A rhamnose-binding lectin from *Rhodnius prolixus* and the impact of its silencing on gut bacterial microbiota and *Trypanosoma cruzi*

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Abstract

Lectins are ubiquitous proteins involved in the immune defenses of different organisms and mainly responsible for non-self-recognition and agglutination reactions. This work describes molecular and biological characterization of a rhamnose-binding lectin (RBL) from *Rhodnius prolixus*, which possesses a 21 amino acid signal peptide and a mature protein of 34.6 kDa. The *in-silico* analysis of the primary and secondary structures of RpLec revealed a lectin domain fully conserved among previous insects studied. The three-dimensional homology model of RpLec was similar to other RBL-lectins. Docking predictions with the monosaccharides showed rhamnose and galactose-binding sites comparable to Latrophilin-1 and N-Acetylgalactosamine-binding in a different site. The effects of RpLec gene silencing on levels of infecting *Trypanosoma cruzi* Dm 28c and intestinal bacterial populations in the *R. prolixus* midgut were studied by injecting RpLec dsRNA into the *R. prolixus* hemocoel. Whereas *T. cruzi* numbers remained unchanged compared with the controls, numbers of bacteria increased significantly. The silencing also induced the up regulation of the *R. prolixus defC* (defensin) expression gene. These results with RpLec reveal the potential importance of this little studied molecule in the insect vector immune response and homeostasis of the gut bacterial microbiota.

Key words: *Rhodnius prolixus*, *Trypanosoma cruzi*, microbiota, rhamnose-binding lectin, Latrophilin, defensin.

1. Introduction

Rhodnius prolixus, Stal, 1859 (Hemiptera: Heteroptera: Reduviidae), is a hematophagous insect during all stages of development in both sexes (Wigglesworth, 1974). Each molt process is associated with a single blood meal facilitating study of the insect's metabolism between each molt by the addition of biotic and abiotic factors during artificial feeding (Wigglesworth, 1974; Sant'Anna et al., 2017; Azambuja et al., 2017). *R. prolixus* is one of the main vectors of *Trypanosoma cruzi,* a protozoan parasite that infects seven orders of mammals (Araújo et al., 2009; Jansen and Roque, 2010). The epidemiological network includes humans, in which it causes Chagas' disease in almost 7 million people worldwide (Roque et al., 2008; Rengifo-Correa et al., 2017; WHO, 2019).

Like all other triatomine species, *Rhodnius. prolixus* also acquires *T. cruzi* by sucking blood from an infected mammal containing bloodstream trypomastigotes (Dias, 1934). Epimastigogenesis (Kessler et al., 2017) and erythrocytes lysis begin in the stomach (anterior midgut) of triatomines, which is considered a potentially hostile environment for *T. cruzi* survival. (Azambuja et al., 1989; Kollien and Schaub, 2000; Cortez et al., 2012; Ferreira et al., 2016). Initially, parasites begin colonizing the intestine (posterior midgut or small intestine) with the epimastigotes adhering to the perimicrovillar membrane and multiplying rapidly (Guitierrez and Burgos, 1978, Gonzalez et al., 1999; 2006). Subsequently, the flagellates reach the rectum, adhere to the wax surface of the epithelium, and undergo metacyclogenesis (Schmidt et al., 1998). The metacyclic

trypomastigotes are then released, together with feces and urine, during or after ~~the~~ subsequent blood feeding (Kollien and Schaub, 2000; Gonçalves et al., 2018).

In the *R. prolixus* gut, the parasite interact with components from the blood meal (Nogueira et al., 2015; Moreira et al., 2018), as well as with factors synthesized by the insect gut (Azambuja et al., 1999; Alves et al., 2007; Garcia et al., 2010; Waniek et al., 2011; Vieira et al., 2016), and also the constituents from the vector gut microbiota (Azambuja et al., 2004; Castro et al., 2012; Diaz et al., 2016; Teotônio et al. 2019). Antimicrobial peptide activities during infection have also been reported in the *R. prolixus* gut, as well as the abundance of the respective mRNAs trancripts (Lopez et al. 2003, Ursic Bedoya et al., 2007; Vieira etal., 2014; Salcedo-Porras et al., 2019). For example, Vieira et al. (2016) showed that *T. cruzi* infection enhances defensin C gene expression in the triatomine midgut and a decrease of some cultivable microbiota species.

Little is known about the role of lectins present in the *R. prolixus* gut and their influence on physiological processes and their interaction with microorganisms (Pereira et al., 1981; Mello et al., 1996). It has been shown that agglutination of *T. cruzi* epimastigotes by *R. prolixus* gut extracts depends on the parasite strain which in turn may be related to variations in the membrane sugar composition of these strains (Mello, 1996; Araújo et al., 2002). In addition, a lectin has been previously purified from the hemolymph of *R. prolixus* whose agglutination was inhibited by various galactose-type sugars (Ratcliffe et al., 1996). This agglutination activity is apparently involved in interaction with trypanosomatids infecting the insect (Mello et al., 1995, 1996). The lectin was also shown to activate mobilization and aggregation of hemocytes in response to incubation with *Trypanosoma rangeli in vitro* (Mello et al.,

1999). A sequence of 16 amino acid residues of the amino terminus of this *R. prolixus* lectin (RpLec) was previously obtained (Mello et al., 1999), but its presence in the *R. prolixus* gut as well as any site of synthesis remained undetermined.

