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Identification and functional studies of sand fly's immune-related genes

Identifikace a funkční studie imunitních genů flebotomů

Doctoral thesis

Supervisor: Dr. Erich Loza Telleria

Prague, 2024

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I declare that this PhD thesis was written by myself, and that information sources and literature were cited properly. The results presented within this thesis were accomplished by me or in the collaboration with co-authors of the presented papers. This work nor the particular data have been used as a final work towards any other university degree.

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I declare that Barbora Vomáčková Kykalová substantially contributed to the experimental work in five projects presented in her PhD thesis. Among those, she had a principal role in the writing of three publications. Her contributions are stated in pages 23 and 24 of the present text.

Prohlašuji, že Barbora Vomáčková Kykalová se významně podílela na experimentální práci v pěti projektech prezentovaných v rámci její disertační práce. Mezi nimi měla hlavní roli při psaní tří publikací. Její podíly na práci jsou definovány v rámci této práce na straně 23-24.

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Dr. Erich Loza Telleria

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ABSTRACT

Phlebotomine sand flies (Diptera, Psychodidae) from genera *Phlebotomus* and *Lutzomyia* are proven vectors of *Leishmania* (Kinetoplastea, Trypanosomatida), causative agent of leishmaniases, tropical neglected diseases. To contribute on creating new control strategies we investigated the molecular aspects of interaction between the vector and pathogen on the immunity level.

Sand fly innate immunity is based on cellular and humoral events which work synergistically to secure effective protection against pathogens. Here we present our research on humoral aspects of sand fly immunity, specifically on main humoral pathways (Toll, Imd, and Jak-STAT), their genes and function under different conditions especially during *Leishmania* development in sand fly midgut.

We have described gene expression profiles of Toll and Imd – related genes in *Phlebotomus papatasi* larvae fed with different microbe loads and in adult females infected with *Leishmania major*. We have identified three antimicrobial peptides (AMPs) in *P. papatasi* and followed their expression profiles during parasite infection and described a gut-specific defensin upregulated by *Leishmania* infection. We have proven that the knockdown of *defensin* genes in *P. papatasi* supports *Leishmania* infection and negatively affects sand fly survival. Moreover, we identified a correlation between Imd pathway and expression of AMPs by silencing *relish* resulting in reduced expression of some AMPs. We discovered that *Leishmania* lipophosphoglycans or bacterial liposaccharides trigger the expression of AMPs, whereas *attacin* showed the earliest and most dramatic changes in both studied species, *P. papatasi* and *Lutzomyia longipalpis*. We have also investigated the role of Jak-STAT pathway during *Leishmania* infection in *L. longipalpis*. While in LL5 cell line, a co-culturing with *Leishmania infantum* led to overexpression of negative regulators of the pathway, the parasitic infection of adult females did not lead to significant change in Jak-STAT related genes. However, use of the gene silencing of STAT transcriptional factor in females reduced the gene expression of inducible oxide synthase and Dual oxidase leading to increased *Leishmania* growth.

Lastly, we attempted to establish a CRISPR-Cas9 gene editing protocol in our laboratory. With two different approaches using CRISPR plasmid constructs and direct sgRNA + Cas9 injection, we injected more than 14,000 sand fly's embryos. Unfortunately, we were not able to establish any edited sand fly line. However, we have made important steps on which future experiments can be built.

ABSTRAKT – CZ

Flebotomové (Diptera, Psychodidae) z rodů *Phlebotomus* a *Lutzomyia* jsou přenašeči parazitů *Leishmania* (Kinetoplastea, Trypanosomatida), původců leishmaniózy. Ve snaze přispět k vytvoření nových strategií kontroly přenosu tohoto onemocnění, jsme se zaměřili jsme se na molekulární aspekty interakce mezi imunitou vektoru a patogenem.

Imunita flebotomů se skládá z buněčné a humorální složky, které fungují synergicky, aby zajistily účinnou ochranu proti patogenům. Náš výzkum byl zaměřený na humorální složku imunity flebotomů, konkrétně na její hlavní dráhy (Toll, Imd a Jak-STAT), jejich geny a fungování za různých podmínek, zejména během parazitární infekce.

U *Phlebotomus papatasi* jsme popsali profily exprese genů Toll a Imd drah během různé bakteriální zátěže u larev a během infekce *Leishmania major* u dospělých samic. Dále jsme identifikovali geny pro tři antimikrobiální peptidy (AMP) a sledovali jejich expresi při parazitární infekci. To umožnilo identifikaci, defensinu, který byl specifický pro střevo a jehož exprese byla zvýšena během leishmaniové infekce. Prokázali jsme, že umlčení defensinových genů u *P. papatasi* vede k silnější infekci leishmaniemi a negativně ovlivňuje přežití flebotomů. Popsali jsme korelaci mezi Imd dráhou a expresí AMP pomocí umlčení transkripčního faktoru, relish. Zjistili jsme, že lipofosfoglykan leishmanií a bakteriální liposacharidy, mohou vyvolat zvýšenou expresi efektorových molekul, přičemž *attacin* vykazoval nejčasnější a nejdramatičtější změny u obou studovaných druhů, *P. papatasi* a *Lutzomyia longipalpis*. Také jsme zkoumali roli Jak-STAT humorální dráhy během infekce *Leishmania infantum* u *L. longipalpis*. Zatímco u buněčné linie LL5 vedla společná kultivace s *L. infantum* k nadměrné expresi negativních regulátorů dráhy, infekce dospělých samic nevedla k významné změně testovaných genů. Přesto, při použití genového umlčení transkripčního faktoru STAT, jsme zaznamenali sníženou genovou expresi inducibilní oxid syntázy a duální oxidázy, což vedlo k nárůstu parazitů.

Dále jsme se v našich laboratorních podmínkách pokusili zavést protokol pro editaci genomu pomocí CRISPR-Cas9 systému. Se dvěma různými přístupy využívajícími plasmidové konstrukty a přímou injekci sgRNA + Cas9 jsme injikovali více než 14 000 embryí flebotomů. Přesto, že se nám nepodařilo úspěšně detekovat vyřazení genu (knockout), učinili jsme důležité kroky pro budoucí experimenty.

INTRODUCTION

Diseases transmitted by arthropods account to significant part of all infective diseases (17 %) worldwide while afflicting mostly poor areas and negatively affecting quality of life of millions of people. Moreover, these vector-borne diseases are directly responsible for 700 000 deaths annually mostly in children under 5 years (WHO, 2023). Leishmaniases are vector-borne disease caused by over 20 species of protozoan parasites from genus *Leishmania*. The three main forms of leishmaniasis are: visceral, cutaneous, and mucocutaneous. Every year, occurs up to 1 000 000 new cases mostly in poor areas with poor housing and sanitary conditions (WHO, 2023).

The transmission of most *Leishmania* species is exclusively tied to sand flies (Diptera, Psychodidae) with one confirmed exception when biting midges (Diptera, Ceratopogonidae) are included in transmission of parasite belonging to subgenus *Mundinia* (Dougall et al., 2011; Bečvář et al., 2021). There are around 1000 known sand fly species of which roughly 10 % is proven or suspected vectors of *Leishmania* (reviewed by Cecílio et al., 2022). In this thesis we focus on 2 sand fly species which play crucial role in the *Leishmania* cycle.

Phlebotomus papatasi is an Old World species and a main vector of *Leishmania major*, causative agent of cutaneous leishmaniasis (Akhoundi et al., 2016; Cecílio et al., 2022). *Lutzomyia longipalpis* is, on the other hand, a New World sand fly susceptible to infection of various *Leishmania* species with the main importance in transmission of *Leishmania infantum*, parasites causing visceral leishmaniasis (Rêgo & Soares, 2021). From the point of view of *Leishmania*-vector interactions and transmission, *L. longipalpis* is referred as a permissive vector, enabling development of distinct *Leishmania* species, while *P. papatasi* is referred as a restrictive (or specific) vector with a remarkable specificity for *L. major* (Volf & Myskova, 2007). Different vector competence is caused by mechanism of the parasite's attachment to the sand fly gut. While in the permissive vectors parasites attach due to mucin like-protein present on midgut epithelium (Myšková et al., 2016), in restrictive species the interaction is mediated by binding a lipophosphoglycan (LPG) molecules from *Leishmania* surface to a specific midgut receptor, β-galactoside binding family of lectins (Kamhawi, 2006; Volf & Myskova, 2007; Dostálová & Volf, 2012).

Leishmania development in the vector is relatively well studied but the knowledge about sand fly immunity and its interactions with parasites is very limited in comparison to other insect vectors such as *Anopheles* sp., *Aedes* sp. mosquitoes. Since the *Leishmania* cycle in sand fly

vector is limited to the gut, it is also necessary to consider mutual interactions with the intestinal microbiota. We decided to focus on characterization of humoral immunity in sand flies and searching for candidate molecules with a potential use in genetic vector-control strategies.

Treatments traditionally used to cure leishmaniases (e.g. amphotericin and antimonials) may have significant side effects and are burdensome for the human body (e.g. cardiotoxicity, kidney failure, anaemia, fever, hypotension.). In addition, the treatment requires an immunocompetent system because itself it does not eliminate the parasite from the body, therefore there is a risk of the disease relapses when immunosuppression occurs. Immunosuppression may occur as a result of different events such as concomitant diseases (e.g. AIDS, cancer, immune deficiency syndromes) or malnutrition (Aronson et al., 2017; WHO, 2023). Effective prevention of the disease transmission includes the vector control, which is based mainly on use of insecticides which can have an effect even on environment, non-target species and there is a risk of resistance rise (Purusothaman et al. 2021). There were 47 sand fly populations described with reported resistance including significant *Leishmania* vectors. In India, *P. papatasi* and *Phlebotomus argentipes* populations were repeatedly resistant to dichlorodiphenyltrichloroethane (DDT), *P. papatasi* showed resistant also to other insecticide such as carbamate propoxur, dieldrin or malathion (reviewed by Rocha et al., 2020). Insecticide resistances were also observed in many populations of *L. longipalpis*, e.g. resistance to deltamethrin (Balaska et al., 2021). Genetic approaches which may reduce vector population or vector competence are a good alternative of vector control strategies.

1. Insect immunity

Bearing in mind that we have a very good understanding of immune defence and molecular insights in some models such as *Drosophila melanogaster* or *Anopheles* sp., this chapter will discuss the insect's immunity mainly from perspective of these model organisms.

Despite of lack of adaptive and highly specific immune response, found only in vertebrates, insects rely successfully on innate immunity. Many of the innate mechanisms are conserved between insects and mammals (Buchmann, 2014) and were firstly described in insect. Subsequently, they were discovered and studied in mammals, which proves the importance of studying the insect immunity also from evolutionary aspect.

In the first line for protection there are physical barriers, such as the insect exoskeleton and epithelia that are in contact with external environment. Together with local and systemic mechanisms, they act against invading pathogens (Lemaitre & Hoffmann, 2007). Insect's innate immunity is composed by cellular and humoral components that work in synergy to create effective protection. The cellular response is mediated by freely circulating blood cells, namely haemocytes. Haemocytes circulate in haemolymph, but in case of infection or injury they can cluster and adhere at the injured tissue or directly on invading pathogens (Lackie, 1988). The haemocytes of Diptera can be divided into three main cell types according to their function and structure: plasmacytes are mainly responsible for phagocytosis and are also the most abundant; lamellocytes provide encapsulation and neutralization of especially multicellular pathogen, that are not possible to phagocyte; and crystal cells are the least abundant and take part in melanisation process (reviewed by Lemaitre & Hoffmann, 2007).

Humoral immunity in invertebrates is composed of various processes that maintain homeostasis. These includes clotting/coagulation system; phenoloxidase (PO) activation and melanisation; lysozyme; and humoral immune signalling pathways leading to production of antimicrobial peptides (AMPs) (Lemaitre & Hoffmann, 2007; Tsakas & Marmaras, 2010). Insect haemolymph clotting prevent from pathogen entry and infection, and from loss of haemolymph by creation of clot (Dushay, 2009). Melanisation play a role in immunity and healing but also in sclerotization and colouration processes. It relies on prophenoloxidase activation of PO cascade. The pathogen is then killed either by toxic compounds from cascade or encapsulated and phagocyted (Cerenius et al., 2010; Sheehan et al., 2018). Lysozymes are hydrolysing a glycosidic linkage between N-acetylmuramic acid and N-acetylglucosamine in bacterial peptidoglycans. Their synthesis is mainly induced by bacteria infection because

lysozymes have bacteriostatic and bacteriolytic effects (Fujita, 2004). Finaly, the humoral immune response leads to production of different AMPs. Such plethora of molecular events are controlled by regulatory pathways such as Toll, Imd or Jak-STAT pathway that play crucial roles in many immune-related and developmental events (Sheehan et al., 2018).

The activation of innate immunity is mediated by Pathogen-associated molecular pattern molecules (PAMPs). These microorganism-related molecules are recognized by Pattern Recognition Receptors (PRRs) which bears the immune response (Tang et al., 2012). Many diverse groups of PAMPs can be recognized such as bacteria peptidoglycans (PG), lipopolysaccharides (LPS) or lipoteichoic acid (LTA), fungal betaglucans or mannans, or parasite surface molecules such as *Leishmania* LPG.

1.1. Humoral immune signalling pathways

1.1.1. Toll pathway

The molecular components of Toll pathway were firstly identified in the 80's during the series of genetic screens investigating *Drosophila* development. For example, the *Toll* gene was discovered to be responsible for dorsoventral polarity in *Drosophila* embryo (Nusslein-Vollhard & Wieschaus, 1980; Valanne et al., 2011). It is derived maternally by signal-transduction pathway producing a nuclear morphogen gradient. Nevertheless, Toll has also a role as membrane receptor in the centre of cascade leading to dorsoventral axis formation (reviewed by Kimbrell & Beutler, 2001). The developmental role of Toll pathway will not be further described at the expense of immune-related role which has greater significance in our research.

In 1995 a connection of *Toll* gene with immune activation was found when a *Drosophila* haemocyte cell line with overexpression of *Toll* has significantly increased transcription of *cecropin A1*, an antibacterial peptide gene (Rosetto et al., 1995).

Concomitantly to the discovers in *Drosophila*, human-related research also started, and in 1997, a human homologue of *Drosophila* Toll protein with function in signal activation of innate immunity was described (Medzhitov et al., 1997). The research done on human's Toll Like Receptors consolidated *Drosophila* Toll pathway as an evolutionary conserved cascade.

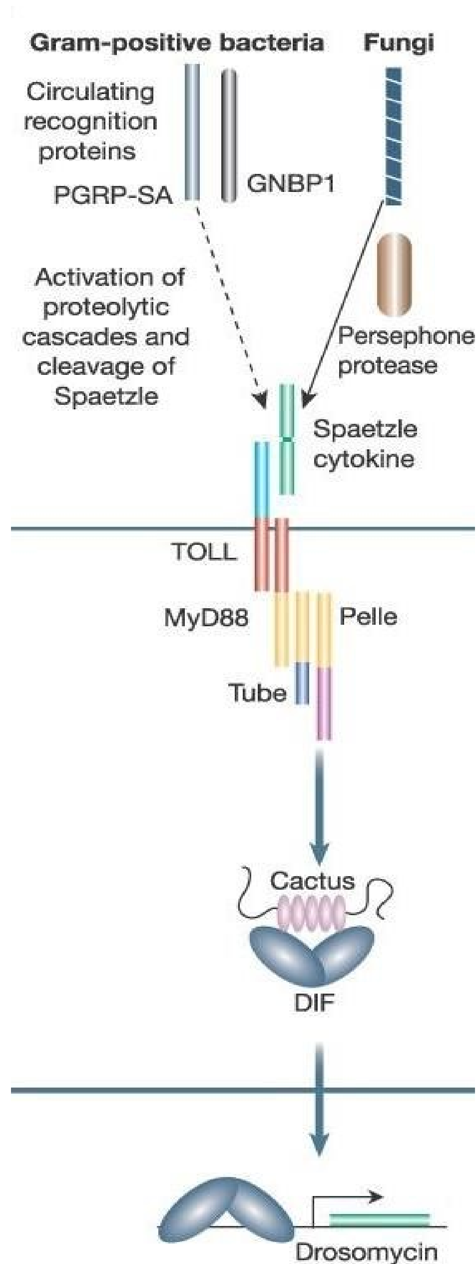

Figure 1 - Toll signalling pathway.

The Toll pathway scheme represents microbes' PAMPs recognition by insect's PRR at the extracellular environment, regulation of the pathway by a cascade of molecular events including cactus (a repressor of the pathway) in the intracellular environment, translocation of transcription factor DIF/Dorsal to the cell nucleus, and expression of effector-molecule genes such as the antimicrobial peptide Drosomycin.

The figure was extracted from Hoffmann, 2003

The activation of Toll pathway-derived immune response relies on the recognition of danger signals. It is mediated by PRRs which recognize specific molecular structures on pathogens, apoptotic or damaged cells. Extracellular recognition leads to activation of protease cascade which activates cytokine like molecule, Spätzle (Spaetzle) a Toll receptor ligand. Spätzle is encoded by maternal effect gene, and its activation is necessary in both, development, and immune response (Morisato & Anderson, 1994; Hoffmann, 2003). To achieve a biological activity of Spätzle, its cleavage induced by early pathogen infection and subsequent proteolytic cascade is required. In the mature form, it binds as a dimer to an ectodomain of Toll receptor leading to subsequent signalling events (Weber et al., 2003; Parthier et al., 2014). In the next step, the Toll receptor binds to adaptor protein called MyD88. MyD88-Tube-Pelle complex is formed, and the next signal event leads to the phosphorylation and degradation of the *Drosophila* IkB factor Cactus (Horng & Medzhitov, 2001; Valanne et al., 2011). Cactus is an inhibitory protein associated with NF-kB proteins such as Dorsal-related immunity factor (DIF) and Dorsal, and its phosphorylation (mediated by Pelle kinase) and degradation is necessary for translocation of DIF and/or Dorsal to nucleus (Hoffmann, 1995; Nicolas et al., 1998). Finally, the translocation of transcriptional factors into nucleus leads to transcription of set of various target genes. Toll signalling pathway is displayed in Figure1.

As the research in *Drosophila* has shown, the Toll signalling pathway is active especially against the G+ bacterial and fungal infections. But it was also proven that in mosquito the Toll pathway has its role in many

others immune defence strategies. The participation on antiviral defence has been reported, for example, when the Toll pathway activation in *Aedes aegypti* leaded to transcription of a set of related genes which are involved in inhibition of Dengue virus (DENV) proliferation (Xi et al., 2008; Pan et al., 2012). Toll pathway has also the antiparasitic role in mosquitoes. In *Ae. aegypti* infected by avian parasite *Plasmodium gallinaceum*, the Toll pathway protected from infection indirectly by silencing the negative regulator of the pathway, *cactus* (Zou et al., 2008). The antiparasitic role was also shown in *Anopheles* sp. mosquito and mammalian *Plasmodium berghei* combination, when the overactivation through the *cactus* silencing resulted in significant decreased parasite burden (Frolet et al., 2006; Garver et al., 2009).

Incomparably less has been so far investigated in sand fly Toll pathway. In *L. longipalpis*, two genes associated with Toll pathway, *cactus* and *dorsal*, were identified and further studied upon different conditions (Tinoco-Nunes et al., 2016). The activation of Toll pathway was studied in *L. longipalpis* LL5 embryonic cell line by associated genes expression after silencing of the repressor, gene, *cactus*. The authors also reported a related activation of Toll pathway after various challenges. An increased expression of Toll-related genes, *cactus* and *dorsal*, were described after *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces marcescens*, *Saccharomyces cerevisiae* , and *L. infantum* infection showing the involvement of the pathway in the defence against bacterial, fungal, and parasitic infections (Tinoco-Nunes et al., 2016). In more recent study, two different cell lines (LL5 and Lulo) were used in *Wolbachia* infection. The early infection in LL5 cell line led to increased expression of *dorsal* indicating the Toll pathway involvement, but in the stably-infected Lulo lines the expression was lower in comparison to non-infected lines (Da Silva Gonçalves et al., 2019). While in *L. longipalpis* the association of Toll pathway with bacterial and parasite infection was proven, such information was lacking for *P. papatasi*.

We were interested in the Toll-mediated production of AMPs in *P. papatasi* larvae and adult sand flies. Indirectly through the expression levels of identified transcriptional factor, *dorsal*, we investigated the Toll pathway involvement in the gut response of larvae exposed different microbial intake and adult females infected with *Leishmania* parasites. This information partly uncover the role of Toll pathway in *P. papatasi* and is included in the publication presented as a result in this thesis (Kykalová et al., 2021 = PUBLICATION 1).

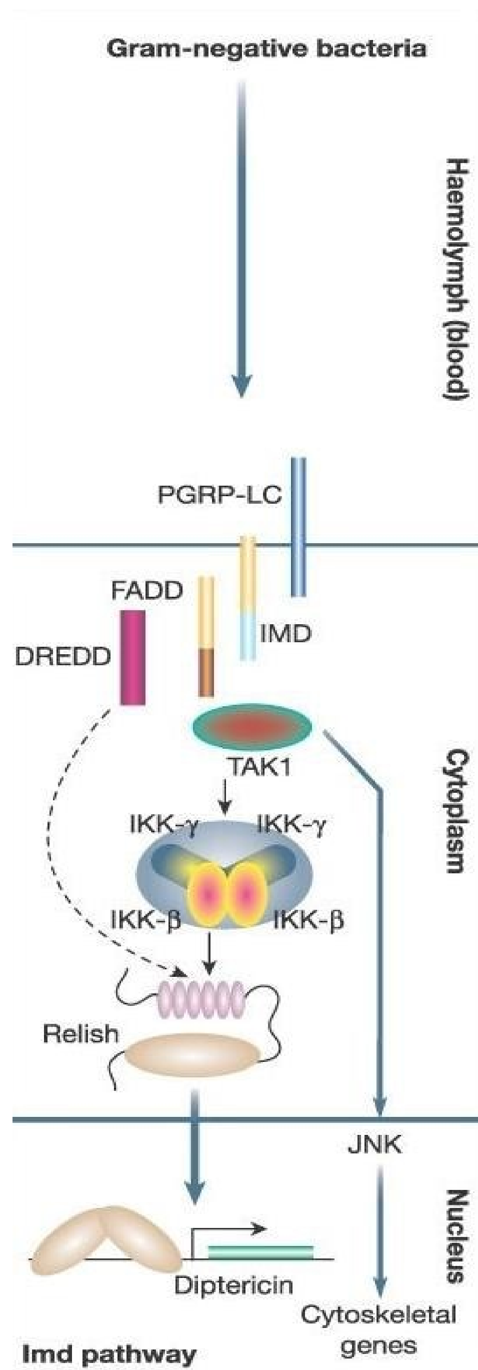

Figure 2 - Imd signalling pathway.

PGRP recognizes PAMPs, signalling complex in cell cytoplasm including relish (a repressor of the pathway), translocation of transcriptional complex into the nucleus, and expression of effector molecule genes such as antimicrobial peptide Diptericin.

The figure was extracted from Hofmann, 2003

1.1.2. Immune deficiency (Imd) pathway

The immune deficiency (Imd) pathway is another regulatory signalling cascade in mechanism of insect innate immunity. The Imd pathway was firstly discovered by Bruno Lemaitre and his colleagues in 1995 in the experiments with fruit fly mutants. Using the Bc (Black Cell) cell line with second chromosome mutation they discovered that the expression of most of the investigated AMP genes was impaired. As a first, they described a different mutation but very close to Bc mutation and they called it an immune deficiency (Imd) mutation in the used line and proved its connection with impaired AMPs expression (Lemaitre et al., 1995). A discovery of other members of the pathway followed shortly after (reviewed by Kleino & Silverman, 2014). Like a previously described Toll pathway, the activation of Imd pathway relies on the recognition of non-self/pathogenic molecules using PRRs. In particular, the activation requires a member of PGRP (Peptidoglycan Recognition Protein) family (Hoffmann, 2003). The binding of pathogen surface molecules on PGRP-LC (large) leads to recruitment of a signalling complex. This complex consists of Imd, adaptor protein dFadd and caspase Dredd (Georgel et al., 2001). Dredd caspase cleaves the Imd and thereby create new binding sites, which leads to activation of Tab2/Tak1 complex responsible for activation of IKK complex (by phosphorylation) (reviewed by Myllymäki & Rämet, 2014). The NF-kB protein (transcription factor) in this pathway is called Relish. Whereas the DIF/Dorsal (transcriptional factors of Toll pathway) have an associated inhibitory protein that is independent molecule which associate with Dorsal during the signalling cascade activation and allows the Dorsal to be phosphorylated,

Relish has its own inhibitory domain. An endoproteolytic cleavage mediated by active IKK complex is necessary to allow a nuclear translocation of Rel homology domain (Stöven et al., 2000). This cleavage event is also a target of Caspar, negative regulatory protein which is responsible for blocking of nuclear translocation (Kim et al., 2006). It also has been shown that the Dredd caspase can associate directly with Relish and play role as a Relish endoprotease. It is called a rapid signal-dependent proteolysis of Relish when the Dredd caspase claves Relish into two parts while there persist both cleaved products. One of them translocates into the nucleus, and the other stays in the cytoplasm (Stöven et al., 2003). Once the Rel homology domain is delivered into nucleus, it leads to expression of pathway associated genes, such as various AMPs. The Imd signalling pathway is displayed in Figure 2.

The activation of Imd pathway was firstly described by expression of linked AMPs related to bacterial challenge using *Drosophila* cell lines (Lemaitre et al., 1995). The antibacterial function such as production of diptericin, attacin, drosocin, cecropin, and defensin of this pathway is the best described, and it is primarily but not exclusively target against G^- bacteria activated by bacteria PG (Hoffmann, 2003; Myllymäki et al., 2014).

In mosquitoes, the Imd pathway is involved as a defence strategy against various pathogens. For example, in *Ae. aegypti* it is an important component of antifungal defence after *Beauveria bassiana* and *Isaria javanica* infection. Interestingly in this study, they also observed an tissue and fungal-strain specific AMPs induction besides the systemic response coming from the fat body, (Ramirez et al., 2019). The pathway is also induced by arboviruses such as DENV or O'nyong'nyong virus and play an important role in the antiviral immunity by producing downstream AMPs (reviewed by Cheng et al., 2016). Furthermore, the parasitic activation of the pathway was described in mosquitoes as well. While the Toll pathway was active in *Anophles* sp. mosquitoes toward the mammalian *P. berghei* infection, the Imd pathway was mostly efficient against human *Plasmodium falciparum* infection (Garver et al., 2009). The silencing of *caspar*, an inhibitory protein of Imd pathway, led to almost complete protection of *Anopheles gambiae*, *Anopheles stephensi,* and *Anopheles albimanus* against *P. falciparum* parasite infection (Garver et al., 2009). Lately, it was found that the pathway is most efficient toward ookinete stage of parasite and again confirmed its potential in control strategies (Garver et al., 2012).

Limited number of studies involving the Imd pathway compounds were also done on sand flies. Heerman and colleagues investigated expression profiles of various immune-related genes in

larval stages of *L. longipalpis* upon bacterial infections. The genes connected with Imd pathway, *IMD* and *Pirk* (another negative regulator of Imd pathway) showed an upregulation after infection by the bacterial species *Pantonea agglomerans* and *Bacillus subtilis* (Heerman et al., 2015). A changed expression of Imd-related genes, *caspar* and *relish* was also reported in *L. longipalpis* LL5 embryonic cell line after bacterial challenges (Tinoco-Nunes et al., 2016). Moreover, *L. longipalpis* females expressed *caspar* differentially when fed on G^- and G^+ bacterial species (Telleria et al., 2012). These results indicate the involvement of Imd pathway in balancing the antibacterial defence in sand flies. An importance of Imd pathway in sand fly antibacterial defence was also reported by Louradour et al., when the knockout (KO) of *relish* (the sole transcriptional factor of Imd pathway) in *P. papatasi* using CRISPR/Cas9 editing technique caused a high susceptibility of mutants to bacterial infections (Louradour et al., 2019). Correlation of Imd pathway and anti-*Leishmania* defence was also demonstrated when *L. longipalpis* LL5 cell lines reported an overexpression of *relish* after *L. infantum* infection but *caspar* stayed unchanged (Tinoco-Nunes et al., 2016). *Lutzomyia longipalpis* females infected by *L. infantum* or *Leishmania mexicana* and silenced in *caspar* gene, showed significant reduction of parasites in both species. It indicates that Imd pathway has a role in the control of parasites, but during unchanged conditions (non-use of gene silencing), it has no effect on disruption of parasite cycle (Telleria et al., 2012). The study with *relish*-KO *P. papatasi* flies also reported the correlation with *L. major* infection when mutant flies showed high susceptibility to infection (Louradour et al., 2019). Nevertheless, this work did not investigate further the *relish* expression during *Leishmania* infection and its correlation with the downstream effector molecules.

In our work we decided to follow the *relish* levels in infected females but also in larvae under different microbe intake (Kykalová et al., 2021 = PUBLICATION 1). Moreover, we were also interested in the comparison of *relish* expression in gut and carcass (rest of body without head and legs) under colony condition and possible connection between *relish* / Imd pathway and expression of specific AMPs. For this purpose, we used *relish*-silencing approach with following investigation of expression levels of three *P. papatasi* AMPs in gut and carcass tissues. These experiments create part of presented thesis in PUBLICATION 2 = Vomáčková Kykalová et al., 2023.

1.1.3. Jak-STAT signalling pathway

In addition to Toll and Imd pathways, the Janus kinase/signal transducer and activator of transcription (Jak-STAT) pathway is also a key component of insect's innate immunity. The pathway was originally discovered and described in vertebrate system when studying how interferons lead to the activation of transcriptional factor (reviewed by Hu et al., 2021). Lately, the related molecules have been identified in invertebrates including *Drosophila* (Zeidler et al., 2000). Originally in the vertebrates, it has been reported that the Jak-STAT signalling pathway has a role in various events connected to immunity, tumour formation, cell division and death. In *Drosophila*, this pathway was shown to be involved in sex determination during the earliest process of embryonic development. Gonads can express Jak-STAT ligand Unpaired (see below) in a male-specific manner leading to activation of the Jak-STAT pathway in male germ cells at the time of gonad formation. Some other components are necessary for embryonic segmentation, larval haematopoiesis, a signalling activity across eye and ommatidial polarity determination, and of course immunity (reviewed by Zeidler et al., 2000).

The first evidence of involvement of this pathway in innate insect immunity has been reported from *A. gambiae* after bacterial challenge (Barillas-Mury et al., 1999). With the help of whole-genome techniques, the main components of the Jak-STAT pathway in insect models were identified. Focusing on *Drosophila*, there are four essential components: the ligand Unpaired (Upd), the receptor Domeless (Dome), the associated Jak tyrosine kinase Hopscotch (Hop), and the substrate, STAT (Stat92E) (reviewed by Bang, 2019). The main components of the pathway in *Drosophila* were evolutionary conserved and the homologues can be found in mammals and human.

