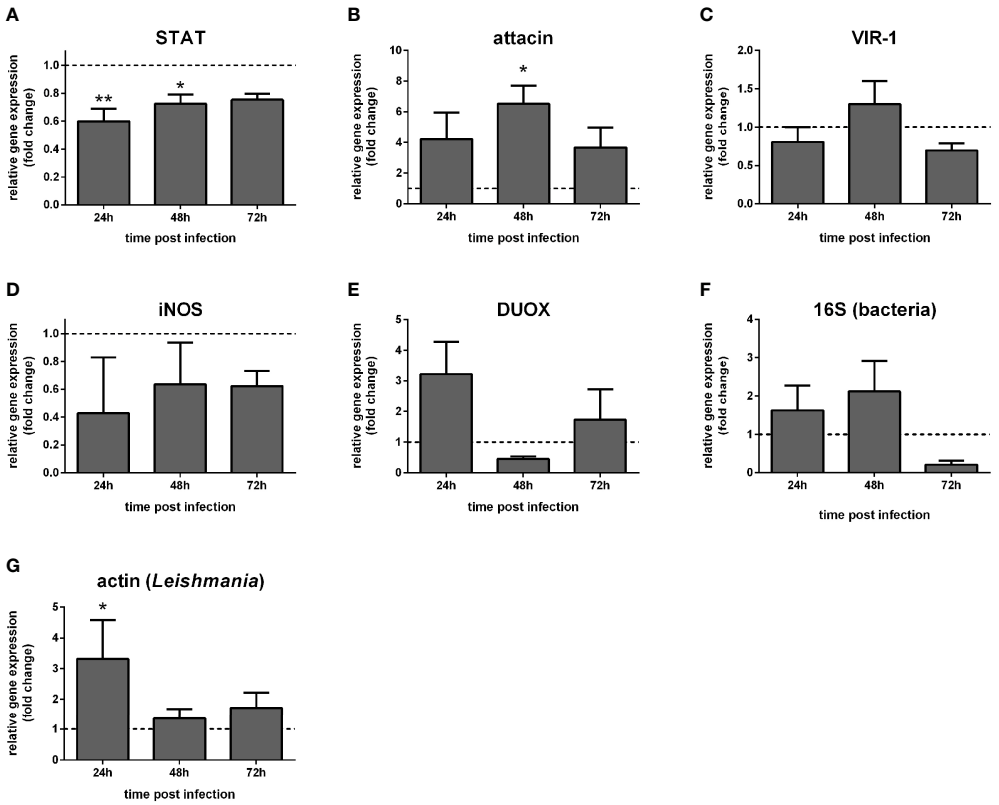

FIGURE 6 | Gene expression of Jak-STAT-related genes in dissected guts of STAT-silenced *L. longipalpis* and infected with *L. infantum*: *L. longipalpis* relative gene expression was calculated compared to the endogenous reference gene RP49. **(A)** silencing of the STAT-like gene; **(B–E)** expression of indicated genes after the STAT-like gene silencing; quantification of bacteria **(F)** and *Leishmania* **(G)**. **(A–F)** relative expression in STAT-like dsRNA injected females (y-axis) was expressed as fold change compared to the control group of LacZ dsRNA injected females. Samples were collected at 24 h, 48 h, and 72 h post-infection (x-axis). Vertical bars represent the mean with standard error (SEM) of 3 biological replicates. Significant differences were calculated using two-way ANOVA (*$p < 0.05$; **$p < 0.01$).

and later shown to have Toll and IMD mediated immune response against bacteria, yeast, and *Leishmania* (6, 9). Previously, these cells were shown to respond efficiently to dsRNA transfection (6), and PIAS was efficiently silenced in the current experimental setting. Although the inhibitory action of PIAS occurs at the protein level, the suppression of PIAS could result in a consequent effect on the transcription of other pathway molecules, possibly by a feedback loop.