In the present work, the full sequence of RpLec was identified by alignment with the sequences deduced by nucleotide sequencing obtained from the *R. prolixus* digestive tract transcriptome (Ribeiro et al., 2014). This information was used to characterize RpLec as a rhamnose-binding lectin (RBL) with the 3D conserved structure then compared to other proteins with RBL carbohydrate recognition domain (RBL-CRD), such as sea urchin egg lectin (SUEL) and Latrophilin, a type of adhesion G-protein-coupled receptor (GPCR) (Tateno et al., 2002a, b.). In addition, the expression of the RpLec gene in the stomach, intestine and fat body of *R. prolixus* infected with *T. cruzi* was quantified. Finally, RpLec gene expression was silenced to evaluate the effects on numbers of *T. cruzi,* on the bacterial microbiota, and on the synthesis of defensin C in the *R. prolixus* gut.

2. Materials and methods

2.1. Trypanosoma cruzi maintenance

Trypanosoma cruzi Dm 28c (TcI) epimastigotes were grown in McNeal, Novy and Nicolle medium with a liver infusion tryptose overlay supplemented with 10% fetal calf serum (Chiari and Camargo, 1984). For insect infection, parasites near the end of the exponential growth phase were washed in phosphate-buffered saline (10 mM sodium phosphate buffer, plus 0.15 M sodium chloride, pH 7.2) and then diluted in citrated,

heat-inactivated-plasma plus rabbit erythrocytes. For all *R. prolixus* infection experiments, parasites were adjusted in the blood meal to 1 x 10^6 parasites/ml.

2.2. Insect feeding and dissection

Fifth instar nymphs were used in all experiments. Approximately two weeks after molting, these insects were fed on blood, or blood containing parasites prepared as described above, using an artificial membrane feeding apparatus (Azambuja and Garcia, 1997). The stomach, intestine and fat body were used in experiments and for each sample, three replicates were prepared (n=3), each one containing tissues of five nymphs. Bled insects were dissected carefully by dorsal excision of the abdomen under a stereo microscope using fine forceps. The samples were briefly washed in 0,9% NaCl and remnants of other tissues (as tracheae and Malphigian tubules) removed for facilitating excision of the entire digestive tract. The body cavity was then extensively cleaned to remove other tissues or remnants of cuticle and washed again in saline to avoid contamination. The fat body adhering on the cuticle inner face was carefully collected and, as with the other tissues used, immediately frozen in liquid nitrogen and stored at -70°C until use.

2.3. Identification, phylogenetic similarities and structural analyzes of RpLec

The RpLec sequence (Mello et al., 1999) was submitted to VectorBase (https://www.vectorbase.org/) to obtain the complete DNA sequence for the gene encoding this protein. Proposed gene names and putative functional annotations were also adopted from the database (Giraldo-Calderón et al., 2015).

Sequence identity was assessed with BLAST X at the web servers of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1997). The deduced lectin amino acid sequences were aligned using ClustalW version 2.1 (Larkin et al., 2007). Predicted signal-peptide cleavage sites were calculated using SignalP version 3.0 (Petersen et al., 2011). The predicted isoelectric points and molecular masses were determined with the Compute pI/MW tool on http://www.expasy.org/tools, whereas secondary structure prediction was calculated by using "Jpred4" designed by Drozdetskiy et al. (2015) and available at http://www.compbio.dundee.ac.uk/jpred/.

Protein similarity was also analyzed by comparison with sequences deposited in the Uniprot database (https://www.uniprot.org/), aligned by the ClustalW method and then an array of distances was constructed. For this array, a phylogenetic tree was created using the Neighbor Joining Method within the Mega program parameters (Saitou and Nei, 1987, Kumar et al. 2018). The tree was built using only insect vectors of important human pathogens and limited to seven dipteran (holometabolous) species and seven hemipteran (hemimetabolous) proteins with the highest similarity indices.

The initial homology models of RpLec were built using the Swiss-MODEL server and Swiss-PDB viewer programs (http://swissmodel.expasy.org/and http://www.expasy.org/spdbv/), as described by Wermelinger et al. (2009). The crystal structure of a rhamnose-binding lectin, SUEL-I, from the toxopneustid sea urchin *Toxopneustes pileolus* (PDB 5H4S) was used as a template with the highest degree of identity to RpLec (41.97%; Hatakeyama et al., 2017).

The structure derived from homology modeling was submitted to the validation process, using the QMEAN scoring function (Benkert et al., 2011) and the PROCHECK program (Laskowski et al., 1993). The 3D alignment between the RpLec homology model and the Gal lectin domain of mouse Latrophilin-1 GPCR NMR structure (PDB 2jx9) was performed with the Swiss-PDB viewer program and additional interactions sites were predicted using a 3DLigandSite program (Wass et al., 2010). The electrostatic potential map (MEP) prediction was also produced in the Swiss PDB viewer program and generated in the range from -1.6 kT/e (deepest red color) < 0.0 kT/e (white) < 1.6 kT/e (deepest blue color) superimposed onto a molecular surface of constant electron density of 0.002 e/au3. Each point of the three-dimensional molecular surface map expresses the electrostatic interaction energy value evaluated with a probe atom of positive unitary charge, providing an indication of the overall molecular size and location of attractive (negative) or repulsive (positive) electrostatic potentials shown in red and blue, respectively (Guex and Peitsch, 1997).