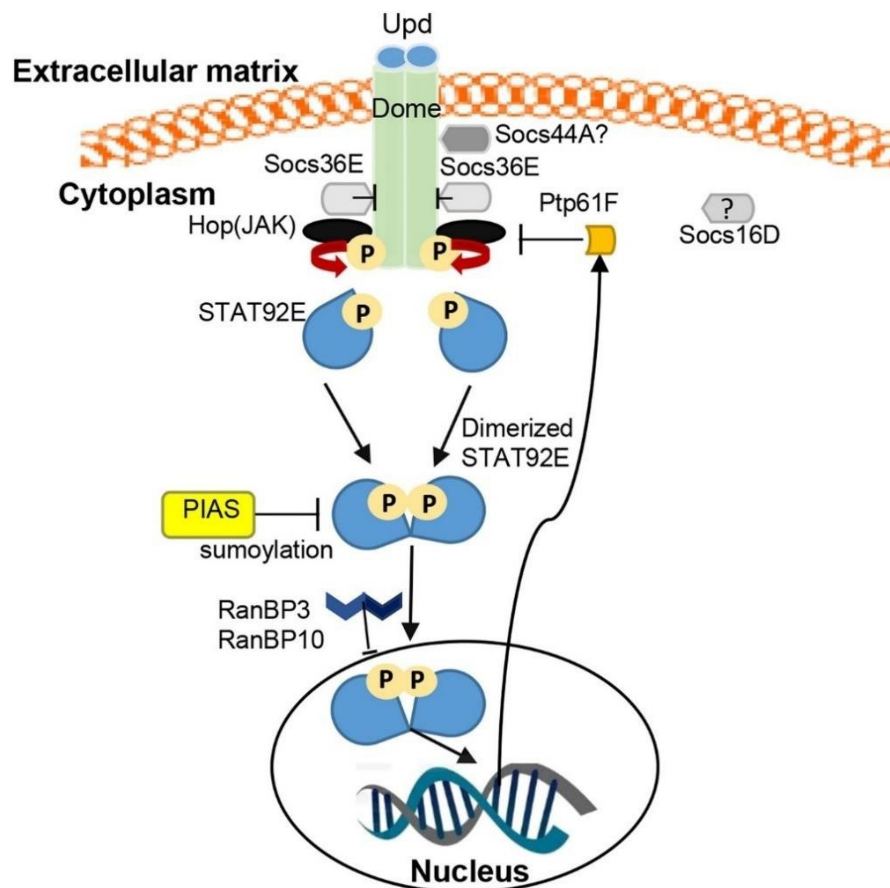

Figure 3 - Jak-STAT signalling pathway.

Transmembrane receptor Dome receives signal from extracellular environment, Jak-STAT signal is amplified in cytoplasm with regulation by e.g. PIAS. Phosphorylated STAT (transcriptional factor) is translocated into the nucleus, and target genes are transcribed.

The figure was extracted from Bang, 2019.

The Upd ligands are cytokine-like extracellular glycoproteins, having three of them been identified in *Drosophila*. The way of the pathway's activation is followed. The signalling goes through the ligands via single-pass receptor Dome inducing its dimerization. Dome has an extracellular domain with cytokine binding motif and cytoplasmic domains containing Jak-binding motif. The Jak kinase phosphorylates the Dome receptor and thereby amplifies the Jak-STAT signal. Only one STAT transcription factor serves as a substrate for Jak kinase in *Drosophila* sp. and its phosphorylation leads to dimers formation and translocation into nucleus where the transcription of target genes occurs (Myllymäki & Rämet, 2014; Bang, 2019). Considering that the Jak-STAT pathway is a multifunctional signalling pathway with a role in multiple developmental and immune events, it must be strictly regulated. This regulation is mediated by multiple mechanisms in *Drosophila* which share similarities with mammal's regulation. The main regulators of the pathway are SOCS (suppressor of the cytokine

signalling), PIAS (protein inhibitor of activated STATs), PTP (protein tyrosine phosphatase) and ET (eye transformer) but here are more molecules with potential or proven role in regulatory mechanisms of this pathway. SOCS regulates the pathway on the level of negative feedback loop interacting with both, STAT and Jak. It serves as a pseudo-Jak substrate. In *Drosophila* genome, three of these molecules have been found but only two of them have proven regulatory function. PIAS affects the pathway via interaction with STAT and coactivators by promoting SUMOylation which is the process when SUMO (small ubiquitin-related modifier) proteins attach to target protein causing regulation protein function. Its abundance and activity are essential for blood cells and eye development in *Drosophila*. Other regulators are PTP which regulate the pathway on kinase level via dephosphorylation of tyrosine residues and work in the system of negative feedback loop. Last regulator mentioned here is ET. This regulator is associated with dome receptor and STAT substrate (Zeidler et al., 2000; Myllymäki & Rämet, 2014; Bang, 2019).

Many studies of role of Jak-STAT pathway in development and immunity have been done on *Drosophila* and on mosquitoes. For example, an antiviral immunity of Jak-STAT pathway was shown in mosquito vectors, particularly, it has a role in the immune response of *Ae. aegypti* toward ZIKA virus (Angleró-Rodríguez et al., 2017). Also, the Dengue virus infection was controlled in *Ae. aegypti* by STAT-regulated effectors (Souza-Neto et al., 2009). Another antiviral activity was observed in *Culex* mosquito from, where an ortholog of *Vago* gene (gene connected with antiviral response in *Drosophila*), was upregulated after West Nile Virus challenge and this peptide restricted the WNV by activation of Jak-STAT pathway (Paradkar et al., 2012).

Beside the antiviral activity, Jak-STAT mediated immune response toward fungus and parasites was also observed in mosquitoes. For example, in *Ae. aegypti* the infection of entomopathogenic fungus *Beauveria bassiana* was controlled by the pathway's effector genes (Dong et al., 2012). *Anopheles stephensi* limits *Plasmodium* parasites by Jak-STAT mediated Nitric Oxide Synthase (NOS) expression and enzyme activity (Luckhart et al., 1998). And in *Anopheles aquasalis*, the early infection of *Plasmodium vivax* was also under control of Jak-STAT pathway (Bahia et al., 2011).

In sand flies, two studies covering the Jak-STAT pathway and its role in sand fly immunity were published previously. Bacterial infection was shown to have effect on some Jak-STAT-related gene expression, using a LL5 embryonic cell line, an early *Wolbachia* infection led to

increased expression of inducible Nitric Oxide Synthase (iNOS) resulting in decrease of *Wolbachia* detection (da Silva Goncalves et al., 2019). In *L. longipalpis* larvae infected with *P. agglomerans* an increase expression of *Domme* gene was observed after 24 hours post infection as a possible homeostatic response to damage by the larvae immune response (Heerman et al., 2015). Though, any further investigation of Jak-STAT pathway role during *Leishmania* infection was missing.

We studied the role of this pathway by gene expression profiles of related genes in both, LL5 cell line and adult females. To have broader view we tracked four related genes: *PIAS, PTP, STAT* and *VIR-1* and we also used a gene silencing approach in cell line and adult females before *Leishmania* infection to investigate role of *PIAS* and *STAT* gene. This study is summarized in PUBLICATION 4 (Telleria et al., 2021a).

1.2. Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are key effector elements of innate immunity. In insect, they work in synergy with other innate immune's mechanisms to provide an effective protection against variety of pathogens (reviewed by Stączek et al., 2023). First AMP was discovered in 1980's by Steiner et al. in the haemolymph of *Hyalophora cecropia* and it has been named as cecropin (Steiner, et al., 1981) and since then hundreds of others were discovered and described (https://aps.unmc.edu/).

In general, AMPs are oligopeptides with varying number of amino acids and sharing some common elements such as low molecular weight, positive net charge in physiological pH, hydrophobicity and structure (mostly amphiphilic α-helices or harpin-like ß-sheets or mixed) (reviewed by Bahar & Ren, 2013). Classes or groups of AMPs can be characterized by many distinctive features such as structure, amino acid sequence, mechanism of action, or targets.

The main mode of AMPs action is based on a cell membrane disrupting mechanism. The cationic peptides interact with the anionic components of the bacterial membrane which leads to increased permeability of the membrane, lysis, cell content leakage, and cell death (Zhang et al., 2021). In addition to disrupting cell membranes, AMPs actions include alterations in translation, biosynthesis of cell wall and nucleic acids or cell division (Dho et al., 2023).

Based on amino acid sequence and structure, insect's AMPs are divided into 3 big groups: defensins, cecropins, and AMPs with an overrepresentation of proline and/or glycine residues such as glycine-rich attacins (Wu et al., 2018).

Defensins are small (4 kDa) proteins containing 6-8 cysteine residues and 3-4 intramolecular disulfide bridges (Cociancich et al., 1993; Cong et al., 2013). Defensins have strong antimicrobial activity reported against both, G+ and G- bacteria (Hoffmann & Hetru, 1992). Defensins mostly act on the bacterial membrane causing perforation (Bulet et al., 1999).

Cecropins were the very first AMPs isolated. They are linear peptides with α-helix structure and missing cysteine residues (Wu et al., 2018). They have a broad range of targets, G+ and G- bacteria, fungi, and parasites (Owens & Schweizer, 2011). Also cecropins act on the bacterial membrane causing pore formation, membrane depolarization and cell death (Brady et al., 2019).

Attacins are relatively large (~190 aa) glycine-rich heterogenous group of AMPs with random coil secondary structure and lack of cysteine bridges (Buonocore et al., 2021). Attacins also interact with bacterial membrane aiming on major outer membrane protein in dividing G- bacteria. They block the protein synthesis which leads to disruption of the membrane (Wu et al., 2018)

AMPs have irreplaceable role in the insect's immunity, aiming on bacteria, viruses, fungi, and parasites. The synthesis of AMPs is concentrated predominantly in the fat body then they are released into the haemolymph (Meister et al., 1997). Beside this central AMPs synthesis, a side-specific immune defence including the local AMPs production was also observed (Buchon et al., 2009; Andoh et al., 2018). Thus showing that AMPs play role in both, systemic and side-specific defence.

Various research was done on sand fly's AMPs covering especially their role in *Leishmania* infection but also during bacterial challenge. Most of the sand fly's AMP genes were identified in *L. longipalpis* such as genes for *attacin, cecropin*, and four different *defensins* (Telleria et al., 2013; Telleria et al., 2021b). One of the studies investigate the role of AMPs in larvae and adults during different feeding regiments and during *Leishmania* infection in adult females. Expression of two *defensins* (Def2, Def4) and *attacin* was triggered in larvae in voracious L3 stage corresponding to an abundant presence of bacteria. Adult females infected by *L. infantum* reported increased expression levels of *attacin*, *cecropin,* and *defensin* (Def2) in response to parasite presence but not indicating any interference with *Leishmania* development in sand fly midgut (Telleria, et al., 2021b). This information is precious evidence of involvement of AMPs in sand fly immune response. Such studies were missing in *P. papatasi* although it is an important Old-World vector of *L. major*.

We were interested in AMPs in *P. papatasi* and their expression dynamics in the gut tissue during development and *Leishmania* infection. Bearing in mind that sand fly digestive tract is the only location of *Leishmania* cycle, and it is also a place where bacteria abundance is balanced, we also hypothesized if any of the investigated AMPs could be gut-specific. We were able to answer this question in two publications that are part of presented thesis (Kykalová et al., 2021 = PUBLICATION 1; Vomáčková Kykalová et al., 2023 = PUBLICATION 2). Besides, we were also interested in the effect of PAMPs derived from *Leishmania* and bacteria on sand fly AMPs levels. We investigated a response of gene expression of two *defensins* and *attacin* in *P. papatasi* and *L. longipalpis* to experimental feeding with two different *Leishmania* LPG and bacterial LPS. Obtained results are summarized in PUBLICATION 3 of this thesis (Vomáčková Kykalová et al., 2024 (IJPara23_415)– in press).

2. Reverse genetic approaches in gene-function studies

Reverse genetics is a collective name for techniques that allow to study the gene function by impairing on the nucleic acid levels and analysing the phenotypic effect using genetic engineering aiming specific nucleic acid sequence within the gene. Various approaches were established to study the biological function of genes such as mutagenesis by directed deletions and point mutations on the DNA or gene silencing by RNA interference (RNAi) to influence the mRNA levels. In the following chapters a knockdown by RNAi-mediated gene silencing and knockout by CRISPR-Cas9 techniques will be introduced in more detail.

2.1. RNAi-mediated gene knockdown

Gene silencing or knockdown of a gene is a process when the expression of that gene is significantly reduced. The gene knockdown can be achieved by RNA interference, a naturally occurring mechanism of antiviral defence and gene regulation.

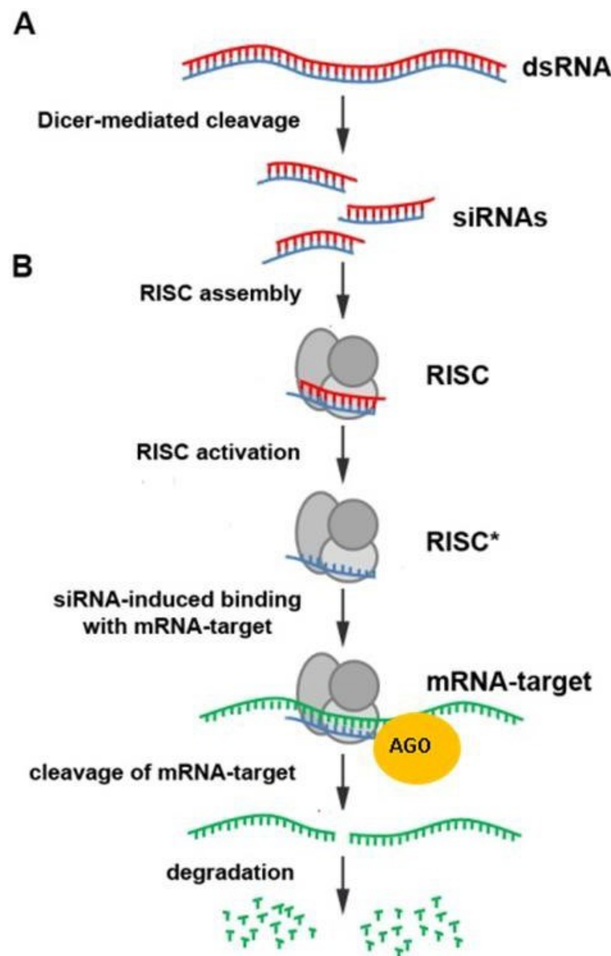

Figure 4 - scheme of RNAi

A -Dicer enzyme mediated cleavage of exogenous/endogenous dsRNA resulting in miRNAs or siRNAs.

B - RNAs in association with RISC and AGO lead to degradation of target mRNA.

The figure was modified from Petrova et al., 2003.

RNAi is a defence mechanism responsive to double stranded RNA aiming exogenous and/or endogenous nucleic acids and thereby provide antiviral defence and/or regulation of gene expression, respectively. The principle of RNAi is cleavage of mRNA by molecular events in the cell (Figure 4). It all starts when dsRNA is recognized by ribonuclease RNA II enzyme called Dicer or Dicer-like enzyme. This enzyme processes the cleavage of dsRNA resulting in two types of small regulatory dsRNAs: microRNAs (miRNAs); and small interfering RNAs (siRNAs). Small RNAs are in the next step associated with RNA-induce silencing complex (RISC) during which the double strand winds and as a single strand RNA connects to target mRNA in the cell. The resulting dsRNA is degraded by nucleolytic enzyme Argonaute (AGO) (Ding & Voinnet, 2007; Saurabh et al., 2014; Hung & Slotkin, 2021). The understanding of RNAi created a powerful tool for reverse genetic studies. An artificially prepared short dsRNAs is used as template for RNAi and therefore targeted gene silencing. This tool allows to study the gene function and participation in biological processes. Gene silencing has been used countless times in studies on *Drosophila* and mosquito genes including immune-related genes (e.g. Flatt et al., 2008; Molina-Cruz et al., 2008; Antonova et al., 2009; Magalhaes et al., 2010; Yan et al., 2022).

In sand flies, RNAi-mediated gene silencing also allowed to study genes involved in immunity and/or parasite defence strategies. Targeting the *P. papatasi* gut chitinase significantly reduced the number of *L. major* parasites in the sand fly midgut. Gut- chitinase has presumptive role in maturation and degradation of peritrophic matrix and therefore its silencing may lead to

entrapment of parasites in endoperitrophic space (Coutinho-Abreu et al., 2010). It has also been published that knockdown of *L. longipalpis catalase* (ROS regulatory gene) negatively affects *L. mexicana* infection in the vector midgut by reducing parasite population (Diaz-Albiter et al., 2012). A different study done on *L. longipalpis* showed that silencing of *caspar* gene (negative regulator of Imd pathway) leading to significantly decreased number of *Leishmania* parasites in treated sand flies (Telleria et al., 2012).

In our research we also utilized the RNAi-mediated gene silencing to bring new knowledge in immune-related gene function. To uncover some details of the role of Jak-STAT pathway during *Leishmania* infection in *L. longipalpis*, gene silencing of *STAT* gene was performed (Telleria, et al., 2021a = PUBLICATION 4).

All previous studies followed the protocol when firstly the gene was silenced, and parasite infection followed. In our study done on *P. papatasi* investigating role of *defensin* genes during *L. major* infection, we decided to change the order of these steps to increase the silencing effect during the crucial part of parasite cycle in the vector, we silenced defensins in infected *P. papatasi* females. We also focused on silencing effect in the insect gut instead of whole body. These unique adaptations and obtained results are part of PUBLICATION 2 of this thesis (Vomáčková Kykalová et al., 2023).

2.2. CRISPR-Cas9 gene knockout

In contrast to RNAi silencing, gene editing is a process when specific sequence of DNA is changed permanently. The sequence can be changed by cutting at specific sites, removing, or inserting DNA sequences. Nowadays, the most powerful tool for gene editing is a CRISPR-Cas9 method. The beginnings of discovery of this groundbreaking technology date back to 1987 when clustered regularly interspaced short palindromic repeats (CRISPR) were first discovered by Ishino's research group. A lot more discoveries were needed to present a CRISPR-Cas9 as a tool for genetic engineering and a great merit on in had Emmanuelle Charpentier and Jennifer Doudna who has been awarded with Nobel Prize in Chemistry in 2022 (reviewed by Gostimskaya, 2022).

CRISPR-Cas systems serve as an adaptive immunity in bacteria and are sorted in classes, types, and subtypes. Only type II CRISPR-Cas9 system has been used in gene editing (Hryhorowicz et al., 2017; Nidhi et al., 2021). There are basically two components necessary for gene editing: a single guide RNA (sg/gRNA); and a CRISPR-associated (Cas) endonuclease. The sgRNA however must meet certain requirements. The sgRNA must be composed by two short RNA

sequences: a CRISPR RNA (crRNA) which is complementary to the target DNA sequence; and a transactivating crRNA (tracrRNA) playing a role in maturing of crRNA and creating the biding site for a Cas endonuclease (Deltcheva et al., 2011; Hryhorowicz et al., 2017).

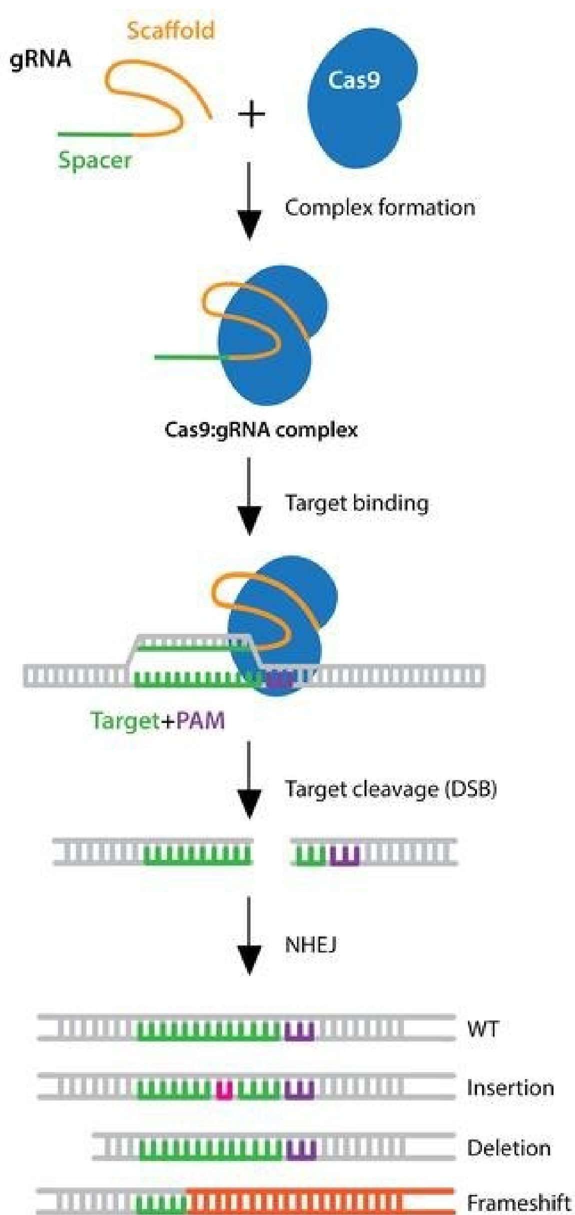

Figure 5 - Scheme of CRISPR-Cas9 gene knock-out.

gRNA and Cas9 endonuclease form a complex that binds on target sequence and results in double stranded break and non-homologous end joining repairing pathway.

The figure was extracted from www.addgene.org/guides/crispr/.

In our experiments, we aimed on creating knockouts by using CRISPR-Cas9 systems. The sgRNA guides the Cas endonuclease to the target site of the gene where creates double stranded break (DSB). The target is approx. 20 nucleotides genomic sequence which must be unique in the genome and is connected to Protospacer Adjacent Motif (PAM) specific for selected Cas endonuclease. Nowadays, there is a number of software helping to design the most promising sgRNA for target gene, such as CHOPCHOP; CRISPOR; Cas-OFFinder; etc. (Dalvie et al., 2022). Once the DBS occurs one of two repair pathways takes place. First and more frequent is non-homologous end joining (NHEJ) pathway causing small changes (insertion or deletion) at the DBS site. The second pathway is homology directed repair (HDR) pathway. It is less frequent, very precise, and is a high-fidelity repair mechanism that can only occur when a homologous DNA sequence is present in the nucleus (Shin & Oh, 2020).

CRISPR-Cas9 gene manipulation quickly became an object of vector-borne diseases control strategies with a mosquito's transmitted diseases in the first place. For this reason, the majority of research was done on mosquitoes (e.g. Dong et al., 2015; Hammond et al., 2016; Hammond et al., 2017; Dong et al., 2018; O'leary & Adelman, 2020). Some studies

were focused on vector immune-related genes as well. Dong et al. studied the role of *FREP1* gene from a family of fibrogen domain immunolectins in *A. gambiae. FREP1* showed to be involved in *Plasmodium* ookinete's invasion of the vector's gut epithelium, whereas *FREP1* knockouts mosquitoes showed a supressed *P. falciparum* and *P. berghei* infection. The deletion of the gene also had an negative effect on mosquito's fitness (Dong et al., 2018). Another study was done on *Ae. aegypt*i and its juvenile hormone-binding protein (mJHBP) regulating innate immune responses including humoral and cellular events. Knockout of mJHBP led to immunosuppression of mosquitoes such as delayed expression of AMPs – *defensin A* and *cecropin A* (Kim et al., 2020). Lastly, the research was focused on *A. stephensi's* Leucin-Rich protein (LRIM1) which is involved in regulation of *Plasmodium* development in the vector. Knocking out the *LRIM1* gene resulted in gut microbial dysbiosis, affected reproduction and reduced sporogony of *P. falciparum* in infected females (Inbar et al., 2021).

CRISPR-Cas9 gene manipulations represent powerful and uncomplicated tool for gene editing in many organisms, therefore our effort was to apply it on sand flies as well. However, for many reasons (small size; egg laying in organic material; fragility; etc.) the implementation of this technique to the sand fly research became more challenging than other insect models. Martin-Martin et al. described in detail the protocol adapted for sand fly embryo microinjection and CRISPR-Cas9 manipulation in 2018. Their protocol describes detailed methodology for setting up a sand fly embryo injection taking in account the sand fly life cycle (Martin-Martin et al., 2018). The only successful CRISPR-Cas9 mutagenesis done on sand flies was published in 2019 by Louradour et al.. They were able to knockout the *relish* gene (transcription factor of Imd pathway) in *P. papatasi* and successfully establish heterozygotes mutants. The edited flies were further used in infection experiments showing greater loads of parasites as well as high susceptibility to bacteria (Louradour et al., 2019). It ought to be mentioned that *relish* was the only successful gene knockout out of more than 5 targets they aimed (Isabelle Louradour; personal communication).

Our group carried out extensive experiments on *P. papatasi* and *L. longipalpis* which unfortunately did not lead to successful establishment of edited sand fly strain. Nevertheless, even our unsuccessful attempts are steps forward in gene editing in sand flies. For that reason, part of our experiments and obtained observation are presented in the thesis as SUPPLEMENTARY FILE 1 composed by unpublished results.

OBJECTIVES

Leishmaniases are diseases of great importance, belonging to the group of Neglected Tropical Diseases. It means they afflict hundreds of thousands of people annually, mainly in low-income populations in developing regions. To fight the disease, vector control and/or transmission control strategies are current important topics in *Leishmania* and sand fly research. In general, this project aims to contribute to extension of knowledge between sand fly's immune system and parasites with the focus on humoral pathways and effector molecules. Such knowledge may help in finding potential candidate molecules for genetic approaches in vector control strategies and deepening the understanding of *Leishmania*-sand fly interaction.

Specific aims were following:

- To identify immune-related molecules in two significant *Leishmania* vector species (*L. longipalpis* and *P. papatasi*) with potential role in the vector-parasite interaction.
- To characterize expression profiles of these immune-related molecules upon different experimental challenges such as bacteria rich food, *Leishmania* infection, pathogen surface molecules.
- To study the selected gene's function using RNAi-mediated gene silencing approach.
- To establish the CRISPR-Cas9 gene editing technique in our laboratory (see Supplementary file 1).

LIST OF PUBLICATIONS

PUBLICATION 1

Kykalová, B.; Tichá, L.; Volf, P.; Loza Telleria, E. (2021). ***Phlebotomus papatasi* Antimicrobial Peptides in Larvae and Females and a Gut-Specific Defensin Upregulated by *Leishmania major* Infection**. *Microorganisms*, 9, 2307.

My contribution:

Performing experiments (experimental feeding of larvae and adults; dissections and sample collection; sample processing; qPCR); data analyses and interpretation; and writing.

PUBLICATION 2

Vomáčková Kykalová, B., Sassù, F., Volf, P., & Telleria, E. L. (2023). **RNAi-mediated gene silencing of *Phlebotomus papatasi* defensins favors *Leishmania major* infection**. *Frontiers in Physiology*, 14, 1182141

My contribution:

Designing of experiments; performing experiments (experimental infection; dissections and sample collection; sample processing; qPCR); data analyses and interpretation; and writing.

PUBLICATION 3 (in press)

Vomáčková Kykalová, B., Sassú, F., Rego, F. D., Soares, R. P., Volf, P., & Telleria, E. L. (2024). **Pathogen-associated molecular patterns (PAMPs) derived from *Leishmania* and bacteria increase gene expression of antimicrobial peptides and gut surface proteins in sand flies** *International Journal for Parasitology* (in press, Manuscript Number: IJPara23_415)

My contribution:

Performing experiments (experimental feeding of larvae and adults; dissections and sample collection; sample processing; qPCR focused on AMPs); AMPs' data analyses and interpretation; and writing.

PUBLICATION 4

Telleria, E. L., Azevedo-Brito, D. A., Kykalová, B., Tinoco-Nunes, B., Pitaluga, A. N., Volf, P., & Traub-Csekö, Y. M. (2021). ***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.** *Frontiers in Tropical Diseases*, *2*, 747820.

My contribution:

Performing experiments infection experiments with adult sand flies (experimental infections; dissections and sample collection; sample processing; qPCR); data analyses and interpretation; text reviewing.

PUBLICATION 1

Kykalová, B.; Tichá, L.; Volf, P.; Loza Telleria, E. (2021).

***Phlebotomus papatasi* Antimicrobial Peptides in Larvae and Females and a Gut-Specific Defensin Upregulated by *Leishmania major* Infection**.

Microorganisms, 9(10), 2307,

microorganisms

MDPI

Article

Phlebotomus papatasi Antimicrobial Peptides in Larvae and Females and a Gut-Specific Defensin Upregulated by *Leishmania major* Infection

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Abstract: *Phlebotomus papatasi* is the vector of *Leishmania major*, causing cutaneous leishmaniasis in the Old World. We investigated whether *P. papatasi* immunity genes were expressed toward *L. major*, commensal gut microbes, or a combination of both. We focused on sand fly transcription factors dorsal and relish and antimicrobial peptides (AMPs) attacin and defensin and assessed their relative gene expression by qPCR. Sand fly larvae were fed food with different bacterial loads. Relish and AMPs gene expressions were higher in L3 and early L4 larval instars, while bacteria 16S rRNA increased in late L4 larval instar, all fed rich-microbe food compared to the control group fed autoclaved food. Sand fly females were treated with an antibiotic cocktail to deplete gut bacteria and were experimentally infected by Leishmania. Compared to non-infected females, dorsal and defensin were upregulated at early and late infection stages, respectively. An earlier increase of defensin was observed in infected females when bacteria recolonized the gut after the removal of antibiotics. Interestingly, this defensin gene expression occurred specifically in midguts but not in other tissues of females and larvae. A gut-specific defensin gene upregulated by *L. major* infection, in combination with gut-bacteria, is a promising molecular target for parasite control strategies.

Keywords: sand fly; insect immunity; gut-specific response; defensin; *Leishmania*

Citation: Kykalová, B.; Tichá, L.; Volf, P.; Loza Telleria, E. *Phlebotomus papatasi* Antimicrobial Peptides in Larvae and Females and a Gut-Specific Defensin Upregulated by *Leishmania major* Infection. *Microorganisms* **2021**, *9*, 2307. https://doi.org/10.3390/microorganisms9112307

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1. Introduction

Phlebotomine sand flies (Diptera: Psychodidae) belonging to *Lutzomyia* and *Phlebotomus* genera are proven vectors of *Leishmania* parasites (reviewed by [1]), causing 700,000 to one million new cases of leishmaniasis every year [2]. *Phlebotomus papatasi* is dispersed across Mediterranean European countries, North Africa, the Middle East, and Central Asia. It transmits *Leishmania major*, one of the etiological agents of cutaneous leishmaniasis (reviewed by [3]). Nevertheless, migration and environmental changes constantly shape the ecoepidemiology of leishmaniases [2].

During the cycle in the sand fly vector (reviewed by [4,5]), *Leishmania* parasites coexist with a diverse microbial community that may interfere with the parasite establishment in *P. papatasi* [6–13]. Concomitantly, the sand fly immune response is adjusted to the presence of commensal and other possible harmful microbes (reviewed by [14]).

In insects, the activation of the Toll and immune deficiency (IMD) pathways occurs when transmembrane receptors, such as Toll-like receptors and peptidoglycan recognition proteins (PGRP), recognize pathogen-associated molecular patterns (PAMPs) [15]. Once these receptors are activated, a sequence of intracellular signaling events occurs, involving regulatory proteases and kinases, resulting in the translocation of transcription factors (e.g., dorsal and relish) to the nucleus and transcription of effector molecules such as antimicrobial peptides (AMPs) [15].

Regarding sand flies, the IMD pathway is involved in the response to *Leishmania*. For instance, the upregulation of this pathway in *Lutzomyia longipalpis*, through the knockdown

of its repressor caspar, reduced *Leishmania infantum* and *Leishmania mexicana* survival [16]. In addition, the depletion of the pathway through the knockout of the transcription factor relish resulted in an increase in *L. major* and gut bacteria in the *P. papatasi* gut [17]. The sand fly AMPs are potentially responsible for a deleterious effect on the parasite. For example, a recombinant defensin peptide encoded by the *Phlebotomus duboscqi* defensin gene has effective activity against *L. major* promastigotes [18]. Moreover, the suppression of a defensin gene in *L. longipalpis*, mediated by RNAi, slightly increased *L. infantum* detection [19]. These results suggest that sand flies express defensins potentially driven toward the parasites.

