



Research paper

Diversity of astacin-like metalloproteases identified by transcriptomic analysis in Peruvian *Loxosceles laeta* spider venom and *in vitro* activity characterization

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ARTICLE INFO

Article history:

Received 17 April 2019

Accepted 28 August 2019

Available online 30 August 2019

Keywords:

Loxosceles laeta

Transcriptome

Metalloprotease

Spider venom

Loxosceles astacin-like metalloproteases

LALP

ABSTRACT

Loxosceles spiders are found in almost all countries of South America. In Peru, *Loxosceles laeta* species is the main responsible for the accidents caused by poisonous animals, being known as “killer spiders”, due to the large number of fatal accidents observed. Astacin-like metalloproteases, named LALPs (*Loxosceles* astacin-like metalloproteases) are highly expressed in *Loxosceles* spiders venom gland. These proteases may be involved in hemorrhage and venom spreading, being relevant to the envenoming process. Thus, the aim of this work was to analyze Peruvian *L. laeta* venom gland transcripts using bioinformatics tools, focusing on LALPs. A cDNA library from Peruvian *L. laeta* venom glands was constructed and sequenced by MiSeq (Illumina) sequencer. After assembly, the resulting sequences were annotated, seeking out for similarity with previously described LALPs. Nine possible LALPs isoforms from Peruvian *L. laeta* venom were identified and the results were validated by *in silico* and *in vitro* experiments. This study contributes to a better understanding of the molecular diversity of *Loxosceles* venom and provide insights about the action of LALPs.

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1. Introduction

Accidents with venomous animals, such as spiders, scorpions and snakes, are considered clinical emergencies in several tropical countries [1]. *Loxosceles* spiders are distributed worldwide, with occurrences in the Americas, Africa, Europe and Oceania. Approximately 100 different species of this genus have been described in South America [2]. *Loxosceles laeta* species can be found in Brazil, Argentina, Chile and Peru. This species is responsible for the highest number of accidents caused by poisonous animals in Peru and are

known as “killer spiders” due to the large number of fatal accidents caused [3].

In Brazil, the Sistema de Informação de Agravos de Notificação (SINAN) is a repository of all envenoming cases reported to the government, including accidents with spiders of *Loxosceles* genus. In Peru, the Instituto Nacional de Salud also collects information about accidents with this spider. In other countries, such as Chile and Argentina, although there are many cases of envenoming by *Loxosceles*, a notification system is absent, and accidents are only reported in isolated epidemiological scientific articles. According to the Centro Nacional de Epidemiología, Prevención y Control Enfermedades, accidents with *Loxosceles laeta* almost tripled in Peru in 2016, reaching 1995 cases and 4 deaths in this year, while in Brazil, in the same period, SINAN notified 7465 cases, with 5 deaths. However, the accidents reported in Brazil can be caused by several

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Loxosceles species, including *Loxosceles intermedia*, *L. laeta*, *Loxosceles gaucho* and *Loxosceles similis*, and it is not possible to precisely quantify the number of cases specifically caused by *L. laeta*. In addition, differently from Brazil, the notification of envenoming by *Loxosceles* is not compulsory in Peru, which impairs the real cases quantification in this country.

Loxoscelism, the set of signs and symptoms caused by spiders of *Loxosceles* genus, occurs in two different forms: cutaneous and cutaneous-hemolytic. In most cases, envenoming victims develop the cutaneous loxoscelism, which is characterized by initial mild symptoms that can progress to necrosis at the bite site which detaches and leaves an ulcer of slow and difficult healing [4–7]. Cutaneous-hemolytic loxoscelism is observed in 1–16% of the cases, depending on the region of the bite. This loxoscelism form is commonly caused by *L. laeta* spiders (Heinecken & Lowe, 1832) and presents clinical manifestations related to intravascular hemolysis. Cases can progress to acute renal failure, loxoscelism main cause of death (1–3% of the cases) [8,9]. In Peru, cutaneous-hemolytic loxoscelism can account for as much as 26% of the cases [10]. In Brazil, cases of loxoscelism caused by *L. laeta* are concentrated in the South region, mainly in the Santa Catarina state. The cutaneous form is diagnosed in most patients, while cutaneous-hemolytic loxoscelism is observed in 13% of cases [11]. Although accidents caused by *L. laeta* bites in Peru and Brazil are theoretically provoked by the same species, differences in the clinical picture can be observed, with those occurring in Peru being generally more severe. Experimental studies also suggest that Peruvian *L. laeta* venom is indeed more toxic than its Brazilian equivalent [12].

Loxosceles spider venom is a complex mixture of proteic and peptidic toxins, with a predominantly low molecular weight profile ranging from 1 to 40 kDa [13]. This venom has been extensively studied and different toxins families have been identified, such as hyaluronidases, phospholipases D, serine proteases, insecticidal peptides and metalloproteases [14,15].

The presence of proteolytic enzymes within *Loxosceles* venoms have been previously acknowledged, being identified both serine [16] and metalloproteases [17]. *L. intermedia* venom metalloproteases were later identified as members of the astacin family [18]. *Loxosceles* astacin-like protease 1 (LALP1), from *L. intermedia* venom, was the first astacin to be described as a venom component. LALP family was further characterized by Trevisan-Silva et al. [19], and a second isoform, named LALP3, was later cloned and characterized [20]. Astacins have also been described as highly present in spiders' digestive fluids, with a role in extra-oral prey digestion, but sharing limited similarity with venom astacins [21–23].

Astacins are zinc-dependent metalloproteases, belonging to the metzincin superfamily. They are secreted as zymogens, with a relatively short pro-peptide. These proteases are characterized by the zinc-binding motif (HEXXHXXGXXH) and a conserved methionine residue beneath the metal active site within a Met-turn (SXMXY), with a tyrosine residue that might be involved in substrate fixation [24,25]. Astacins are found in diverse organisms, from bacteria to mammals, with different functions such as digestion, extracellular matrix processing, tissue differentiation, growth factor activation and general protein degradation [26,27].

LALPs have been reported to act upon matrix proteins, such as fibronectin and fibrinogen, and in particular, the basal lamina, degrading entactin and heparan sulfate proteoglycan [17,28]. Therefore, their role in hemorrhage, delayed wound healing, venom spreading through the intercellular compartment and vascular permeability caused by *Loxosceles* venom has been suggested [29]. LALP from *L. intermedia* venom has been previously associated to endothelial cell adhesion impairment [18], which can trigger cell death [30], contributing to the damage caused by *Loxosceles* venoms. However, the exact role of LALPs in *Loxosceles*

envenoming has not been completely disclosed.

Considering the severity of cases involving Peruvian *L. laeta* spiders and the lack of information concerning LALPs from this venom, these proteins were chosen as the focus of this study. Our main goal was to describe and characterize LALPs expressed on the venom gland of Peruvian *L. laeta* spiders using RNA-seq transcriptomic analysis, *in vitro* techniques and *in silico* tools.

2. Material and methods

2.1. Animals, venoms and antivenoms

New Zealand rabbits (4 kg) were obtained and maintained at Centro de Bioterismo of Instituto de Ciências Biológicas of Universidade Federal de Minas Gerais (UFMG) and received food and water *ad libitum*. Experimental protocols were performed after approval by the Ethics Committee in Animal Experimentation of UFMG (388/2017-CETEA/UFMG).