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted using an SV Total RNA Isolation System (Promega, Madison, WI, USA) following the manufacturer's instructions. Each sample was eluted with 60 µl of ultrapure nuclease-free water. RNA concentrations were measured on a Nano-Drop2000 photometer (Thermo Fisher Scientific, Waltham, MA, USA). For cDNA synthesis, 0.5-1 µg of total RNA and the GoScript™ Reverse Transcription System (Promega) were used following the manufacturer's instructions. All cDNA samples used for quantitative real-time PCR in the present study were diluted to 5 ng/µl.

2.5. Real-Time quantitative PCR (qPCR)

Real-Time quantitative polymerase chain reactions (qPCR), to determine the relative abundance of RpLec cDNA 6 and 12 day after feeding, were performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with a PDTIS/FIOCRUZ Real-Time PCR Platform RPT-09A. Oligonucleotide primers for the gene encoding the lectin were designed using the Primer3/BLAST primer designing tool (Primer3/BLAST) on the NCBI homepage (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Specific primers (calibrators and targets) used in the present study are listed in Table 1. Each sample was analyzed in duplicate and the mean value calculated. The final volume in each well was 20 µl and contained 10 ng cDNA, primer pairs (0.25 µM) and GoTaq® qPCR Master Mix (Promega). The qPCRs were carried out under the following conditions: initiation step of 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min and a final melting curve analysis. Negative controls included for each target did not contain the cDNA template. Transcript abundance was analyzed by the ΔΔCt method (Livak and Schmittgen, 2001) and normalized with α-tubulin and GAPDH encoding genes (Paim et al., 2012). Data were analyzed by the Expression Suite software, version 1.1 (Thermo Fisher Scientific).

2.6. Double-strand RNA (dsRNA) synthesis and silencing

Linear templates for synthesis of β-*lactamase* control (Takekata et al., 2012) and RpLec target dsRNAs were amplified by PCR using oligonucleotides (Table 1) containing T7 promoter regions at the 5'-ends. PCR conditions were as follows: initiation step of 94°C for 10 min, 35 cycles of 94° for 25 sec, 55°C for 30 sec and 72°C for 45 sec. Primer specificity was verified by separating the PCR-product on GelRed™ (Biotum, CA, USA)

stained 1% agarose gels. Amplicons which contain T7-promoters at each 5' end were purified from the PCR-reaction using a NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). dsRNA was synthesized with the T7 RiboMAX™ Express RNAi System (Promega) following the manufacturer's instructions. Subsequently, the dsRNA was purified using the NucleoSpin® RNA kit (Macherey-Nagel, USA). In addition, the dsRNA quality was verified by agarose electrophoresis, as described above. Insects were inoculated into the coxal membrane of the mesothorax using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific, PA, USA). In preliminary experiments, insects were inoculated with 1.5 µg and 3 µg (inoculation of 1.5 µg on two consecutive days) to test the most efficient quantity to use. The inoculation of 3 µg caused a high mortality rate; therefore 1.5 µg of dsRNA was used in all further experiments. Blood supply was offered one day after inoculation, as it ~~was the procedure that~~ led to lower non-specific mortality due to the leakage of hemolymph or stomach contents. Then, for the *T. cruzi* experiments, insects were inoculated with dsRNA ~~and~~, followed by an infected blood meal the following day. Subsequently, the intestines were excised 7 and 21 days after feeding (DAF) with three biological replicates prepared containing tissues of five insects. Vieira et al. (2014, 2015, 2016) also used 7 DAF in analyzes of trypanosomes infection, antibacterial activity and DefC expression in R. prolixus and 21 DAF was also used by Dias et al.(2015a), to be near the end of the 5th instar just prior to molting. To analyze the impact of RpLec silencing on *T. cruzi* and intestinal bacteria their relative abundance was quantified by RT-qPCR using the universal primers *16SrDNAfwd*/*16SrDNArev* (Soares et al., 2015) and *Tc18SF*/*Tc18SR* (Table 1) and the amplification protocol shown above. Analysis was undertaken of the cDNA obtained from ~~the intestine,~~ the intestinal region~~,~~ in which

both the parasites and bacteria are abundant. Oligonucleotides used for the analysis of *T. cruzi* were designed, as described above, using the 18S rRNA sequence of the Dm28 strain (GenBank accession no. AF245382.1) and tested accordingly.

3. Results

3.1. Identification of the RpLec DNA sequence

The search using the 16 amino acid sequence of the amino terminus of RpLec showed an identity of 81.25% with the *R. prolixus* protein RPRC011880 (Table 2). The analysis of the amino acid sequence showed that RPRC011880 (named RpLec in the present work) is a rhamnose-binding lectin (RBL) encoding gene of *R. prolixus* with an open reading frame of 1008 nucleotides encoding a protein of 336 amino acids. The nucleotide sequence revealed a 72 nucleotide 5'-UTR that precedes the putative translation start site and a 32 3'-UTR including the stop codon (TAA).