The Jak-STAT pathway has many key regulatory molecules, including repressors such as SOCS and PTP61F, that could be expressed to counterbalance PIAS silencing. While the putative SOCS showed no significant changes, PTP61F expression was increased after PIAS was silenced. PIAS downmodulation suggests that the pathway was activated, and PTP61F may be expressed to compensate for PIAS reduction. PTPs can dephosphorylate STAT tyrosine residues, therefore preventing this transcription factor translocation to the nucleus and consequently negatively regulating the pathway-dependent genes (reviewed in 60). STAT-like expression was not correlated with PIAS suppression or the increase of PTP61F. This finding shows that STAT expression was not directly related to PIAS silencing. Thus, the possibility of existing a feedback loop between PIAS and STAT is scarce. Attacin and VIR-1 showed no direct correlation with the repressors. Together these results suggest that the Jak-STAT pathway in LL5 cells was balanced between PIAS and PTP61F repressors.

4.3 Effect of *Leishmania* Challenge on Jak-STAT-Related Genes in LL5 Cells

It was previously shown that the interaction with *Leishmania* parasites caused dramatic changes to *L. longipalpis* embryonic Lulo cells (61, 62). In LL5 cells, we previously observed that the interaction with *Leishmania* triggered the expression of two transcription factors, dorsal and relish, involved in the Toll and IMD pathways, respectively, with a subsequent moderate effect on AMPs expression (6). We used the LL5 co-cultured with *Leishmania* to investigate whether the parasite interaction would trigger the Jak-STAT pathway. We observed that the expression of PIAS and PTP61F was increased after the second day, showing that the co-culture setting repressed the pathway. The possibility of a feedback loop influencing the STAT-like transcription was also considered in this experimental setting. We observed that STAT expression was not reduced, as the repressor was upregulated, but its expression was increased at 24 h post

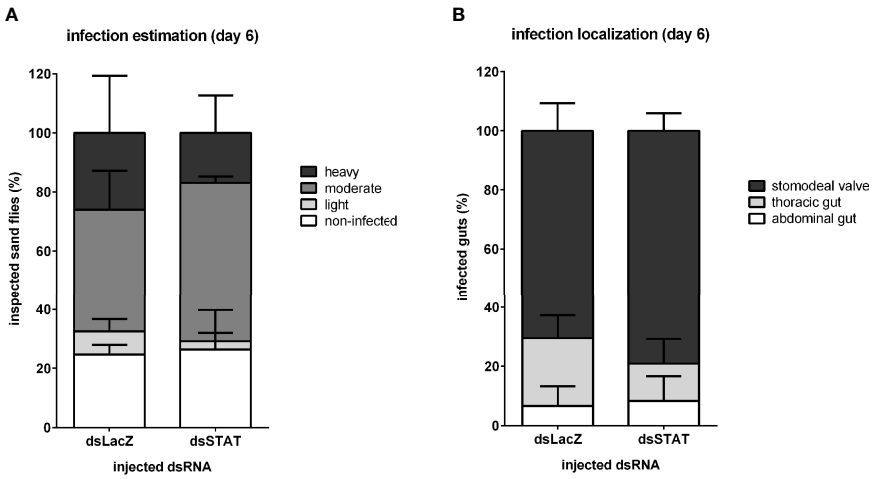

FIGURE 7 | *Leishmania* infection development in STAT-like-silenced *L. longipalpis*: Infection intensity and development at late infection in dsRNA-injected sand flies. **(A)** The y-axis represents the percentage of all individually inspected insects (minimum of 20 sand flies in each dsRNA injected group). Bar colors indicate infection intensity: non-infected (white), with light (light grey), moderate (mid grey), and heavy (dark grey) infections. **(B)** The y-axis represents the percentage of infected insects used in infection progress evaluation in the gut. Bar colors indicate sand fly gut localization: parasites reached the stomodeal valve (dark grey), thoracic gut (light grey), or stayed in the abdominal gut (white). The x-axis represents dsRNA injected groups. No significant differences were found between experimental and control groups (two-way ANOVA).