A cDNA library from venom gland transcripts was built from five female *L. laeta* spider. Spiders were fed three days before venom gland extraction. Glands were collected in Lima, Peru, and processed in the Laboratório de Biotecnologia e Marcadores Moleculares (ICB-UFMG). *L. intermedia*, *L. laeta* and *L. gaucho* venoms samples were provided by Dr. João Carlos Minozzo, from the Centro de Pesquisa e Produção de Imunobiológicos (CPPI) in Paraná, Brazil. Peruvian *L. laeta* venom was provided by Dr. Cesar Bonilla, from Instituto Nacional de Salud of Peru. Lyophilized samples were stored at –20 °C in the dark until use. Prior to experimentation, venom was solubilized in water and protein concentration was determined by the Bradford method using bovine serum albumin as standard [31].

Recombinant LALP1 used in the immunization protocol was provided by Dr. Silvio Sanchez Veiga from Universidade Federal do Paraná, Brazil.

2.2. Immunization protocol

An adult female rabbit was immunized with a recombinant form of LALP-1 (recLALP-1) expressed in *E. coli* in order to obtain anti-recLALP-1 antibodies. Pre-immune (PI) serum was collected and one week later the animal received an initial subcutaneous (s.c.) injection of 400 µg of recLALP1 in Montanide adjuvant. Three booster injections (s.c.) were administered at 2-weeks interval, using the same recLALP1 dose in Montanide adjuvant and blood sample was collected 10 days after the fourth dose for antibody titer analysis. After 17 days, a final dose (400 µg) of recLALP1 in Montanide adjuvant was administered and 10 days later blood sample was obtained.

2.3. Immunoassays

2.3.1. Indirect ELISA

Microtiter plates were coated with 100 µL of a solution containing 0.5 µg/well of either *L. intermedia*, *L. gaucho*, Brazilian *L. laeta* or Peruvian *L. laeta* venoms in 0.02 M sodium bicarbonate buffer, pH 9.6 and incubated overnight at 4 °C. After washing, the plate was blocked with 100 µL/well of a solution containing 3% of skimmed non-fat milk in Phosphate Buffer Saline (PBS) and incubated at 37 °C for 1 h. The plate was washed, and the anti-recLALP-1 serum produced was added in serial dilutions (1:50 to 1:6400) in 0.01% of milk-PBS in a final volume of 100 µL and incubated for 1 h at 37 °C. The plate was washed and the secondary antibody (anti-rabbit peroxidase, diluted 1:4000 in 0.01% of milk-PBS) was added in a final volume of 100 µL. After 1 h of incubation at 37 °C, the reaction was detected using peroxidase chromogenic substrate o-

phenylenediamine dihydrochloride (OPD) (Sigma) and incubated in the dark for 30 min. The reaction was stopped by adding 25 µL of 2 M H₂SO₄. Absorbance values were determined at 490 nm using an ELISA plate reader (BIORAD, 680 models). All assays were performed in triplicate. Means of the results from three independent experiments were calculated and plotted.

2.3.2. Electrophoresis and Western Blot

Samples containing 10 µg of *L. intermedia*, Brazilian *L. laeta* or Peruvian *L. laeta* venom were diluted in sample buffer under non-reducing conditions and subjected to SDS-PAGE on 15% polyacrylamide gels. After electrophoresis, protein bands were visualized by silver staining. For Western Blot, gel was electrotransferred to a nitrocellulose membrane overnight. To confirm protein transference, the membrane was stained with Ponceau and then washed with distilled water. The membrane was blocked with blocking buffer for 1 h, washed three times for 5 min with PBS-Tween 0.05% and incubated with rabbit serum anti-recLALP-1 diluted in blocking buffer (1:400) for 1 h and 30 min at room temperature. The membrane was washed three times with PBS-Tween 0.05% and anti-rabbit IgG conjugated to peroxidase in blocking buffer (1:2500) was added for 1 h at room temperature. After additional washes, blots immunoreactivity was detected using DAB/chloronaphthol substrate, according to manufacturer's instructions. Immunoreactive band density was calculated by image analysis using ImageJ 1.51.

2.4. Glycosylation detection

For the identification of possible protein glycosylation, a blot was performed using the same initial steps reported in the previous section, however, 20 µg of each venom was applied in the initial gel electrophoresis. After protein transfer, the membrane was saturated for 2 h by incubation with TBS (0.01 M Tris, 0.15 M NaCl, 0.3% Tween 20). Subsequently, the membrane was incubated for a further 2 h with Concanavalin A (ConA 50 mg/mL) lectin solution diluted in lectin buffer (TBS 0.05% Tween 20, 1 mM MnCl₂). The membrane was incubated with peroxidase in lectin buffer (50 µg/mL) for another 2 h and developed with H₂O₂ and 4-chloro-1, alpha-n-naphthol.

2.5. Enzymatic activities

2.5.1. Fibrinogenolytic activity

Proteolytic activity of Brazilian and Peruvian *L. laeta* venoms was determined by a fibrinogen (Fg) digestion assay. Fibrinogen was diluted at final concentration of 2.5 mg/mL in 25 mM Tris-HCl buffer containing 0.15 M NaCl (pH 7.4). Three micrograms of each venom were added to 0.5 mL of fibrinogen at a molar ratio of venom to substrate of 1/52 (w/w) and incubated for 16 h at 37 °C. At the end of this period, aliquots containing 50 µL of these samples were mixed with equal volume of denaturing solution (10 M urea, 4% β-mercaptoethanol, 4% SDS) at room temperature for another 16 h. Then, 20 µL of the sample was added to 20 µL of sample buffer under reducing conditions. After boiling for 5 min, 35 µL of that solution was applied in 10% polyacrylamide gel and stained with Coomassie blue.

A time-dependent fibrinogenolytic activity assay was also performed using the same samples and conditions previously described and the following incubation time points: 1, 2, 4, 6, 8, 10, 12 and 16 h. Each aliquot received 25 µL of denaturing solution and 35 µL of each sample was loaded into two 10% polyacrylamide gels. The density of fibrinogen bands was measured by pixel quantification using Image J 1.51 software in order to estimate degradation. Means of the results from two independent experiments were

calculated and plotted.

2.5.2. Gelatinase/collagenase activity

The ability of Brazilian and Peruvian *L. laeta* venoms to degrade gelatin was determined by fluorescence in 96-well microtiter plate using EnzChek Gelatinase/Collagenase assay kit by Invitrogen. In each well, 80 µL of reaction buffer (0.5 M Tris-HCl, 1.5 M NaCl, 50 mM CaCl₂, 2 mM sodium azide, pH 7.6) and 20 µL of DQ gelatin from pig skin conjugated with fluorescein were added. The negative control received additional 100 µL of reaction buffer and the positive control received 100 µL of Collagenase Type IV from *Clostridium histolyticum*. Serial dilutions of the aforementioned venoms (12–0.37 µg) were made in reaction buffer at a 100 µL volume and added to the wells. The microplate was incubated at room temperature and protected from light for 24 h. Fluorescence was measured at 495 nm for absorption and 515 nm for emission using Fluorescence Reader (Varioskan Flash Multimode Reader - Thermo Scientific). The assay was performed in duplicate. Means of the results from two independent experiments were calculated and plotted.