The deduced protein sequence of RpLec possesses an N-terminal signal peptide cleavage site between Gly21 and Glu22. The predicted theoretical molecular mass of the mature molecule is 34.6 kDa with a theoretical isoelectric point of 8.09. In the deduced amino acid sequence and secondary structure of RpLec, there are conserved α-helix and β-sheet structures, fourteen cysteine residues, presumably forming seven disulfide bridges, and also tandem repetitions of the domains and consensus sequences YGR (Tyr-Gly-Arg), DPC (Asp-Pro-Cys) and KYL (Lys-Tyr-Leu) (YGR, DPC, KYL) characteristic of rhamnose-binding lectins, such as Latrophilin-1 from mice (Figs. 1, 3B) (Hosono et al., 1999; Varonakis et al., 2008; Carneiro et al., 2015). The sequence analysis revealed that RpLec is more similar to the lectins of the hemipteran,

Halyomorpha halys, of the coleopteran, *Aethina tumida,* and of the A chain of *Mus musculus* Latrophilin, than to the hymenopteran, *Cephus cinctus* (Fig 1, 3B).

3.2. Phylogenetic and structural analyzes of RpLec

The search for insect vector proteins with RBL-CRD was carried out at the UniProt website. In this server, putative protein sequences are also deposited and deduced from transcriptome or genomics analyzes, many of which have yet to be fully characterized. The amino acid sequence of RPRC011880A/RpLec, showed 100% homology with the proteins T1I6F9 and R4G4Z0 deposited in UniProt. The first was obtained by a genomic analysis and is still uncharacterized. The other, R4G4Z0, was the one identified in initial analysis, and had been derived from the digestive tube transcriptome of *R. prolixus* and characterized as a putative galactoside-binding lectin (Ribeiro et al., 2014).

The phylogenetic tree was built with fourteen proteins from insect vectors, including seven proteins selected from different species of holometabolous insects with the highest identities with RpLec and seven from hemimetabolous vectors. The theoretical tree showed two main branches separating the seven proteins of the different species of the order Diptera (holometabolous) from the seven proteins from five species of vectors from the order Hemiptera (hemimetabolous), among them RpLec/R4G4Z0 (Fig. 2). R4G4Z0 with 100% homology with RpLec was characterized as a putative galactoside-binding lectin by Ribeiro et al., (2014). For the hemipteran branch, *Triatoma infestans* and *R. prolixus* contributed two proteins each. Although all fourteen proteins in the tree contain RBL-CDB sequences in their structures, in the

database some were uncharacterized (5) or characterized as galactoside-binding proteins (4); RBL (2); SUEL (2) or Latrophilin (1) (Fig. 2).

The homology model of RpLec showed an overall structure with a unique distribution of amino acids (acid, basic, polar and non-polar) that leads to an electrostatic distribution with negative and positive regions (Fig. 3A). This biochemical feature is characteristic of proteins that present several binding abilities such as lectins (Abreu et al., 2006; Chikalovets et al., 2020) and enzymes (*eg*. thrombin) (Castro et al., 2011; Bond, 2019).

The overall 3D-fold of RpLec has a β-sandwich structural arrangement with two antiparallel sheets composed of amino acids 21-270 (β1), 29-35 (β2), 40-50 (β3), 84-90 (β4) and 104-113 (β5), enclosing the hydrophobic core (Fig. 3B, 4A). The three carbohydrate- binding domains of RpLec protrude outwards (Fig. 4A, B). Each domain consists of approximately 100 amino acids residue, with a secondary structure distribution of five β strands, a single long α-helix, and one small helical element. The rhamnose recognition domains of RpLec are structurally similar and the superimposition with conserved domains of Latrophilin (PDB 2JX9) showed the same molecular environment for binding carbohydrates located on an exposed pocket primarily formed by loop2 (Fig. 3B).

Docking evaluation of the lectin-L-rhamnose complex revealed first the carbohydrate-binding site of domain 1 of RpLec. In domain 1, bound L-rhamnose forms hydrogen bonds with Glu27, Arg50, Asn92, Asp97 and Lys 104 at the 2-, 3-, and 4-hydroxy groups (Figs.3B, 4A insets). The L-rhamnose also binds in domains 2 and 3, thus leading to a 1:3 ratio complex (1 lectin to 3 rhamnoses). The *in-silico* analysis also revealed domain

1 and 3 of RpLec exclusive interaction of Asn92 with rhamnose that is not present in domain 2 and in the Latrophilin binding complex structure (Fig. 4A).

The interaction predictions calculated with the 3DLigandSite software reinforced the previous literature data that reported that RpLec also interacts with β-D- galactose (β-D-Gal) and N-Acetylgalactosamine (GalNAc) (Fig. 4 insets). Since the RpLec binding site was similar to those of rhamnose-binding lectin CSL3 (PDB 2ZX4), and mouse Latrophilin-1 GPCR Gal-lectin domain (PDB 2JXA), two docking complexes using the RpLec homology model and these carbohydrates were constructed. The first, confirmed that β-D-Gal had the same L-rhamnose-binding site, while the second revealed a different GalNAc-binding site of RpLec comprised of the N-terminal site and a long α-helix of RpLec domain 1 (Fig. 3A, B).