These studies showed that the IMD pathway and AMPs could affect both *Leishmania* and gut bacteria. Nevertheless, it is not yet clear whether the parasite *per se* triggers the sand fly immune response and if such a response would be triggered specifically in the sand fly gut. In the present study, we focused on analyzing the effects of microbe-rich larvae food with a particular interest in the expression of genes mediated by Toll and IMD pathways. In addition, we depleted the gut bacterial community of adult females to assess the *P. papatasi* female immune response to *L. major*. Our results provide information on *P. papatasi* expression of dorsal and relish transcription factors and attacin and defensin AMPs.

2. Materials and Methods

2.1. *P. papatasi* Immunity Genes

Two transcription factor sequences that contain the rel homology domain (RHD) were selected [20]. The *Phlebotomus papatasi* relish transcription factor gene was previously identified [17]. Dorsal transcription factor, attacin, and defensin were identified by similarity using *L. longipalpis* sequences [21] as a query to search on the *P. papatasi* RNAseq database publicly available from the Vector Base website (www.vectorbase.org, accessed on 10 September 2018), using blast search tools. Partial coding sequences were amplified by PCR using *P. papatasi* cDNA as a template and were sequenced. Similarities between *P. papatasi* sequences and other insect vectors were assessed by the MUSCLE multiple sequence alignment tool [22] built-in Geneious 7.1.9 software (Biomatters, Auckland, New Zealand). This was followed by phylogram analysis using the Maximum Likelihood method with a bootstrap value of 400 repetitions in MEGA X 10.0.5 software [23]. The best substitution model was estimated using MEGA X software using the lowest Bayesian Information Criterion (BIC) score. The Whelan and Goldman (WAG) model [24] was used in dorsal, relish, and defensin analyses, while the Le_Gascuel (LG) model [25] was used in the attacin analysis.

2.2. *L. major* Culture

Leishmania major parasites (FV1 MHOH/IL/80/Friedlin) were cultivated in Medium 199 (Sigma–Aldrich, Saint Louis, MI, USA) at 23 °C, supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Carlsbad, CA, USA), 1 % BME vitamins (Sigma–Aldrich), 2 % of sterile urine, and 250 µg/mL amikacin (Medopharm, Pozorice, Czech Republic). Propagation of *L. major* promastigote culture had up to five passages before the sand fly experimental infections.

2.3. *P. papatasi* Colony Rearing

The *Phlebotomus papatasi* colony, established from sand flies caught in Turkey in 2005, was maintained under standard conditions [26]. Larvae were kept in plastic pots filled with plaster of Paris and fed larvae food made from composted rabbit feces. Three- to seven-day-old females were fed anesthetized mice and were transferred to plaster-lined pots for oviposition four or five days after blood-feeding. Larvae and adult insects were kept at 26 °C.

2.4. Larvae Experimental Feeding

Larval food made from composted rabbit feces [26,27] was divided into two parts, one part was sterilized in an autoclave, and another part was kept unaltered, henceforth referred to as microbe-rich food. Both were collected from the same batch of composted food; therefore, they had same initial composition. We did not make an identification of the bacteriome present in the types of larvae food used in our experiments. Both autoclaved (control group) and microbe-rich food were kept at 4 °C until use. Rearing pots from the two different feeding regimens were kept separately, and fresh food was added three times a week. Observation of larvae development and emerged adults was recorded following colony maintenance routine three times per week. A suspension of each type of larvae food was plated on Luria Bertani (LB) agar medium and incubated for 48 h at 25 °C to estimate the bacterial load. Larvae of the second (L2), third (L3), and early- and late-fourth (L4) instars were dissected, and the guts were collected in pools of 15 individuals from each instar. The experiment was repeated three times. First instar larvae were not sampled due to their diminutive size.

2.5. Depletion of Sand Fly Gut Bacteria

An antibiotic cocktail (AtbC) composed of 100 units/mL of penicillin (BB Pharma, Martin, Slovakia), 50 μg/mL of gentamicin (Sandoz, Boucherville, Canada), and 4 μg/mL of clindamycin (Sigma–Aldrich), adapted from [28], was used to deplete the bacteria community of sand fly female guts. The AtbC was added to 30 % sucrose solution, and 100 μL of the mixture was offered to the recently emerged females *ad libitum* in small Petri dishes. The AtbC-sucrose mixture was changed daily during the experiments.

Bacterial depletion was checked after one week of AtbC treatment on sucrose meal (prior blood-feeding) and five days post blood-feeding of the control and experimental groups. Insects were surface-cleaned twice in a 70 % ethanol bath and rinsed in a sterile saline solution before dissections. Pools of 10 dissected guts from AtbC-treated or non-treated females were homogenized in 100 μL of fresh sterile saline solution and plated on a blood-agar medium. Colony-forming units (CFUs) were counted after 48 h of incubation at 25 °C.

2.6. Leishmania Experimental Infection

One control (blood-fed, non-infected) and two (infected) experimental feeding groups were prepared using seven-day AtbC-treated *P. papatasi*. The control group was fed blood and sucrose meal, both containing AtbC. Female sand flies in the experimental groups were fed defibrinated sheep blood (LabMediaServis, Jaromer, Czech Republic) seeded with 10^6 *L. major* promastigotes/mL. In one experimental group, AtbC was added to the infected blood meal and to the sucrose meal offered *ad libitum* to the sand flies after infectious feeding. A second experimental group received the infected blood meal and sucrose meal without AtbC. The suppression of AtbC allows bacteria to recolonize the sand fly gut (Table 1).

Table 1. AtbC treatment and *Leishmania* experimental infection.

Sand Fly Groups	AtbC in Sucrose Meal before Blood Meal	AtbC in Blood Meal	*Leishmania* in Blood Meal	AtbC in Sucrose Meal after Blood Meal
Control group	+	+	-	+
Experimental group 1 (EG1)	+	+	+	+
Experimental group 2 (EG2)	+	−	+	−

Sand fly samples were collected at different time points post blood-feeding. Guts and corresponding carcasses were stored at −70 °C in lysis buffer until RNA extraction.

2.7. RNA Extraction, cDNA Synthesis, and PCR

Total RNA was extracted from pools of 10 dissected guts or carcasses using the High Pure RNA Tissue Kit (Roche, Pleasanton, CA, USA) according to the manufacturer's instructions. The RNase-free DNase I (Thermo Fisher) digestion step at 1 U/µg of RNA was used to clear possible genomic DNA traces. Synthesis of cDNA was carried using a Transcriptor First Strand cDNA Synthesis Kit (Roche) following the manufacturer's instructions.

Conventional PCR targeting the *P. papatasi* actin gene (Table 2) was used to test successful cDNA synthesis. Reactions were done according to the EmeraldAmp GT PCR Master Mix (TaKaRa, Shiga, Japan) instructions. Cycling conditions were as follows: 95 °C for 3 min; 34 amplification cycles (95 °C for 30 s; 60 °C for 30 s, 72 °C for 1 min); and 72 °C for 5 min. The same conditions were used with defensin primers (Table 2) for detection in different tissues. Amplicons were visualized on 1.5 % agarose gel.

2.8. Relative Gene Expression by qPCR

Expression of sand fly immunity genes, *Leishmania* actin, and bacteria 16S rRNA were determined by qPCR in a LightCycler 480 thermocycler (Roche) with gene-specific oligonucleotides (Table 2) and SYBR Green I Master (Roche). The cycling conditions were as follows: 95 °C for 10 min enzyme activation, 45 amplification cycles (95 °C for 10 s, 60 °C for 20 s; 72 °C for 45 s). Relative gene expression was calculated in comparison to the *P. papatasi* reference gene actin (PPAI004850-RA) and ribosomal protein L8 (PPAI008202-RA) and expressed as the fold change in comparison to the autoclaved-fed or blood-fed control groups [29].

2.9. Leishmania Infection Estimation and Morphometrics

Leishmania development in sand fly vectors was examined by light microscopy in 20 sand fly guts 144 h post infection (day 6 PI), a time when the blood meal was digested and defecated. Under the conditions used, most *P. papatasi* females defecated on day 4 PI [30]. Guts were dissected in saline solution (NaCl 0.9 %), covered with a thin glass slide, and examined under a 40× magnification objective lens. Parasite abundance was estimated and classified as low (less than 100 parasites), moderate (between 100 and 1000 parasites), or heavily infected (more than 1000 parasites) [31]. The localization of parasites in the gut (abdominal or thoracic gut, cardia, and colonized stomodeal valve) was recorded to evaluate infection progress, following previously published methods [32]. Samples were collected from two independent experiments.

Parasite developmental stages were assessed by morphometric methods. Images of 250 randomly selected promastigotes were captured from sand fly gut smears on Giemsa-stained glass slides under a 100× magnification objective lens. Cell width, length, and flagellum were measured using the microscope scale plugin in ImageJ 1.52a software [33]. *Leishmania* developmental stages were categorized as procyclic promastigotes (body length < 14 µm and flagellar length ≤ body length), elongated nectomonads (body length ≥ 14 µm), metacyclic promastigotes (body length < 14 µm and flagellar length ≥ 2× body length), and leptomonads (short nectomonads = remaining parasites) [32,34]. Samples were collected from two independent experiments.

Table 2. Oligonucleotides.

Reference	Gene	Sequence
Nadkarni et al. 2002 [35]	Bacteria 16S rRNA	5^t TCCTACGGGAGGCAGCAGT 3^t 5^t GGACTACCAGGGTATCTAATCCTGTT 3^t
Di-Blasi et al. 2015 [36]	*Leishmania* actin	5^t GTCGTCGATAAAGCCGAAGGTGGTT 3^t 5^t TTGGGCCAGACTCGTCGTACTCGCT 3^t
(PPAI004850)	*P. papatasi* actin	5^t GCACATCCCTGGAGAAATCCTAT 3^t 5^t GGAAAGATGGCTGGAAGAGAGAT 3^t
(PPAI003791)	*P. papatasi* attacin	5^t GCCATTTCTGCTGCGTACTC 3^t 5^t GAGGCACCAAGTACACGACA 3^t
(PPAI004256)	*P. papatasi* defensin	5^t GCCCGGTTAAAGACGATGTAAAG 3^t 5^t AGTTGGTCCAAGGATATCGCAAG 3^t
(PPAI001149)	*P. papatasi* dorsal	5^t GCTGCAAATCCTGCAAAGA 3^t 5^t CCCAAGGAGGTCACAGGTTA 3^t
Louradour et al. 2019 [17]	*P. papatasi* relish	5^t ATCCATCCTTTATGCAACCG 3^t 5^t GCCTTTGAGTCGCAGTATCC 3^t
(PPAI008202)	*P. papatasi* ribosomal protein L8	5^t GACATGGATACCTCAAGGGAGTC 3^t 5^t TTGCGGATCTTATAGCGATAGGG 3^t

VectorBase gene identification shown in parenthesis.

2.10. Statistical Analysis

Ordinary two-way ANOVA with Sidak's correction for multiple comparisons test built-in GraphPad Prism software (version 6.07) (GraphPad Software Inc., San Diego, CA, USA) was used to calculate significant differences in gene expression results obtained by qPCR, and infection estimation and localization were obtained by light microscopy observation.

3. Results

3.1. Transcription Factors and Antimicrobial Peptide Genes

The two *P. papatasi* transcription factors belong to the nuclear factor-kappa B (NF-κB) superfamily, namely, dorsal (PPAI001149) and relish (PPAI012820). The dorsal amino acid sequence contains a rel homology domain (RHD) and a rel homology dimerization domain (RHDD) (Figure 1A). The relish amino acid sequence also contains an ankyrin repeat domain in addition to RHD, RHDD (Figure 1B). The *Phlebotomus papatasi* dorsal sequence shares close similarity to the *L. longipalpis* dorsal sequence forming a sister branch with the *Aedes* and *Anopheles* dorsal sequences (Figure 1C). The relish sequence was closely grouped with the *L. longipalpis* sequence and formed a sister clade with sequences from *Aedes* and *Culex* identified as NF-κB (Figure 1C).

The two *P. papatasi* AMPs identified by similarity with the *L. longipalpis* genes are attacin (PPAI003791) and defensin (PPAI004256). The attacin deduced amino acid sequence contains the corresponding superfamily domain (Figure 2A). The defensin sequence contains the Defensin-2 superfamily domain (Figure 2B). The phylogenetic analysis showed that *P. papatasi* defensin (PPAI004256) grouped with *L. longipalpis* defensin 4 (LlDef4), but it was separated from a clade containing *L. longipalpis* defensin 2 (LlDef2) and *P. duboscqi* sequences (Figure 2C).

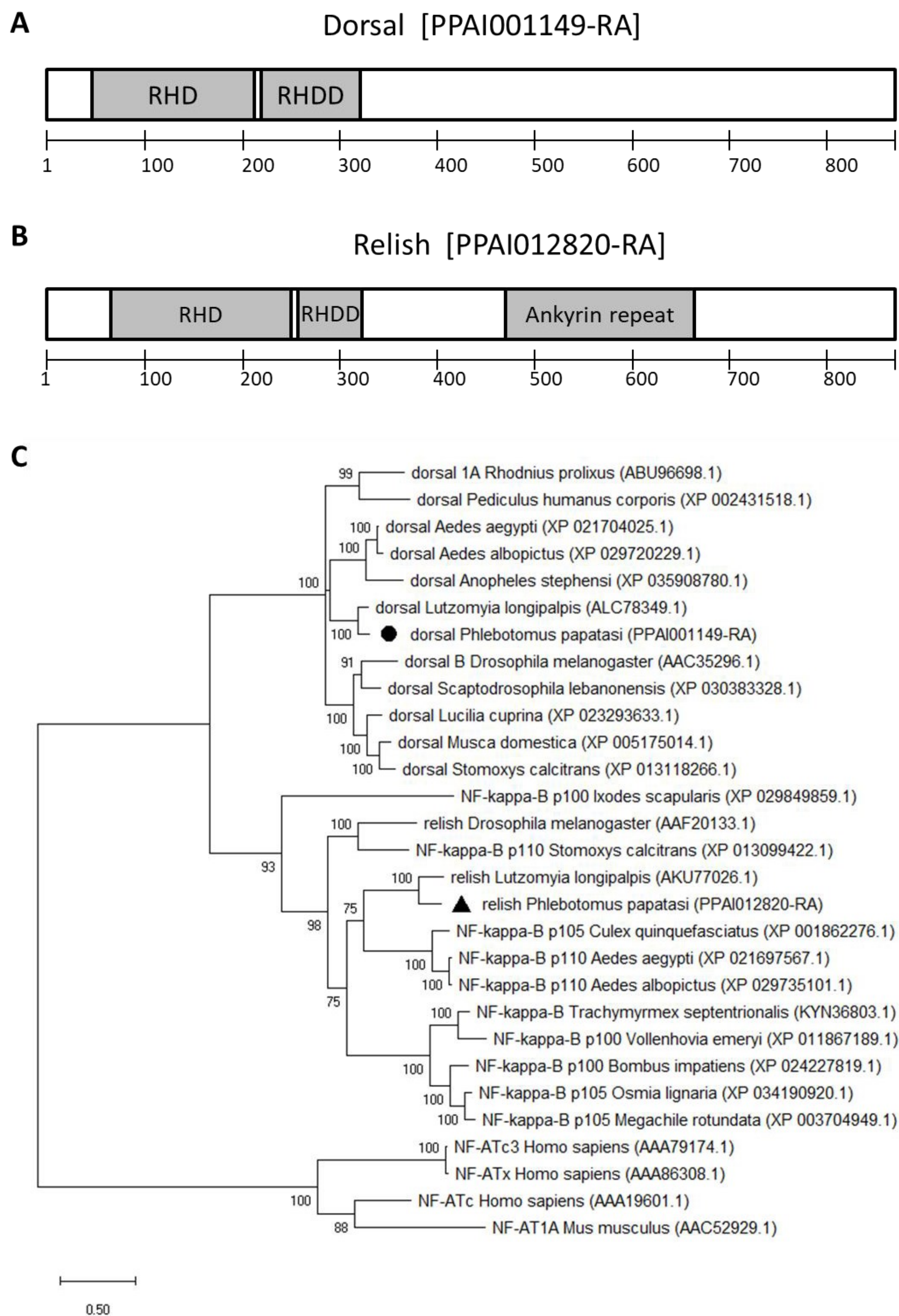

Figure 1. *P. papatasi* dorsal and relish amino acid sequences. (**A**,**B**)- Signature domains identified on the amino acid sequence: grey boxes indicate the rel homology domain (RHD), rel homology dimerization domain (RHDD), and ankyrin repeat domain; numeric scales indicate amino acid positions. (**C**)- Phylogram of amino acid sequences from *P. papatasi* and other organisms' transcription factors inferred by the Maximum Likelihood method, with the WAG model and Gamma distribution. Mammalian nuclear factor of activated T-cells (NF-AT) sequences were used as an outgroup. Numbers on branch nodes indicate bootstrap values higher than 50 %. *Phlebotomus papatasi* dorsal and relish sequences are indicated by a black circle and triangle, respectively. Species names are followed by corresponding VectorBase (*P. papatasi*) or GenBank (other species) accession numbers; the scale bar indicates the number of substitutions per site.

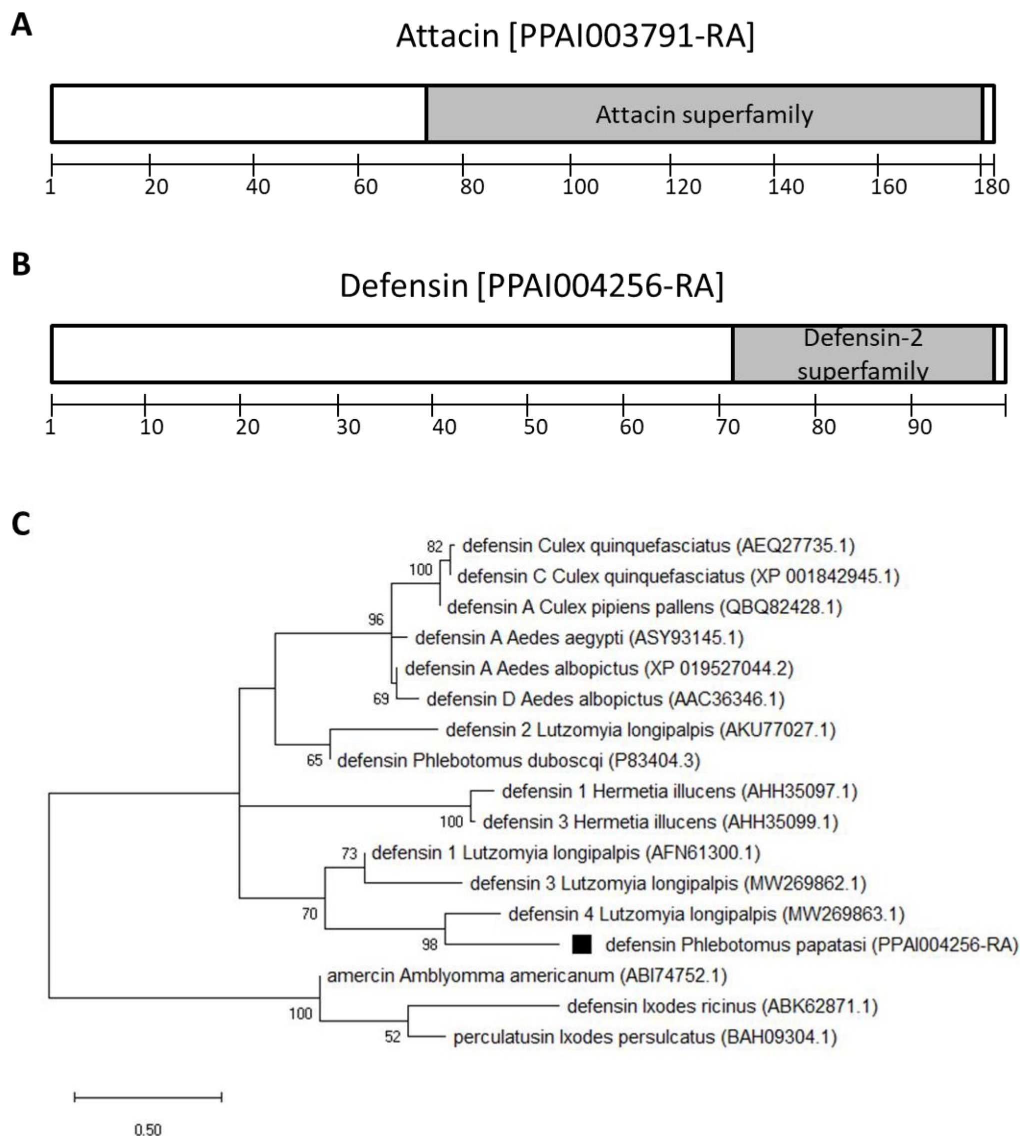

Figure 2. *P. papatasi* AMP amino acid sequences. (**A**,**B**)- Signature domains identified on the amino acid sequences: grey boxes indicate AMP superfamily domains; numeric scales indicate amino acid positions. (**C**)- Phylogram of defensin amino acid sequences from *P. papatasi* and other arthropods inferred by the Maximum Likelihood method, with the WAG model and Gamma distribution. Tick AMPs sequences were used as an outgroup. Numbers on branch nodes indicate bootstrap values higher than 50 %. The *Phlebotomus papatasi* defensin sequence is indicated by a black square; species names are followed by corresponding Vector Base (*P. papatasi*) or GenBank (other species) accession numbers; the scale bar indicates the number of substitutions per site.

For all *P. papatasi* genes used in our experiments, we designed gene-specific primers within each coding sequence used in PCR amplifications. Amplicons were sequenced to confirm the targeted gene. In our current approach, we were not able to distinguish differences between *P. papatasi* sequences derived from VectorBase (sand flies originated from Israel) and from our colony (originated from Turkey).

3.2. Expression of Immunity Genes in Larval Guts

We wanted to determine whether the selected *P. papatasi* immunity genes would be expressed throughout development and altered under different diet conditions in guts dissected from various larval instars. The larval growth period was slightly delayed in the group fed autoclaved food between the L3 and early-L4 stages, but no difference was observed between the early-L4 and late-L4 stages (Table S1). Nevertheless, there was no noticeable difference in the larvae size and survival rates, neither in the size or number of emerged adults between the two groups of reared larvae. When we plated a suspension of food samples on LB agar, we observed no bacterial growth in the sample of autoclaved food while massive/significant bacterial growth was present in the microbe-rich food sample. There was no significant modulation in the gene expression of dorsal in the group fed microbe-rich food compared to the group fed autoclaved food (Figure 3A). On the other hand, the expression of other immunity molecules was increased in larvae fed microbe-rich food. Particularly, relish was increased in the early L4 stage (Figure 3B), attacin was increased in the L3 and early L4 stages (Figure 3C), while defensin was increased in the L3 stage (Figure 3D). The relative gene expression of bacterial 16S increased significantly in the late L4 stage (Figure 3E).

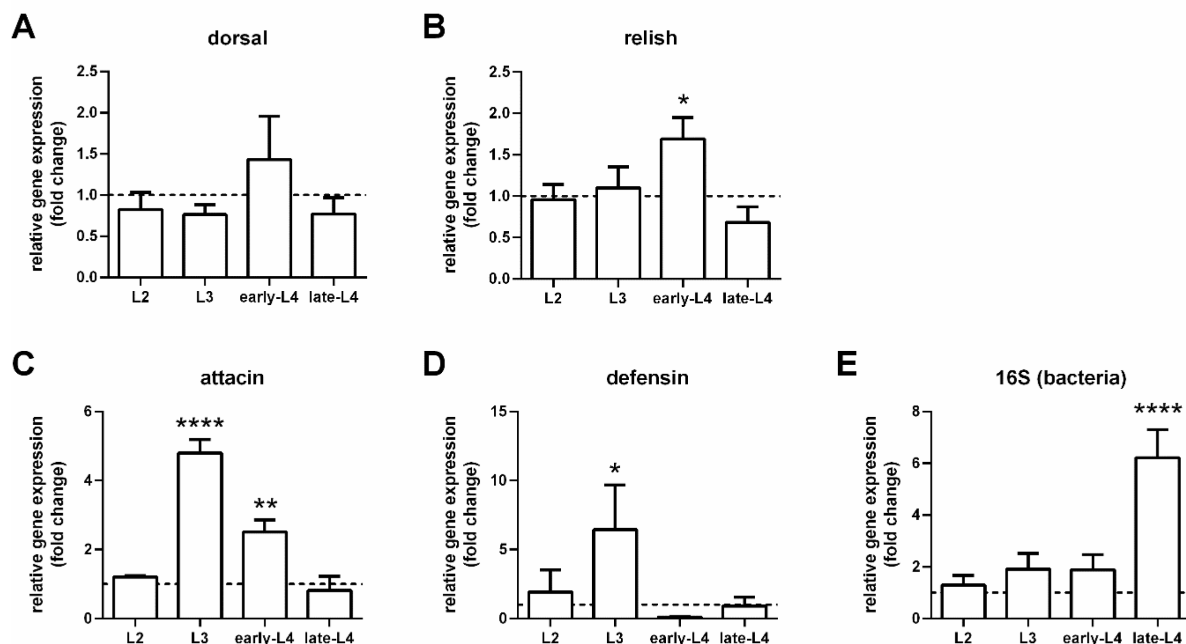

Figure 3. Relative gene expression of *P. papatasi* immunity genes and bacteria detection in dissected guts of larvae fed microbe-rich food. (**A**) Dorsal; (**B**) relish; (**C**) attacin; (**D**) defensin; (**E**) bacteria 16S rRNA. The y-axis represents the relative gene expression of larvae fed microbe-rich food plotted as fold change values compared to the control group fed autoclaved food (dotted line). The x-axis indicates larval stages. Vertical bars represent the average values of three independent experiments, and error bars represent the standard error. Two-way ANOVA was conducted to determine significant differences (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$).

3.3. Expression of Immunity Genes in Infected Females with Depleted Gut Bacteria

We first tested the efficiency of AtbC in blood-fed sand flies. We evaluated the CFUs from dissected guts from sand flies five days post blood-feeding, when all females had eliminated the digested blood content. The CFUs were significantly reduced in AtbC-treated in comparison to the non-treated group (Figure S1).

We also tested if AtbC treatment would interfere with the parasite development in the sand flies. On the sixth day post infection, we assessed the intensity and progression of infection in AtbC-treated (+AtbC) sand flies compared to the non-treated (-AtbC) group. There was no significant difference in infection intensity and localization levels in the sand fly gut (Figure 4A,B). In addition, we analyzed the parasite developmental forms on gut smears, and we did not observe significant differences between the +AtbC- and -AtbC groups (Figure 4C).

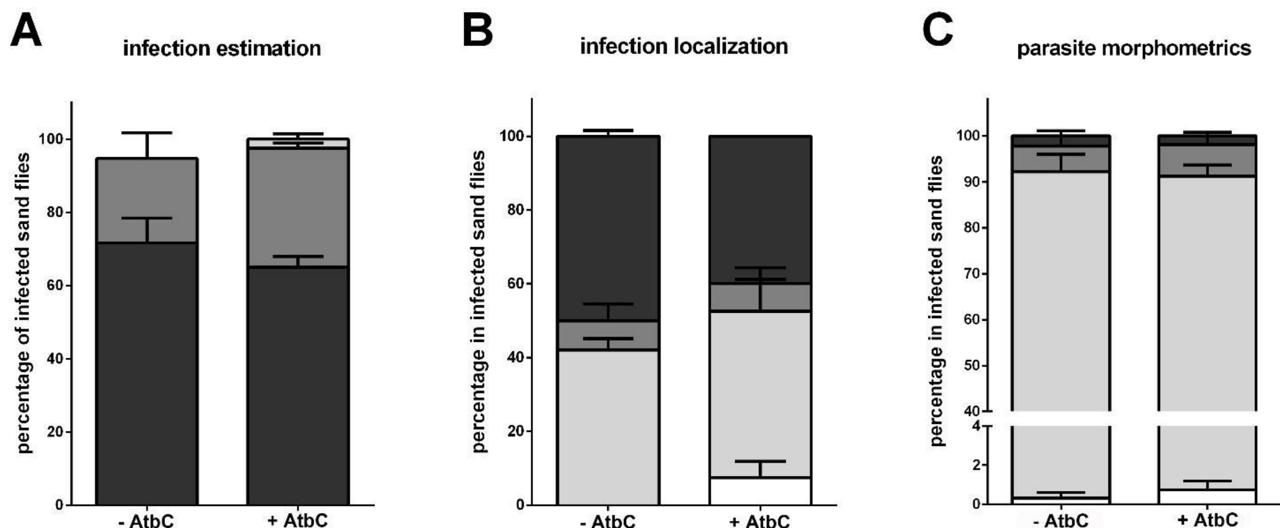

Figure 4. *Leishmania* infection intensity, localization, and development on the sixth day. (**A**) Infection intensity estimation. The y-axis represents the percentage of all individually inspected insects (a total of 40 sand flies in each group). Bar colors indicate infection intensity: light (light grey), moderate (mid grey), heavy (dark grey). (**B**) Infection progression in the sand fly gut. The y-axis represents the percentage of infected insects. Bar colors indicate sand fly gut localization: abdominal gut (white); thoracic gut (light grey); cardia (mid grey); stomodeal valve (dark grey). (**C**) Parasite development in the sand fly gut. The y-axis represents the percentage of analyzed parasites. Bar colors indicate parasite developmental forms: procyclic promastigote (white); elongated nectomonad (light grey); leptomonad (mid grey); metacyclic promastigote (dark grey). The x-axis represents AtbC-treated (+AtbC) and non-treated (-AtbC) groups. Vertical bars represent the average values of two independent experiments, and error bars represent the standard error. No significant differences were found (two-way ANOVA).

We hypothesized that the sand fly immune response could be specifically induced by *L. major*. To address this possibility, we used *P. papatasi* sand flies and assessed the expression of dorsal, relish, attacin, and defensin genes in females infected by *Leishmania* (experimental group 1–EG1) compared to the non-infected control group. Both control and experimental groups were AtbC-treated before and after experimental feeding.

In dissected guts of the *Leishmania*-infected group EG1, dorsal expression was increased at 48 h while relish showed no significant expression changes compared to the AtbC-treated blood-fed control group (Figure 5A,B). Attacin did not show significant changes while defensin expression significantly increased at 144 h post infection (Figure 5C,D). *Leishmania* detection showed that it increased at 72 h post infection compared to the parasite loads detected on the first day post infection (Figure 5E).

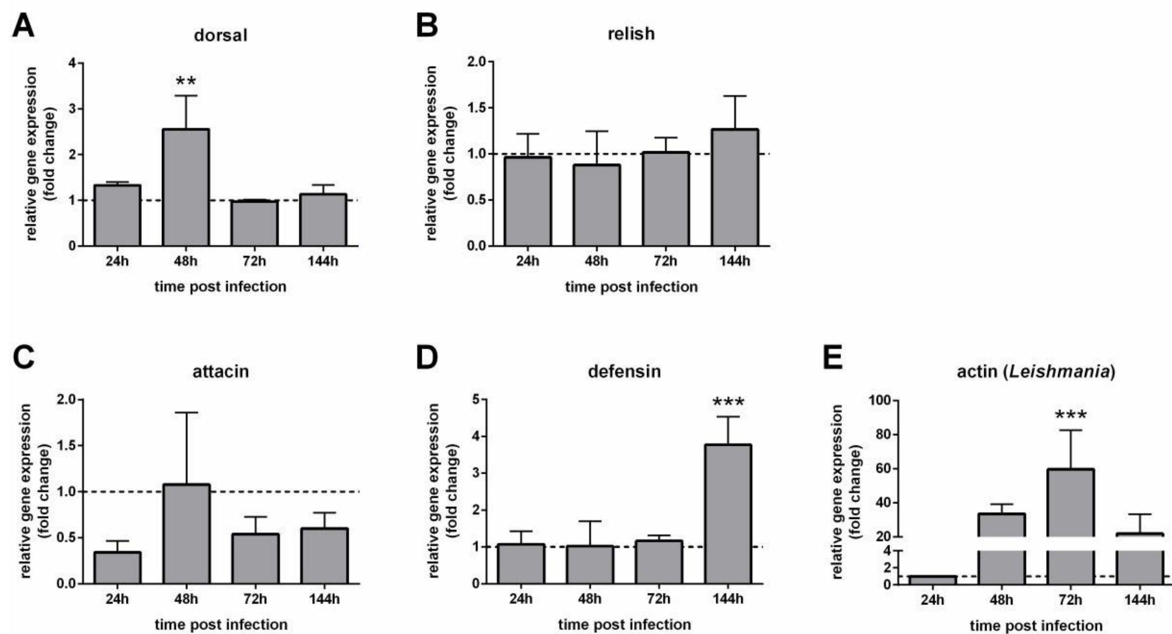

Figure 5. Relative gene expression of *P. papatasi* immunity genes in *Leishmania*-infected females with depleted gut bacteria (EG1) (**A**) Dorsal; (**B**) relish; (**C**) attacin; (**D**) defensin; (**E**) *Leishmania* actin. The y-axis represents relative gene expression as fold change values of *Leishmania*-infected females treated with AtbC (EG1) in comparison to the non-infected control group also treated with AtbC (dotted line) collected at the corresponding time points (**A**–**D**); *Leishmania* detection was expressed in comparison to 24 h (**E**). The x-axis indicates females collected at different times post infection. Vertical bars represent the average values of three independent experiments, and error bars represent the standard error. Two-way ANOVA was performed to determine significant differences (** $p < 0.01$; *** $p < 0.001$).