2.6. Bioinformatics analysis

2.6.1. Transcriptome sequencing

A cDNA library from Peruvian *L. laeta* venom glands was constructed using the TruSeq™ RNA Sample Prep Kit v3 Set A (Illumina) following the manufacturer's instructions. The library was quantified and applied to the sequencing cartridge after denaturing. Library sequencing was performed by MiSeq (Illumina) sequencer using two sequencing cartridges by the paired-end technique, generating 600 cycles (300 for reads 3'5' and 300 for reads 5'3'), which resulted in a fastq file containing the bases and values of raw reads.

2.6.2. Processing and assembling of reads

Raw sequence quality was evaluated using the FastQC program. Parameters such as reads total amount, GC content and variable length of the sequences were analyzed, allowing results improvement when necessary [32].

Reads with Phred quality score below 30 were trimmed using the program Trimmomatic by sliding window [33]. After trimming, a second sequence quality analysis was performed by FastQC to visualize the result and confirm the completion of this step.

Once the quality analysis presented satisfactory results, reads were assembled without the use of a reference genome, by a *de novo* assembly. This assembly was carried out on the Sagarana server of the Instituto de Ciências Biológicas of the Universidade Federal de Minas Gerais using Trinity software [34], in which assemblies with different values of k-mers (18–32) were used to define k-mer through the CLC Genomics Workbench 10 (<https://www.qiagenbioinformatics.com>), where they were also translated.

2.6.3. In silico sequences analysis

After assembling, the resulting sequences were annotated using the BlastP tool [35], seeking out for similarity with previously described LALPs. For this, "Loxosceles" and "metalloproteases" were used as search terms, in addition to sequence quality parameters, such as a minimum alignment size of 250 amino acids (corresponding to the mean size of LALP1), identity of 50% or more and a maximum e-value of 0.0001. The best quality sequences, which contained greater similarity to metalloproteases, were selected and analyzed for the presence of the essential elements of metalloproteases: zinc-binding motif (HEXXHXXGXXHE) and met-turn (MXY). In addition, to compare these sequences regarding similarity, identity and percentage of gaps, the Emboss Stretcher

(https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/) [36] program was used.

Alignment of the retrieved Peruvian *L. laeta* LALP sequences (LALP-LP - *Loxosceles* astacin-like proteases – *laeta* Peru) with astacins from *Astacus astacus* (Astacin-P07584), *L. intermedia* (LALP1-Li, LALP2-Li and LALP3-Li), Brazilian *L. laeta* (LALP4-Li), *L. gaucho* (LALP5-Lg), Brazilian *L. laeta* (LLAE), *Parasteatoda tepidariorum* (XP), *Nephila clavipes* (PRD), *Stegodyphus mimosarum* (KFM), *Tityus serrulatus* (CDJ) and *Centruroides sculpturatus* (XP_023232109.1) available from the Uniprot [37] (<http://www.uniprot.org/uniprot/>) database was performed by Clustal Omega program (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

For physicochemical analysis, the program Expasy-Protparam (<https://web.expasy.org/protparam/>) was firstly used [38]. SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) [39] was used for determining signal peptides and ProP program determined putative propeptides (<http://www.cbs.dtu.dk/services/ProP/>) [40]. NetNGlyc program (<http://www.cbs.dtu.dk/services/NetNGlyc/>) predicted N-glycosylation sites.

2.6.4. Phylogenetic analysis

A phylogenetic tree representing the possible evolutionary history of LALP-LPs was constructed using MEGA X [41], using the alignment of astacins from *Astacus astacus*, *L. intermedia* (LALP1, LALP2 and LALP3), Brazilian *L. laeta* (LALP4), *L. gaucho* (LALP5), Brazilian *L. laeta* (LLAE), *Parasteatoda tepidariorum* (XP), *Nephila clavipes* (PRD), *Stegodyphus mimosarum* (KFM), *Tityus serrulatus* (CDJ) and *Centruroides sculpturatus* (XP_023232109.1) using the maximum-likelihood model, with bootstrap of 1000 replicates.

2.6.5. Molecular modeling

The Protein Fold Recognition Server (Phyre2) program (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>) [42] was used to model LALP-LPs using astacin from *Astacus astacus* as reference [43]. After the modeling, the result was analyzed by the PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrodinger, LLC - <https://pymol.org/2/>) and Swiss-PdbViewer 4.0 (<https://spdbv.vital-it.ch>).

3. Results

3.1. Immunoassays

In order to verify the possible presence of LALPs in the Peruvian *L. laeta*, Brazilian *L. laeta*, *L. intermedia* and *L. gaucho* spiders venoms, a screening with anti-recLALP-1 rabbit sera was performed by ELISA, as shown in Fig. 1. This assay demonstrated high reactivity between the Peruvian *L. laeta* venom and anti-recLALP-1 serum, with significant statistical difference until 1:400 dilution, when compared to other venoms.

To verify whether the observed difference in the immunoreactivity between Peruvian and Brazilian *L. laeta* venoms was indeed due to the distinct content of LALPs, a Western Blot was performed using anti-recLALP-1 rabbit sera (Fig. 2). LiV (*L. intermedia* venom) was used as a positive control and pre-immune serum as a negative control (data not shown). Results showed an intense probing of protein bands with molecular weight around 24 kDa (the molecular weight range for LALPs), for all tested venoms (Fig. 2). Moreover, Peruvian *L. laeta* venom presented an additional band recognized by anti-recLALP-1, when compared to the Brazilian species venom, indicating that the same species from different geographical locations may present different content of LALPs or LALPs with different post-translational modifications. Densitometry analysis of the anti-rLALP1 recognized bands presented the following results: *L. intermedia*: 3922.012 px/area, Brazilian *L. laeta*: 6577.468 px/area and Peruvian *L. laeta*: 6910.882 px/area, confirming that Peruvian *L. laeta* venom seems to have more immunoreactive proteins with anti-rLALP1 sera.

We next analyzed if the extra immunorecognized band in Peruvian *L. laeta* venom was due to different glycosylation patterns. For that aim, a blot was performed using Concanavalin A lectin (ConA) solution, a reagent that binds specifically to glycidic motifs (Fig. 3). After probing the membrane containing the transferred proteins with ConA solution, although it was not possible to visualize two distinct bands as in the Western Blot assay, there was a more intense staining of protein bands with molecular weight between 20 kDa and 25 kDa in the Peruvian *L. laeta* venom than in the Brazilian *L. laeta* venom (Fig. 2C), which supports the hypothesis of higher glycosylation of LALPs in Peruvian venom, when compared to its Brazilian equivalent.