3.3. RpLec transcript abundance in infected and non infected R. prolixus tissues

After feeding of *R. prolixus* 5th instar nymphs with blood containing *T. cruzi* or blood alone, the transcript abundance of the gene encoding RpLec was analyzed in the stomach, intestine and fat body tissues (Fig. 5A-C). There was no significant difference in the RpLec transcript abundance between *T. cruzi* infected and non - infected *R. prolixus* tissues. In the stomach ($p < 0.001$, $p < 0.01$) and intestine ($p < 0.05$, $p < 0.01$), the RpLec transcript abundance was significantly greater 12 DAF in both infected and not infected insects and about 1-fold higher in the stomach and 3-fold in the intestine tissues, respectively (Fig. 5A, B). Comparing stomach, intestine and fat body of uninfected insects (Fig. 5D), RpLec transcript abundance was significantly higher in the intestine than stomach 6 DAF ($p < 0.05$) and in the stomach than fat body at 12 DAF (p

< 0.05). Except in the stomach at 12 DAF (ca. 2.5-fold higher than 6 DAF), the lectin transcript abundance was comparable in the analyzed control tissues (Fig. 5D).

3.4. Silencing of the RpLec encoding gene by dsRNA inoculation

After inoculation of control and target dsRNAs into *R. prolixus,* the transcript abundances of the RpLec encoding mRNA, and the relative *T. cruzi* and bacterial loads were analyzed by qPCR in the insect intestine tissues (Fig. 6). In both 7 DAF (ca. 32-fold) and 21 DAF (ca. 14-fold) the RpLec encoding mRNA was significantly ($p < 0.00001$ and $p < 0.05$, respectively) reduced compared to the negative control (Fig. 6A, B). *T. cruzi* rRNA transcript abundance was slightly, but not significantly, higher at 7 DAF than at 21 DAF when it was 1-fold lower (Fig. 6C, D). The relative bacteria load was about 4-fold higher than the negative control, but not significantly, at 7 DAF in the lectin dsRNA inoculated insects. In insects at 21 DAF, however, bacterial rRNA was significantly higher ($p < 0.05$) in lectin dsRNA inoculated *R. prolixus* 5th instar nymphs (Fig. 6E, F) compared with the controls. Analysis of the *R. prolixus* defensin C gene (*defC*) showed no significant difference in transcript abundance between insects in which RpLec was silenced and the negative controls bugs at 7 DAF whereas at 21 DAF the *defC* gene expression was significantly ($p < 0.01$, 3.5-fold) upregulated (Fig. 7A, B).

4. Discussion

Lectins are ubiquitous molecules found in many plants and animals, and responsible for various functions in immunity such as opsonization of non-self cells or molecules by binding to exposed glycans (Macedo et al., 2015; Xue et al., 2015; Wang et al., 2018;,

Xia et al., 2018). Some lectins also have antibacterial activity by forming pores in bacterial membranes (Mukherjee et al., 2014; Arasu et al. 2017; Procopio et al., 2017). In the present study, the lectin originally isolated from the hemolymph of *R. prolixus* (RpLec) (Ratcliffe et al., 1996) was identified as a rhamnose-binding lectin (RBL) since it has the conserved motifs, YGR, DPC and KYL, commonly found in such molecules (Terada et al., 2007; Carneiro et al., 2015). Previously, RBLs have been described mainly in echinoderms and fish but are also present in ascidians and other invertebrates including annelids and bivalves (Gasparini et al., 2008; Ballarin et al., 2013). Whereas they are typically conserved with eight cysteine residues forming four disulfide bridges (Ballarin et al., 2013; Cammarata et al., 2014; Carneiro et al., 2015), RpLec has additional cysteine residues that may be important for the lectin native conformation.

Rhamnose binding lectins bind L-rhamnose and α-galactose with no need for Ca^{2+} or thiol clusters for their haemagglutinating activity (Carneiro et al., 2015). Recognition of carbohydrates containing galactose residues has already been demonstrated by RBLs from other invertebrates including sea urchins (Carneiro et al., 2015; Hatakeyama et al., 2017) and fish (Cammarata et al., 2014). Originally, RpLec was purified by affinity chromatograph using a galactose column and then classified as a galectin-like hemagglutinin (Ratcliffe et al., 1996). Previously, the RpLec hemagglutination activity was shown to be inhibited by D - (+) - galactose, lactose, and D - (-) – galactosamine (Ratcliffe et al., 1996).