Leishmania challenge. These results are not sufficient to assess a direct correlation between the expression of PIAS or PTP61F with STAT-like transcription. Still, the STAT increase may be a result of a reduced expression of downstream effector molecules. A simultaneous increase between repressor and transcription factor also occurred in *L. longipalpis* Toll and IMD pathways (6).

Attacin expression was unaltered under co-culture with *Leishmania*, but VIR-1 was downregulated during the three time points investigated. It is in agreement with the upregulation of PIAS and PTP61F. VIR-1 is actively expressed in *Drosophila* S2 cells (19), and *A. aegypti* Aag2 cells (63) in response to DCV and *Wolbachia* infections, respectively, and might be under the effect of other signaling pathways. The downregulation of VIR-1 after a parasitic challenge was not previously shown in insect cells. However, it may result from the shedding of parasite exosomes containing virulence factors (reviewed in 64), as seen in *L. infantum* in culture (65). For instance, *Leishmania* secretes virulence factors such as elongation factor 1 alpha (EF1-alpha) (66) and the widely studied metalloprotease GP63 to activate the macrophage tyrosine phosphatases, which in turn represses the Toll and Jak-STAT pathways in the host cell (67, 68). Therefore, it is reasonable to consider that the parasite may cause suppression in the insect cell immune response. These results suggest that the Jak-STAT pathway in LL5 cells was repressed by *L. infantum* with a downstream suppression of VIR-1.

4.4 Effect of *Leishmania* Infection on Jak-STAT-Related Genes in *L. longipalpis* Females

We also tested the Jak-STAT pathway activation upon parasite infection using adult *L. longipalpis* as a model. Our results showed that the *Leishmania* infection in the sand fly caused no significant changes in the expression of the putative pathway repressors PIAS, SOCS, PTP61F, and STAT-like. Such nondramatic changes were also reported in the latest transcriptome study of *L. longipalpis* infected with *Leishmania*, where PIAS expression had no statistically significant modulation (45). Interestingly, in a recent investigation on *P. papatasi* infected with different trypanosomatid species (*Leishmania major*, *Leishmania donovani*, and *Herpetomonas muscarum*), a STAT transcript was reduced as blood digestion occurred. Still, its expression pattern did not show significant differences when compared to non-infected sand flies (69).

In our present study, attacin expression was reduced at the end of the blood digestion process, when the peritrophic matrix is degraded, and parasites get in close contact with the sand fly gut epithelium (70, 71). Therefore it is possible that attacin reduction was induced by the parasite, not through interfering with the Jak-STAT pathway, but possibly by Toll or IMD pathways as previously shown in *Drosophila* (72, 73). Our analysis of bacteria during the infection indicated no dramatic changes when compared to the non-fed females. The highest *Leishmania* detection occurred on the second day post-infection when multiplicative forms of the parasite are abundant (74). These results suggest that *Leishmania* infection did not significantly alter Jak-STAT-related gene expression in *L. longipalpis*.

4.5 Depletion of Commensal Gut Bacteria in *Leishmania*-Infected Sand Flies and Its Effect on Jak-STAT-Related Genes

Commensal microbiota harbored in the insect gut may add another level of complexity inherent to the *L. longipalpis* study

model. We tested if the gut bacterial community created additive stimuli to the Jak-STAT mediated response during the parasite infection. We depleted bacteria by adding a combination of antibiotics to the sand fly sucrose- and blood-meals. Several antibiotic combinations were used in sand flies to deplete gut bacteria (8, 75–79). We chose a combination of penicillin, streptomycin, and gentamicin, and we tested its efficiency on sucrose-fed females in a pilot experiment (80). The antibiotic treatment effectively depleted gut bacteria up to the fourth day of treatment compared to a non-treated group, detected on LB-agar plates seeded with sand fly gut homogenates. Therefore, we used this same antibiotic treatment in our infection experiments.