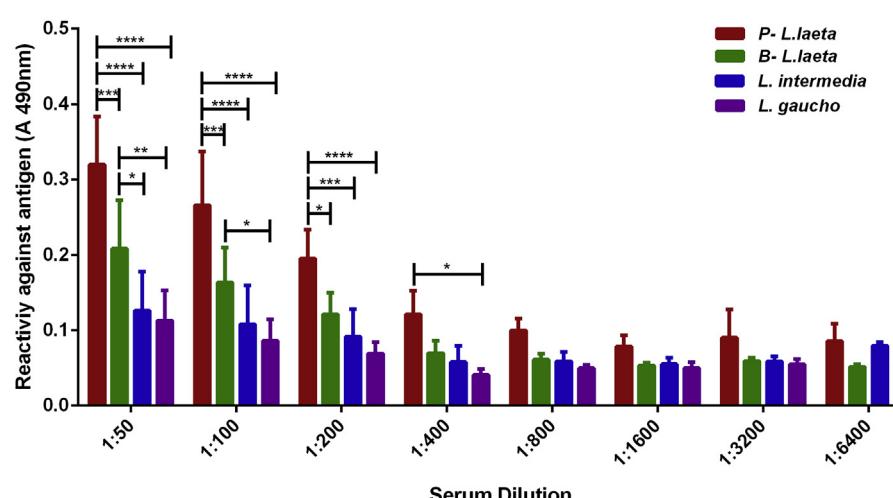


Fig. 1. *Loxosceles* venoms immunoreactivity against anti-recLALP1 serum by ELISA. The plates were coated with 0.5 µg/mL of venoms from either Peruvian *L. laeta*, Brazilian *L. laeta*, *L. intermedia* or *L. gaucho*. Anti-recLALP-1 rabbit serum was added in serial dilutions (1:50 to 1:6400). Samples absorbance was determined at 490 nm (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). Values shown represent means of the duplicates plus standard errors.

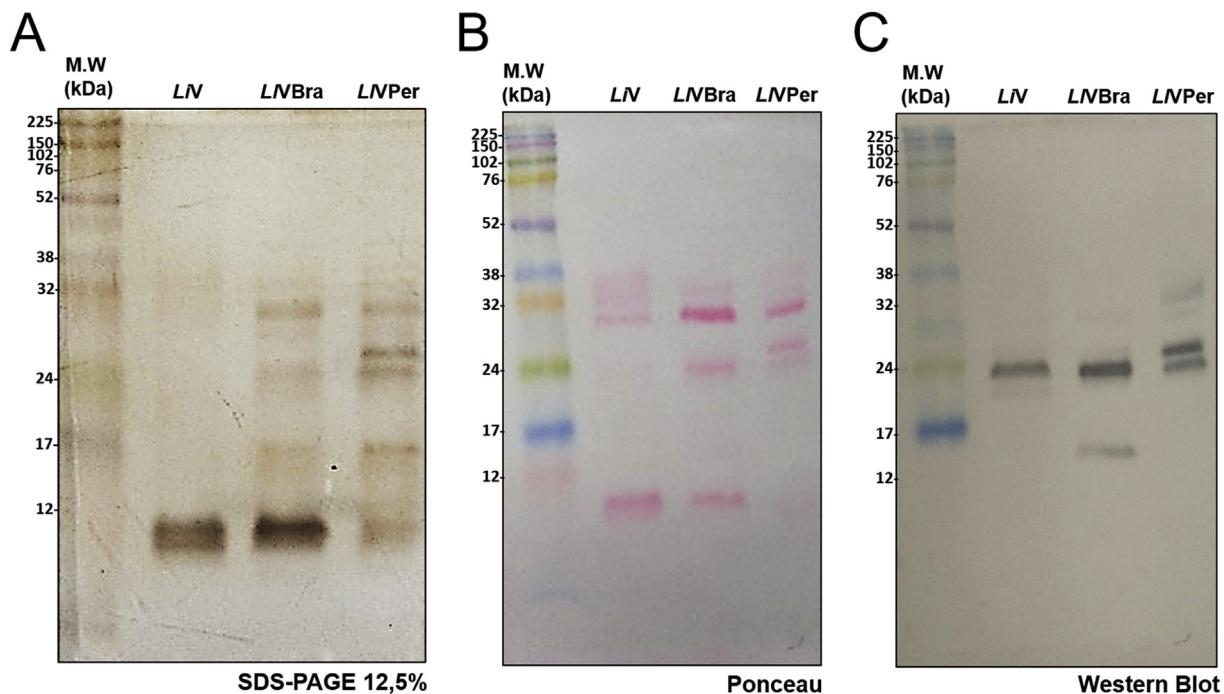


Fig. 2. Electrophoresis and Western Blot of *L. intermedia* (*LIV*) and *L. laeta* venoms from Brazil (*LIVBra*) and Peru (*LIVPer*). Samples with 10 µg of *L. intermedia*, Brazilian *L. laeta* or Peruvian *L. laeta* venom were subjected to SDS-PAGE on 15% polyacrylamide gels and the membrane was incubated with rabbit serum anti-recLALP-1 diluted in blocking buffer (1:400). (A) SDS-PAGE. (B) Transferred membrane stained with Ponceau for temporary staining of proteins and (C) Membrane probed with anti-recLALP1.

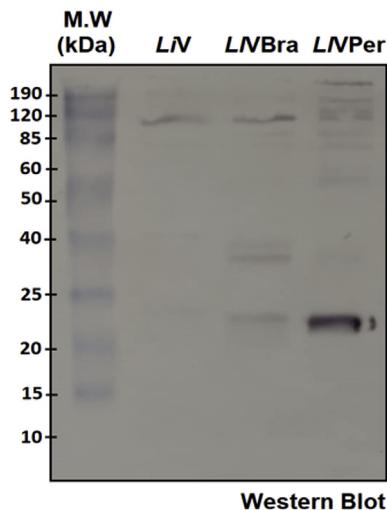


Fig. 3. Western Blot of *L. intermedia* and *L. laeta* venoms from Brazil and Peru for the identification of the glycosylation. Samples with 20 µg of *L. intermedia*, Brazilian *L. laeta* or Peruvian *L. laeta* venom were subjected to SDS-PAGE on 15% polyacrylamide gels and the membrane was incubated with Concanavalin A lectin solution (ConA 50 µg/mL) diluted in TBS with Tween 20 0.05% and 1 mM MnCl₂.

3.2. Enzymatic activities

Taken together, the assays previously performed in this work may suggest a varied content of LALPs in different *Loxosceles* venoms. To verify if this could reflect in different venom proteolytic activity, a fibrinogenolytic assay was performed. Fibrinogen consists of three polypeptide chains α , $\beta\beta$, and γ that can be visualized as separate bands after gel staining. The change in these bands density upon venom incubation may indicate the presence of proteolytic enzymes. Most enzymes with fibrinogenolytic action

preferentially degrade the α subunit, followed by $\beta\beta$ [44]. When a given sample has fibrinogen degradation activity, bands referring to the different subunits of fibrinogen will be less intense or completely disappear, when analyzed in SDS-PAGE under reducing conditions. Bands of lower molecular weight related to degradation products may also be present in the lower part of the gel. Fig. 4A shows the degradation of the α subunit of fibrinogen by both Peruvian and Brazilian *L. laeta* venoms after 16 h of incubation. In a time-dependent assay (Fig. 4B), the degradation of the fibrinogen α subunit was followed over the hours. Pixels intensity of the α bands was measured for each time point and the results suggest that both Peruvian and Brazilian *L. laeta* venoms presented different increases in activity throughout the time.

To further characterize and compare the proteolytic activity of Peruvian and Brazilian *L. laeta* venoms, their capacity to degrade gelatin/collagen (Fig. 5) was evaluated using the commercial Enz-Check kit. This assay demonstrated that Peruvian *L. laeta* venom had a greater gelatinase/collagenase activity or a higher enzyme quantity than the Brazilian venom, in all tested concentrations, being the difference statistically significant.