In the present analysis, RpLec tertiary structure was classified as a tandem repeat-type protein containing two or three tandemly repeated CRDs in a single polypeptide, similar to SUEL-related lectins from fish eggs (Tateno, 2010). Similar to other members

of the RBL group, RpLec domains have three conserved sequences related to the lectin biological activity (YGR, DPC and KYL) (Tateno et al., 2010). The *in-silico* comparison of rhamnose and galactose interactions with RpLec revealed L-rhamnose with more interactions than galactose and similar to Latrophilin-1 and SUEL-lectins (Vakonakis, 2008). Also, the docking data of RpLec with GalNAc indicated another binding site, so that additional functional interactions can be possible. Indeed, in mice, Latrophilin has additional roles in adhesion and is considered the prototype molecule for the G-protein coupled receptor family and may be involved in angiogenesis (Serban et al., 2015). This RpLec structural data may assist in exploring the evolution and biological roles of these proteins in different insect species.

Database (UniProt) analyses showed that the RBL-CRDs are found in bacteria, plants, invertebrates and vertebrates, including humans. These recognition domains are present also in amino acid sequences of insect vector proteins, some have already been characterized such as RBLs, SUEL-type or galactoside binding proteins (Ribeiro et al., 2014; Tateno et al., 2010), but the majority, deposited in databases, are classified as uncharacterized proteins. In the phylogenetic tree (Fig 2), only two proteins had been described as RBL (A0A161N012 and AOA224XD11), identified from the transcriptomes of *Triatoma infestans* (Calderón-Fernandés et al., 2017) and *Panstrongylus lignarius* (Nevoa et al., 2018).

These proteins with RBL-CRD may be freely circulating, anchored directly to membranes or on their surfaces. They are involved in adhesion and recognition, with roles in innate immunity and other biological processes (Watanabe et al., 2009; Franchi et al., 2011; Ballarin et al., 2013; Monk et al., 2015). The role of these proteins has been neglected in research on insect immunity, parasite/vector interactions (King,

2020) and methods for vector-borne disease control using bacterial microbiota (Huang et al., 2020).

The silencing of RpLec in the present study did not significantly alter the parasite load in the intestine of *R. prolixus,* and apparently it has no crucial role in the initial development of *T. cruzi* in *R. prolixus*. A longer term study might reveal a negative effect of RpLec silencing as there is a slight, although not significant, decrease in the parasite numbers at 21 DAF. Allen et al. (2013) have described L-rhamnose as a component of a gp72 phosphoglycan in the *T. cruzi* epimastigotes flagellar attachment zone, in low abundance and only at this stage of parasite development. Consequently, RpLec could be involved in parasite attachment to the intestinal wall (Allen et al., 2013), but the marginal effects of silencing~~e~~ indicate that this is not a crucial aspect of the infection cycle. However, future studies could evaluate if RpLec silencing would induce any effect on the develompment of *T. cruzi* strains for which the insect is refractory to infection (Azambuja et al, 1989; Mello et al., 1996; Araujo et al., 2014). The role of lectins in bacterial control has been extensively demonstrated in many vertebrates and invertebrates, including insects (Zhan et al., 2016). The function of lectins in the recognition of non-self, agglutination and lysis of bacteria has also been widely reported (Wang et al., 2012; Zhang et al., 2014; Dias et al., 2015b; Zhan et al., 2016). Thus, one explanation for the recorded increases in bacteria and ~~the~~ *defC* expression in the *R. prolixus* intestine after RpLec gene silencing is that the RpLec has important antibacterial activity and that silencing reduces this ability so that the bacteria can develop unhindered. The increase in the *defC* expression could be the insect's response to the extra bacterial peptidoglycan and polysaccharides present in the gut and which compensates for the loss of the RpLec defensive role. There is

however, strong evidence that RBLs are multifunctional with an important role in the control of bacteria by agglutination and growth inhibition (Ma et al., 1997; Tateno et al., 2002a, b; Ballarin et al., 2013; Camarata et al., 2014; Ng et al., 2014).

For example, Ng et al. (2014) showed in horseshoe crabs, *Tachypleus tridentataus,* that a recombinant RBL not only binds to *Pseudomonas aeruginosa* PAO1 to inhibit growth, but also to medically important strains of *Streptococcus pneumoniae* and *Klebsiella pneumonia.* The binding was specific and mediated by interaction with the pathogen associated molecular patterns (PAMPS) on both Gram-positive and -negative bacterial surfaces (Ng et al., 2014). These PAMPs include capsular or lipo - polysaccharides and lipoteichoic acid with L-rhamnose or D-rhamnose present in the polysaccharides of Gram-positive or Gram-negative bacteria (Mistou et al., 2016; Mishra and Balaji, 2019). Rhamnose is present in all plants, is uncommon in invertebrates and vertebrates but is a common sugar in the cell wall and capsule of many bacteria. The *R. prolixus* RBL could therefore represent a unique and specific antibacterial factor previously only reported in one other arthropod, the horseshoe crab, *T. tridentatus* (Ng et al., 2014).

