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Lipidomic alterations of *in vitro* macrophage infection by *L. infantum* and *L. amazonensis*†

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Particular lipid profiles have been found in two different protozoa of the Leishmania genus. *Leishmania infantum*, a visceral leishmaniasis causative agent and *Leishmania amazonensis*, a cutaneous leishmaniasis, reveal distinctive lipid contents of phosphatidylethanolamine and phosphatidylserine plasmalogens, sphingolipids, phosphatidylinositols, phosphatidylcholine, and phosphatidylethanolamine, which have been shown to be related to species, life-cycle of the parasite, and macrophage infection. *L. infantum* displayed a higher content of phosphatidylethanolamine plasmalogens than *L. amazonensis*, which may help to differentiate their unique clinical manifestations. Phosphatidylserines plasmalogens are also found to be an important lipid class for the intracellular form of the parasite. Our findings also reveal lipid classes that may be involved in visceralization pathways and parasite differentiation.

Introduction

The leishmaniases are a group of diseases caused by more than 20 different *Leishmania* species. They are protozoan parasites transmitted to hosts by the bites of their sandfly vectors. Depending mainly on the *Leishmania* species, the disease can evolve into three major clinical forms: cutaneous, mucocutaneous, and visceral.[1] Close to 0.4 million and 1.2 million cases of visceral (VL) and cutaneous leishmaniasis (CL), respectively, occur each year in 98 countries and three territories where these diseases are endemic.[2] Despite their widespread distribution, over 90% of global VL cases occur in only six countries (India, Bangladesh, Sudan, South Sudan, Ethiopia, and Brazil), whereas most cases (70–75%) of CL occur in ten countries (Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica, and Peru).[2] Challenges to control the disease include the remoteness of, and difficult access to endemic areas, dangerous environments in some of those areas and the difficulties of transporting diagnostic tests and medicines for case management.

During the life cycle, *Leishmania* differentiates from the extracellular promastigote to the intracellular amastigote form. Flagellated promastigotes develop in the midgut of sandflies, which are inoculated in humans during the blood meal. Promastigotes then differentiate to amastigotes that multiply inside the macrophage lysosome.[3]

The differences in clinical manifestations are in general mainly associated with the parasite characteristics. They have adapted to replicate in distinct host macrophage environments and whereas some species remain contained in cutaneous lesions, others such as *L. infantum* disseminate into visceral organs causing VL.[4] *L. amazonensis* is widely recognized as a causative agent of CL in Latin America, especially in the Amazon region. Some cases are however reported where this parasite can also disseminate to produce atypical VL with hepatitis and lymphadenopathy.[5] Many models are used to mimic natural infection of leishmaniasis in an attempt to understand the disease and its spread to visceral organs, which could involve the movement of infected cells. Despite all knowledge of the genome, protein, and lipids of several *Leishmania* species, the route used by protozoa to eventually reach visceral organs is still poorly understood.

Different emerging approaches are using omics as a powerful tool to analyze the diversity of *Leishmania* isolates.[6] These studies have improved the understanding of pathogenesis of leishmaniasis,[7] and are contributing to unveiling details of the intimate relations between hosts, parasites, and vectors. Compared with genomic and proteomic techniques, metabolomic approaches can provide a clearer picture of the phenotype of a biological system since metabolites are products from gene transcription and translation (proteins).[8] Recent studies found that lipids comprised the largest class of identified metabolites and the phospholipids and sphingolipids contain

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the main differences observed between most parasite life-cycle phenotypes.[9,10] These two lipid classes are also both abundant and critical to virulence and viability in *Leishmania* species.[11]

Lipids can be accessed by standard tools, such as direct mass spectrometry (MS) analysis, but for highly complex matrices such as lipid extracts, coupling MS to chromatographic systems is essential since it enhances the number of detected compounds, sensitivity, and selectivity.[12] We investigated principal lipid compositions related to the differentiation of two *Leishmania* species in culture as well as *in vitro* macrophage infection: *L. infantum*, a visceral well-studied protozoon; and *L. amazonensis*, a cutaneous poorly studied protozoon. We used liquid chromatography coupled with mass spectrometry (LC-MS) to evaluate lipids that could be involved in the development of visceral leishmaniasis.