3.3. Bioinformatics analysis

3.3.1. Processing and assembling of the transcriptome

Since Peruvian *L. laeta* venom seems to present a more intense proteolytic activity, the presence and identity of LALPs expressed in their venom glands was investigated through RNA-seq analysis.

From the files provided by sequencing (BioSample accession: SAMN12002796/SRA: SRS4898300), 11,870,865 reads were obtained, with sequences ranging from 35 to 301 bases and with a GC content of 41% when analyzed by the program FastQC. As commonly done in the Illumina platform sequencing, the quality of results demonstrated the need for sequences improvement, as it was observed a decrease in the reads quality in the final portion.

The trimming process was performed using the Trimmomatic

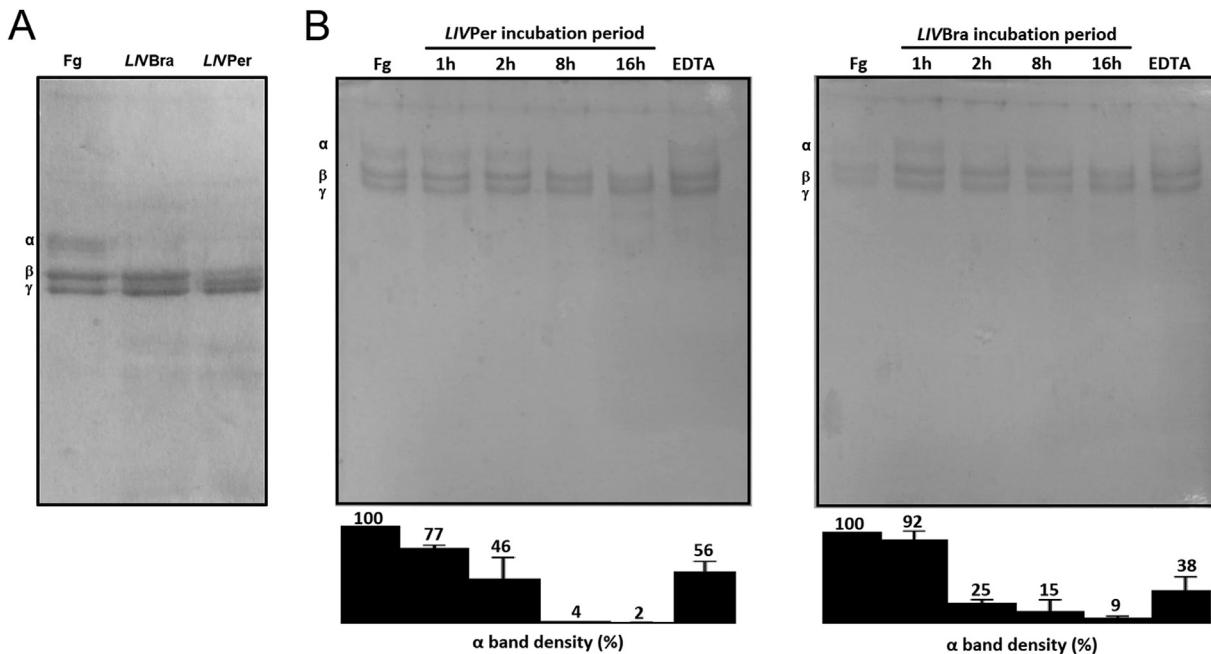


Fig. 4. Fibrinogenolytic activity of Brazilian and Peruvian *L. laeta*. Samples containing 3 µg of each venom were added to 0.5 mL of fibrinogen and incubated for the following incubation time points: 1, 2, 4, 6, 8, 10, 12 and 16 h at 37 °C. The solution was mixed to an equal volume of denaturing solution and incubated at room temperature for another 16 h. This solution (35 µL) was applied to a 10% polyacrylamide gel. EDTA was used as control (last lane). Fg: Undegraded fibrinogen; LIVBra: Fibrinogen incubated with Brazilian *L. laeta* venom; LIVPer: Fibrinogen incubated with Peruvian *L. laeta* venom (A) Fixed-time assay (16 h of incubation). (B) Time-dependent assay. In the lower part of the figure, the graph shows the percentage of band density for each time point, compared to fibrinogen (Fg) control, considered as 100%, analyzed with ImageJ 1.51.

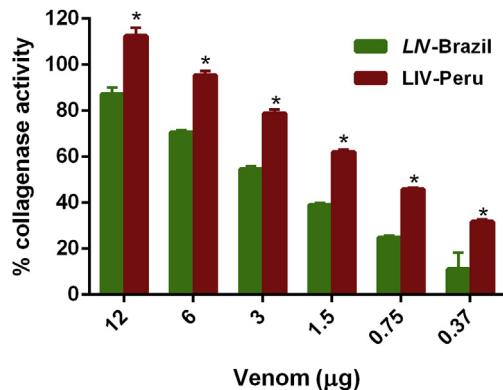


Fig. 5. EnzCheck Gelatinase/Collagenase assay of venoms from Brazilian and Peruvian *Loxosceles laeta*. Results are shown as percentage of collagenase activity, compared with the kit's positive control, in relation to venom amount. Plotted values are means ± SEM of two independent experiments (*p < 0.05).

program, obtaining 10,289,208 reads in file R1 and 10,203,328 reads in file R2, with average quality over 40:20 (Size:Phred). This result was then analyzed again using the FastQC program, in which it was observed a final Phred quality value of maximum 38, ensuring a correct base call probability greater than 99.90%.

Since a reference genome is absent in the literature, a *de novo* assembly was performed [34]. Ten assemblies were performed and among them, CLC Genomics Workbench 10 program selected the k-mer 22 as the best match and the most expressive, presenting a total of 143,237 contigs (Fig. 6).

3.3.2. Physicochemical analysis and multiple alignment analysis

After BlastP annotation, a sequence alignment, using the program Clustal Omega was made to locate the essential elements of metalloproteases (zinc-binding motif (HEXXHXXXGXXHE) and met-

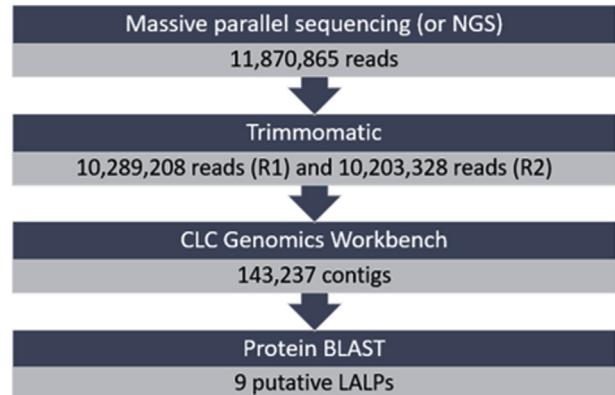


Fig. 6. Massive parallel sequencing (or NGS) and bioinformatic analysis workflow. The transcriptome of Peruvian *Loxosceles laeta* revealed by massive parallel sequencing generated 11,870,865 reads. These reads were trimmed and assembled in 143,237 reads, resulting in 9 putative astacine-like metalloproteases (LALP-LPs).

turn (MXY)). After this analysis, it was possible to select nine putative LALPs sequences from *L. laeta* Peru transcriptome (LALP-LPs). In addition to the established parameters described above, the identified sequences had high identity values with LALP1 from *L. intermedia* (77%–84%). Sequence similarities among the nine sequences and with LALP1 were defined by Emboss Stretcher Pairwise alignment (https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/) and it is presented in Fig. 7. Identity percentages ranging from 73 to 98% were observed among the sequences.