The enhanced expression of *R. prolixus defC* following RpLec silencing, may represent the insect's response to the increase in the bacterial population of the gut. Some microbiota bacteria, however, may be resistant to this host peptide, increasing only due to the reduction of competing bacteria in the same environment. There is evidence that invading pathogens/parasites in the gut interact with AMPs and the microbiome and can alter the habitat (eg. pH), and favor the growth of alternative bacterial species and the upregulation of AMPs (Manko et al., 2017; Pero et al., 2019). Thus, for diverse gut pathogens as *Helicobacter pylori* and *Giardia duodenalis,* the microbiomes are modulated and there is an enhanced production of defensin (Manko

et al., 2017; Pero et al., 2019). These are similar immune response reactions to those recorded after *T. cruzi* infections in *R. prolixus* with the parasite apparently unaffected by changes in gut microbiota and defensin gene expression (Castro et al., 2012; Vieira et al., 2016). The upregulation of *R. prolixus defC* following silencing of RpLec may not be just a response to the enlarged gut-bacteria population but be related to the multifunctional roles of defensins. In many vertebrates, they are associated with the gut epithelium and to the regulation of the microbiome and guard against inflammation and disease (Sankaran-Walters et al., 2017; Meade and O'Farrelly, 2019). Silencing of genes encoding c-type lectins in other insects significantly decreases ~~the insect's~~their immune response (Eleftherianos et al., 2006, Schnitger et al., 2009). The increase of the bacterial population in the present study might indicate a similar role of RpLec in the intestinal tract. The positive regulation of defC seems to contradict this assumption, which, however, cannot be completely ruled out. Unfortunately, there are no previous studies on the effects of silencing RBLs, although RNAi against the expression of the latrophilin gene (with a RBL carbohydrate recognition domain like RpLec) in *Tribolium castaneum* disrupted the development and reproduction of the insects and suppressed the IMD pathway (Gao et al., 2017, 2018). In addition, the role of G-Protein coupled receptors, like latrophilin, in the mechanisms of innate immunity, the recognition of LPS, the balance of the intestinal microbiota and in the response to infection by pathogenic microorganisms has also been reported in insects and mammals (Pan et al., 2018; Watnick and Jugder, 2019). Thus, the effect of RpLec silencing on physiological processes and immune recognition pathways needs to be assessed in more detail in *R. prolixus.* Finally, it is also necessary to evaluate any non-specific silencing of non-target putative proteins with CDR /RBL sequences also

identified in the *R. prolixus* genome as RPRC011885, RPRC009089, and RPRC009090, but without any evidence yet of transcriptional activity.

In conclusion, a unique rhamnose-specific lectin, RpLec, has been identified for the first time from *R. prolixus,* and its structural characteristics have been described. The effects of silencing the RpLec gene on infection of the insect vector by *T. cruzi* **has** been determined and the possible role of this molecule in the insect host defenses should be studied further.

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Table 1: The oligonucleotides designed and selected during this study were used to quantify, by qPCR, the relative abundance of *R. prolixus* RpLec, *T. cruzi* 18s rRNA and bacteria 16S rRNA. Both α-tubulin and GAPDH encoding genes were used as controls. Primers were also developed for amplification of the template sequence and subsequent insertion of the T7 sequence for production of RpLec dsRNA.

	Primers*	**Sequence (5'-3')**	**Amplicon Size (pb)**	**References**
	To qPCR:			
1	*TUB For*	TTTCCTCGATCACTGCTTCC	129	Paim et al., 2012
	TUB Rev	CGGAAATAACTGGGGCATAA		
2	*GAPDH For*	GATGGCGCCCAGTACATAGT	111	Paim et al., 2012
	GAPDH Rev	AGCTGACGGGGCTGTTATTA		
3	*qLF1*	ATGAGTGCGTGCCAAGTACA	148	Present Paper
	qLR1	ACTCGGTTCCTCATAGGGCA		
4	*Tc18SF*	CAAGCGGCTGGGTGGTTATT	134	Present Paper
	Tc18SR	AGACCGAAGTCTGCCAACAA		
5	*16SrDNAfwd*	AGAGTTTGATCCTGGCTCAG		Soares et al. 2015
	16SrDNArev	CATGCTGCCTCCCGTAGGAGT		
6	*RPDEFC-F*	CAGTACAGTCCTAATACCTAGCC	136	Vieira et al., 2016
	RPDEFC-R	TGGGCATCATCTAATTGATGTTGAGAA		
	To dsRNA (T7 underlined):			
7	pGβlacmT7-F1	TAATACGACTCACTATAGGGAGACCACGTCGCCGCATACACTATTCTC	504	Takekata et al., 2012
	pGβlacmT7-R1	TAATACGACTCACTATAGGGAGACCACCTACGATACGGGAGGGCTTAC		
8	iLF1	GTAATACGACTCACTATAGGGAGGTGAGCAAAAGTCCTCCG	663	Present Paper
	iLR1	GTAATACGACTCACTATAGGGCACAAGGAGTAGCCAGGGTT		

* **Controls**: 1- *β-tubulin*, 2- *GAPDH*, 7- *β-lactamase*. **Targets**:3 and 8- *RpLec*, 4- *T. cruzi 18S rRNA*, 5- bacteria *16SrDNA*. 6- *R. prolixus DefC*

Table 2: Alignment of the RpLec nucleotide sequence (Mello et al. 1999) with the homologous protein RPRC011880 found in VectorBase. The sequence of RpLec begins at the first amino acid as it was obtained from the purified mature protein. In contrast, that of RPRC011880 starts at the 22nd amino acid, as it was deduced from a *R. prolixus* transcriptome with the signal peptide. The three divergent amino acids in the comparison are highlighted in bold.