3.4. Immunity Genes and Infection Progression in Infected Females with Recovered Gut Bacteria

To test if the recolonization of gut bacteria would modify the immune response in the *Leishmania* infected sand fly gut, we removed the AtbC treatment from a group of sand flies (experimental group 2–EG2).

We assessed the effect of removing AtbC on bacterial detection in the sand fly guts. We seeded gut homogenates on blood agar plates and observed an increase in CFUs at 72 h and 144 h post infection in the recovered bacterial group compared to the AtbC-treated group (Figure S2A). This increase was also detected by the relative expression of bacterial 16S rRNA (Figure S2B).

When we compared the EG2-infected group with recovered gut bacteria to the blood-fed control group, there were no significant differences in the expression levels of the transcriptional factors dorsal and relish (Figure 6A,B). At 72 h post infection, attacin expression was variable, therefore showing a non-significant increase (Figure 6C); nevertheless, defensin was significantly increased at the same time point (Figure 6D). The expression of the *Leishmania* actin gene used to assess the parasite detection showed that the parasite increase at 72 h was quite variable, with no significant difference compared to the parasite loads 24 h post infection (Figure 6E).

We also compared the gene expression data of *Leishmania*-infected sand flies with recovered bacteria (EG2) compared to the infected group constantly treated with AtbC (EG1). The relative gene expressions of all five analyzed genes were not significantly altered (Figure S3).

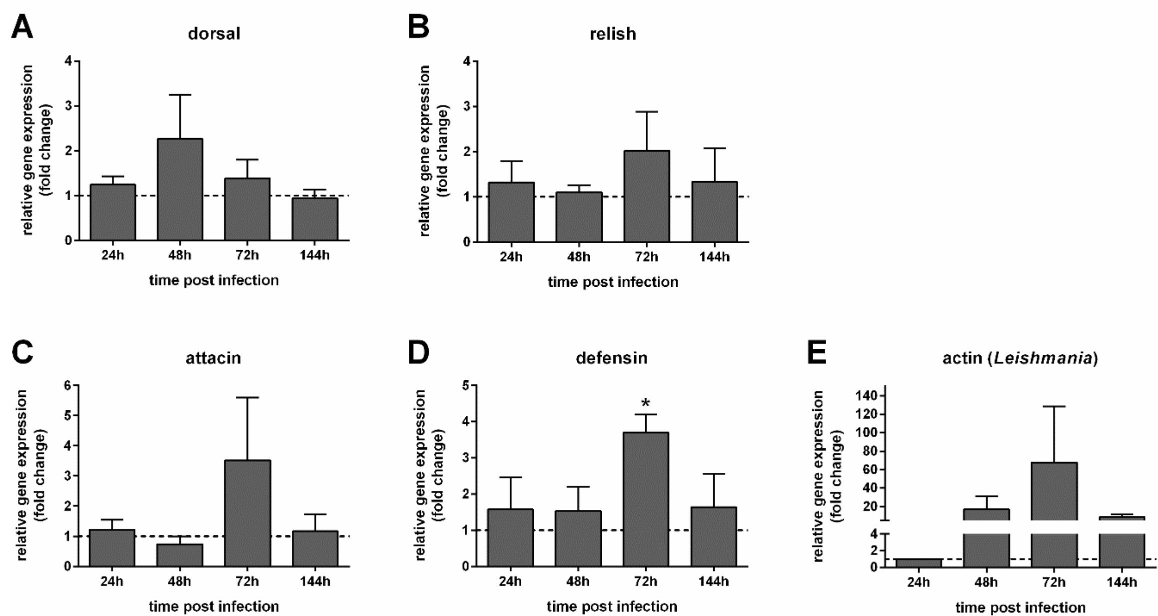

Figure 6. Relative gene expression of immunity genes in *Leishmania*-infected females with recovered gut bacteria (EG2). (**A**) Dorsal; (**B**) relish; (**C**) attacin; (**D**) defensin; (**E**) *Leishmania* actin. The y-axis represents the relative gene expression as the fold change values of *Leishmania*-infected females with recovered gut microbiota (EG2) in comparison to the non-infected control group also treated with AtbC (dotted line) collected at the corresponding time points (**A**–**D**); *Leishmania* detection was expressed in comparison to 24 h (**E**). The x-axis represents females collected at different time points post infection. Vertical bars represent the average values of three independent experiments, and error bars represent the standard error. A two-way ANOVA was performed to determine significant differences (* $p < 0.05$).

3.5. Defensin Gut-Specific Expression

We investigated if the *P. papatasi* defensin gene was also expressed in other sand fly tissues in larvae and adult females. qPCR detected defensin amplification only in guts and not in other tissues. Therefore, we performed non-quantitative PCR using dissected tissues, followed by electrophoresis in 1.5 % agarose gel for visual representation. In larvae, we observed defensin amplification in the guts of various instars but not in carcasses (Figure 7A). In blood-fed females, defensin expression was found in midguts dissected 24 and 144 h post blood meal (Figure 7A). However, no defensin expression was observed in other tissues such as the head, thorax, Malpighian tubules, ovaries, or posterior end of the abdomen of blood-fed females (Figure 7B).

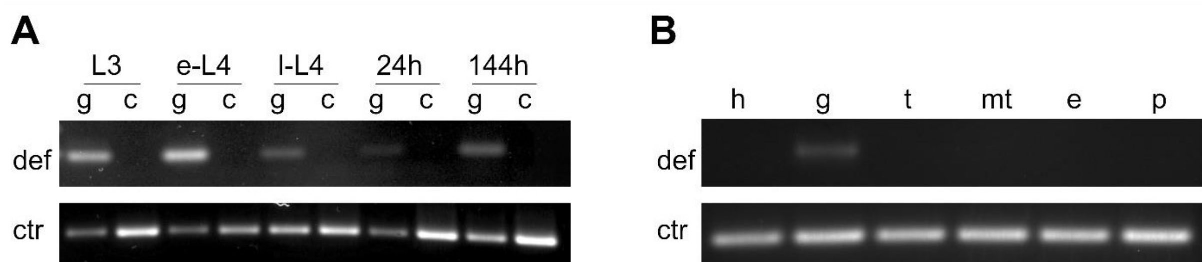

Figure 7. PCR amplification of *P. papatasi* defensin gene in different tissues. (**A**) Defensin PCR from guts (g) and carcasses (c) from L3, early L4 (e-L4), late L4 (l-L4) larval stages, and from females collected 24 h and 144 h post infection. (**B**) Defensin PCR from heads (h), guts (g), thorax (t), Malpighian tubules (mt), eggs (e), and posterior end of the abdomen of females collected on day four (96 h) post blood feeding. Representative images of electrophoresis of defensin (def) and control (ctr) PCR products in 1.5 % agarose gel are shown.

4. Discussion

We selected two transcription factors genes belonging to Toll and IMD pathways (dorsal and relish) and two AMPs (attacin and defensin) to tackle the questions we raised regarding the *P. papatasi* immune response toward the changes in gut bacteria and *L. major*.

We searched for the gene sequences in public databases and used their predicted amino acid sequences to identify signature domains to support gene identification. *Phlebotomus papatasi* dorsal and relish have RHD and RHDD domains characteristic of the NF-κB superfamily [37,38]. The relish sequence contains a C-terminal ankyrin-repeat domain that is characteristic of the 'NF-κB protein' sub-family. Dorsal belong to the 'rel protein' sub-family sequences that lack the ankyrin-repeat domain [39–41]. Both *P. papatasi* dorsal and relish sequences are similar to those previously identified in *L. longipalpis* [21]. They form a group closely related to mosquitoes in both cases while less similar to other flies such as *Drosophila* and *Stomoxys*. Phylogenetic analyses of these transcription factors commonly show mosquito sequences forming a separate branch from *Drosophila* species [42] and more distantly related to other insects such as *Nasonia vitripennis* and *Tribolium castaneum* species [43]. These findings suggest that these sand fly and mosquito transcription factors evolved from a common ancestor.

The *P. papatasi* AMPs attacin and defensin sequences have the signature domains of their respective protein families. The attacin family signature domain contains a proline-rich propeptide (N-terminus) and two glycine-rich domains (C-terminus) [44]. In a previous study, the *P. papatasi* attacin sequence was shown to be closely related to *L. longipalpis* and *Nyssomyia neivai* [19]. In addition, the dipteran attacin group formed separately from the coleopteran and lepidopteran groups [45]. The insect defensins motif has six conserved cysteines responsible for intra-chain disulfide bonds [46]. It was also previously reported that the sand fly defensin group was divided into two branches, one containing the *L. longipalpis* defensin 1, 3, and 4, (LlDef1, LlDef3, and LlDef4) and another containing the LlDef2 and *N. neivai* defensin 2 [19]. Interestingly, the *P. duboscqi* defensin was previously shown to be closely related to the black soldier fly *Hermetia illucens* [47], and in our current analysis, it grouped with the *L. longipalpis* defensin 2. On the other hand, the *P. papatasi* defensin sequence investigated in the present study (PPAI004256) is closely related to the LlDef4, forming a separate branch from the *P. duboscqi* defensin. Together, these studies show that sand fly defensins form diverse groups, but it is unclear which selection pressures acted on their diversification.

In insects, feeding on an enriched microbe food triggers the expression of a complex gene set. For instance, in the larvae of *Trichoplusia ni* moth, feeding on a food mixture containing *Micrococcus luteus* (Gram-positive) and *Escherichia coli* (Gram-negative) can trigger the expression of a group of effector molecules involved in the larvae immune response including AMPs [48]. Similarly, in the larvae of *L. longipalpis*, feeding on *Bacillus subtilis* (Gram-positive) or *Pantoea agglomerans* (Gram-negative) also modulates genes coding for receptors, regulators, and effector molecules of immunity pathways [49]. Therefore, we explored the effect of ingested microbes present in the larvae food on the *P. papatasi* immune response. We cannot exclude possible effects on larvae immunity derived from nutrient processing or absorption caused by the differences in the microbial composition of the food [50]. Nevertheless, our experimental approach is supported by the fact that both food types originated from the same batch, thus having the same initial nutrient composition. In addition, there was no noticeable difference in development between the two larval groups. Dorsal, the transcription factor of the Toll pathway, showed no increased expression under the microbe-rich larvae rearing while relish, associated with the IMD pathway, was upregulated in the actively eating L4 stage. Under this experimental condition, we cannot rule out the participation of the Toll pathway in the regulation of *P. papatasi* AMPs, but its contribution may be reduced. On the other hand, the IMD pathway has a more prominent role through relish expression.

Attacin was highly expressed in the *P. papatasi* L3 and early L4 stages fed microbe-rich food. These stages are voracious [49,51], thus indicating that attacin is necessary for

controlling the increased ingestion of bacterial content in the food. Curiously, defensin was increased in the L3 stage but not in early L4. This may have occurred as a counterbalance between effector molecules or due to changes in bacterial diversity within the larvae gut. Similarly, *L. longipalpis* larvae adjust their AMPs expression throughout their developmental stages with a more evident increase in attacin and two defensin genes (LlDef2 and LlDef4) in L3 larvae compared to the non-feeding larval stage [19]. In addition, AMPs expression in *L. longipalpis* L3 larvae was also adjusted according to the microbial challenge offered through artificial feeding. The ingestion of *B. subtilis* or *P. agglomerans* reduced attacin as early as 12 h, while *P. agglomerans* increased LlDef1 at 24 h post feeding [49]. Together, these findings indicate that sand fly larvae adjust the AMPs expression to balance various bacteria. Sand fly attacins may be responsible for balancing loads of general ingested bacteria, while defensins are tuned to compose a more refined response. This balance is crucial for sand flies since their breeding sites are rich in microorganisms and decomposing material [52–54].

Our study was not focused on changes in bacterial diversity. Nevertheless, we observed that the overall bacterial load was slightly increased in *P. papatasi* L3 and early L4, indicating that the sand fly immune response, possibly through attacin expression, controlled the bacteria abundance in these larval stages. In the late L4 stage (a non-feeding stage), the detection of bacteria was highly increased in the group fed microbe-rich compared to the group fed autoclaved food, thus suggesting that a given bacterial population resists after the peaks of AMPs. These remaining bacteria may survive transstadially and contribute to the colonization of the gut in the adult stage, similar to *P. duboscqi* that carried *Ochrobactrum* sp. from the larvae to the pupae and adult stages [55]. Therefore, the efficient balance of the microbial community in the larvae gut can interfere directly in the adult stage, with a possible impact on sand fly fitness.

We treated the adult sand flies with AtbC before the parasite infection to deplete their gut bacteria. In previous studies, AtbC alone showed no deleterious effect on the parasites *Leishmania donovani* and *L. infantum* or the sand fly *L. longipalpis* survival [28,56]. In our initial trials, AtbC did not cause negative effects on *L. major* culture or *P. papatasi* survival. Nevertheless, changes in the vectors' gut microbial environment may have a distinct outcome. The reduction of bacteria interfered negatively with the progression of *L. infantum* infection in *L. longipalpis*, evidenced by the reduction of metacyclic promastigotes [28]. Under our conditions, our choice of AtbC did not eliminate bacteria but significantly reduced them. In addition, the progression of *L. major* infection in *P. papatasi* evaluated at the sixth day post infection was not significantly altered in the AtbC-treated group. In both groups, parasites multiplied and migrated anteriorly from the abdominal midgut to the thoracic part and the stomodeal valve, and a small percentage of metacyclic forms could be detected in both AtbC-treated and non-treated groups, as observed in previous studies [32,57]. Indeed, the induced variation of the gut microbiota may offer a considerable challenge to the parasite, but the outcome of this balance reveals the potentials of the *Leishmania* adaptability.

In the context of sand fly and *Leishmania* interactions, it is relevant to highlight that the ingested parasites remain inside the *P. papatasi* peritrophic matrix (PM) during the early phase of infection [58,59]. The PM poses a physical barrier between the parasite and its vector, and it could level down the effect of the parasite over the sand fly immune response. Nevertheless, we did not rule out the possibility that secreted parasite molecules and exosomes [60–62] could affect the sand fly immune response during the early phase of infection when the PM is formed and then degraded. Therefore, we analyzed the *P. papatasi* gene expression before and after PM degradation.

We used the bacteria-depleted sand fly model to investigate the sand fly immune response to *L. major*. One experimental group and the control group were AtbC-treated throughout the experiment. The experimental group was infected by *L. major*, while the control group was blood-fed. Dorsal was upregulated in infected sand flies at a time when attacin was highly variable, and this points to a possible connection between this

transcription factor and the effector molecule. Relish showed no significant modulation except for a slight increase on the sixth day when defensin was upregulated, which may also indicate the connection between these molecules. *Phlebotomus papatasi* attacin was slightly but not significantly downregulated at three out of four time points analyzed, indicating that the *L. major* infection caused this subtle reduction. Nevertheless, this variability may also be a result of an under-detected variation of AtbC-resistant bacteria. Moreover, attacin was possibly regulated by another pathway such as Jak-STAT, as similarly reported in *Drosophila* [63,64].

In our experiments, the most significant difference in gene expression levels happened with defensin. The upregulation of this AMP occurred 144 h post *Leishmania* infection in the AtbC-treated group, in the late phase of infection when parasites migrate to the anterior part of the digestive tract and colonize the stomodeal valve. The modulation of defensin genes also occurred in *L. longipalpis*, where both of them were reported to be modulated after the parasitic infection. LlDef1 was reduced after *L. mexicana* [65], and LlDef2 was increased after *L. infantum* infection [19]. In addition, the *P. duboscqi* defensin, which is more similar to LlDef2, was increased by *L. major* infection [18]. In *P. papatasi*, the PPAI004256 defensin, more similar to LlDef4, had different fold changes in gene expression after *L. major* or *L. donovani* infection [66]. Variability of vector's immune responses according to different parasite species was previously described in *Anopheles* mosquitoes. The IMD-mediated response was the most effective against *Plasmodium falciparum*, while the Toll-mediated response was more effective against *Plasmodium berghei* [67–70]. It is possible that the sand fly immune response also adjusted to the different *Leishmania* species, thus adding another range of molecular events that will interfere with the sand fly permissiveness or restrictiveness in hosting other parasite species.

The parasite increase on the third day post infection reflects the natural multiplication of procyclic and its transformation into nectomonad promastigotes, which happens before the termination of blood digestion and elimination of gut contents by defecation (reviewed by [4,71]). Under our colony conditions, the *P. papatasi* blood digestion and defecation processes ended between the fourth and fifth days post blood ingestion [58]. These results indicate that the parasite infection in our experimental setting followed the commonly observed pattern, thus indicating that the parasite cycle was adjusted to changes in the sand fly microbial gut community.

To determine whether the sand fly immune response would be altered by the additive effect of *Leishmania* infection and restored gut bacteria, we removed the AtbC treatment during infection and from the sucrose meal in a second experimental group, thus allowing bacteria to multiply and recolonize the sand fly gut. There is a possibility that part of the bacterial community originates in adults from strains carried transstadially from larval and pupal stages [55]. Nevertheless, most are acquired from environmental bacteria that opportunistically colonize their hosts' guts (reviewed by [72]). Sand flies probe surfaces with their mouthparts during their feeding process, and this habit allows new microbes to be ingested and colonize the sand flies' guts. These studies support the hypothesis that the gut environment would be readily recolonized after antibiotics are metabolized by sand flies.

Indeed, we observed an increase in bacteria based on CFU calculation and qPCR detection in the second sand fly group where the AtbC treatment was interrupted during the experimental infection. This increase indicates that bacteria were reintroduced by sucrose feeding and surface probing or they resisted the AtbC-treatment and regrew in the sand fly gut. In this additional experimental setting, dorsal was slightly but not significantly increased, as seen in the fully AtbC-treated group. The relish expression was not significantly altered, but there was a slight increase at 72 h post feeding, indicating an earlier activation of the IMD pathway. Attacin had a highly variable and slightly increased expression at 72 h post feeding in the recovered-bacteria group. This variable expression occurred one day later than what was observed in the AtbC-treated group. This delayed attacin expression may reflect *L. major* infection reducing the pace of bacteria

recolonization as similarly occurred in *L. longipalpis* infected by *L. mexicana*, which protected the sand fly from the entomopathogenic *Serratia marcescens* [73]. These findings indicate that both parasites and bacteria face a certain level of competition to survive in the sand fly gut. Possible beneficial outcomes may occur, but this complex interaction is not yet fully explored.

Interestingly, defensin increased earlier in the bacteria-recovered group, indicating that the combination of the parasite with bacteria resulted in its earlier regulation. The upregulation of defensin was observed at a time when relish was slight increased, which corresponds with the findings in AtbC-treated females about a possible connection between these two molecules. Simultaneously, the parasite detection at 72 h was variable and slightly increased, reflecting the complex and dynamic balance within the gut microbiota. For example, the reintroduction of *Lysinibacillus* or *Serratia* strains in *L. longipalpis* infected by *Leishmania chagasi* (syn. *L. infantum*) reduced the number of parasites. At the same time, *Pseudocitrobacter* had no deleterious effect on *Leishmania amazonensis* infection in the sand fly gut [74]. Our results indicate that the combination of increased bacteria and *L. major* resulted in an earlier modulation of *P. papatasi* immunity in the gut. It is possible that bacterial growth was detected as a more imminent threat, thus inducing the AMPs gene expression. These results reflect the complex dynamics between sand fly immunity and gut-residing organisms.

We plotted the relative gene expression of AtbC-treated-infected (EG1) and the bacteria-recovered (EG2) sand fly groups for comparison purposes. This alternative way of visualizing our results revealed that in the context of *L. major*-infected sand flies, the addition of bacteria did not cause a significant change in the *P. papatasi* immune response, suggesting that the *L. major* infection buffered the response against bacteria or protected the sand fly from pathogenic bacterial regrowth. This finding correlates with the protection created by *L. infantum* against *S. marcescens* infection in *L. longipalpis* mentioned above [73]. It is possible that the parasite produces molecules that compete for sand fly receptors in the gut or secrete virulent factors that would inhibit part of the bacterial community.

Some AMPs can be expressed in the fat body of insects and secreted into the hemolymph [75,76]. In addition, others can be expressed in specific tissues [70] constitutively or due to an injury-type of stimuli [77]. In *Drosophila*, the gut immune response is mediated by the IMD and Jak-STAT pathways, but AMPs can be expressed in the gut or systemically [78]. In *P. duboscqi*, a defensin peptide was identified in the hemolymph [18], while in *L. longipalpis*, defensin mRNAs were expressed in reproductive organs [79], female guts [80], or in female whole-body samples [19,65]. In addition, a *L. longipalpis* defensin gene was expressed differentially depending on the route of infection [65]. Especially for hematophagous insects, the digestive track is mostly exposed to pathogens that may be present in the ingested blood.

In the current study, among the two investigated AMPs, we identified a defensin gene exclusively expressed in gut tissue in larvae and adult females, indicating that this defensin is responsible for the specific protection of the digestive tract of *P. papatasi*. Furthermore, the defensin gene expression was upregulated by *L. major* infection. An additional load of bacteria can trigger an earlier peak of expression before completing the sand fly digestion process. The moment before defecation is strategic for parasite survival because parasites are multiplying and preparing to colonize the sand fly gut.

Very little is known about the action of defensins against *Leishmania*. One interesting study using a plant defensin PvD1 from *Phaseolus vulgaris* showed inhibitory activity against *L. amazonensis* [81]. In in vitro assays, this plant defensin inhibited promastigote proliferation and caused cytoplasmic fragmentation, the formation of multiple cytoplasmic vacuoles, and cell membrane permeabilization [81]. Although we do not discard any potential antiparasitic effects of defensins, the *P. papatasi* defensin promotor sequence is a very interesting candidate for coupling to a foreign gene coding for a parasite-killing molecule. Such a molecular construct could lead to efficient expression of an anti-leishmanial molecule

in a gene-edited *P. papatasi*. Such a molecular construct may lead to efficient strategies to control parasite survival inside the vector's gut.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9112307/s1, Table S1: L3 and L4 larval growth period under different feeding regimens; Figure S1: Effects of AtbC-treatment on CFUs from sugar-fed *P. papatasi* dissected guts; Figure S2: Effects of AtbC-treatment based on CFU calculation and qPCR bacteria detection from treated and non-treated females; Figure S3: Relative gene expression of immunity genes in *Leishmania*-infected females with recovered gut bacteria compared to infected females treated with AtbC.

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Data Availability Statement: Nucleotide and amino acid sequences used in this study can be found in NCBI GenBank (https://www.ncbi.nlm.nih.gov/ accessed on 5 November 2021) and VectorBase (https://vectorbase.org/vectorbase/app/ accessed on 5 November 2021). Original gel images of Figure 7 were uploaded in FigShare (https://doi.org/10.6084/m9.figshare.16939591.v1).

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RNAi-mediated gene silencing of *Phlebotomus papatasi* defensins favors *Leishmania major* infection

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Introduction: Production of different antimicrobial peptides (AMPs) is one of the insect's prominent defense strategies, regulated mainly by Toll and immune deficiency (IMD) humoral pathways. Here we focused mainly on two AMPs of *Phlebotomus papatasi*, vector of *Leishmania major* parasites, their association with the relish transcription factor and the effective participation on *Leishmania* infection.

Methods and results: We further characterized the role of previously described gut-specific *P. papatasi* defensin (PpDef1) and identified the second defensin (PpDef2) expressed in various sand fly tissues. Using the RNAi-mediated gene silencing, we report that the silencing of *PpDef1* gene or simultaneous silencing of both defensin genes (*PpDef1* and *PpDef2*) resulted in increased parasite levels in the sand fly (detectable by PCR) and higher sand fly mortality. In addition, we knocked down relish, the sole transcription factor of the IMD pathway, to evaluate the association of the IMD pathway with AMPs expression in *P. papatasi*. We demonstrated that the relish gene knockdown reduced the expression of *PpDef2* and *attacin*, another AMP abundantly expressed in the sand fly body.

Conclusions: Altogether, our experiments show the importance of defensins in the sand fly response toward *L. major* and the role of the IMD pathway in regulating AMPs in *P. papatasi*.

KEYWORDS

sand fly, innate immunity, relish, antimicrobial peptides, knockdown, leishmania, defensin

1 Introduction

Antimicrobial peptides (AMPs), including defensins, are prominent effector molecules of innate insect immunity. Their transcription is regulated mainly by two humoral pathways, Toll and immune deficiency (IMD) (Hoffmann, 2004). Very briefly, pathogens are recognized by transmembrane receptors, which leads to numerous signaling events. The signaling cascade terminates by translocation of transcriptional factors dorsal and relish belonging to Toll and IMD pathways, respectively, into the cell nucleus followed by AMPs transcription (De Gregorio et al., 2002).

In insect vectors, the IMD pathway has a role in the innate immune response against parasites. For example, the over-activation of IMD-mediated response in three anopheline mosquitoes caused the reduction of *Plasmodium falciparum* infection (Garver et al., 2009). Similarly, this increased response in the sand fly *Lutzomyia longipalpis* caused the reduction of *Leishmania* parasites (Telleria et al., 2012). The IMD-mediated response is controlled by

the nuclear factor-kappa B (NF-κB) protein sub-family members, also known as relish proteins in arthropods (Dushay et al., 1996; Huguet et al., 1997). The importance of relish in controlling the response against parasites was shown when the knockout of relish in the sand fly *Phlebotomus papatasi* caused increased numbers of *Leishmania major* parasites and bacteria loads in the sand fly gut (Louradour et al., 2019).

Insects have a broad repertoire of AMP molecules acting in synergy to effectively control a plethora of infectious agents, although they are often targeted for specific microorganisms (Bulet et al., 1999). In the present work, we focused on defensins, small 4-kDa peptides with 6 cysteines and 3 intramolecular disulfide bridges (Cociancich et al., 1993). Insect defensins act against a wide spectrum of Gram-positive (G+) and Gram-negative (G-) bacteria and fungi. Their mechanisms of action involve membrane perforation, blocking the ion channel formation, or targeting specific pathogen structures (Bulet et al., 1999). Defensins, however, have anti-protozoan activity as well; for example, purified defensins from the blow fly *Phormia terranovae* and dragonfly *Aeschna cyanea* acted against *Plasmodium gallinaceum* by reducing the oocysts number in mosquitoes and altering sporozoite morphology (Shahabuddin et al., 1998). In addition, a recombinant defensin produced from *Triatoma pallidipennis* showed *in vitro* lytic activity on *Trypanosoma* and *Leishmania* parasites (Díaz-Garrido et al., 2021).

We are interested in the *P. papatasi* study model because it stands out as a main vector of *L. major* parasites, causing cutaneous leishmaniasis and affecting hundreds of thousands of human lives yearly (Akhoundi et al., 2016). Understanding how the sand fly reacts to *Leishmania* infection may reveal alternative targets for transmission control strategies. Several studies have been developed in this direction, but many aspects of this multifactor relationship remain uncovered (Telleria et al., 2018). In *Phlebotomus duboscqi*, another vector of *L. major*, a recombinant defensin showed *in vitro* antiparasitic activity against *Leishmania* promastigotes (Boulanger et al., 2004). In *L. longipalpis,* a vector of *L. infantum* in the Americas, the gene expression of AMPs defensin2 (LlDef2), attacin (LlAtt), and cecropin (LlCec) was increased during *Leishmania infantum* infection, and the systemic silencing of *LlDef2* gene resulted in a slight change in *L. infantum* detection (Telleria et al., 2021b). Although the development of *Leishmania* parasites in the sand fly is restricted to the gut, the parasitic infection seems to trigger a systemic response produced in fat body cells.

Interestingly, recent results of our team also reveal a tissue-specific response in sand flies: the gene expression of a gut-specific *P. papatasi* defensin (PpDef1) was increased after *L. major* infection in bacteria-depleted sand flies (Kykalová et al., 2021). However, it is unknown whether the IMD pathway regulates this defensin and if PpDef1has any role in controlling the parasites in the sand fly gut. To address these questions, we silenced relish by RNAi-mediated gene silencing and followed the AMPs expression by qPCR. We investigated these immunity gene expressions in dissected guts or carcasses. The Toll pathway can also regulate AMPs expression (Hoffmann, 2004; Tinoco-Nunes et al., 2016). Nevertheless, we did not address it in the present study. We also silenced two defensin genes in bacteria-depleted sand flies to evaluate the effect on *L. major* infection.

2 Materials and methods

2.1 Sand flies and antibiotic treatment

Phlebotomus papatasi colony was established from field-caught sand flies from Turkey in 2005. Sand flies were kept under standard conditions at 26°C, 45% relative humidity, and 14 h light/10 h dark photoperiod (Volf and Volfova, 2011). Adult sand flies were fed on a 30% sucrose solution offered on cotton wool. For experimental infections, adult females were fed on a 30% sucrose solution containing an antibiotic cocktail (AtbC) to deplete gut bacteria. AtbC was adapted from (Kelly et al., 2017): 100 units/mL of penicillin (BB Pharma, Martin, Slovakia), 50 μg/mL of gentamicin (Sandoz, Boucherville, Canada), and 4 μg/mL of clindamycin (Sigma–Aldrich, Saint Louis, MI, United States). The bacteria depleted sand flies were used to access the immune response caused mainly by *L. major*. The efficiency of our choice of AtbC and a possible interference with parasite development in the vector was previously addressed in this parasite-vector pair by our team, and no negative correlation between parasite development and AtbC was detected (Kykalová et al., 2021).

2.2 Parasites and experimental infections of sand flies

Leishmania major parasites (FV1 MHOH/IL/80/Friedlin) were cultivated in Medium199 (Sigma–Aldrich) at 23°C, supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Carlsbad, CA, United States), 1% BME vitamins (Sigma–Aldrich), 2% of sterile urine, and 250 μg/mL amikacin (Medopharm, Pozorice, Czech Republic). Adult females had access to AtbC in sucrose solution served *ad libitum* and changed daily for 5 days after eclosion, during the experimental infection and after infection. On day 5, females were fed through chicken skin membrane on defibrinated sheep blood (LabMediaServis, Jaromer, Czech Republic) with AtbC, seeded with 10^6 *L. major* promastigotes/mL. Blood-fed females were separated, kept under the same conditions described above, and dissected at different time intervals (indicated in figure legends). Guts (without Malpighian tubules) and carcasses (i.e., all other tissues) were dissected in sterile saline solution, collected in pools of 10, and stored at −80°C until processing.