Sequences alignment of the previously described LALPs and the nine sequences found in the present work was performed by Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) (Fig. 8). Relevant amino acids, signal peptides and propeptides were identified using SignalP and ProP and highlighted in the alignment

	LALP1	LALP-LP1	LALP-LP2	LALP-LP3	LALP-LP4	LALP-LP5	LALP-LP6	LALP-LP7	LALP-LP8	LALP-LP9
LALP-LP1	77	100	88	78	84	84	82	86	88	73
LALP-LP2	75	88	100	80	93	93	91	85	91	79
LALP-LP3	81	78	80	100	79	79	77	78	77	88
LALP-LP4	75	84	93	79	100	98	97	91	90	85
LALP-LP5	75	84	93	79	98	100	97	91	89	85
LALP-LP6	74	82	91	77	97	97	100	90	88	87
LALP-LP7	77	86	85	78	91	91	90	100	88	81
LALP-LP8	73	88	91	77	90	89	88	88	100	81
LALP-LP9	75	73	79	88	85	85	87	81	81	100

Fig. 7. Identity among the possible Peruvian *L. laeta* LALPs sequences (LALP-LPs) identified by BlastP and *L. intermedia* (LALP1). The percentage of similarity was estimated using the pairwise sequence alignment toll EMBOSS Stretcher.

(orange and pink). It was observed that the nine LALP-LPs share similar signal peptides and propeptides, which differs from the ones described for LALPs 1 to 3. Disulfide bridges locations in LALP-LPs were predicted by similarity with astacin from *Astacus astacus* and in the described LALPs. The presence of the essential elements metal-binding motif and met-turn were also observed in all sequences, in addition to the active site in all 15 sequences and the N-glycosylation sites according to NetNGlyc. Furthermore, a repetitive gap was observed in all LALP-LPs, located close to amino acid number 70, marked in red. All LALP-LP sequences do not have a final portion of final amino acids when compared to the other LALPs.

3.3.3. Phylogenetic analysis

Using the sequence alignment of astacin from *Astacus astacus*, previously described LALPs (1–5), astacins from Brazilian *L. laeta*, *Parasteatoda tepidariorum*, *Nephila clavipes*, *Stegodyphus mimosarum*, *Tityus serrulatus*, *Centruroides sculpturatus* and the putative sequences identified in this study (LALP-LP), a phylogenetic tree using MEGA X was inferred. The tree constructed using the maximum likelihood method with 1000 bootstrap replicates, shows three large groups: One with the astacins from Brazilian *L. laeta*, LALP2 and LALP3, a second group with the nine LALP-LPs, LALP1, LALP4, LALP5 and two astacins from Brazilian *L. laeta*, and the last group with astacin from *Astacus astacus*, *Parasteatoda tepidariorum*, *Nephila clavipes*, *Stegodyphus mimosarum*, *Tityus serrulatus* and *Centruroides sculpturatus* (Fig. 9). The clade that groups together all LALP-LPs has a bootstrap of 97% confidence, and consistent with the proximity between the tested species, LALP4, identified from Brazilian *L. laeta* venom, nests closer to LALP-LPs than to the other sequences. In addition, a probable separation of LALP-LPs sequences by representation in three internal nodes or hypothetical taxonomic units: LALP-LP1, LALP-LP7 and LALP-LP8; LALP-LP2, LALP-LP4, LALP-LP5 and LALP-LP6; LALP-LP3 and LALP-LP9, leading us to believe in possible differences between these sequences identified.

3.3.4. Molecular modeling

The Phyre2 program was used for 3D structure modeling of the nine putative LALP-LPs using astacin from *Astacus astacus* as template, and the available results were analyzed in PyMOL. The z-score of the nine models was 4.07, representing that these data are above the expected average. In addition, the identities between the sequences were greater than 70%, and cysteines are well aligned, ensuring that although differences are found, these models are sufficiently reliable.

The essential elements of metalloproteases (zinc-binding motif and met-turn) where marked in yellow in Fig. 10A, where it is observed that the proteoforms are very similar to each other, but

small differences in some amino acids can be seen in the overlap in Fig. 10B, especially in the upper right area, corresponding approximately to residues of number 100. In addition, a superimposition of LALP-LPs structures with LALP1 allowed the identification of the gap visualized in the alignment, besides showing differences between these sequences in Fig. 10C. This gap is present in all LALP-LPs with small differences between them, as is the case of LALP-LP3 (Fig. 10C), which has a gap of only seven amino acids (three times less than other LALP-LPs).

4. Discussion

Although metalloproteases have been long described as important toxic agents in *Loxosceles* venoms, acting on hemostasis and other events related to the most severe manifestation of this kind of accidents, very few studies have been performed to characterize these proteins. *L. laeta* venom seems to be one of the most toxic among the genera [45]. Furthermore, Peruvian population of this species appears to be related to a higher proportion of envenoming systemic complications [10]. Taking this into account, this study aimed to characterize the content of *Loxosceles*-astacin-like proteases (LALPs) in the Peruvian *L. laeta* venom.

Anti-recLALP-1 rabbit serum reactivity detected by ELISA revealed the possible presence of this enzyme in the venoms of the three most medically relevant *Loxosceles* species of Brazil (*L. laeta*, *L. intermedia* and *L. gaucho*) plus *L. laeta* from Peru (Fig. 1). The higher reactivity of anti-recLALP-1 rabbit serum with Peruvian *L. laeta* venom, with significant statistical difference when compared to the other tested *Loxosceles* venoms, corroborates a previous work which shows that the LALP family is larger and more complex than expected. This comparison demonstrates differences between venoms biological activities of closely related species [46]. As this class of toxins may play an important role in hemorrhage and in venom spreading, the higher content of this enzyme in Peruvian *L. laeta* venom, as verified in the blots and enzymatic activities, may be related to its increased venom toxicity. However, this hypothesis remains to be tested and confirmed, since it is necessary to consider the presence and differential content analysis of other venom components.