Protein	Start(5')	**Amino acids**															End (3')
RpLec	1	E	Q	K	S	**T**	V	A	**A**	E	H	D	K	L	T	I	**I**	16
RPRC011880	22	E	Q	K	S	**S**	V	A	**C**	E	H	D	K	L	T	I	**S**	37
		*	*	*	*		*	*		*	*	*	*	*	*	*		

Figures Legends

Fig. 1. Sequence alignment and secondary structure prediction of *R. prolixus* rhamnose-binding lectin (RpLec) with different insect lectins using ClustalW (2.1) and JPRED4 designed by Drozdetskiy et al. (2015). It shows Identical (*) and conserved amino acids (:) whereas the conserved cysteine residues involved in disulfide bridges are boxed in cyan; the amino acids related to the helix secondary structure are in red and those for the secondary sheet structure are in green. Characteristic consensus rhamnose-binding lectin amino acid sequences (YGR, DPC, KYL) are boxed in yellow. GenBank accession numbers for the analyzed sequences: XP_014280987– *Halyomorpha halys*, XP_019866668 – *Aethina tumida*, XP_024940390 – *Cephus cinctus.*

Fig. 2. Phylogenetic tree analysis of RpLec (R4G4Z0) from *R. prolixus* and 13 proteins from different species of insect vectors (7 holometabolous and 5 hemimetabolous): *Anopheles stephensi*, *Anopheles darlingi*, *Phlebotomus papatasi*, *Culex quinquefasciatus*, *Aedes aegypti*, *Aedes albopictus*, *Glossina morsitans*, *Rhodnius prolixus*, *Rhodnius neglectus*, *Panstrongylus lignarius*, *Triatoma infestans* (2x) and *Triatoma dimidiata*. All proteins conserved the rhamnose binding lectin carbohydrate recognition domains (RBL-CRDs). The percentage of trees associated with this group tested by boostraping was shown near the branches. The scale bar indicates the number of amino acid substitutions per site. The tree was elaborated using the Neighbor Joining Method and the code to search and identify each protein is expressed in brackets after the name of each species of insect vector

Fig. 3. Theoretical analysis of the 3D model of RpLec. A) Distribution of the amino acids residues according to their chemical characteristics (acid - red, basic - blue, polar – yellow and non-polar - white) (upper) and electrostatic potential map (negative – red and positive – blue) (lower), B) Amino acid alignment and superposition model of the three carbohydrate recognition domains of RpLec (YGR - Tyr-Gly-Arg, DPC - Asp-Pro-Cys and KYL - Lys-Tyr-Leu) with lectin domain of mouse Latrophilin-1 (pink, PDB=2JX9). The inset shows the superposition of the rhamnose (orange) -binding domains of RpLec (domain 1, blue) and Latrophilin 1 (pink).

Fig. 4. Theoretical analysis of the 3D model of the recognition carbohydrate domains of RpLec. A) RpLec/L-rhamnose complex in secondary structure representation (alpha-helix - red and beta-sheet - green) with three L-rhamnoses interacting with the RpLec binding regions. The inset (CPK colors) shows the amino acids residues from the carbohydrate-binding site of RpLec (domain 1) interacting with L-rhamnose (orange). B) Alignment of complexes of RpLec with L-galactose (Gal - cyan) and N-Acetylgalactosamine (GalNAc – pink). RpLec is shown in secondary structure representation (alpha-helices - red and beta-sheets - green) whereas the ligands are in CPK. The insets show the residues from the RpLec carbohydrate-binding site that interact with galactose and GalNAc, respectively.

Fig. 5. Relative RpLec transcript abundance in A) stomach (ST), B) intestine (IN) and C) fat body (FB) tissues of *R. prolixus* 5^{th} instar nymphs at 6 and 12 days after feeding

(DAF) with blood containing *T. cruzi* (grey bars) or blood alone (white bars, negative control). (D) Comparison of RpLec transcript abundance in stomach, intestine and fat body tissues in *R. prolixus* 5th instars fed with blood only 6 (dotted bar) and 12 (striped bar) DAF. Significantly different samples are highlighted by horizontal bars (***p < 0.001, **p < 0.001, *p < 0.05).

Fig. 6. Relative transcript abundance of RpLec (A, B), or *T. cruzi* rRNA (C, D) or bacterial rRNA (E, F) in the intestine at 7 (A, C, E) or 21 (B, D, F) days after inoculation with RpLec (white columns, target) or β-lactamase (black columns, negative control - NC) dsRNA in *R. prolixus* 5th instar nymphs Significant differences of samples are highlighted by a horizontal bar (****p < 0.00001, *p < 0.05).

Fig. 7. Relative transcript abundance of *R. prolixus defC* RNA after inoculation with RpLec (target) or β-lactamase (negative control, NC) dsRNA in *R. prolixus* 5th instar nymphs. For the analysis, *R. prolixus* intestine tissues were used dissected 7 (A) or 21 (B) days after dsRNA inoculation. Significant differences of samples are highlighted by a horizontal bar (**p < 0.01).