2.3 *P. papatasi* AMPs and relish gene sequences

Phlebotomus papatasi relish (PpRel) (PPAI012820), attacin (PpAtt) (PPAI003791), and PpDef1 (PPAI004256) gene sequences were previously identified (Louradour et al., 2019; Kykalová et al., 2021; Sloan et al., 2021) and are available from the Vector Base website (Amos

TABLE 1 Oligonucleotides.

Gene name	Reference	Sequence
Actin (PpAct)	Kykalová et al. (2021)	5′ GCACATCCCTGGAGAAATCCTAT 3′
(PPAI004850)		5′ GGAAAGATGGCTGGAAGAGAGAT 3′
Ribosomal protein L8 (PpRibL8)	Kykalová et al. (2021)	5′ GACATGGATACCTCAAGGGAGTC 3′
(PPAI008202)		5′ TTGCGGATCTTATAGCGATAGGG 3′
Relish (PpRel)	Louradour et al. (2019)	5′ GGAGCTTCCGTTCCCATCAA 3′
(PPAI012820)		5′ TCGTCCTCTCGAATAGCCCA 3′
Attacin (PpAtt)	Kykalová et al. (2021)	5′ GCCATTTCTGCTGCGTACTC 3′
(PPAI003791)		5′ GAGGCACCAAGTACACGACA 3′
Defensin1 (PpDef1)	Kykalová et al. (2021)	5′ GCCCGGTTAAAGACGATGTAAAG 3′
(PPAI004256)		5′ AGTTGGTCCAAGGATATCGCAAG 3′
Defensin2 (PpDef2)	present study	5′ ATTCACGCCAAAAACGAGCC 3′
(PPAI010650)		5′ CGATACAATGGGCAGCACAAG 3′
PpDef2 confirmation	present study	5′ TGCGTACGTTCTTGGTAGTAGT 3′
(PPAI010650)		5′ TGTGCAGACAGCCTTTGA 3′
Leishmania actin	Di-Blasi et al. (2015)	5′ GTCGTCGATAAAGCCGAAGGTGGTT 3′
		5′ TTGGGCCAGACTCGTCGTACTCGCT 3′
dsRel (PpRel)	present study	5′ TAATACGACTCACTATAGGGAGAACTCTTCTGACATTCCCCTGAC 3′
		5′ TAATACGACTCACTATAGGGAGATTTGATGGGAACGGAAGCTCCC 3′
dsDef1 (PpDef1)	present study	5′ TAATACGACTCACTATAGGGAGATGTAAAGGACCCTGTGGAGGA 3′
		5′ TAATACGACTCACTATAGGGAGAATCATCGCACCATCCTCCTG 3′
dsDef2 (PpDef2)	present study	5′ TAATACGACTCACTATAGGGAGACTTGTCGTTGTTGTGGGAG 3′
		5′ TAATACGACTCACTATAGGGAGAAAGCAGCATGACCAACTC 3′
dsLacZ (LacZ)	(adapted from Molina-Cruz et al., (2008)	5′ TAATACGACTCACTATAGGGAGATATCCGCTCACAATTCCACA 3′
		5′ TAATACGACTCACTATAGGGAGAGAGTCAGTGAGCGAGGAAGC 3′

Accession numbers of gene sequences obtained from VectorBase database are indicated below gene names.
*Underlined nucleotides indicate T7 Polymerase Promoter.
Bold characters indicate gene name followed by gene abbreviation.

et al., 2022). A second *P. papatasi* defensin sequence (PpDef2) was identified by similarity using the *L. longipalpis* defensin sequences (Telleria et al., 2021b) as a query to search on the *P. papatasi* RNAseq database publicly available from the Vector Base using blast search tools. The PpDef2 was amplified by PCR (Table 1) and *P. papatasi* cDNA template and sequenced for confirmation.

The conserved domain present in the PpDef2 amino acid sequence was identified using the InterPro (Blum et al., 2021) and the NCBI Conserved Domain Database (Lu et al., 2020) tools to support its identification. Similarities between the PpDef2 and other insect defensins were assessed by the MUSCLE multiple sequence alignment tool (Edgar, 2004) built-in Geneious 7.1.9 software (Biomatters, Auckland, New Zealand). A phylogram analysis was performed using the Maximum Likelihood method and Whelan and Goldman model (Whelan and Goldman, 2001), allowing for evolutionarily invariable sites (WAG + I) with a bootstrap value of 400 repetitions in MEGA X 10.0.5 software (Kumar et al., 2018).

2.4 dsRNA synthesis and microinjections in the sand flies

Templates for dsRNA synthesis were amplified by PCR using *P. papatasi* cDNA and gene-specific primers for PpRel, PpDef1, and PpDef2 containing the T7 promoter sequence on the 5′ends (Table 1). The template for control dsRNA was amplified from p-GEM-T Easy plasmid (Promega, Madison, WI, United States) using dsLacZ primers (Table 1). The PCR cycling conditions were as follows: 95°C for 3°min; 34 amplification cycles (95°C for 30°s; 60°C for 30°s, 72°C for 1 min); and 72°C for 5 min. Amplicons were visualized on 1% agarose gel and purified using E.Z.N.A. Gel

Extraction kit (Omega Bio-tek, Norcross, GA, United States). Purified templates were used in dsRNA synthesis using the MEGAscript RNAi Kit (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. Gene-specific dsRNA were lyophilized and resuspended in sterile H_2O to a final concentration of 4.5 µg/µL.

For gene silencing, dsRNA was manually microinjected using Nanoject II microinjector (Drummond, Broomall, PA, United States) into the thorax of adult sand fly females anesthetized on ice (Sant'Anna et al., 2008). To test the efficiency of gene silencing, 1–2°days old sucrose-fed colony females were microinjected with 32 nL (150 ng) of dsRNA specific for *PpRel* (dsRel), *PpDef1* (dsDef1), *PpDef2* (dsDef2) genes, and a non-related LacZ dsRNA (dsLacZ) as a control. After confirming the efficient gene silencing, that in our hands is usually achieved between first and third days post dsRNA injection, the dsRNA is used in further experimental conditions. Preliminary experiments showed that sugar and blood feedings do not interfere with gene silencing by RNAi pathway.

In addition, to study the role of defensins during *L. major* infection, 3-day infected females were separated into three groups. The first experimental group was injected with 64 nL (300 ng) of dsDef1 to silence the gut-specific defensin. The second group was injected with a mixture of 32 nL (150 ng) of dsDef1 plus 32 nL (150 ng) of dsDef2 (dsDef1+2) to create an additive effect of silencing another defensin that was systemically expressed. The third group (control) was injected with 64 nL (300 ng) of dsLacZ. The choice of injecting dsRNA in 3-day infected females was based on two factors. First, to match the period of efficient gene silencing with the time when PpDef1 is differentially expressed in the sand fly gut (72 h or 144 h post infection) (Kykalová et al., 2021). Second, to match with the crucial moment in *Leishmania* cycle in *P. papatasi* when blood digestion ends, and the parasites migrate to the anterior part of the sand fly gut (between 72°h and 96 h.post infection) (Dillon and Lane, 1993; Pruzinova et al., 2015).

2.5 RNA extraction and cDNA synthesis

Total RNA was extracted from the samples stored at −80°C using E.Z.N.A. Total RNA Kit I (Omega Bio-tek) following the manufacturer's instructions. A DNA digestion step with RNase-free DNase I (Thermo Fisher Scientific, Waltham, MA, United States) was included to clean RNA from possible DNA residues. DNA-free RNA templates were used in a cDNA synthesis reaction with anchored-oligo $(dT)_{18}$ primer using a Transcriptor First Strand cDNA Synthesis Kit (Roche Life Science, Rotkreuz, Switzerland) following the manufacturer's instructions.

PCR amplification of *P. papatasi* actin (Table 1) was carried out to control cDNA synthesis using the same cycling conditions mentioned above. Amplicons were visualized on 1% agarose gel.

2.6 Relative gene-expression analysis

Quantitative PCR (qPCR) was prepared with cDNA samples, gene-specific primers (Table 1), and SYBR Green PCR Master mix (Roche) to detect the expressions of investigated genes using a LightCycler 480 thermocycler (Roche). Relative gene expression was calculated relative to *P. papatasi* endogenous control genes *actin* (*PpAct*) (PPAI004850) and *60S ribosomal protein subunit L8* (*PpRibL8*) (PPAI008202) using the following cycling conditions: 95°C for 10 min enzyme activation, 45 amplification cycles (95°C for 10°s, 60°C for 20°s; 72°C for 45 s) (Kykalová et al., 2021). Expression levels were expressed as the fold change compared to the control groups.

2.7 *L. major* development in *P. papatasi*

Sand fly guts were examined 6 days post parasite infection under a light microscope to determine the parasite loads and localization. Day 6 of infection represents a late stage when the defecation process is finished, and parasites start to migrate to the thoracic midgut and the stomodeal valve (Dostálová and Volf, 2012). In addition, this time point corresponds with the third day post dsRNA injection, when the gene silencing effects can still be detected (Sant'Anna et al., 2008; Sant'Anna et al., 2009; Coutinho-Abreu et al., 2010b; Telleria et al., 2012; Di-Blasi et al., 2019). A total of 65 females per group (dsDef1, dsDef1+2, and dsLacZ) from three independent experiments were dissected in sterile saline solution (NaCl 0.9%) and inspected under a ×40 magnification objective lens. Parasite loads in the gut were classified as light (below 100 parasites per gut), medium (between 100 and 1,000 parasites per gut), and heavy (above 1,000 parasites per gut), as it was previously described (Myskova et al., 2008). Also, the localization of parasites was evaluated and recorded in the abdominal midgut, thoracic midgut, cardia, and stomodeal valve (Sádlová et al., 2010).

Smears of randomly selected gut samples were prepared on glass slides for assessing the parasite development stages. Smears of individual guts were fixed with methanol and Giemsa-stained. Images of fifty randomly selected parasites per slide, 200 parasites from each group, were captured under a ×100 magnification objective lens in an Olympus BX51 microscope (OLYMPUS, Tokyo, Japan). Parasite cell width, length, and flagellum were measured using the microscope scale plugin in ImageJ 1.52a software (Abràmoff et al., 2004). Parasite morphological forms were identified according to previously published criteria as procyclic promastigotes (body length < 14 µm and flagellar length ≤ body length), elongated nectomonads (body length ≥ 14 µm), metacyclic promastigotes (body length < 14 µm and flagellar length ≥ 2× body length), and leptomonads (short nectomonads = remaining parasites) (Sádlová et al., 2010).

2.8 Mortality rate

To evaluate the possible negative effect of defensins silencing after *L. major* infection, numbers of alive and dead sand flies from experimental (dsDef1 and dsDef1+dsDef2) and control (dsLacZ) groups were recorded during three consecutive days post intrathoracic microinjections, and mortality rate for each group

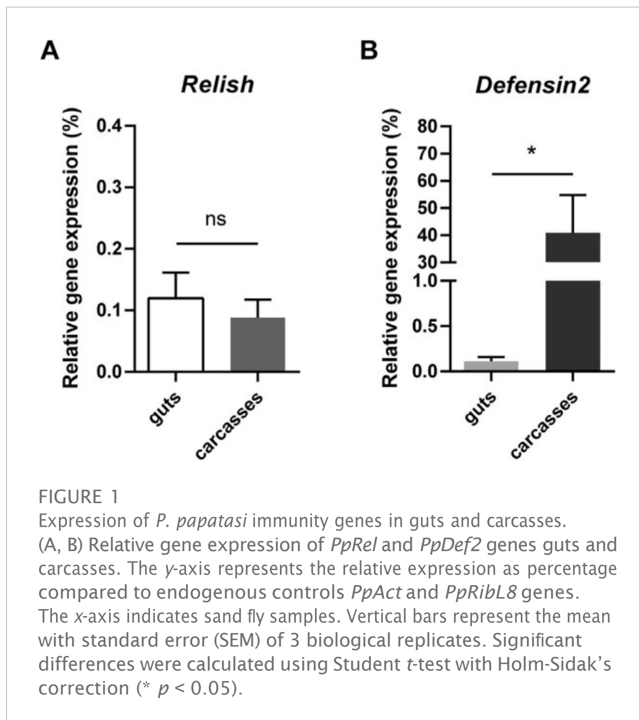

FIGURE 1
Expression of *P. papatasi* immunity genes in guts and carcasses. (A, B) Relative gene expression of *PpRel* and *PpDef2* genes guts and carcasses. The *y*-axis represents the relative expression as percentage compared to endogenous controls *PpAct* and *PpRibL8* genes. The *x*-axis indicates sand fly samples. Vertical bars represent the mean with standard error (SEM) of 3 biological replicates. Significant differences were calculated using Student *t*-test with Holm-Sidak's correction (* $p < 0.05$).

was calculated. Each of the three independent experiments contained a minimum of 50 female sand flies per group.

2.9 Statistical analysis

Statistical analyses and graphs were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, United States). Student *t*-test was applied to calculate significant differences in gene expression levels in the gut compared to carcass samples obtained from a single time point. Ordinary two-way ANOVA was applied to calculate significant differences in gene expression levels at several time points in gut and carcass samples from experimental groups compared to a control group. Two-way ANOVA was also applied to calculate significant differences in mortality rates between experimental (dsDef1 and dsDef1+2) and control (dsLacZ) groups. Both student *t*-test and two-way ANOVA methods were applied with Holm-Sidak correction for multiple comparisons. For analyzing several infection parameters, contingency tables were created, and Chi-square was applied to test significant differences between experimental and control groups injected with dsRNA. Subsequently, Fisher's test was used for each category between the experimental and control group. Additional details were included in figure legends.

3 Results

3.1 *P. papatasi* defensin2 and relish expression in the sand fly tissues

Previously, the PpDef1 (PPAI004256) was identified as a gut-specific defensin (Kykalová et al., 2021). We searched for other defensin-coding sequences in the VectorBase database and identified the PPAI010650 sequence, here named PpDef2. The translated amino acid sequence has 98 residues, including six cysteine residues characteristic of the defensin superfamily signature domain (Supplementary Figure S1A). The phylogenetic analysis showed that the PpDef2 amino acid sequence is closely related to *P. duboscqi* defensin (P83404.3) and *L. longipalpis* LlDef2 (AKU77027.1). On the other hand, the gut-specific PpDef1 and *L. longipalpis* defensin4 (MW269863.1) form a separate clade (Supplementary Figure S1B). The PpDef2 gene expression in *P. papatasi* guts was highly variable after the *L. major* infection. Although it was slightly increased at 24 h and reduced at 72 h post-infection, none of the analyzed time points showed significant differences compared to the non-infected control group (Supplementary Figure S1C).

We explored whether *PpRel* and *PpDef2* genes have gut-specific expressions. Both were expressed in dissected guts and carcasses of females from the colony (sucrose-fed). *PpRel* gene had similar expression levels when compared between guts and carcasses (Figure 1A). On the other hand, *PpDef2* gene was expressed significantly higher in carcasses than in guts (Figure 1B).

3.2 Relish correlation with AMPs expression

We hypothesized that the IMD pathway controlled the two defensins through relish transcription factor. To test this hypothesis, we injected 150 ng of PpRel dsRNA (dsRel) into sugar-fed *P. papatasi* females. This amount of dsRNA was previously used with good efficiency in sand flies, but no significant changes were observed in PpRel expression in guts (Figure 2A). On the other hand, a significant reduction occurred in carcasses at 24 h (Figure 2B). Then, we selected the sand fly samples that showed low PpRel expression collected at 24 h (carcass) and 48 h (gut) post dsRel injection to test if there was an alteration of expression in the AMPs genes. In sand flies injected with 150 ng of dsRel, there were no significant changes in AMPs gene expression in the selected gut samples (Figure 2C). At the same time, there was a significant reduction in the expression of *PpDef2* and *PpAtt* genes in the carcasses (Figure 2D).

3.3 Gene silencing of defensins and its effect on *Leishmania* infections

In a first experimental setting, we injected a standard volume 32 nL (150 ng) of dsRNA intrathoracically into sugar-fed non-infected *P. papatasi* females to silence both defensins. While the injection of the dsDef1 did not result in successful gene silencing in the sand fly gut (Figure 3A), the injection of dsDef2 resulted in a reduced expression of *PpDef2* gene (Figure 3B) in the sand fly gut on the first day post-injection. For our control of dsRNA injection and gene silencing, we followed the *PpDef2* gene expression in carcasses and we detected a significant reduction on the first day post dsDef2 injection (Supplementary Figure S2). No significant changes were observed in *PpDef1* expression in sand fly guts after dsDef2 injection (Supplementary Figure S3A), nor in *PpDef2* after dsDef1 injection (Supplementary Figure S3B).

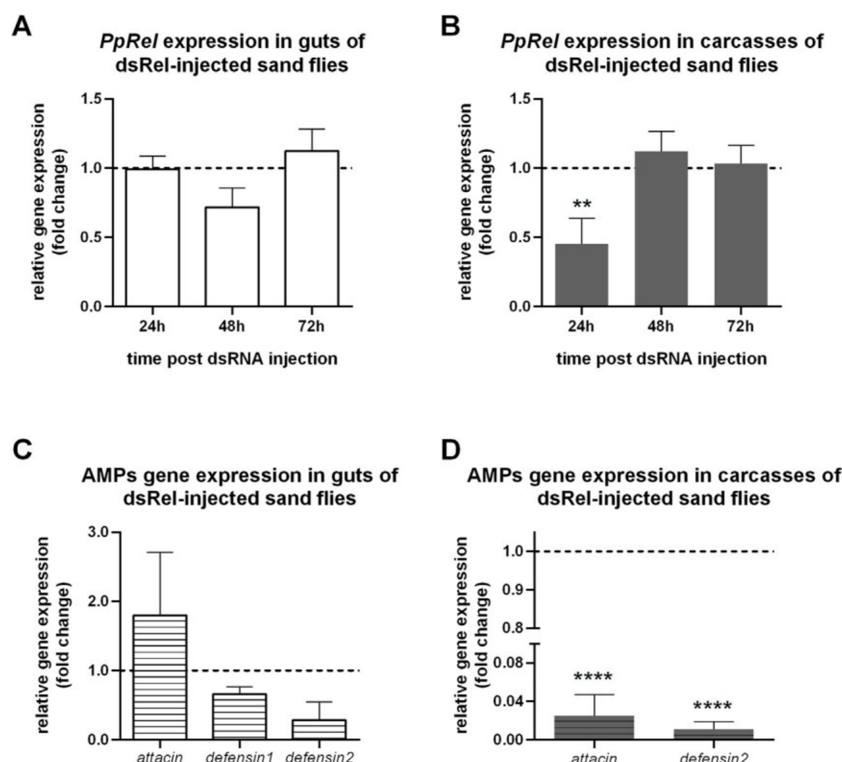

FIGURE 2
Expression of *P. papatasi* immunity genes after silencing of relish. (A, B) The relative expression of *PpRel* gene in dsRel-injected sand flies is represented in the *y*-axis, and time points when samples were collected post dsRNA injection are indicated in the *x*-axis. (C, D) The relative gene expression of AMPs at 24 h post dsRel injection is represented in the *y*-axis, and AMPs gene names are indicated in the *x*-axis. (A, C) White background color indicates guts. (B, D) Grey background color indicates carcasses samples. The relative expression was normalized to endogenous controls *PpAct* and *PpRibL8* genes and expressed as fold change compared to the dsLacZ control group collected at each correspondent time point (dotted line). Vertical bars represent the mean with standard error (SEM) of 3 biological replicates. Significant differences were calculated using two-way ANOVA with Holm-Sidak's correction (**$p < 0.01$; ****$p < 0.0001$).

In a different experimental setting, on the third day post *Leishmania* infection, we increased the injected volume to double of dsDef1 (64 nL; 300 ng) in *P. papatasi* females. The higher volume of injected dsRNA resulted in similar survival rate as the standard volume. Significant silencing of *PpDef1* gene was observed on days 1 and 3 post-injection (Figure 3C). For comparison purposes, we tested the combined silencing of both *PpDef1* and *PpDef2* genes (considering the maximum volume that can be injected intrathoracically) by injecting 32 nL (150 ng) of each dsRNA into infected *P. papatasi* females. The silencing of both defensins in the sand fly gut was significant on days 1 and 3 post-injection (Figure 3D).

We hypothesized that defensins could have a role in the *L. major* cycle in the gut of *P. papatasi*. Therefore, we silenced them independently using 300 ng of gene-specific dsRNA, or concurrently using a mixture of 150 ng of each defensin dsRNA in infected sand flies. To assess the effect on *Leishmania* parasites we used sand flies treated with AtbC to eliminate the influence of the natural sand fly gut bacteria. We estimated the parasite abundance by the gene expression of the parasite actin in the dsDef1 and dsDef1+2. On day 6 post- infection, parasite numbers in both experimental groups were significantly increased compared to the control group inoculated by dsLacZ (Figure 4A).

Mortality was recorded during 3 days after dsRNA microinjections in infected sand flies to detect a possible negative effect of defensin silencing on *P. papatasi* survival. The mortality was slightly higher in both experimental groups during the entire course of experiments, but the most noticeable difference was observed during late-stage infections. On day 6, mortality reached roughly 15% in the control group, while in dsDef1 was more than 42%, and in dsDef1+2 was 34%. Statistical significance indicating a negative effect of silencing the defensin genes in infected flies was found in dsDef1 and dsDef1+2 injected group (Figure 4B).

In addition to the molecular detection of the parasite, we assessed the effect of the defensins gene silencing on the *Leishmania* infection load, parasite localization *in situ* and morphology by light microscopy. This method provides quick assessable information and it is applicable even to low-intensity infection after the blood digestion is completed (Myskova et al., 2008). A slight increase in moderate infections and a concomitant decrease in heavy infections in the dsDef1 injected group were not statistically significant (Figure 5A). A similar percentage of

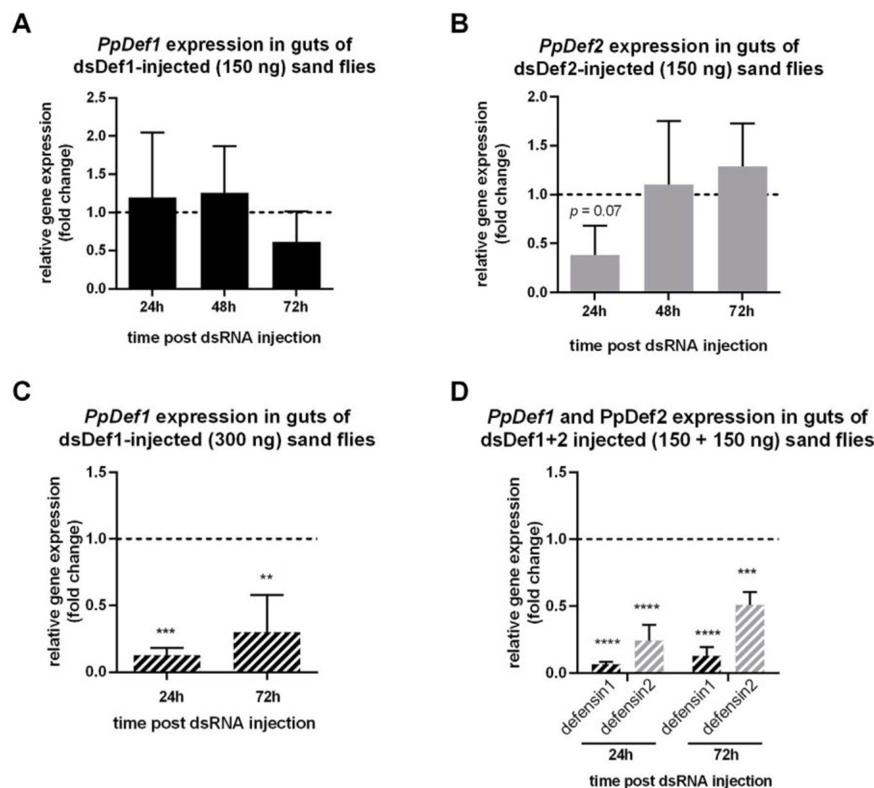

FIGURE 3
Gene expression of *P. papatasi* defensins genes after dsRNA injections. The relative expression of *PpDef1* (black) and *PpDef2* (light grey) genes in dsRNA-injected sand flies is represented in the *y*-axis. Time points when samples were collected post dsRNA injection are indicated in the *x*-axis. (A, B) Expression of *PpDef1* and *PpDef2* genes after 150 ng of gene-specific dsRNA in sugar-fed non-infected flies. (C, D) Expression of *PpDef1* and *PpDef2* genes in *Leishmania*-infected flies (white stripes) after 300 ng of dsDef1 or dsDef1+2 mixture. In infected sand flies, 24 h and 72 h post dsRNA injection correspond to day 4 and 6 post infection. The relative expression was normalized to endogenous control genes *PpAct* and *PpRibL8* and expressed as fold change compared to the dsLacZ control group collected at each correspondent time point (dotted line). Vertical bars represent the mean with standard error (SEM) of 3 biological replicates. Significant differences were calculated using two-way ANOVA with Holm-Sidak's correction (**$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

moderate and heavy infected sand flies were found between dsDef1+2 and the control group (Figure 5A).

In the two experimental and the control groups, parasites were able to colonize the stomodeal valve. In the control group, the stomodeal valve was infected in approximately 50% of examined infections in all groups (Figure 5B). Interestingly, in the control and dsDef1+2 groups, some of the infections remained localized only in abdominal gut, while in dsDef1, all inspected infections colonized also the thoracic parts of the gut (Figure 5B).

The parasite morphometric data analysis showed no significant difference among the procyclic and metacyclic promastigote forms between the dsDef1, dsDef1+2, and dsLacZ groups. Nevertheless, there was a significant ($p = 0.0055$) decrease in the percentage of the elongated nectomonads and a consequent increase in leptomonads in the dsDef1+2 sand flies compared to the control group (Figure 5C).

4 Discussion

Sand flies, like other insects, rely on innate immune mechanisms to fight potentially harmful microbial and viral challenges (Telleria et al., 2018). Nevertheless, it is not yet clear how the sand fly immunity affects *Leishmania* parasites. In this study, we focused mainly on two AMPs, a previously reported gut-specific defensin1 (Kykalová et al., 2021) and newly identified defensin2, their association with the relish transcription factor, and their role during *Leishmania* infection.

Based on phylogram analyses, the newly identified *P. papatasi* defensin2 (PpDef2) grouped with *L. longipalpis* defensin2 and *P. duboscqi* defensin. Moreover, *P. papatasi* defensin2 forms a wider clade with mosquito and other insect defensins. On the other hand, *P. papatasi* defensin1 (PpDef1) formed a group with *L. longipalpis* defensin4, which is enclosed in a clade with two other *L. longipalpis* defensins (Kykalová et al., 2021). These findings indicate a broad spectrum of insect defensins within the same sand fly species and their similarity across insect species.

We found that *PpDef2* gene was expressed in the gut and the rest of the body (carcasses), which includes the fat body. Nevertheless, a significantly higher expression was found in the carcasses which suggests a distribution of PpDef2 across various sand fly tissues. In contrast, the previously reported *PpDef1* gene was expressed exclusively in the *P. papatasi* gut and not detected in other tissues (Kykalová et al., 2021). Similarly, in *Anopheles gambiae*,

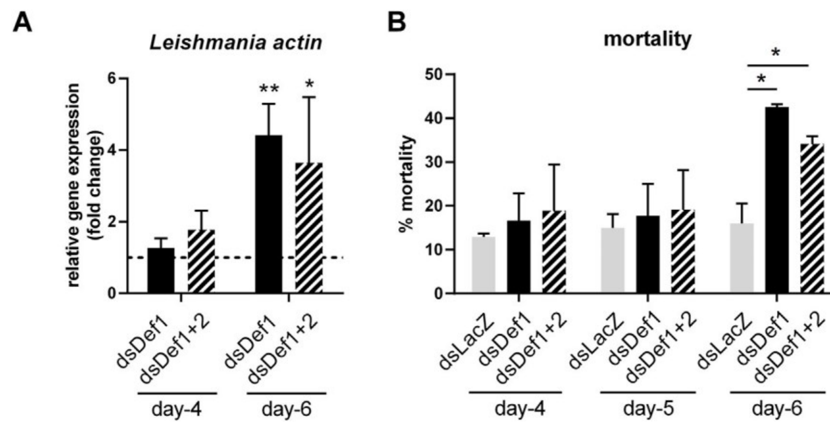

FIGURE 4
Leishmania actin expression and mortality after dsRNA injections in *Leishmania*-infected sand flies. (A) *Leishmania* actin expression in dsDef1 and dsDef1+2 injected groups are represented by black color and striped bars, respectively. The relative gene expression was normalized to endogenous control genes *PpAct* and *PpRibL8* and expressed as fold change compared to the dsLacZ control group collected at each correspondent time point (dotted line). (B) Mortality levels are expressed as a percentage in comparison to the total number of live sand flies (*y*-axis) in each of the 3 days post dsRNA injection (*x*-axis). Vertical bars represent the mean with standard error (SEM) of 3 biological replicates. Each experiment consisted by minimum of 50 female sand flies in each experimental and control groups. Significant differences were calculated using two-way ANOVA with Holm-Sidak's correction (*$p < 0.05$; **$p < 0.01$).

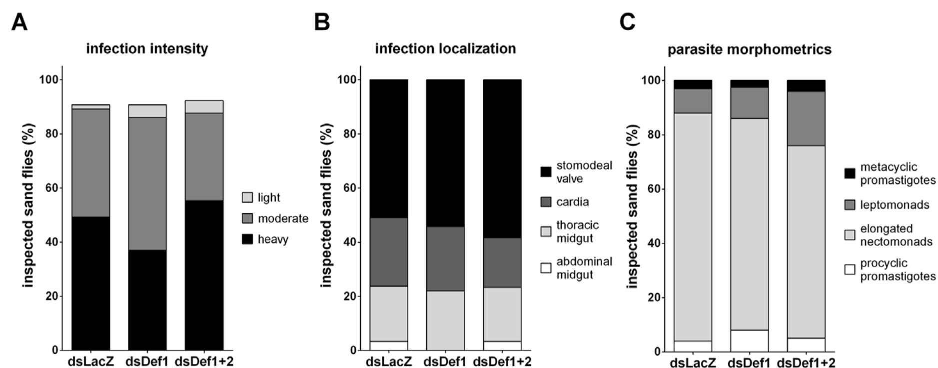

FIGURE 5
Effect of defensins silencing in the progress of *Leishmania* on the sixth day post-infection. (A) Infection intensity estimation with the *y*-axis representing the percentage of all individually inspected insects (minimum of 60 sand flies in each group). Bar colors indicate infection intensity. (B) Infection progress in the sand fly gut with the *y*-axis representing the percentage of infected insects. Bar colors indicate sand fly gut localization. (C) Parasite development in the sand fly gut with the *y*-axis representing the percentage of analyzed parasites. Bar colors indicate parasite developmental forms. The *x*-axis represents dsRNA-injected groups. Vertical bars represent the average values of three independent experiments. Significant differences were analyzed by Chi-square using both experimental and control groups. Fisher's test was used between the experimental and control groups to assess the differences within each category.

the AMP gambicin was highly expressed in the anterior midgut while less expressed in other segments of the mosquito digestive tract (Vizioli et al., 2001). In *Rhodnius prolixus*, the defensin A, but not defensin B, was highly expressed in the fat body and less expressed in the anterior midgut when infected by *Trypanosoma cruzi* (Vieira et al., 2016). These findings indicate that some AMPs are expressed differently depending on the tissue or organ. The gut- specific PpDef1, or other AMP, may compensate for the low expression of PpDef2 in the gut. For example, *Drosophila* and *Tenebrio* insects used AMPs synergy to resist different pathogenic challenges (Zanchi et al., 2017; Hanson et al., 2019), but the such possibility was never investigated in sand flies.