Bands with the expected molecular weight for LALPs were identified in the SDS-PAGE (Fig. 2A). Western Blot with anti-recLALP-1 rabbit serum (Fig. 2C) confirmed the presence of LALPs with an approximate molecular weight of 24 kDa in all three tested venoms, however, Peruvian *L. laeta* venom showed an additional reactive band of approximately 24 kDa. This extra band may reflect enhanced glycosylation patterns, since two putative N-glycosylation sites were identified in the LALP-LPs sequences found herein, according to NetNGlyc prediction (Fig. 8). In the ConAblot analysis, an intense band with molecular weight between 20 kDa and 25 kDa

Fig. 8. Alignment of astacins from *Astacus astacus* (Astacin-P07584), *L. intermedia* (LALP1-Li, LALP2-Li and LALP3-Li), Brazilian *L. laeta* (LALP4-Li), *L. gaucho* (LALP5-Lg), Brazilian *L. laeta* (LLAE), *Parasteatoda tepidariorum* (XP), *Nephila clavipes* (PRD), *Stegodyphus mimosarum* (KFM), *Tityus serrulatus* (CDJ), *Centruroides sculpturatus* (XP_023232109.1) and Peruvian *L. laeta* (LALP-LPs) identified in the present work by Clustal Omega. (Orange) Signal peptide identified by Expasy - SignalP; (Pink) Propeptides by ProP; (Blue) Disulfide bridges predicted by similarity with astacin from *Astacus astacus*; (Yellow) Essential elements of metalloproteases - Met-turn; (Green) Essential elements of metalloproteases - Zin-binding motif; (Purple) N-glycosylation sites identified by the NetNGlyc; (Red) Repetitive Gap in the middle and at the end of the sequences in LALP-LPs

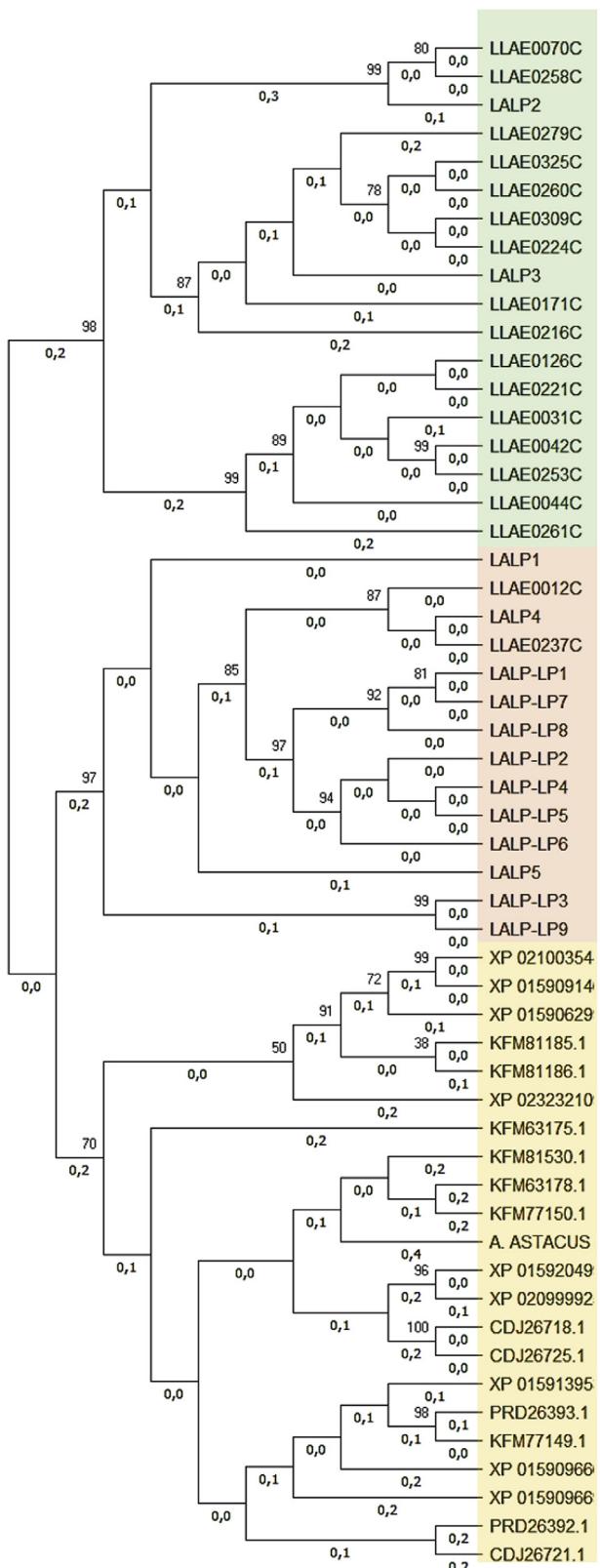


Fig. 9. Maximum likelihood phylogenetic tree with 1000 bootstrap replicates by Mega X. Phylogeny of astacins from *Astacus astacus* (A. ASTACUS), *L. intermedia* (LALP1, LALP2 and LALP3), Brazilian *L. laeta* (LALP4), Brazilian *L. laeta* (LLAE), *Parasteatoda tepidariorum* (XP), *Nephila clavipes* (PRD), *Stegodyphus mimosarum* (KFM), *Tityus serrulatus* (CDJ), *Centruroides sculpturatus* (XP_023232109.1) and Peruvian *L. laeta* (LALP-LPs). Numbers above the branches represent bootstrap support from parsimony analysis. Numbers below the branches represent posterior probabilities of nodes in their right. Parsimony bootstrap values < 70 are not shown.

was evidenced in Peruvian *L. laeta* venom, corroborating the higher glycosylation of astacin-like metalloproteases from Peruvian than Brazilian *L. laeta* venom (Fig. 3). A study has previously detected glycosylations in proteases from *L. intermedia* venom, which were relevant to their enzymatic activity [47].

In the fixed-time fibrinogenolytic activity assay (Fig. 4A), both Peruvian and Brazilian *L. laeta* venoms succeeded in degrading the $\text{A}\alpha$ subunit of fibrinogen, endorsing a previous work which showed that *L. intermedia* venom also presents a metalloprotease proteoform that exhibits fibrinogenolytic activity [17]. The time-dependent fibrinogenolytic activity assay was performed to follow the degradation of the $\text{A}\alpha$ subunit along the hours, allowing to observe that Peruvian *L. laeta* venom possibly degrades the fibrinogen in a greater speed and quantity than the Brazilian venom. A previous study has shown that *L. laeta* venom has an enhanced fibrinogenolytic activity compared to others *Loxosceles* species venoms [48]. This observation corroborates our results, as it demonstrates that spiders of the same genus may have different venom proteolytic activities. The fibrinogenolytic activity of *Loxosceles* venoms can be related to intravascular coagulation, which is responsible for the most severe symptoms of brown spider envenomation and death [29]. Taking this into account, Peruvian *L. laeta* venom may be more fibrinogenolytic and can possibly induce this disorder more intensely, explaining the higher rate of systemic symptoms occurring in Peru, when compared to Brazil.

Previous works demonstrated that *L. intermedia* and *L. laeta* venoms presented gelatinolytic proteases [17,49]. By assessing the gelatinolytic potential of *L. laeta* venoms using the EnzChek™ Gelatinase/Collagenase Assay Kit, we observed a significant difference between the gelatinolytic activity of the Brazilian and Peruvian *L. laeta* venoms. Using the same kit, other work demonstrated that indeed the gelatinolytic activity between three species of *Loxosceles* presented differential intensities, being *L. laeta* the venom with the highest activity [46]. In addition, a previous work has proposed that the *L. intermedia* venom metalloproteases activity is associated to venom deleterious effects, since gelatin is a byproduct of collagen, and it is possible that this activity is related to collagen degradation, an important event in the typical skin injury caused by this spider [29].