None of the four genes investigated in the antibiotic-treated group showed significant differential expression compared to the non-treated control group. Nevertheless, there was a slight increase in the expression of the repressor PIAS and the STAT-like transcription factor at 24 h post-infection. This effect can be credited to the parasite. As the infection progressed, attacin showed a quite variable expression toward 144 h, and VIR-1 was highly variable in all time points investigated. This variability suggests that differences in the progress of *Leishmania* infection may be the cause. In addition, these changes may be associated with changes in parasite development caused by bacteria depletion. Kelly et al. (75) showed that depletion of bacteria caused by antibiotics impaired the development of *L. infantum* metacyclic forms on late-phase infection. Therefore, our results indicate that the Jak-STAT mediated response may be slightly changed by the reduction of gut bacteria but not playing the primary role.

4.6 Effect of STAT-Like Knockdown on Downstream Effector Genes in Non-Infected Sand Flies

One alternative to investigating the role of the Jak-STAT pathway in *L. longipalpis* response upon infection is to interfere with downstream transcription of effector molecules. For that, we silenced the STAT-like transcription factor using dsRNA, which also reduced the gene expression of VIR-1 and iNOS in non-infected insects on the first day post-injection. The iNOS reduction was maintained on the second day, indicating a direct correlation with STAT silencing. On the other hand, VIR-1 expression increased on the second day and reduced on the third day suggesting that other transcription factor may be involved in its regulation. Interestingly, while iNOS expression was downregulated, DUOX was upregulated on the first day. DUOX is responsible for the production of superoxide (H_2O_2) and is modulated by the Hedgehog (Hh) signaling pathway (81) which is vital for controlling gut infections in *Drosophila* (82). This finding reflects a possible balance between these two genes that are oxidative stress-inducing molecules.

The overall detection of bacteria in STAT silenced sand flies during the three days investigated did not significantly change compared to the control group and reflects the maintained balance between iNOS and DUOX expression. However, the attacin expression upregulation may be caused by another pathway such as Toll or IMD as indicated in our previous studies (6, 41) thus posing an additional microbial control. These results indicate that the *L. longipalpis* putative STAT is associated with the iNOS expression, while attacin and VIR-1 had no direct correlation with STAT silencing in sucrose-fed females. Since the Jak-STAT pathway could be differently activated in the fat body (83) and in gut cells (84), as is the case in *Drosophila*, we decided to investigate the pathway-related gene expression in *L. longipalpis* separated carcasses and dissected guts.

4.7 Effect of STAT-Like Knockdown in *Leishmania*-Infected Sand Flies

We also hypothesized that the suppression of the pathway would facilitate the development of the parasite in its vector. We observed that STAT silencing was achieved in sand fly carcasses on the first two days post-infection. We also observed the increase of attacin expression similar to what was observed in our results with non-infected STAT-silenced, possibly being regulated by the Toll and IMD pathways as mentioned above. This silencing also caused a reduction in iNOS. The consistent decrease in iNOS expression in STAT-silenced insects supports the idea that iNOS is under Jak-STAT regulation, as seen in *A. gambiae* (55). Nevertheless, attacin and VIR-1 expressions were not likely to be under the regulation of the STAT-like transcription factor.

The reduction in the putative STAT expression was achieved in dissected guts, but silencing levels were not as intense as in carcasses. Attacin expression was increased at 48 h post-infection, similar to carcasses, indicating the upregulation of this AMP occurred systemically. Although attacin expression was raised, it did not cause an effect against *Leishmania*. VIR-1 expression did not alter after STAT silencing in guts similar to what was seen in carcasses, thus adding more evidence that it is not regulated through this transcription factor in *L. longipalpis*. iNOS expression was shown to be quite variable in the first two days, and DUOX modulation also followed a variable pattern, possibly associated to iNOS modulation. The bacterial detection was not significantly altered, similar to what was seen in the non-infected and silenced sand flies, indicating that molecules associated to the putative STAT had a limited effect on the bacteria balance, and vice versa.