During *Leishmania* infection, these two defensins showed a different expression pattern. While *PpDef2* did not show significant changes in the present study, *PpDef1* was upregulated on day 6 post-infection (Kykalová et al., 2021). These findings suggest that *PpDef1* and *PpDef2* genes were not concurrently expressed in the infected sand fly gut. These different defensins may be under the control of different transcription factors, therefore, under the control of different regulatory pathways. In *P. papatasi*, knockout of relish using CRISPR/ Cas9 genome editing increased susceptibility to *Leishmania* infection (Louradour et al., 2019). However, the direct correlation between relish and the downstream expression of effector molecules in sand flies remained unknown.

We observed that the expression of *PpRel* gene was similar in both guts and carcasses samples, indicating that the IMD pathway can be regulated across *P. papatasi* body. Our results showed a significant reduction of *PpAtt* and *PpDef2* genes in the carcasses when *PpRel* was silenced with 150 ng of dsRNA, a commonly used amount of dsRNA in sand flies (Sant'Anna et al., 2008; Sant'Anna et al., 2009; Diaz-Albiter et al., 2011; Telleria et al., 2012; Telleria et al., 2021b; Telleria et al., 2021a; Di-Blasi et al., 2019). Nevertheless, *PpRel* silencing was not significantly reached in the guts with this same amount of dsRel. The subsequent *PpAtt* expression was quite variable, and defensins' expression was observed with a slightly reduced expression of *PpDef2*. Our findings indicate that the *PpRel* silencing correlated to the downregulation of AMPs in carcasses, but this correlation remained unclear in the gut tissues. The correlation between relish knockdown and the suppressed AMPs production has been repeatedly reported in other insects such as *Galleria mellonella* (Sarvari et al., 2020) and *Octodonta nipae* (Sanda et al., 2019), indicating an evident conserved function. In *Tenebrio molitor*, the relish-silencing led to reduced levels of *attacin2* in the fat body and hemocytes but increased levels in the gut (Keshavarz et al., 2020). Therefore, in sand flies, the relish correlation with AMPs may also differ depending on the organs or tissues.

In addition to relish, we aimed to knock down the defensins genes in the adult sand flies. In the first set of experiments, we used 32.2 nL of 4.5 μg/μL dsRNA (150 ng). When injecting this initial amount of dsDef1 or dsDef2 in colony sand flies, we did not observe a reducing effect on the expression of the gut-specific *PpDef1* gene, while PpDef2 was significantly silenced in carcasses with the same dsRNA amount. This result indicated that 150 ng of dsRNA injected in *P. papatasi* was insufficient for obtaining a significant gene silencing in the gut tissue.

Consequently, to silence the defensins in sand fly guts during *Leishmania* experimental infections, we increased the amount of injected dsRNA to 64.4 nL of 4.5 μg/μL dsRNA (300 ng), and we obtained a significant silencing of *PpDef1* gene in guts. The additional strategy of injecting the same volume but composed of a mixture of two different dsRNA, 150 ng of each dsDef1 and dsDef2, was used for comparison purposes and resulted in the silencing of both defensin genes. These results suggest that a larger volume allowed a better distribution of injected dsRNA within the sand fly body, with the consequent silencing effect in the insect gut. Nevertheless, an opposite trend was previously reported when silencing another *P. papatasi* gene. A more efficient silencing was obtained with a lower concentration and volume (23 nL of 3.5 μg/μL) of a chitinase dsRNA (Coutinho-Abreu et al., 2010a). Together these findings support the hypotheses that different aspects such as dsRNA distribution, RNAi target region, or transcription turnover may be at play in successful gene knockdown (Pancoska et al., 2004; Dornseifer et al., 2015; Svoboda, 2020).

We hypothesized if defensins knockdown in infected females may influence sand fly mortality. In our experiments, the mortality rate on day 3 slightly increased in the dsLacZ control, while in both experimental groups (dsDef1 and dsDef1+2) increased sharply with significant differences. These findings are similar to defensins' suppression in *A. gambiae* and *T. molitor* leading to the reduced viability of insects after G+, with a less remarkable effect after G-bacterial infection (Blandin et al., 2002; Zanchi et al., 2017). Therefore, the outcome of defensins' activity varies with the pathogenic challenge.

Focusing on the possible effect of suppressed sand fly defensins on *Leishmania*, we investigated parasite levels based on the relative gene expression of a constitutive parasite gene in bacteria-depleted females. The depletion of bacteria in the infection experiments was chosen to reduce the additional effect of gut bacteria on immunity. The use of antibiotic treatment may have distinct effect on the parasite development. For example, antibiotic treatment alone had no deleterious effect on the parasites *Leishmania donovani* in *L. longipalpis* (Dey et al., 2018). On the other hand, reducing bacteria interfered negatively with *L. infantum* infection progress in the same sand fly, evidenced by the reduction of metacyclic promastigotes (Kelly et al., 2017). Under our experimental conditions, the choice of AtbC treatment reduced significantly the bacteria load in the sand fly gut and had no detectable effect on *L. major* development in *P. papatasi* (Kykalová et al., 2021). Therefore, it is plausible to consider that different factors such as choice of antibiotic combination, parasite, sand fly species and its commensal microbiota are influencing the effect of the antibiotic treatment.

Based on qRT-PCR results, we showed that the absence of defensins led to significantly increased levels of *Leishmania* in the insects silenced with PpDef1 or PpDef1+2 dsRNA. This trend was not observed in *Leishmania*-infected *L. longipalpis* females with *LlDef2* gene silenced (Telleria et al., 2021b). Nevertheless, several different factors must be considered. In our experiments, *P. papatasi* females were injected with dsRNA 3 days post *Leishmania* infection; therefore, the silencing effect lasted until 72 h post dsRNA injection corresponding to day 6 of infection, when the defecation process was finished. Differently, in the previous study done with *L. longipalpis,* the females were injected with dsRNA prior to the infection, with the silencing effect lasting until day 3 of infection when the *Leishmania* levels were measured by qPCR (Telleria et al., 2021b). Interestingly, the absence of attacin resulted in increased levels of *Trypanosoma* parasite in *Glossina morsitans morsitans* (Hu and Aksoy, 2006). Analogously, the overexpression of defensin A and cecropin A reduced number of *P. gallinaceum* oocyst in the infected *A. aegypti* females (Kokoza et al., 2010). On the other hand, in *A. gambiae* infected by *Plasmodium berghei*, the vector viability and the development of the parasite in the gut were not affected in the suppression of a defensin (Blandin et al., 2002). Altogether these observations highlight the differences that reflect the complex tunning of AMPs in regulating parasitic insect infection.

To help understanding the effects of defensins silencing in the development of the parasite inside the vector, we evaluated individually dissected gut using light microscopy. No statistically significant differences were observed in the localization of the parasite within the vector's gut and infection intensity, despite the increased percentage of moderate infection in the dsDef1 silenced group. Similar negative results were previously observed in *L. longipalpis* infected by *L. infantum*: silencing of *LlDef2* gene did not result in significant differences in intensity, localization, and parasite morphology (Telleria et al., 2021b). Nevertheless, here in *P. papatasi* dsDef1 injected group, we observed that all insects had infections in the thoracic parts of the gut on day 6, suggesting that the parasites were developing faster than the other groups. Based on parasite morphology, we conclude that the absence of both defensins may provide room for the multiplication of the parasites when the non-multiplying elongated nectomonads forms were reduced at the expense of multiplying leptomonads.

It is possible that the PpDef2 gene silencing in the carcass had an influencing effect on the outcome of *Leishmania* infection in the gut. AMPs are readily induced in the gut but also in hemocytes after a potential risk, leading to other subsequent molecular signals that activate a broader systemic immune response (Krautz et al., 2014; Manniello et al., 2021). In addition, different pathway regulatory events may take place. For example, in *Drosophila*, the expression of the AMP drosomycin is regulated by the IMD pathway in response to tracheal epithelium infection. However, the Toll pathway regulates it during a systemic response (Ferrandon et al., 1998; Tzou et al., 2000). These findings revealed distinct regulatory mechanisms in insects for systemic and local induction of AMPs genes. We emphasize that the gene silencing detected by qPCR indicates the decrease in mRNA levels, but not peptide levels. While complete suppression of mRNA and protein levels may not be obtained through RNAi-mediated gene silencing, significant differences between experimental and control groups can be attributed to the gene-specific mRNA suppression.

In summary, *Phlebotomus papatasi* has at least two defensin genes; one is gut-specific (*PpDef1*), and the newly investigated *PpDef2* is expressed throughout the sand fly tissues. The IMD pathway transcription factor relish regulated the expression of two investigated AMPs (PpAtt and PpDef2). We adjusted the dsRNA microinjections protocol and obtained successful gene silencing in the sand fly gut. The suppression of *PpDef1* or the combination of both defensins genes led to increased *L. major* loads and higher sand fly mortality. Moreover, we demonstrated the importance of defensins in the *P. papatasi* response toward *L. major*.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

Conceptualization, methodology, and validation, BV and ET; investigation, formal analysis, data curation, visualization, and writing—original draft preparation, BV, FS, and ET; writing—review and editing, PV and ET; supervision, ET; funding acquisition, resources, and project administration, BV, PV, and ET. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1182141/full#supplementary-material

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PUBLICATION 3

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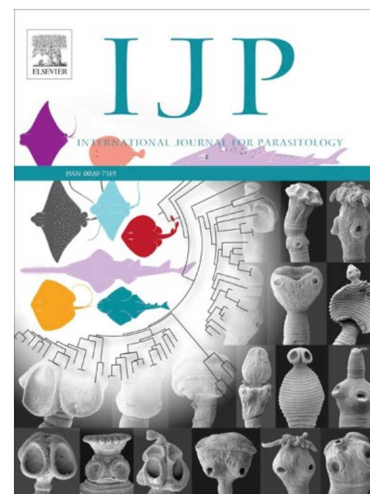

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Pathogen-associated molecular patterns (PAMPs) derived from *Leishmania* and bacteria increase gene expression of antimicrobial peptides and gut surface proteins in sand flies

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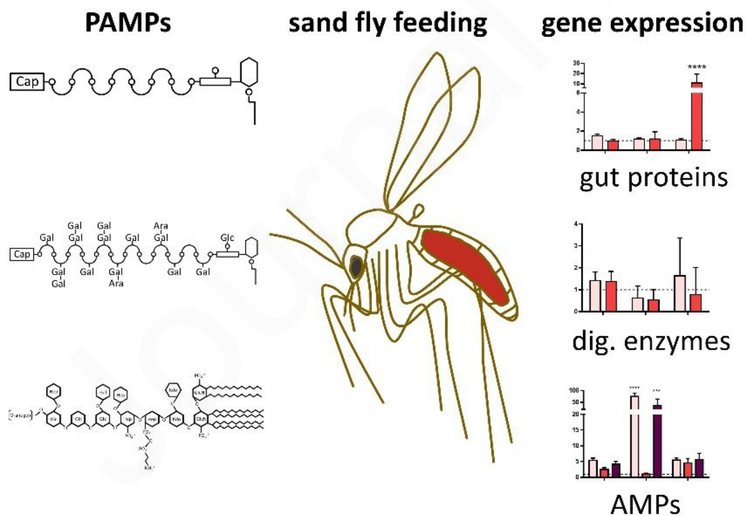
Graphical Abstract



Highlights

- Microbe surface molecules can upregulate a mucin-like gene in *Lutzomyia longipalpis*
- Expression of the attacin gene is triggered as a generic immune response in sand flies
- Sand fly defensin gene expression is tuned, depending on the ingested microbe molecule

Abstract

The interaction between pathogens and vectors' physiology can impact parasite transmission. Studying this interaction at the molecular level can help in developing control strategies. We study leishmaniases, diseases caused by *Leishmania* parasites transmitted by sand fly vectors, posing a significant global public health concern. Lipophosphoglycan (LPG), the major surface glycoconjugate of *Leishmania*, has been described to have several roles throughout the parasite's life cycle, both in the insect and vertebrate hosts. In addition, the sand fly midgut possesses a rich microbiota expressing lipopolysaccharides (LPS). However, the effect of LPG and LPS on the gene expression of sand fly midgut proteins or immunity effectors has not yet been documented. We experimentally fed *Lutzomyia longipalpis* and *Phlebotomus papatasi* sand flies with blood containing purified LPG from *Leishmania infantum*, *Leishmania major*, or LPS from *Escherichia coli*. The effect on the expression of genes encoding gut proteins galectin and mucin, digestive enzymes trypsin and chymotrypsin, and antimicrobial peptides (AMPs) attacin and defensins was assessed by quantitative PCR (qPCR). The gene expression of a mucin-like protein in *L. longipalpis* was increased by *L. infantum* LPG and *E. coli* LPS. The gene expression of a galectin was increased in *L. longipalpis* by *L. major* LPG, and in *P. papatasi* by *E. coli* LPS. Nevertheless, the gene expression of trypsins and chymotrypsins did not significantly change. On the other hand, both *L. infantum* and *L. major* LPG significantly enhanced expression of the AMP attacin in both sand fly species and defensin in *L. longipalpis*. In addition, *E. coli* LPS increased the expression of attacin and defensin in *L. longipalpis*. Our study showed that *Leishmania* LPG and *E. coli* LPS differentially modulate the expression of sand fly genes involved in gut maintenance and defence. This suggests that the glycoconjugates from microbiota or *Leishmania* may increase the vector's immune response and the gene expression of a gut coating protein in a permissive vector.

Keywords: *Leishmania* LPG; Bacteria LPS; PAMPs; *Lutzomyia*; *Phlebotomus*; Gut protein; Digestion; Innate immunity

1. Introduction

Pathogen-associated molecular patterns (PAMPs) are molecules containing highly conserved structural motifs associated with pathogenic infections. These molecules act as ligands for host pattern recognition receptors, triggering both innate immunity and acquired immune responses (Gazzinelli et al., 2004). Notable examples include lipophosphoglycan (LPG) from *Leishmania* parasites and lipopolysaccharide (LPS) from bacteria, complex cell surface glycoconjugates with lipid anchors and intricate saccharide domains (De Assis et al., 2012; Bertani and Ruiz, 2018). These molecules exhibit agonistic activity during the host-pathogen interaction, with *Leishmania* LPG acting as a potent Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) agonist in mammalian hosts (de Veer et al., 2003; Ibraim et al., 2013; Rêgo et al., 2022), while LPS from Gram-negative bacteria serves as a TLR4 agonist with high pro-inflammatory potential (Poltorak et al., 1998). Although the pioneer studies on Toll pathways started in *Drosophila* (Rosetto et al., 1995), there is no information on how PAMPs from pathogens modulate innate immune responses in sand fly vectors of *Leishmania*.

LPG is a multi-virulence factor that allows *Leishmania* attachment to the sand fly gut and resists digestive enzyme activity (Sacks and Kamhawi, 2001). These two events prevent elimination of the parasite during sand fly defecation, thus allowing completion of the infection cycle in the insect. Regarding attachment, a sand fly galectin is the receptor for *Leishmania major* LPG in the midgut of the restrictive vector *Phlebotomus papatasi* (Kamhawi et al., 2004). In contrast, in permissive vectors, attachment does not involve LPG-galectin interaction (Volf and Myskova, 2007; Svárovská et al., 2010). For example, in *Lutzomyia longipalpis*, the parasite-binding molecule is a sand fly mucin-like gut protein (Myšková et al., 2016).

Concerning digestive enzymes, the presence of these parasites was shown to reduce proteolytic activity in the vector's gut. For instance, *L. major* promastigotes reduce protease activity in the *P. papatasi* gut and delay digestion in *Phlebotomus langeroni* (Dillon and Lane, 1993). Similarly, *Leishmania mexicana* and *Leishmania infantum* reduce trypsin activity in *L. longipalpis* (Sant'Anna et al., 2009; Telleria et al., 2010). In contrast, LPG-deficient *L. major* is eliminated during *Phlebotomus duboscqi* digestion, while the addition of protease inhibitors and purified proteophosphoglycans (PPGs) promote parasite survival (Secundino et al., 2010). However, the role of the parasite- or bacteria-shed glycoconjugates on the physiology and innate immunity of the midgut is still an aspect of sand fly-pathogen interaction requiring study.

The commensal bacterial community is important for insect nutrition, development, and protection from potential pathogens (Engel and Moran, 2013); controlling the balance between gut microbiota and insect immune response is crucial. In sand flies, increased quantities of bacteria in the sand fly gut trigger the expression of antimicrobial peptide (AMP) genes in *L. longipalpis* and *P. papatasi* (Telleria et al., 2013; Kykalová et al., 2021). Therefore, factors derived from the bacterial community could initiate an innate immune response in the sand fly midgut and affect the *Leishmania* cycle.

The direct association between the parasite LPG and the sand fly immune response has not yet been investigated. Our previous studies have shown that *Leishmania* infection in the sand fly also triggers the insect immune response. In *L. longipalpis* females, *L. infantum* caused increased gene expression of attacin and defensin antimicrobial peptides (AMPs) (Telleria et al., 2021b). In *P. papatasi* infected with *L. major*, there were increased levels of gut-specific defensin and variable levels of attacin in the sand fly gut (Kykalová et al., 2021).

Here, as a part of a wider study on innate immune mechanisms in sand flies, we artificially fed *L. longipalpis* and *P. papatasi* with blood containing purified glycoconjugates; *Leishmania* LPGs and *Escherichia coli* LPS. Then, the level of expression of key genes was evaluated at the mRNA level. Addressing the functional properties of intact PAMPs bearing polymorphisms in their biochemical structures will help to understand the complex mechanisms underlying the host-pathogen interactions in sand flies.

2. Materials and methods

2.1. Selected sand fly genes

We selected specific gene sequences from sand flies to investigate their expression. Among the gut proteins, we selected a gut-specific galectin (*Pp*Galec, renamed *Pp*GalecA) in *P. papatasi*. This galectin has been previously implicated in the attachment of *L. major* to the sand fly gut (Kamhawi et al., 2004; Abrudan et al., 2013; Labbé et al., 2023). Similarly, the highly similar *L. longipalpis* galectin (Galectin A, renamed *Ll*GalecA in the present study) was also identified (Dillon et al., 2006; Labbé et al., 2023). Additionally, we selected the *L. longipalpis* mucin-like gut protein 19 kDa (LuloG, renamed *Ll*Gprot19 in the present study) that plays a role in *L. infantum* interaction with the *L. longipalpis* gut (Jochim et al., 2008; Myšková et al., 2016). In the case of *P. papatasi*, as there is no highly similar sequence to *Ll*Gprot19, we selected a 13.7 kDa gut protein (*Pp*Gprot13.7) (Ramalho-Ortigão et al., 2007) as the most similar sequence to be used in the gene expression analysis.

Among digestive enzymes, we selected *L. longipalpis* trypsin 1 (*Ll*Tryp1) and *P. papatasi* trypsin 4 (*Pp*Tryp4), which were upregulated after blood ingestion (Ramalho-Ortigão et al., 2007; Telleria et al., 2007; Jochim et al., 2008; Sant'Anna et al., 2009). We also selected *L. longipalpis* chymotrypsin 1A (*Ll*Chym1A) and *P. papatasi* chymotrypsin 2 (*Pp*Chym2), which were downregulated after the parasitic infection (Ramalho-Ortigão et al., 2007; Jochim et al., 2008).

In addition to gut proteins and digestive enzymes, we selected gene sequences encoding AMPs. For *L. longipalpis*, attacin (*Ll*Att) and defensin 2 (*Ll*Def2) were selected; both were upregulated after *L. infantum* infection (Telleria et al., 2021b). Additionally, *L. longipalpis* defensin (*Ll*Def, renamed *Ll*Def1) was selected, which was downregulated after *L. mexicana* (Telleria et al., 2013) but not significantly altered after *L. infantum* infection (Telleria et al., 2021b). In *P. papatasi*, we selected a gut-specific defensin (*Pp*Def, renamed *Pp*Def1), a systemically expressed defensin (*Pp*Def2), and attacin (*Pp*Att), all of which were upregulated after *L. major* infection (Kykalová et al., 2021; Vomáčková Kykalová et al., 2023).

The gene sequences used to design primers for qPCR, together with their corresponding accession numbers, references, and primer sequences, are listed in Table 1.

2.2. Leishmania culture and glycoconjugates

Promastigotes of *L. infantum* (MCAN/BR/89/Ba-262 strain) and *L. major* (MHOM/IL/80/Friedlin strain) promastigotes were cultured in M199 medium (Sigma Aldrich, Burlington, MA, USA) supplemented with 0.005% hemin (Sigma), 40 mM HEPES pH 7.4 (Sigma), 100 µM adenine (Sigma), 4 mM sodium bicarbonate (Sigma), 20 µg/mL of gentamicin (Sigma), and 10% fetal bovine serum (FBS) (Gibco Life Technologies, Grand Island, NY, USA). LPGs from *L. infantum* and *L. major* procyclic parasites were extracted and purified using phenyl-sepharose (Sigma) as reported elsewhere (Soares et al., 2002), with an average yield of 1 mg of glycoconjucates per 1 L of culture (Dubois et al., 1956). The LPG of *L. infantum* (strain Ba262, type I) is devoid of sidechains, whereas that of *L. major* (Friedlin strain) has galactose/arabinose as sidechains (McConville et al., 1990; Cardoso et al., 2020). *Escherichia coli* (serotype O26:B6) LPS was commercially available (SIGMA, Saint Louis, MO, USA). The basic structure of *E. coli* LPS comprises the O-antigen, an oligosaccharide core, and the lipid-A, the endotoxic motif (Bertani and Ruiz, 2018) (Fig. 1).

2.3. Sand fly colonies

Phlebotomus papatasi and *L. longipalpis* females used in the experiments were established from field-caught sand flies from Turkey (Cukurova region) and Brazil (Jacobina), respectively. The insects were kept under standard conditions at 26 °C and a 14:10 h light:dark photoperiod (Volf and Volfova, 2011).

Adult sand flies were fed on a 30% sucrose solution offered on cotton wool, which was changed every other day.

2.4. Sand fly experimental feeding and gut samples

Five days old females were used for experimental feeding through a chicken skin membrane containing defibrinated sheep blood (LabMediaServis, Jaroměř, Czech Republic) mixed with *L. infantum* or *L. major* LPGs, or *E. coli* LPS, to reach a final concentration of 1 μg/mL of blood. Considering that a sand fly can ingest approximately 1 μL of blood (Sant'Anna et al., 2008; Burniston et al., 2010; Valinsky et al., 2014), the amount of LPG or LPS taken by the insect is approximately 1 ng. One nanogram of LPG corresponds to 6.7×10^4 parasite cells (McConville and Bacic, 1990; Ponte-Sucre et al., 2001). Therefore, 1 ng of LPS corresponds to approximately 2.5×10^4 *E. coli* cells (Watson et al., 1977; Zhang et al., 1998). Our preliminary experiments testing two different LPG concentrations (1 and 5 μg/mL) in the blood for sand fly feeding showed that the lowest was sufficient to increase the LlAtt and LlDef2 gene expression (Supplementary Fig. S1). Therefore, we decided to use this lowest concentration in all subsequent sand fly feeding assays. Females from the control group were fed on blood only. We highlight that sand flies harbor resident gut microbiota that may be altered after feeding on blood, either growing from the already present bacteria or introduced from the feeding process (Telleria et al., 2018). Sand fly guts were dissected 24, 48, and 72 h post artificial feeding, corresponding to the period from the main blood digestion to defecation. Blood-fed females were anaesthetized on ice and dissected in a sterile saline solution. For each time point, one pool of 12 guts (midguts plus hind guts) randomly selected from three independent experiments was collected and stored at -80°C until processing. All dissected sand fly guts contained partially digested blood, although at 72 h after feeding, we observed a slight variation in gut content within individuals.

2.5. Total RNA extraction and cDNA synthesis

Pooled gut samples were used for total RNA extraction using an E.Z.N.A. Total RNA Kit I (Omega Bio-tek, Norcross, GA, USA). A DNA digestion step with RNase-free DNase I (Thermo Fisher Scientific, Waltham, MA, USA) was included to eliminate potential DNA residues. cDNA was synthesized from total RNA (up to 1 μg per reaction) using a Transcriptor First Strand cDNA Synthesis Kit and anchored-oligo$(dT)_{18}$ primer (Roche Life Science, Rotkreuz, Switzerland). The protocols were followed according to each manufacturer's instructions.

2.6. Relative gene expression

The relative expression of the selected sand fly genes was assessed from cDNA samples using gene-specific primers (Table 1) and SYBR Green PCR Master mix (Roche) in qPCR performed on a LightCycler 480 thermocycler (Roche). Gene expression was calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) from two technical replicates, relative to endogenous control gene transcripts, actin and ribosomal proteins (Table 1), using the following cycling conditions: 95 ◦C for 10 min enzyme activation, 45 amplification cycles (95 ◦C for 10 s, 60 ◦C for 20 s; 72 ◦C for 45 s) (Kykalová et al., 2021).

2.7. Statistics

Significant differences compared with the control group fed on blood were calculated in GraphPad Prism software (GraphPad Software, La Jolla, USA) using a two-way ANOVA with Dunnett test for multiple comparisons.

3. Results

Our choice of glycoconjugates was based on their distinct structure and potential recognition by sand fly physiology. The *L. infantum* promastigote LPG (Ba262 strain) has no side chains in the repeat unit domain (Coelho-Finamore et al., 2011a, 2011b; De Assis et al., 2012), while the *L. major* promastigote (Friedlin V1 strain) has several side chains with galactose and arabinose residues but no glucose residue (McConville et al., 1992; Volf et al., 2014). On the other hand, the overall structure of *E. coli* LPS is conserved, although some variations on these structural domains may occur among different strains (Bertani and Ruiz, 2018) (Fig. 1).

3.1. Effect of Leishmania or bacteria surface components on gut proteins in L. longipalpis

In the group fed on blood containing *L. infantum* LPG and *E. coli* LPS, the gene expression of *Ll*Gprot19 was significantly increased at 72 h (means: 10.878 and 31.829 fold change, respectively) (Fig. 2A and C). In sand flies fed on blood with *L. major* LPG, the *Ll*GalecA gene expression increased at 48 h (mean: 2.402 fold change) (Fig. 2B).

3.2. Effect of Leishmania or bacteria surface components on gut proteins in P. papatasi

*Pp*GalecA and *Pp*Gprot13.7 were also analyzed. In the group fed on blood containing *L. infantum* and *L. major* LPG, the gene expression of PpGalecA and PpGprot13.7 did not show significant alterations (Fig. 3A and B). Nevertheless, in the group fed on blood with *E. coli* LPS, the PpGalecA gene expression increased at 48 h post-feeding (mean: 2.392 fold change) (Fig. 3C).

3.3. Effect of Leishmania or bacteria surface components on digestive enzymes in L. longipalpis gut

Expression levels of *Ll*Tryp1 and *Ll*Chym1A were observed during 3 days post-feeding. In the groups fed on blood containing LPGs from *L. infantum* or *L. major*, the gene expression of both digestive enzymes did not show significant alterations during the three time points observed (Fig. 4A and B). In the group fed on blood containing *E. coli* LPS, the gene expression of both enzymes was not significantly changed. Nevertheless, expression of the *Ll*Chym1A transcript was increased at 72 h post-feeding compared with the control group fed on blood, although it exhibited high variability within the three biological replicates (0.065, 0.548, and 31.724 fold change) (Fig. 4C).

3.4. Effect of Leishmania or bacteria surface components on digestive enzymes in P. papatasi gut

The expression levels of *Pp*Tryp4 and *Pp*Chym2 were also assessed by qPCR. In the three groups fed on blood containing LPG from *L. infantum* or *L. major*, or *E. coli* LPS, the gene expression of these two digestive enzymes was not significantly altered during the experiments (Fig. 5A-C).

3.5. Effect of Leishmania or bacteria surface components on AMP expression in L. longipalpis gut

The LPG from *L. infantum* (naturally transmitted by *L. longipalpis*) significantly increased the levels of *Ll*Att and *Ll*Def2 at 48 h post-feeding (means: 74.632 and 36.470 fold change, respectively), while the levels of *Ll*Def1 did not significantly differ from the blood-fed control group (Fig. 6A).

In the group fed with blood containing the LPG from *L. major* (not naturally transmitted by *L. longipalpis*), *Ll*Att expression increased readily but not significantly at 24 h, with a significant increase occurring at 48 h p.i. (mean: 62.378 fold change). *Ll*Def1 and *Ll*Def2 did not show any significant changes in expression. Nonetheless, *Ll*Def2 exhibited a non-significant increased expression at 72 h post-feeding (Fig. 6B).

Unlike LPGs, *Ll*Att and *Ll*Def2 showed significantly increased expression at 72 h post-feeding (means: 45.974 and 156.320 fold change, respectively), while *Ll*Def1 did not exhibit any significant changes in expression at the observed time points when exposed to bacteria LPS (Fig. 6C).

3.6. Effect of Leishmania or bacteria surface components on AMP expression in P. papatasi gut

Expression levels of *Pp*Att, the gut-specific *Pp*Def1, and the systemically expressed *Pp*Def2 were observed 3 days after artificial feeding, in the gut samples. In the group fed on blood containing LPG from *L. infantum* (a species that does not infect naturally), *Pp*Att levels were significantly increased at 24 h post-feeding compared with the control group fed on blood (mean: 17.253 fold change). Neither *Pp*Def1 nor *Pp*Def2 showed significant changes in expression levels in *P. papatasi* fed on *L. infantum* LPG (Fig. 7A).

*Pp*Att levels were also overexpressed at 24 h post-feeding in the group fed on blood with LPG from *L. major* (mean: 12.271 fold change). *Pp*Def1 showed variability in this group but did not exhibit significant changes, while *Pp*Def2 showed a non-significant increase with highly variable fold change values (three biological replicates: 0.408, 12.756, and 20.086) at 72 h post-feeding (Fig. 7B).

None of the examined gene transcripts significantly changed expression levels in the *P. papatasi* group fed on blood containing the *E. coli* LPS. However, the *Pp*Att levels exhibited a non-significant increase at 24 h post-feeding (Fig. 7C).

4. Discussion

Leishmania-sand fly interactions represent an intricate part of the parasite cycle. In a natural infection, sand flies ingest the host blood containing amastigote forms of *Leishmania*. These forms are rapidly surrounded by the peritrophic matrix (PM) secreted by the sand fly gut epithelium. After degradation of the PM, the differentiated procyclic forms attach to the surface of the sand fly gut (Pimenta et al., 1997; Dostálová and Volf, 2012; Pruzinova et al., 2015). Considering this direct contact between parasites and sand flies, we focused on how LPG expressed by procyclic forms can influence a few aspects of insect physiologyin in two *Leishmania*-vector models. The LPG structural modification determines the successful attachment of *L. major* to *P. papatasi* gut epithelium in some restrictive pairs of *Leishmania* and sand fly species (Kamhawi et al., 2004). Therefore, LPG from different *Leishmania* spp. with different phosphoglycan side chain structures may affect sand fly gene expression differently.

Moreover, the presence of LPS in insect hemolymph (Kato et al., 1994; Charles and Killian, 2015) and muscle tissue (Potter et al., 2021) results in characteristics of antigenicity.

In our experiments, the sand flies ingested an approximate amount of LPG comparable to the amount of parasites ingested during an experimental infection with *L. infantum* or *L. major* (Kimblin et al., 2008; Telleria et al., 2013; Martín-Martín et al., 2015; Vaselek et al., 2020; Ashwin et al., 2021; Kykalová et al., 2021). In addition, the amount of ingested LPS corresponds to the number of colony-forming unit (CFU) detected in sand fly guts (Hurwitz et al., 2011; Peterkova-Koci et al., 2012; Kelly et al., 2017; Louradour et al., 2017). Therefore, the combination of the resident microbiota and ingested LPG or LPS is comparable to common experimental conditions and not expected to harm the insects. Indeed, we did not observe a significant difference in mortality rates between experimental and control groups. Different combinations of

LPG and LPS would reveal other interesting aspects of the sand fly response to PAMPs. However, the current experimental design focused on how the insect gene expression responds to the individual PAMPs.