Transcriptomic analysis identified nine possible LALP sequences from Peruvian *L. laeta* (LALP-LPs) venom glands. The alignment performed with astacin from *Astacus astacus*, LALPs 1 to 5, the nine LALP-LPs and other organisms, like spiders and scorpions, showed the presence of important sequence information to characterize these sequences. In addition, the alignment showed a repetitive gap that can be observed in all LALP-LPs, located around amino acid number 70 and at the end of all sequences. This may indicate a possible structural difference for LALP-LPs when compared to the previous LALPs described. This structural difference can account for the enhanced proteolytic activity detected in Peruvian *L. laeta* venom, however this remains to be confirmed. Ideally, the purification and sequencing of an isoform of LALP from Peruvian *L. laeta* venom or the cloning of a LALP sequence from its venom glands could further confirm the presence of this gap. It cannot be ruled out that data generated by transcriptome *de novo* assemblies without a reference genome, as shown in here, can present some obstacles, generating chimeras, fragmented genes, unassembled alleles and assembled paralogues [50]. Moreover, despite the identification of nine putative LALPs transcripts in our transcriptomic analysis, not necessarily all of them are translated into proteins [51]. It remains to be elucidated which LALP proteoforms are present in secreted venom.

Loxosceles venom is a complex mixture of bioactive compounds that may act synergistically to generate the observed toxic effects in envenoming. We believe that the present work provides possible

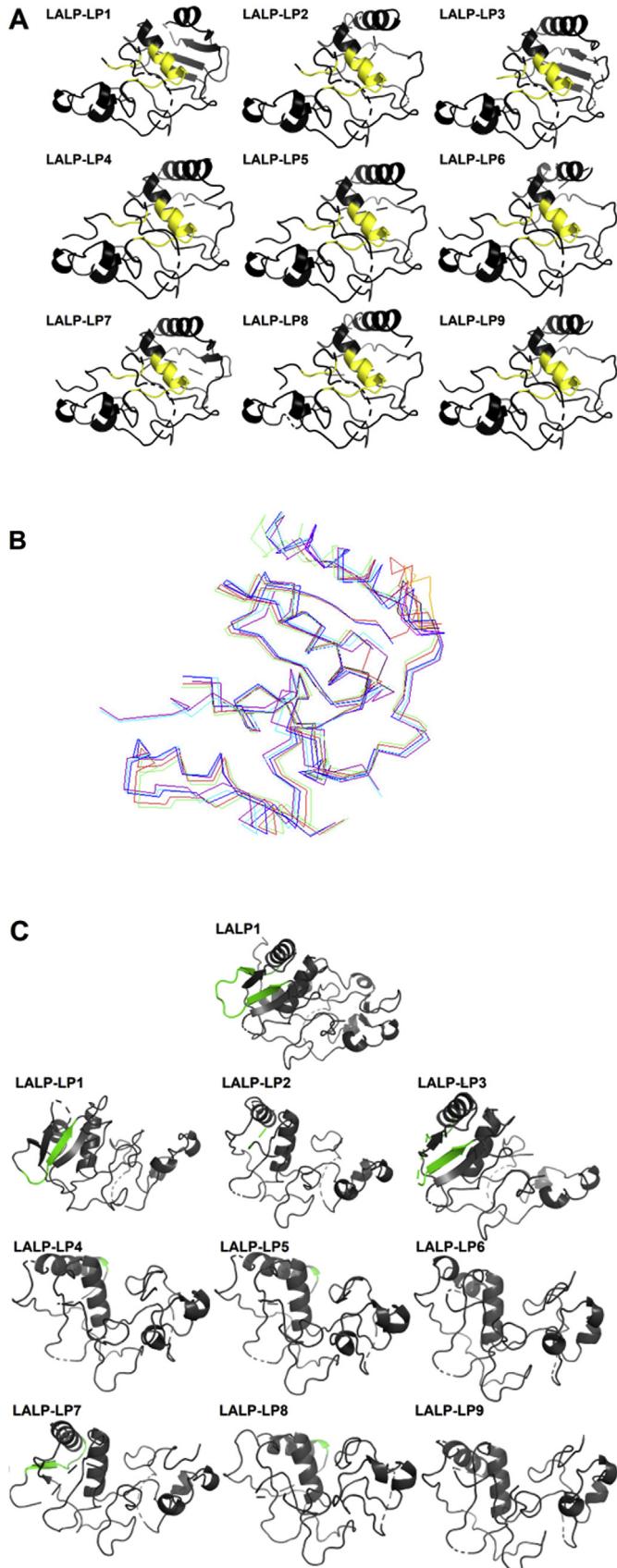


Fig. 10. Molecular modeling of the LALP-LPs identified by transcriptomics using Phyre2 and analysis of the results made by PyMOL and Swiss-PdbViewer 4.0. (A) Modeling of the nine putative LALP-LPs. (Yellow) Essential elements of metalloproteases (HEXXHXXGXHE and MXY). **(B)** Superimposition of structural models of

insights in the understanding of the differences in venoms from geographical variants *L. laeta* species, that may translate into clinical differences in the envenomings.

However, it is important to emphasize that *L. laeta* venom has numerous other components that may also present differences in identity and quantity requiring further comparative studies.

5. Conclusions

The proteolytic activities from Brazilian and Peruvian *L. laeta* venoms showed significant differences. Nine LALPs proteoforms from Peruvian *L. laeta* venom glands were determined for the first time by RNAseq, with similarity to the other LALPs previously identified in spiders of *Loxosceles* genus. The results obtained here suggest that the primary LALP-LPs sequences have significant differences from the LALPs sequences from the venom of other spiders of the same genus.

Following the documentation of LALPs diversity in Peruvian *L. laeta* venom glands, further characterization studies using purified molecules or active recombinant LALP-LPs would help to elucidate the role of these enzymes in loxoscelism, specially concerning its systemic manifestation. Comparison of LALP-LPs molecules sequences and venom content with Brazilian *L. laeta* may provide a molecular basis to explain the toxicity difference among the venoms of these two *L. laeta* populations.

Authors contribution

R.M.S., C.G.D. and C.C.O conceived the design research, analyzed data, and wrote the paper. R.M.S., C.G.D., S.A.L. and F.C.O. performed experiments. R.M.S., C.G.D. and C.C.O. analyzed data and organized figures. P.A.A., A.O.C., C.B. and E.E.G.K. contributed to the design and interpretation of the results. E.K. performed the sequencing of the samples. R. M. S., C.C.O., F.C.O. and C.C.O contributed to manuscript revision.

Declarations of interest

None.

Ethics statement

Experimental protocols were performed after approval by the Ethics Committee in animal Experimentation of Federal University of Minas Gerais (388/2017-CETEA/UFMG).

Declaration of interests

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

Funding sources

This research was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil (CNPq) (no 17/2013, process: 490269/2013-3), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil - CAPES (TOXINOLOGIA no23038000825/2011-63) and by Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Brazil (FAPEMIG) (no PPM: 00270-16).

the nine putative LALP-LPs. (Red) LALP-LP1; (Green) LALP-LP2; (Blue) LALP-LP3; (Yellow) LALP-LP4; (Pink) LALP-LP5; (Light blue) LALP-LP6; (Orange) LALP-LP7; (White) LALP-LP8 and (Purple) LALP-LP9. (C) Identification of gap areas in LALP1 and LALP-LP1 to LALP-LP9. (Green) Area present in LALP1 which is a gap in LALP-LPs.

Acknowledgements

The authors wish to thank Dr. Silvio Sanches Veiga for offering the LALP1 recombinant for the immunization protocol.

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