Most interestingly, on the first day of STAT silencing, there was an increase of *Leishmania* detection within the silenced group, indicating that an effector molecule regulated by this transcription factor was consequently suppressed and favored *Leishmania* survival in this early time. Although iNOS expression was quite variable in guts, it is possible that the nitric oxide production was altered and resulted in a less harmful environment to *Leishmania*. An analogous effect of increased nitric oxide in *L. longipalpis* caused a reduction of parasite detection in TGF-beta gene silenced sand flies (35). These results indicate that the STAT-like transcription factor did not regulate attacin and VIR-1 in the gut of infected females and suggest that the variation in iNOS expression may have favored the parasite on the first day after STAT suppression.

4.8 Effect of STAT-Like Knockdown on the *Leishmania* Infection Development in Sand Flies

We also hypothesized that the silencing effect on the early time of infection could further influence the progress of *L. infantum* infection in the sand fly. We assessed infection intensity on a later phase of infection (6 days post-infection) by light microscopy. The infection estimation in individual sand flies showed that parasitic loads vary, and both silenced and control groups shared approximately the same loads of parasites. In addition, the infection localization showed that both insect groups had similar percentages of insects harboring parasites in the stomodeal valve, thus presenting similar conditions to complete the parasite cycle in the vector. Our results indicate that the favored parasite growth in the early phase of infection had no significant effect on the late infection stage.

4.9 Concluding Remarks

In conclusion, we aimed to understand the role of the Jak-STAT pathway in *L. longipalpis* immune response, more specifically toward *L. infantum* parasites. We identified putative sand fly genes belonging to the main pathway regulators and possible downstream related effector molecules. The SOCS-like may not be involved in regulating the sand fly Jak-STAT pathway. PIAS and PTP61F repressors balanced the pathway in LL5 cells, and the *Leishmania* challenge upregulated the pathway repressors and reduced VIR-1 expression. The parasite infection and the bacteria suppression during the parasite infection in females did not cause significant changes in the Jak-STAT pathway. The putative STAT silencing caused a reduction in iNOS in whole bodies and carcasses, while it had no direct effect on attacin and VIR-1. In addition, parasite detection was increased on the first day post STAT silencing, although it was insufficient to yield a higher-rate infection on late-phase infection.

The present work is the first report on putative sand fly genes involved in the Jak-STAT pathway. It is possible that other STAT proteins not covered in this study may be expressed and could compensate for the knockdown of this STAT-like gene. Similarly, other attacin genes may be expressed under the control of other pathways such as Toll or IMD, but this possibility needs further investigation.

The sand fly immune response is constantly regulated to balance and respond to different microbial challenges [reviewed in (47)]. Simultaneously, *Leishmania* parasites can adapt to these changes expressing a plethora of genes during its cycle (85). We focused on a pathway not explored in sand flies and brought additional information on the complex interaction between the *L. longipalpis* immune response to *L. infantum*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by "Committee on the Ethics of Laboratory Experiments of the Charles University" under permission No. MSMT-8604/2019-6 and "Committee on the Ethics in the use of Animals of the Institute Oswaldo Cruz (CEUA-IOC)" under permission No.CEUA/IOC-005/2019.

AUTHOR CONTRIBUTIONS

Conceptualization, ET, AP, and YT-C.Methodology, ET and AP. Validation, ET. Formal analysis and investigation, ET, DA-B, BK, and BT-N. Resources, YT-C and PV. Data curation, ET and BT-N. Writing-original draft preparation, ET. Writing-review and editing, ET and YT-C. visualization, ET. Supervision and project administration, ET, AP, PV, and YT-C. Funding acquisition, YT-C, ET, and PV. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2021.747820/full#supplementary-material

Supplementary Figure 9 | Summarized workflow of experimental approach and outcomes

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