The gene sequences investigated in the current study were selected according to their role in the parasite interaction with the sand fly. For instance, galectins belong to a family of carbohydrate (glycan) binding proteins that are expressed by a vast group of cells and bind to galactose-containing glycans (Liu and Stowell, 2023). Mucin and mucin-like proteins belong to a large family of O-glycosylated proteins and, in vertebrate guts, compose the primary barrier of defense and a niche for the gut microbiome (Werlang et al., 2019). In sand flies, both *Pp*GalecA (Kamhawi et al., 2004; Abrudan et al., 2013; Labbé et al., 2023) and *Ll*Gprot19 (Jochim et al., 2008; Myšková et al., 2016) were shown to be involved in attachment of the parasite to the sand fly gut. *Ll*GalecA is highly similar to *Pp*GalecA (Labbé et al., 2023), probably sharing biochemical and biological roles in the gut epithelium but these have not yet been investigated. *Pp*Gprot13.7 is the *P. papatasi* sequence most similar to *Ll*Gprot19 (Ramalho-Ortigão et al., 2007; Myšková et al., 2016; Amos et al., 2022) available. Both *Ll*Gprot19 and *Pp*Gprot13.7 amino acid sequences contain a quiver family domain (IPR031424) present in a potassium channel subunit in *Drosophila* (Koh et al., 2008). *Pp*Gprot13.7 has not yet been investigated regarding its role in the sand fly gut. Therefore, no further correlative roles may be speculated.

Chymotrypsins and trypsins are endopeptidases that belong to the serine protease family (Rawlings and Barrett, 1994). In addition to their widely known role in digestion, serine proteases can also be involved in hemolymph coagulation, activation of AMP synthesis, and melanin synthesis in mosquitoes (Gorman and Paskewitz, 2001). The digestive enzymes *Ll*Chym1A and *Pp*Chym2 were selected due to their downregulation after *Leishmania* infection. In contrast, *Ll*Tryp1 (Telleria et al., 2010) and *Pp*Tryp4 (Ramalho-Ortigão et al., 2003) were selected for comparison due to their direct association with blood digestion.

The *L. longipalpis* AMPs *Ll*Att and *Ll*Def2 were selected due to their upregulation after *L. infantum* infection, and *Ll*Def1 was included for comparison since it was not altered (Telleria et al., 2021b). The *P. papatasi* AMPs *Pp*Att, *Pp*Def1, and *Pp*Def2, on the other hand, were all upregulated after *L. major* infection (Kykalová et al., 2021; Vomáčková Kykalová et al., 2023). We hypothesized that *L. longipalpis* gene transcripts *Ll*Gprot19, *Ll*Chym1A, *Ll*Att, and *Ll*Def2, as well as the *P. papatasi* *Pp*GalecA, *Pp*Chym2, *Pp*Att, *Pp*Def1, and *Pp*Def2, could be differentially expressed after sand flies ingest *Leishmania* LPGs. Thus, it addresses whether the LPGs can influence the expression of these transcripts.

We highlight that the relative gene expression levels based on the mRNA levels do not reflect the quantity of corresponding proteins.

Feeding *L. longipalpis* with LPG from *L. major* caused the upregulation of *Ll*GalecA mRNA expression. Although *L. major* does not naturally occur in the Americas, it can colonize the *L. longipalpis* gut in experimental infections (Volf and Myskova, 2007; Cecílio et al., 2020). The upregulation of *Ll*GalecA suggests that *L. major* LPG can affect the expression of this lectin in the *L. longipalpis* gut.

In *P. papatasi*, the *L. infantum* and *L. major* LPGs caused a slight non-significant variation in *Pp*GalecA transcript expression, while it increased after *E. coli* LPS feeding. *Escherichia coli* can be found in *Phlebotomus* sand flies, including *P. papatasi* (Fraihi et al., 2017). Therefore, the *P. papatasi* gut gene expression may be affected by *E. coli* LPS. In addition, galectins can interact directly with bacterial surface glycans such as vertebrate galectin 3 binding to Gram-negative bacteria LPS in a feedback regulation of the inflammatory response (Li et al., 2008; Ayona et al., 2020).

In summary, while the role of *P. papatasi* gut galectin in *L. major* attachment through LPG is well known (Kamhawi et al., 2004), the role of *L. longipalpis* galectins remains scarcely investigated. However, the transcript expression of both sand fly species can be altered depending on the ingested microbe molecule.

Feeding *L. longipalpis* with *L. infantum* LPG or *E. coli* LPS caused the increase in *Ll*Gprot19 mRNA expression. The *Ll*Gprot19 gene sequence (EU124597) was originally identified as a putative 19 kDa protein

(Jochim et al., 2008) and later identified as a mucin-like glycoprotein localized in the *L. longipalpis* midgut epithelial surface (Mysková et al., 2016). The corresponding recombinant protein, with a mass of 45 kDa, exhibited a robust binding to the surface of *L. major* (Mysková et al., 2016). Later investigations involving *L. mexicana* showed that the parasite LPG can bind to this mucin-like protein in *L. longipalpis* (Hall et al., 2020). Therefore, there is an evident interaction between these *Leishmania* LPG and *L. longipalpis* mucin-like proteins.

The *L. infantum* and *L. major* LPGs caused a slight non-significant variation in *P. papatasi* *Pp*Gprot13.7 transcript expression. The *Pp*Gprot13.7 protein sequence (ABV44735) (Ramalho-Ortigão et al., 2007) contains the same quiver conserved domain as in *Ll*Gprot19 (ABV60315) (Jochim et al., 2008). Considering that no further characterization of *Pp*Gprot13.7 is available and its expression was not altered after the feedig with LPGs, it is impossible to infer that *Pp*Gprot13.7 is connected to *Leishmania* infection.

In invertebrates, mucins and mucin-like proteins are found in cuticle-free tissues such as the midgut and the PM (Hegedus et al., 2009; Dias et al., 2018). In many other organisms, they compose a substrate where commensal gut microbiota and pathogens can thrive (Hansson, 2020; Mantle et al., 1989; Wang and Granado, 1997). In *Aedes albopictus*, mucins can be upregulated in the midgut by blood meal (Deng et al., 2020). Our results suggest that in *L. longipalpis*, the *Ll*Gprot19 production is increased in response to *Leishmania*, creating a favourable niche for the parasite to bind.

Earlier transcriptomic studies showed that the expression of some genes coding for digestive enzymes was altered after *Leishmania* infection. For example, *L. major* and *L. infantum* caused a decrease in the expression of some, but not all, chymotrypsins and trypsins from *P. papatasi*, *P. perniciosus*, and *L. longipalpis* transcripts, respectively (Ramalho-Ortigão et al., 2007; Jochim et al., 2008; Dostálová et al., 201;). In our experiments, feeding sand flies with LPGs and LPS did not alter transcript expression of *Ll*Tryp1 and *Pp*Tryp4. Indeed, expression of these genes was reported to be increased by the blood meal but not affected by the parasitic infection (Ramalho-Ortigão et al., 2007; Telleria et al., 2007; Jochim et al., 2008; Sant'Anna et al., 2009). Nevertheless, the non-altered expression of *Ll*Chym1A and *Pp*Chym2 differs from previous transcriptomic studies (Ramalho-Ortigão et al., 2007; Jochim et al., 2008). Therefore, our results indicate that *Leishmania* LPGs are not the molecules involved in the consequent downregulation of LlChym1A and PpChym2 after *Leishmania* infection in *L. longipalpis* and *P. papatasi*, respectively. If the parasite can interfere with the sand fly digestion process, it may occur through an inhibitory effect of the gut enzymatic activity.

The *E. coli* LPS caused a non-significant increase in *Ll*Chym1A expression. Interestingly, some serine proteases may have antibacterial activity. For example, trypsin-like protease was expressed by the flesh fly *Sarcophaga peregrina* during remodelling of the pupa midgut and had an antibacterial effect against Gram-negative and Gram-positive bacteria (Tsuji et al., 1998). Therefore, the variable increase in *Ll*Chym1A after ingestion of *E. coli* LPS may reflect a delayed response of *L. longipalpis* in controlling bacteria in the gut.

LPG from different parasite species causes distinct effects in the mammalian host. For instance, *Leishmania braziliensis* LPG induced a stronger murine macrophage immune response than *L. infantum* LPG due to its biochemical differences on the side chains of the phosphosaccharide-repeat domain (Ibraim et al., 2013). Therefore, we investigated the effect of parasite LPGs on the sand fly innate immune response.

In *L. longipalpis*, we found that *L. infantum* LPG triggers the sand fly immune response through an overexpression of *Ll*Att and *Ll*Def2. These results correspond with previously published studies using *L. longipalpis* infected by *L. infantum*, where *Ll*Att and *Ll*Def2 were upregulated. At the same time, *Ll*Def1 did not show a significant change in transcript expression (Telleria et al., 2021b). Here, in the group fed on blood containing LPG from *L. major*, we also observed a non-significant but intriguing increased expression of *Ll*Att even earlier than in the *L. infantum* LPG group, suggesting that *Ll*Att expression was quickly triggered, especially to non-adapted *Leishmania* spp. Interestingly, *Ll*Def2 gradually increased after *L. major* LPG, suggesting a tailored response to changes in the sand fly gut. Our studies showed that *L. longipalpis* can activate its immune response during *Leishmania* infection, but how the parasite overcomes the sand fly

immune response is still not completely understood. Nevertheless, recent studies showed that *Leishmania* could upregulate immunity repressors associated with Jak-STAT and Toll pathways in the sand fly (Telleria et al., 2021a, 2023), revealing how the balance between the parasite and the sand fly immune response is dynamic and complex.

The ingestion of LPS led to a significantly increased expression of *Ll*Att and *Ll*Def2. Intriguinly, these transcripts were not significantly upregulated in *L. longipalpis* with increased gut bacteria (Telleria et al., 2021b). Attacins can bind to bacterial LPS and increase outer-membrane permeability (Carlsson et al., 1998); therefore, it is plausible to consider that the purified *E. coli* LPS represented an intense stimulus to the sand fly immune response.

We observed that *P. papatasi* attacin (*Pp*Att) levels rise rapidly after exposure to the LPG molecule from both *L. major* and *L. infantum*, which points to *Pp*Att being an AMP acting in the first response after exposure. In the experiments with live parasites, *Pp*Att levels also increased after *L. major* infection (Kykalová et al., 2021).

Curiously, *Pp*Def1 and *Pp*Def2 expression did not alter significantly after LPG and LPS feeding in the current results, while in experimental infections with *L. major*, they were upregulated (Kykalová et al., 2021; Vomáčková Kykalová et al., 2023). Therefore, other parasite molecules expressed during an experimental infection could induce a more evident effect on the expression of these genes.

Feeding the sand flies with *E. coli* LPS resulted in highly variable PpAtt transcript expression levels, but was more intense at earlier time points. In *P. papatasi* larvae, PpAtt is increased by ingesting bacteria-rich food (Kykalová et al., 2021). The ready increase in attacin in *P. papatasi* after exposure to bacterial contents indicates that this AMP has an important role in the vector’s immune response.

In conclusion, the interaction between *Leishmania* parasites and sand flies has many facets. For instance, *L. braziliensis* interaction with *L. longipalpis* cell lines showed that metallo-proteinases such as 65 kDa and 55 kDa heparin-binding proteins from the surface of the parasite (de Castro Cortes et al., 2012a; De Castro Côrtes et al., 2012b) or secreted Gp63 (Telleria et al., 2023) also play an important role in cell attachment or suppression of the insect immune response, respectively.

In the present study, we demonstrated that *Leishmania* LPGs trigger expression of mucin-like proteins in the sand fly gut, particularly near the end of the blood digestion process. On the other hand, the rapidly increased expression of AMPs, especially attacin, was observed in both tested sand fly species. The results of our study provide valuable insights into the intricate molecular mechanisms governing the interaction between *Leishmania* parasites and sand fly vectors.

In addition, glycoconjugates from *Leishmania* and *E. coli* bearing polymorphisms in their carbohydrate motifs affected the expression of gut-related and innate immunity genes in sand flies. The most significant changes were the increase in gene expression of a mucin-like protein in *L. longipalpis*, a permissive vector, and attacin in both sand fly species.

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Legends to figures

Fig. 1. Schematic diagram of PAMPs: *Leishmania infantum* and *Leishmania major* lipophosphoglycans (LPGs) are composed of a cap oligosaccharide represented on the left side, galactose-mannose-phosphate repeating units core oligosaccharide represented in the centre, and a phosphatidylinositol-lipid anchor on the right side of the structure (adapted from Coelho-Finamore et al., 2011b; adapted from McConville et al., 1992). *Leishmania major* LPG contains sugar branch ramification on galactose-mannose-phosphate repeating units. *Escherichia coli* lipopolysaccharides (LPS) structure is composed of the O-antigen represented on the left side, a core oligosaccharide region in the centre, and the lipid-A on the right side of the structure (adapted from Clifton et al., 2013).

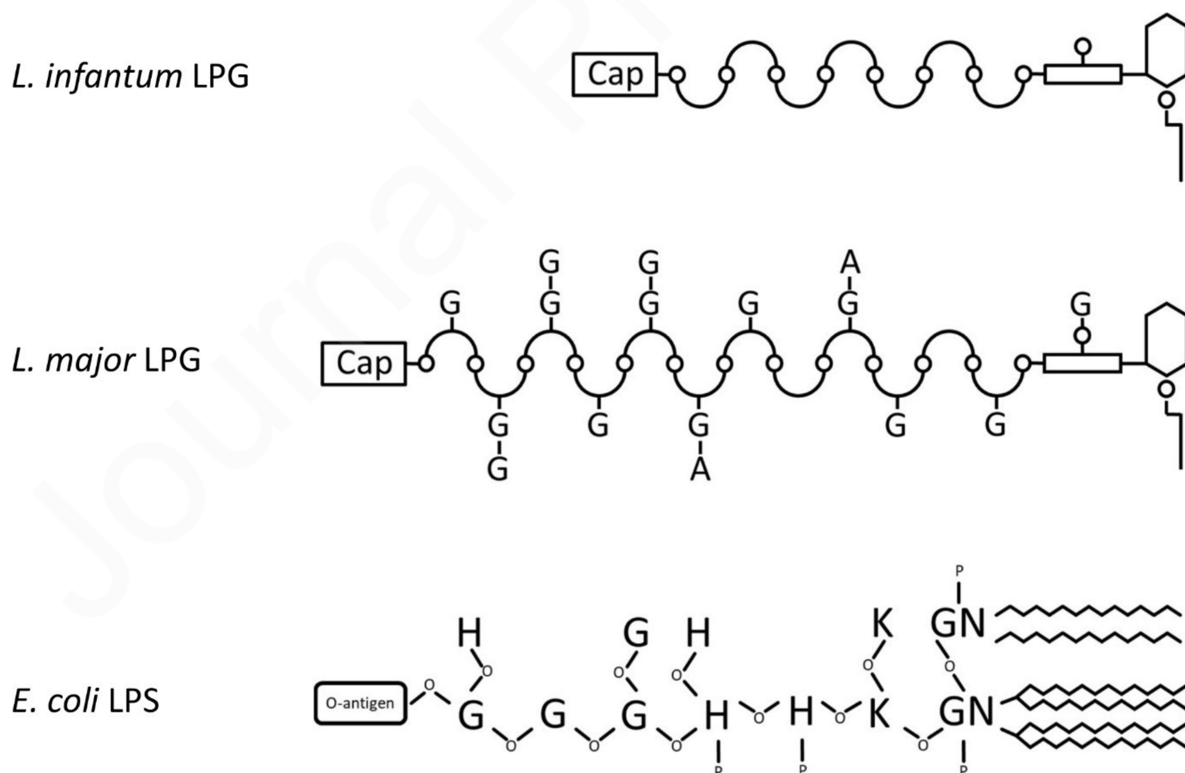


Fig. 2. Expression of midgut proteins in *Lutzomyia longipalpis*. (A) Sand flies fed on blood containing *Leishmania infantum* lipophosphoglycan (LPG); (B) *Leishmania major* LPG; and (C) *Escherichia coli* lipopolysaccharides (LPS). Bars represent the mean of *L. longipalpis* galectin A (*Ll*GalecA) and mucin-like

gut protein 19 kDa (*Ll*Gprot19) relative gene expressions calculated compared with the endogenous control genes. Y-axis indicates relative gene expression expressed as fold change compared with the control group fed on blood (dotted line). Error bars represent standard error of three independent experiments. X-axis indicates the times when guts were dissected after the blood meal. Significant differences were calculated using two-way ANOVA (* $P < 0.05$; **** $P < 0.0001$).

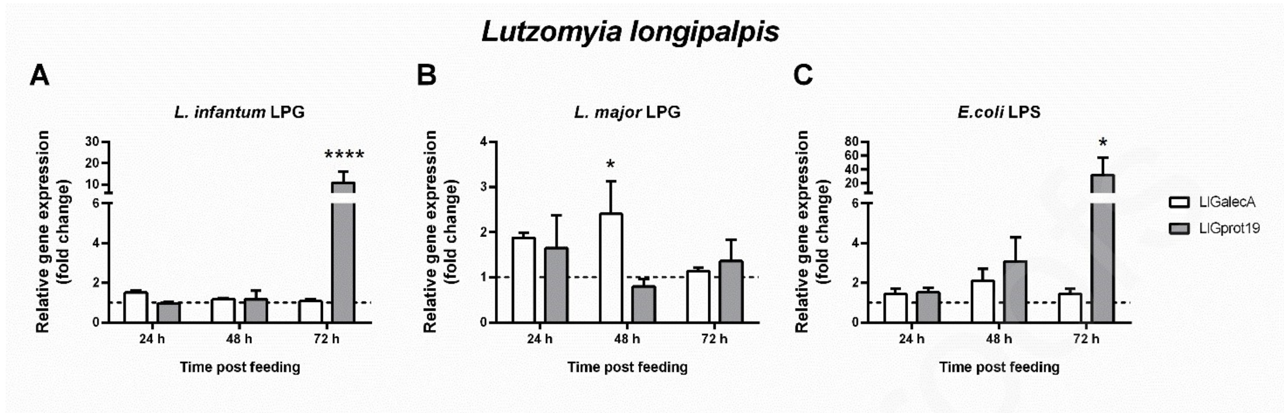


Fig. 3. Expression of midgut proteins in *Phlebotomus papatasi*. (A) Sand flies fed on blood containing *Leishmania infantum* lipophosphoglycan (LPG); (B) *Leishmania major* LPG; and (C) *Escherichia coli* lipopolysaccharides (LPS). Bars represent the mean of *P. papatasi* galectin A (*Pp*GalecA) and 13.7 kDa gut protein (*Pp*Gprot13.7) relative gene expressions calculated compared with the endogenous control genes. Y-axis indicates relative gene expression expressed as fold change compared with the control group fed on blood (dotted line). Error bars represent standard error of three independent experiments. X-axis indicates the times when guts were dissected after the blood meal. Significant differences were calculated using two-way ANOVA (* $P < 0.05$).

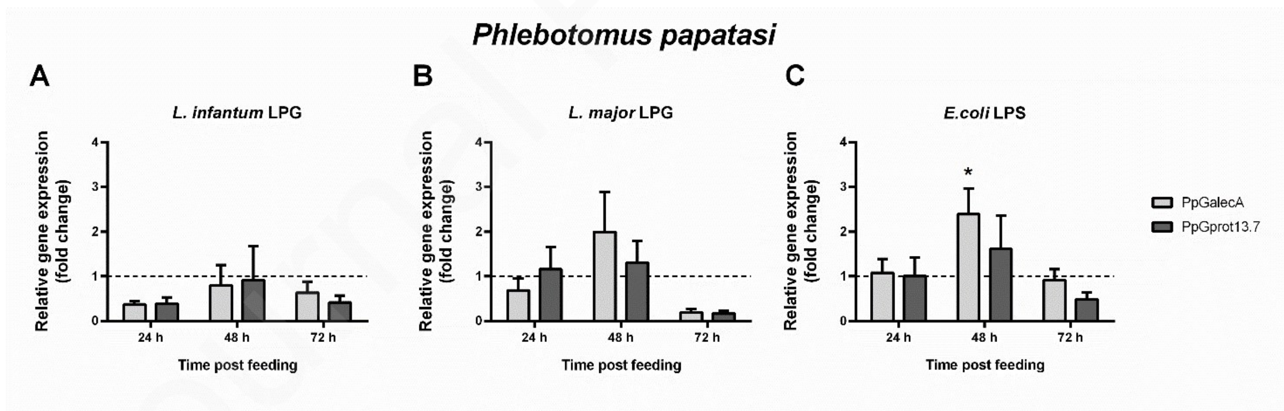


Fig. 4. Expression of midgut digestive enzymes in *Lutzomyia longipalpis*. (A) Sand flies fed on blood containing *Leishmania infantum* lipophosphoglycan (LPG); (B) *Leishmania major* LPG; and (C) *Escherichia coli* lipopolysaccharides (LPS). Bars represent the mean of *L. longipalpis* trypsin 1 (*Ll*Tryp1) and chymotrypsin 1A (*Ll*Chym1A) relative gene expressions calculated compared with the endogenous control genes. Y-axis indicates relative gene expression expressed as fold change compared with the control group fed on blood (dotted line). Error bars represent standard error of three independent experiments. X-axis indicates the times when guts were dissected after the blood meal. No significant differences were found (two-way ANOVA).

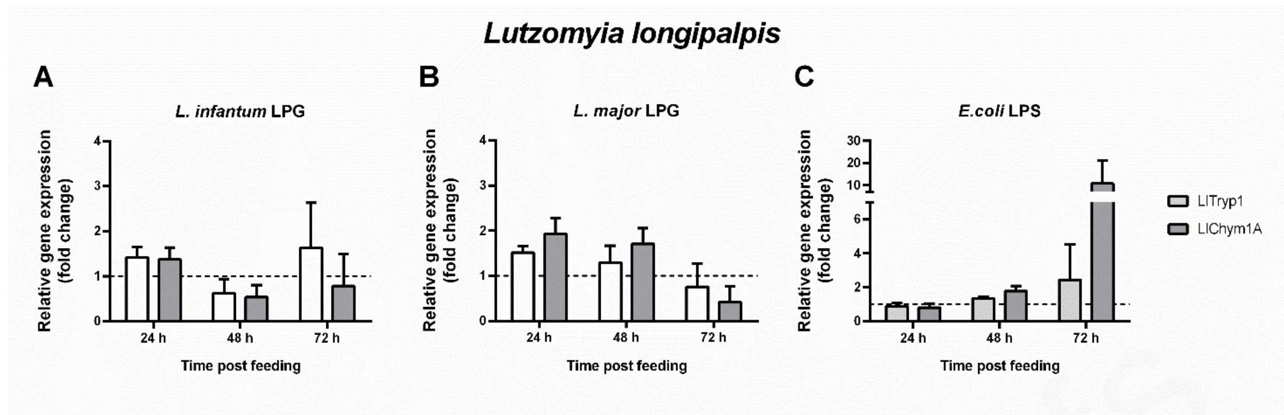


Fig. 5. Expression of midgut digestive enzymes in the *Phlebotomus papatasi*. (A) Sand flies fed on blood containing *Leishmania infantum* lipophosphoglycan (LPG); (B) *Leishmania major* LPG; and (C) *Escherichia coli* lipopolysaccharides (LPS). Bars represent the mean of *P. papatasi* trypsin 4 (*Pp*Tryp4) and chymotrypsin 2 (*Pp*Chym2) relative gene expressions calculated compared with the endogenous control genes. Y-axis indicates relative gene expression expressed as fold change compared with the control group fed on blood (dotted line). Error bars represent standard error of three independent experiments. X-axis indicates the times when guts were dissected after the blood meal. No significant differences were found (two-way ANOVA).

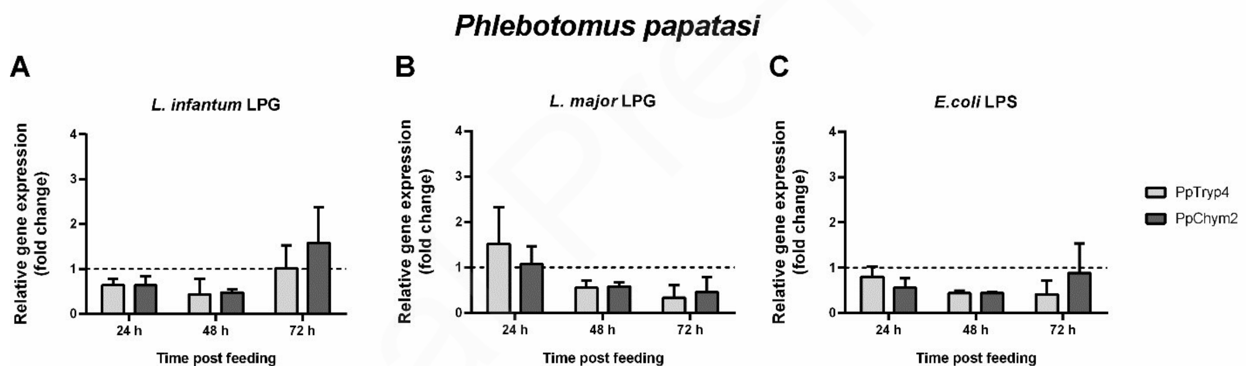


Fig. 6. AMP expression in *Lutzomyia longipalpis* guts. (A) Sand flies fed on blood containing *Leishmania infantum* lipophosphoglycan (LPG); (B) *Leishmania major* LPG; and (C) *Escherichia coli* lipopolysaccharides (LPS). Bars represent the mean of *L. longipalpis* attacin (*Ll*Att), defensin (*Ll*Def1), and defensin 2 (*Ll*Def2) relative gene expressions calculated compared with the endogenous control genes. Y-axis indicates relative gene expression expressed as fold change compared with the control group fed on blood (dotted line). Error bars represent standard error of three independent experiments. X-axis indicates the times when guts were dissected after the blood meal. Significant differences were calculated using two-way ANOVA (* $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$).

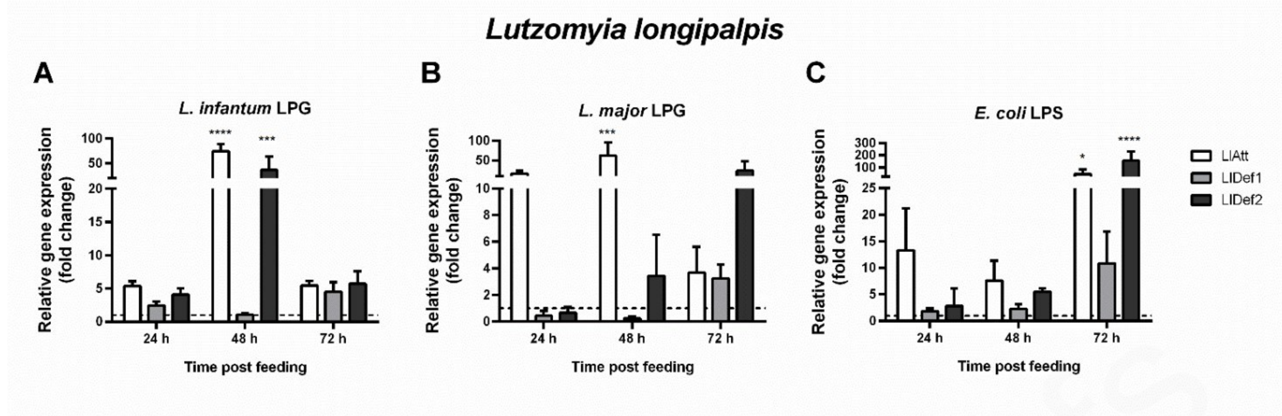
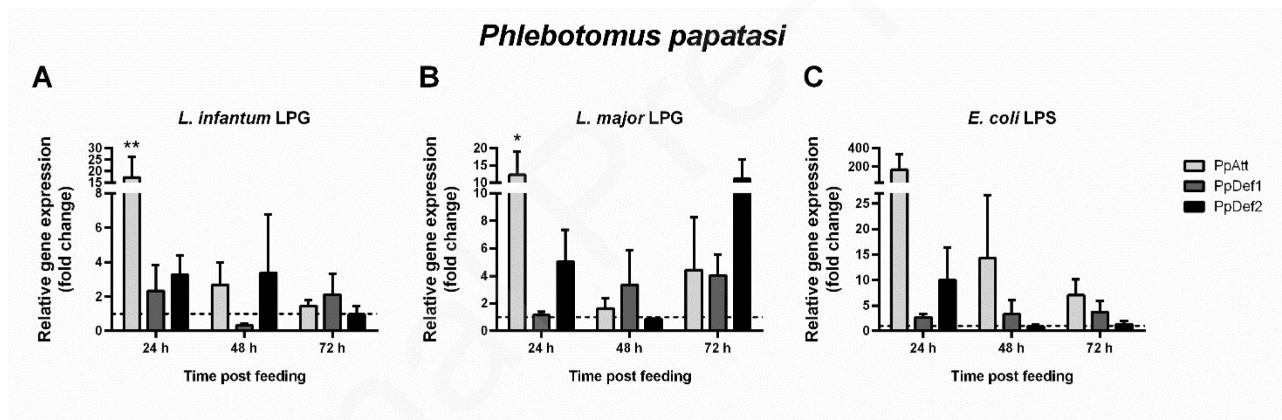


Fig. 7. AMP expression in *Phlebotomus papatasi* guts. (A) Sand flies fed on blood containing *Leishmania infantum* lipophosphoglycan (LPG); (B) *Leishmania major* LPG; and (C) *Escherichia coli* lipopolysaccharides (LPS). Bars represent the mean of *P. papatasi* attacin (*Pp*Att), gut-specific defensin (*Pp*Def1), and systemically expressed defensin (*Pp*Def2) relative gene expressions calculated compared with the endogenous control genes. Y-axis indicates relative gene expression expressed as fold change compared with the control group fed on blood (dotted line). Error bars represent standard error of three independent experiments. X-axis indicates the times when guts were dissected after the blood meal. Significant differences were calculated using two-way ANOVA (* P < 0.05; ** P < 0.01).



Supplementary figure legend

Supplementary Fig. S1. Effect of *Leishmania infantum* dose on AMP expression in the *Lutzomyia longipalpis* gut. AMP expression after feeding on 1 or 5 µg/mL of *L. infantum* lipophosphoglycan (LPG) in the blood meal. (A) *Lutzomyia longipalpis* attacin (*Ll*Att) gene expression. (B) *Lutzomyia longipalpis* defensin 2 (*Ll*Def2) gene expression. Bars represent the mean of AMP relative gene expression calculated compared with the endogenous control genes. Y-axis indicates relative gene expression expressed as fold change compared with the control group fed on blood (dotted line). Error bars represent the standard error of three independent experiments. X-axis indicates the times when guts were dissected after blood meal. Significant differences were calculated using two-way ANOVA (**** P < 0.001).

Table 1. Sand fly gene primers

Sand fly species	Gene name	Reference	Sequence
Lutzomyia longipalpis	Galectin A (*Ll*GalecA) (**LLOJ010541**)	Present work	GAGCTTACCCGTCACCCTATCCA TCCCTTCACTACCACCACATGCC
	Mucin-like gut protein 19 kDa (*Ll*Gprot19) (**EU124597**)	Present work	CAACCACACCATCGACACCTCCT AGTTGAAGACACTGTTGGCGTCG
	Trypsin 1 (*Ll*Tryp1) (**LLOJ008676**)	Present work	TAATACCCAGAGCAGCCAGGAG ATCATTGTCTTTGTAATGCCGC
	Chymotrypsin 1A (*Ll*Chym1A) (**EU124576**)	Present work	CCAACATCTGTGCTGGAGAACCT AACGCCGTAAACCTGAACCTCTC
	Attacin (*Ll*Att) (**KP030755**)	Tinoco-Nunes et al., 2016	ATGGGCATGGCAGCGTCTCT AGGCTGATCCTCTGGGTCCTGT
	Defensin 1 (*Ll*Def1) (**EF491251**)	Telleria et al., 2021b	GCTGCAAATCCTGCAAAGA CCCAAGGAGGTCACAGGTTA
	Defensin 2 (*Ll*Def2) (**KP030758**)	Telleria et al., 2021b	ATCCATCCTTTATGCAACCG

			GCCTTTGAGTCGCAGTA TCC
	Ribosomal protein L8 (*Ll*RibL8) (**LLOJ005437**)	Present work	AGAAGACCCGTGTGAAG CTC TTGTCGATTCTTCCGCCA CC
	Ribosomal protein RP49 (*Ll*RP49) (**LLOJ006773**)	Meireles-Filho et al., 2006	GACCGATATGCCAAGCT AAAGCA GGGGAGCATGTGGCGTG TCTT
Phlebotomus papatasi	Galectin A (*Pp*GalecA) (**AY538600**)	Present work	CCTGGCTACCGGCAAAA ACCTTG CCTCTGCACCAAAACAT TGCCCT
	Mucin-like gut protein 13.7 kDa (*Pp*Gprot13.7) (**PPAI006243**)	Present work	GAAAACTCAGCATGTGG AGATCC CCAGAAGTTCCATTAAC GTGCAA
	Trypsin 4 (*Pp*Tryp4) (**AY128111**)	Present work	CTCGATGGGAGAGTCGT AGGTGG CAGGCTTCTCAAGTTCC AGCAGG
	Chymotrypsin 2 (*Pp*Chym2) (**AY128107**)	Present work	AGCATTAAGAGTCCGGA TCAGT ACCTCAACCAGACCAAC ATCAT
	Attacin (*Pp*Att) (**PPAI003791**)	Kykalová et al., 2021	GCCATTTCTGCTGCGTA CTC GAGGCACCAAGTACACG ACA
	Defensin 1 (*Pp*Def1)	Kykalová et al., 2021	GCCCGGTTAAAGACGAT GTAAAG

(**PPAI004256**)		AGTTGGTCCAAGGATATCGCAAG
Defensin 2 (*Pp*Def2) (**PPAI010650**)	Vomáčková Kykalová et al., 2023	ATTCACGCCAAAAACGAGCC CGATACAATGGGCAGCACAAG
Actin (*Pp*Act) (**PPAI004850**)	Kykalová et al,. 2021	GCACATCCCTGGAGAAATCCTAT GGAAAGATGGCTGGAAGAGAGAT
Ribosomal protein L8 (*Pp*RibL8) (**PPAI008202**)	Kykalová et al., 2021	GACATGGATACCTCAAGGGAGTC TTGCGGATCTTATAGCGATAGGG

Accession numbers of gene sequences from GenBank or VectorBase, from which primers were designed, are indicated below gene names. Newly designed primers for quantitative PCR are indicated as ‘present work’.

PUBLICATION 4

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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 mg/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 mg 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 mg/mL 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/mg of total RNA for removing possible traces of DNA. Up to 1 mg 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 DDC_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

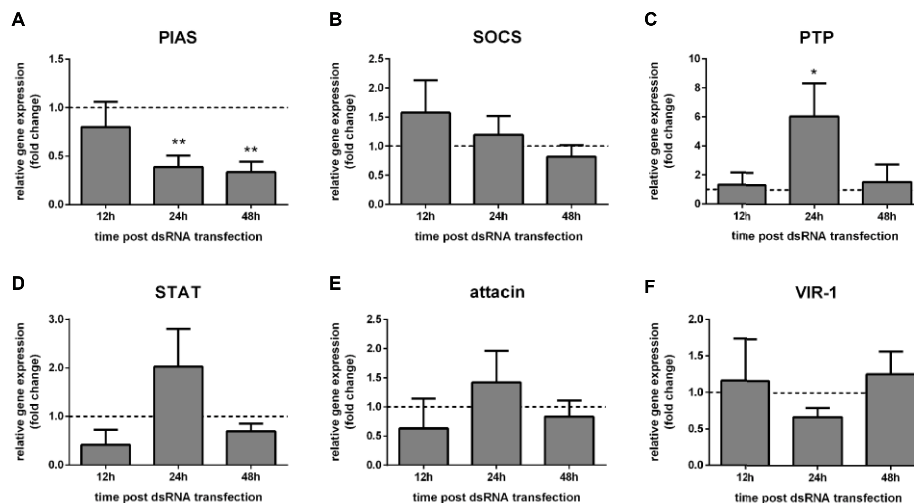

FIGURE 1 | Gene expression of Jak-STAT regulators in LL5 cells after PIAS silencing: *L. longipalpis* relative gene expression was calculated compared to the endogenous reference gene RP49. (A) silencing of the PIAS gene; (B–F) expression of indicated genes after PIAS silencing. (A–F) relative expression 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).

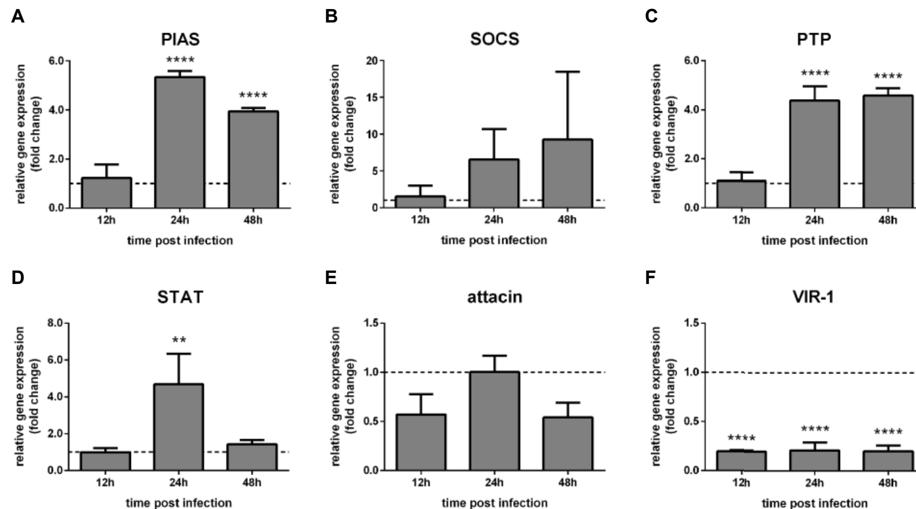

FIGURE 2 | Gene expression of Jak-STAT-related genes in LL5 cells after *Leishmania* challenge: *L. longipalpis* relative gene expression was calculated compared to the endogenous reference gene RP49. (A–F) expression of indicated genes after *Leishmania* co-culture. Relative expression in *Leishmania*-challenged 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

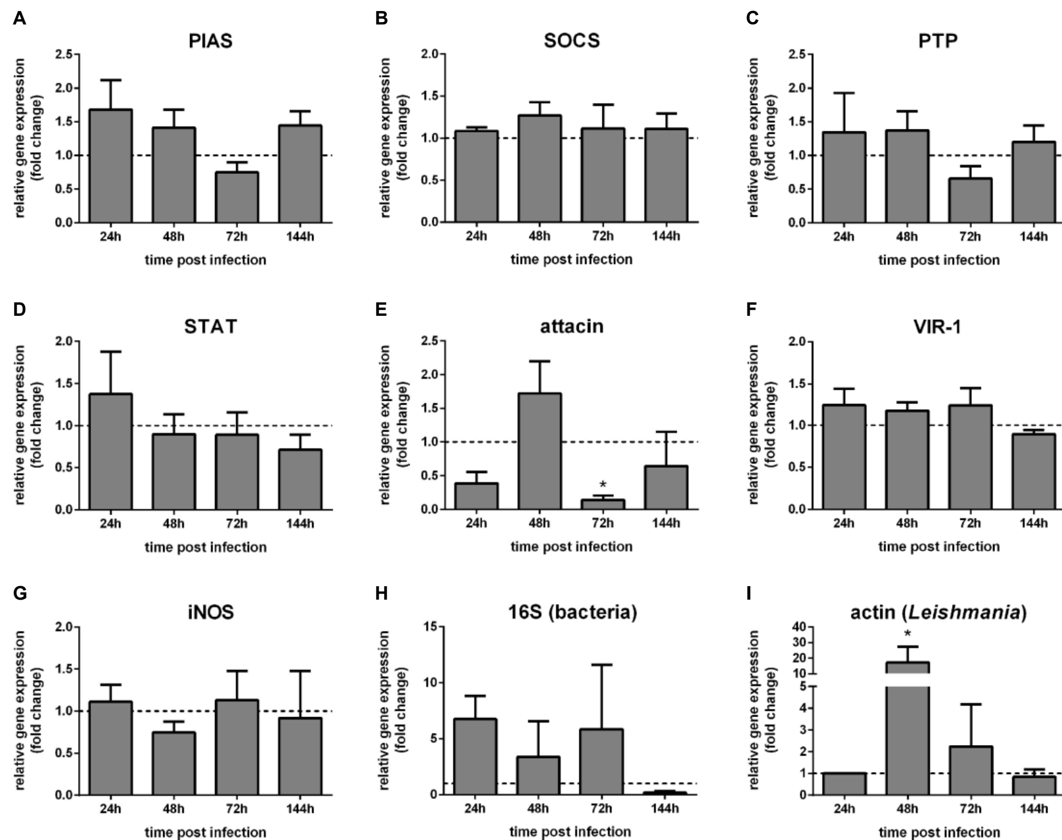

FIGURE 3 | Gene expression of Jak-STAT-related genes in *L. longipalpis* infected with *Leishmania*: *L. longipalpis* relative gene expression was calculated compared to the endogenous reference gene RP49. (A–G) expression of indicated genes after *Leishmania* infection; quantification of bacteria (H) 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.

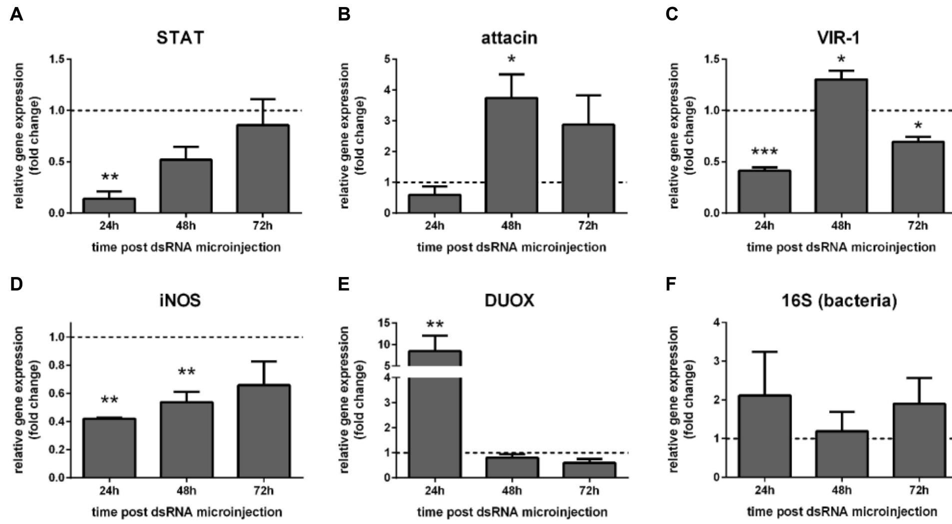

FIGURE 4 | Gene expression of Jak-STAT-related genes in STAT-silenced sucrose fed *L. longipalpis*: *L. longipalpis* relative gene expression was calculated compared to the endogenous reference gene RP49. (A): silencing of the STAT-like gene; (B–E): expression of indicated genes after STAT-like gene 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)

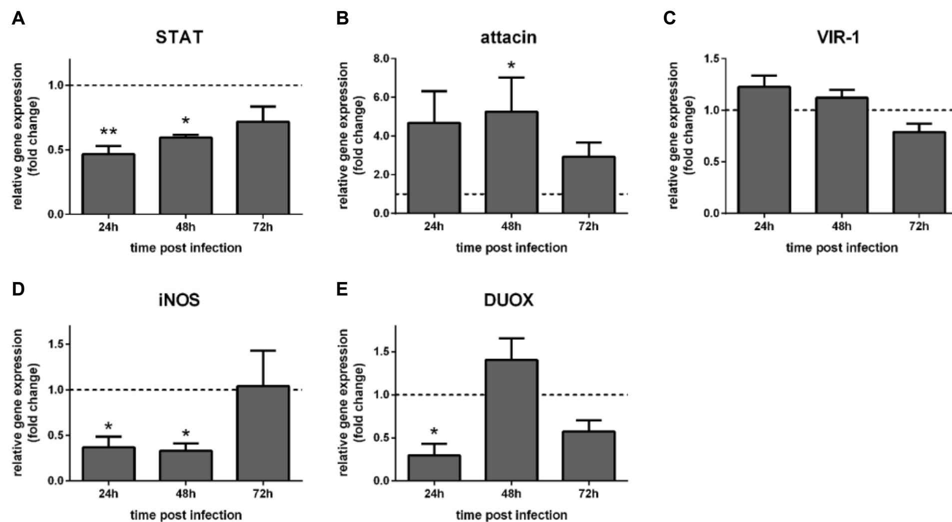

FIGURE 5 | Gene expression of Jak-STAT-related genes in carcasses of STAT-silenced *L. longipalpis* and infected with *L. infantum*: *L. longipalpis* relative gene expression was calculated compared to the endogenous reference gene RP49. (A) silencing of the STAT-like gene; (B–E): expression of indicated 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).

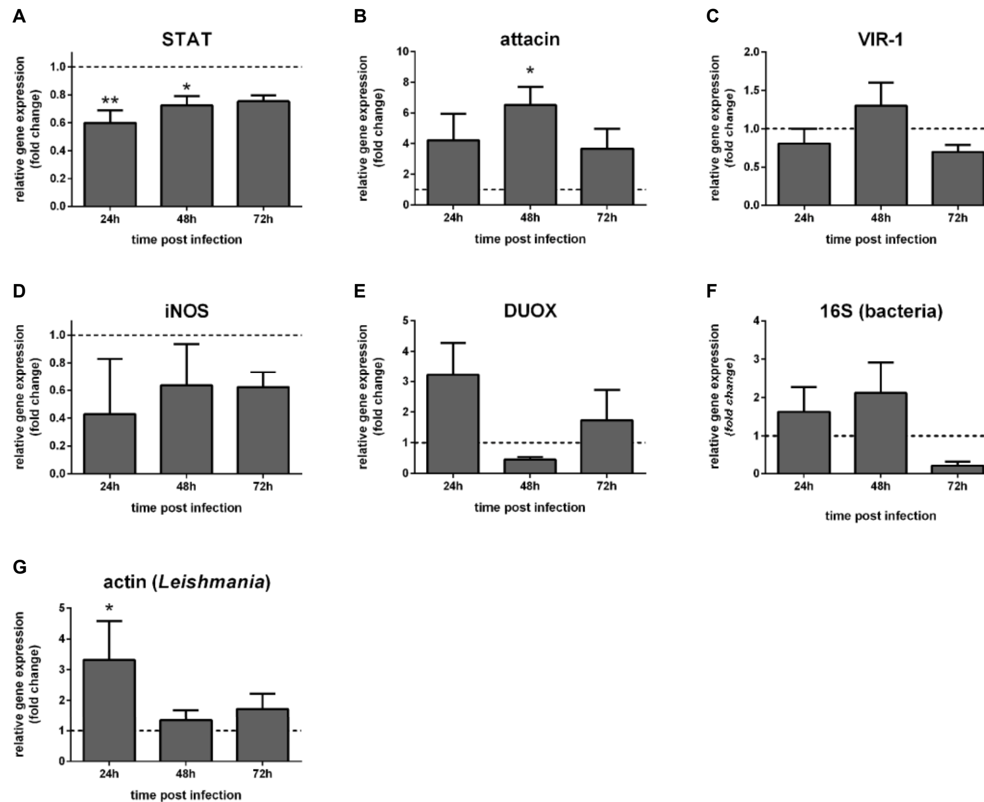

FIGURE 6 | Gene expression of Jak-STAT-related genes in dissected guts of STAT-silenced *L. longipalpis* and infected with *L. infantum*: *L. longipalpis* relative gene expression was calculated compared to the endogenous reference gene RP49. (A) silencing of the STAT-like gene; (B–E) expression of indicated 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

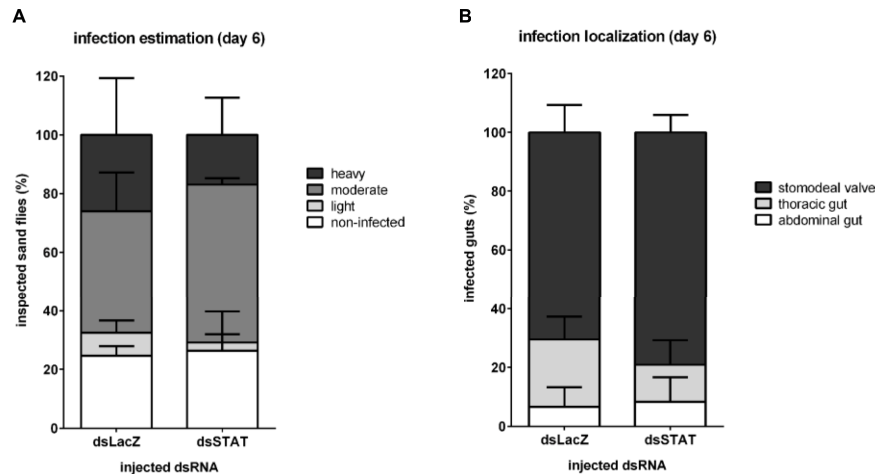

FIGURE 7 | *Leishmania* infection development in STAT-like-silenced *L. longipalpis*: Infection intensity and development at late infection in dsRNA-injected sand flies. (A) The y-axis represents the percentage of all individually inspected insects (minimum of 20 sand flies in each dsRNA injected group). 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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SUMMARY AND CONCLUSIONS

The presented PhD. thesis is composed of results obtained from more than 4 years of research on sand fly immunity and gene editing. We focus our research mainly on immune-related molecules that are potentially involved in the *Leishmania*-vector interactions. It is important to study both, the parasite as a causative agent of the disease and sand flies as their vectors because only a detailed knowledge of this complex relationship can help with developing molecular-based strategies of transmission control.

Our investigation was dedicated to two sand fly species, *P. papatasi* and *L. longipalpis*. Both species are important vectors, and their genomes and transcriptomes are available in public databases. We studied the main immune pathways, Toll and Imd, their transcriptional factors and downstream effector molecules, AMPs. Both pathways were previously showed to be involved in antiparasitic defence, e.g. in *Anopheles*-*Plasmodium* relationship (Frolet et al., 2006; Garver et al., 2009, 2012). We were interested in involvement of Toll and Imd pathway in relation to *L. major* cycle in *P. papatasi* vector.

Firstly, we have selected and identified *P. papatasi* immunity genes. *Relish* transcriptional factor was previously identified (Louradour et al., 2019), *caspar*, *defensin 1, 2* and *attacin* we have identified based on similarities with *L. longipalpis* sequences and confirmed by sequencing (Kykalová et al., 2021 = PUBLICATION 1; Vomáčková Kykalová et al., 2023 = PUBLICATION 2). Using the relative gene expression assessed by qPCR, we followed the gene profiles during *Leishmania* infection. To shield the influence of gut microbiota on the expression of immune genes and to have the possibility to observe the effect mainly caused by the parasitic infection, we treated the adults with antibiotics cocktail leading in significant reduction of gut microbiota. We reported significantly increased expression of *dorsal*, transcriptional factor of Toll pathway, at 48 hours post infection. More interestingly we observed an overexpression of *defensin 1* at later stage of infection (144 hours post infection) when parasites migrate to the anterior part of the sand fly gut and colonize the stomodaeal valve. In the group with recovered gut microbiota, an overexpression of *defensin 1* was observed earlier, at 72 hours post infection indicating the influence of bacteria abundance on gene expression. Expression of other genes such as *relish*, *attacin* and *defensin 2* was not significantly modified during *Leishmania* infection (Kykalová et al., 2021 = PUBLICATION 1; Vomáčková Kykalová et al., 2023 = PUBLICATION 2).

Due to our observation of *defensin 1* gene, we decided to further investigate its role in sand flies focusing *Leishmania* infection. Previously, it has been shown that defensins may have antiprotozoal activity, e.g. defensin from *Phormia terranovae* and *Aechna cyanea* report activity against *P. gallinaceum* (Shahabuddin et al., 1998) or *Triatoma pallidipennis* defensin acts against *Trypanosoma* and *Leishmania* parasites (Diáz-Garrido et al., 2021). First of all, we compared the expression of *defensin 1* in separate tissues (head, gut, thorax, Malpighian tubules, eggs and posterior end of abdomen) and we found out that it is expressed only in the midgut, the tissue where parasite cycle takes place (Kykalová et al., 2021 = PUBLICATION 1).

Based on our result we conclude defensin 1 as a gut-specific AMP with function in local immune defence. We hypothesized that the local production of defensin 1 is compensated in other tissues. Using quantitative qPCR we discovered that *defensin 2* is expressed in other tissues (carcasses) more than 40x more than in the midgut indicating that it may compensate the missing expression of *defensin 1* (Vomáčková Kykalová et al., 2023 = PUBLICATION 2). It has been also observed in other insects that some AMPs are expressed differently depending on the tissue (Vizioli et al., 2001; Dong et al., 2006; Vieira et al., 2016) while working either synergistically or additively (Hanson et al., 2019).

Using the gene-silencing approach we continued our investigation in defensins role during *Leishmania* infection. Firstly, we adapted the protocol to reach the gene silencing in sand fly gut. We increased the amount of injected dsRNA and report a gene silencing in the midgut tissue (Vomáčková Kykalová et al., 2023 = PUBLICATION 2). All previous studies, using gene silencing approach to study the sand flies in connection with a pathogen challenge, followed the same order when first step was gene silencing and second was pathogen challenge (Sant'Anna et al., 2009; Coutinho-Abreu et al., 2010; Telleria et al., 2012; Telleria, et al., 2021a; Telleria, et al., 2021b). Taking in account that gene silencing effect is temporary and can last maximum 72 hours, we decided to change the order to target the later stage of *Leishmania* cycle in the vector. We firstly experimentally infected the *P. papatasi* females with *L. major* and 3 days post infection we microinjected dsRNA in the females and silenced *defensin1* or *defensin1+2*. Thus, the silencing effect lasted across days 4 and 5 (when P. papatasi females defecates and parasites attach to midgut microvilli) till day 6 of infection when parasites migrate anteriorly and colonize the stomodaeal valve (critical part of infection for parasite transmission). qPCR showed that, the silencing of *defensin 1* or *defensin 1+2* led to significantly increased levels of *Leishmania* in comparison to control group (infected females injected with

control LacZ dsRNA). Moreover, we tracked the mortality rates of infected sand flies. In the groups with *defensin* silencing the mortality levels were significantly higher on day 6 compared to the control group. We have proven that suppression of *defensins* in *P. papatasi* favors *Leishmania* infection and negatively affect sand fly fitness (Vomáčková Kykalová et al., 2023 = PUBLICATION 2).

Given that sand fly larvae and pupae live in the microbe-rich environment with lot of detritus, such as animals burrows (Killick-Kendrick, 1999), we were also interested in expression profiles of immune-related genes during development and under different bacterial loads. *Phlebotomus papatasi* larvae were reared under two feeding regiments. One group was fed on regular laboratory food made by composed rabbit faces as a microbe-rich food. Second group was fed on the same food but sterilized by autoclaving referred as control. We follow the expression of the same genes as in adults during the development in guts of larvae fed on microbe-rich food. We found out that expression of *relish*, transcriptional factor of Imd pathway, was increased in early L4 stage, *attacin* was overexpressed in L3 and early L4 stage; *defensin*'s expression was increased in L3 stage and *dorsal* gene was not changed significantly. *Attacin* showed the most significant changes under higher microbe intake, especially in the voracious stages (L3 and early L4) which indicated that this AMP is necessary for controlling the bacterial loads in food (Kykalová et al., 2021 = PUBLICATION 1).

To understand more the expression of AMPs in *P. papatasi*, we decided to evaluate their association with immune pathway. We focused on the Imd pathway which has a sole transcriptional factor, relish. We used RNAi-mediated gene silencing of *relish* in carcasses and guts and follow the consequent expression of AMPs. We were able to reach the successful knockdown in carcasses but unfortunately not in guts where the expression was not reduced significantly. Future experiments using increased volume of dsRNA can result in more efficient silencing (just like we did with *defensin 1*). In the *relish*-silenced samples of sand fly carcasses, we reported significant downregulation of *attacin* and *defensin 2*. *Defensin 1* was not measured in carcasses samples because of its gut-specificity. In the gut sample with reduced *relish* expression, *attacin* levels were very variable and *defensin 2* was reduced but not significantly. Our findings showed the correlation between *relish* knockdown and suppression of AMPs expression in carcases, although this correlation was not confirmed in the guts, (Vomáčková Kykalová et al., 2023 = PUBLICATION 2) We consider that the *relish* correlation with AMPs may differ depending on the organs or tissues as it was previously published in other insect (Keshavarz et al., 2020).

Leishmania lipophospoglycan (LPG), the major surface glycoconjugate of the parasites, has several functions described during *Leishmania* cycle in both, vectors and hosts (Späth et al., 2003). We were interested to know how PAMPs, such as *Leishmania* LPG and bacteria LPS, modulate innate immune responses, more specifically expression of AMPs. We based our scientific approach on previous studies which shown that *Leishmania* infection triggered immune response while *L. longipalpis* reported increased expression of *attacin* and *defensin* after *L. infantum* infection (Telleria, et al., 2021b) and *P. papatasi* reported overexpression of *defensin* after *L. major* infection (Kykalová et al., 2021). Expression dynamics of 3 AMPs (*attacin, defensin 1, defensin 2*) in *P. papatasi* and *L. longipalpis* were analysed after artificial feeding with LPG molecules derived from *L. infantum* or *L. major* or LPS from *E. coli*.

Lutzomyia longipalpis attacin levels were significantly increased after all investigated challenges. LPG derived from naturally transmitted *L. infantum* led to overexpression of *attacin* at 48 hours post feeding as well as LPG from the non-naturally transmitted *L. major* parasites. Differently, LPS ingestion caused increase of *attacin* at 72 hours post feeding. *Lutzomyia longipalpis defensin 2* levels were increased after *L. infantum* LPG at 48 hours post feeding and 72 hours post LPS feeding and did not react to *L. major* LPG. Interestingly, *L. longipalpis defensin 1* did not show any changes after any of investigated challenges. Definitely, *attacin* gene reported the most changes in *L. longipalpis* after PAMPs ingestion. Similarly*, P. papatasi attacin* did show the most detectable modulation after PAMPs feeding. LPG derived from non-naturally transmitted *L. infantum,* and naturally transmitted *L. major* led to overexpression of *attacin* at 24 hours post feeding. Similar trend was observed after LPS feeding, but the increase was not significant. *Defensin 1* and *2* did not show any significant increase but they were variable after *L. major* LPG feeding (Vomáčková Kykalová et al.,2024 accepted for publication= PUBLICATION 3).

Our results indicate that *attacin* expression reacts quickly and universally to investigated PAMPs presence and may be part of the immediate defence. This study also investigated the effect of PAMPs on expression of gut proteins (mucin and galectin) and digestive enzymes (trypsin and chymotrypsin), but the results are not summarized here as they are not related to sand fly immunity.

Inter alia, Jak-STAT pathway can regulate iNOS molecule responsible for nitric oxide production (Kleinert et al., 2004). Nitric oxide has antileishmanial activity and therefore parasites developed mechanism how to supress *iNOS* expression in infected macrophages in

vertebrate hosts (Orsini et al., 2016). We investigated the role of Jak-STAT pathway in sand fly vector during *Leishmania* infection. This collaborative project with FIOCRUZ, Brazil, was done on *L. longipalpis* adult females and LL5 embryonic cell line. Firstly, we did measure Jak-STAT-related genes levels after *Leishmania* challenge in both study models. In LL5 embryonic cell line, an overexpression of *PIAS* and *PTP* (both negative regulators of Jak-STAT pathway) was observed 24 and 48 hours post challenge, meanwhile *SOCS* (third investigated negative regulator) did not show significant changes in expression. Interestingly, even expression of transcriptional factor of the pathway, *STAT*, was increased at 24 hours post challenge. Two more genes were investigated, *attacin* and *VIR-1* as a potential effector molecule downstream of the pathway. While *attacin* levels were not changed, *VIR-1* expression was significantly reduced at all investigated timepoints (12, 24, 48 hours post challenge). An increased expression of two out of three negative regulators indicates the suppression of the pathway as a result of *L. infantum* challenge with consequent suppression of downstream *VIR-1* molecule. On the other hand, increased expression of *STAT* indicates that there is no direct correlation of *PTP* and *PIAS* regulators with *STAT* expression. No similarities were observed when investigating the same genes in the adult females after experimental infection of *L. infantum*. None of the negative regulators (*PIAS, SOCS, PTP*) levels were changed, same as *STAT* transcriptional factor or *VIR-1*. The only gene reporting a significant change was *attacin* with downregulation at 72, hours post infection. We also added *iNOS* to investigated gene set but we did not report any change in expression after *Leishmania* infection.

The decreased expression of *attacin* suggests its linkage to another regulatory pathways, such as Toll or Imd. Using the knockdown approach of selected genes, we investigated the function of *PIAS* in LL5 cell line and *STAT* in adult females. The silencing of *PIAS* in LL5 cell line led to increased expression of *PTP* indicating a compensatory regulation between the repressors. *STAT*-silenced adult females infected by *L. infantum* reported a significant decreased expression of *iNOS* and *DUOX* in the carcasses together with increased levels of *Leishmania* in guts at 24 hours post infection. We conclude that STAT has its role in iNOS regulation in sand flies with a subsequent effect on parasite survival. (Telleria, et al., 2021a = PUBLICATION 4).

Finally, we invested efforts to establish a new sand fly's gene editing method in our laboratory. CRISPR-Cas9 editing system was selected for its versatility, and it has been used in multiple pathogen's vector studies (e.g. Hammond et al., 2016; Dong et al., 2018; Kyrou et al., 2018; Inbar et al., 2021; Chen et al., 2021) but it has only been once published in sand flies (Louradour

et al., 2019). Based on the progress done by our colleagues from LSHTM on *Drosophila* and sand fly's cell lines, we firstly tried a plasmid delivery system of gene editing. A *Drosophila* pDCC6 plasmid adapted for sand flies was used to deliver a sgRNAs together with Cas9 enzyme into embryos. As a second approach we decided for direct injection of sgRNAs and recombinant Cas9 enzyme following the protocol used previously by Louradour et al. (2019) With more than 14,000 injected embryos, knockout of multiple target genes was aimed during our experiments such as *L. longipalpis* olfactory genes (*Gr2, IR8a,* and *Orco*); genes coding for wing's properties (*rudimentary*, *vestigial*); *P. papatasi* genes coding for pigmentation (*ebony*, *cinnabar*) and an immune-related gene (*caspar*).

Only results pertaining to immune-related gene, *caspar*, were summarized in the supplementary file to this thesis due to its focus and unpublishable nature of the results. To increase the chances for gene knockout we simultaneously used 3-4 sgRNAs aiming various exons of the *caspar* gene promising the multiple gene's cut offs. None of the used approaches led to successfully detectable edited sand fly lines, but we were able to identify and partially solve many of the difficulties. For that reason, we think our work done on CRISPR-Cas9 editing system laid the first foundations for future experiments in our laboratory.

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