Material and methods

Parasite culture

L. amazonensis, strain MHOM/BR/67/M2269, and *L. infantum*, strain MHOM/BR/1972/LD were maintained by regular passage in BALB/c mice.[13] *In vitro*, *L. infantum* promastigotes were grown in Schneider medium supplemented with 10% fetal bovine serum (FBS, from SIGMA), 5% filtrated urine and gentamicin at 50 μg mL^{-1}. *L. amazonensis* promastigotes kept in RPMI medium containing 10% FBS and gentamicin at 50 μg mL^{-1} at pH 7.4. In both cases, cultures of promastigotes were maintained at 26 °C. All the animal experiments were performed according to the principles stated in the Brazilian law on animal experiments and were approved by the UNICAMP Committee for Ethics in Animal Research (Protocol 4140-1). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International) and the Brazilian Guide for care and use of animals for scientific and didactic purposes (DBCA).

J774 macrophage culture and infection

Macrophage cell line J774 was obtained from the American Type Culture Collection. It is a mouse BALB/c monocyte macrophage line. Cells were grown in DME medium, supplemented with 10% FBS and 50 μg mL^{-1} gentamicin, at 37 °C and in a humidified atmosphere of 5% CO_2. For *in vitro* Leishmania infections, 2.5×10^6 cells were plated in 6-well plates and maintained for 16 h. After two washes with PBS, promastigotes from both species (*Leishmania amazonensis* or *Leishmania infantum*) were added at ratios of 1 : 5 and 1 : 10 in cell medium. Cocultures were maintained for 16 h at 37 °C in 5% CO_2, 5% O_2, and balanced N_2, followed by two washes to remove non-internalized parasites, and plated in 24-well plates. The wells were carefully washed with sterile saline to avoid releasing macrophages from the bottom and then lipid extraction was performed. Each experimental group was cultured in triplicate.

Lipid extraction

Infected macrophages from *in vitro* infections or parasitic cells in saline solution were centrifuged at 16 000*g* for 5 min at 4 °C. All the pellets were resuspended with 400 μL of methanol (−20 °C) and 400 μL of cold milli-Q water following the addition of 400 μL of chloroform (−20 °C) containing the internal standard (Equilin-2,4,16,16-d_4, 3 ng mL^{-1}, Sigma Aldrich). Then all suspensions were vortexed for 20 min followed by centrifugation at 16 000*g* for 5 min at 4 °C. Finally, 300 μL of the bottom layer (containing the lipids) were removed and stored at −20 °C until analysis.[14]

LC-MS analysis and data extraction

An HPLC (Hewlett Packard, Agilent Technologies 1290 series) coupled to a Q-ToF iFunnel 6550 mass spectrometer fitted with an electrospray ionization (ESI) source was used for the LC-MS analysis, LC was performed on a Titan 1.9 μm column (2.1 × 100 mm, Supelco) equilibrated with 100% A. Mobile phase A was isopropanol : methanol : H_2O (5 : 1 : 4), with 5 mM CH_3COONH_4 and 0.1% of CH_3COOH; and mobile phase B was isopropanol : H_2O (99 : 1) with 5 mM CH_3COONH_4 and 0.1% of CH_3COOH. The samples were eluted with a flow rate of 0.35 mL $min^{-1}$and the following linear gradient: 0–3 min 0% B; 3–5 min, 0% B to 20% B; 5–25 min 20% B to 30% B, 25–35 min 30% B to 95% B. The samples were analyzed in negative ion mode. The mass spectrometer parameters used were: VCap 3000 V; fragmentor voltage at 100 V; OCT 1RF Vpp at 750 V; gas temperature at 290 °C; sheath gas temperature at 350 °C; drying gas at 12 L min^{-1}. Mass spectra were acquired in profile mode and the acquisition range was 100–1500 *m*/*z*. The samples were randomly analyzed.

Raw data were submitted to molecular feature detection in Mass Hunter software using a quality score threshold of 80, and searching for deprotonated, chlorate adduct and acetylated compounds and all detected features were exported as CSV files for further statistical analysis. All the relevant compounds were identified by their exact mass (*m*/*z* values) and by their fragmentation pattern, when possible (Fig. S7 to S15, ESI†).

Statistical analysis

All the statistical analyses were performed through MetaboAnalyst 3.0 (www.metaboanalyst.ca).[15] The matrix data were created using a 0.025 *m*/z tolerance and 30 seconds retention time tolerance. The abundance of each ion was normalized by the internal standard to generate a data matrix that consisted of the *m*/*z*-retention time values and the normalized peak area. Next, unsupervised segregation was assessed with principal components analysis (PCA) using mean-centered scaled data. PCA data were visualized by plotting the PCA scores in which each point represents one sample and the PCA loading in which each point represents an *m*/*z*. The loading plot gives, therefore, an indication of the lipids that most strongly influence the patterns in the score plot. To maximize class discrimination and biomarker identification, the data were further analyzed using the partial least squared discriminant analysis (PLS-DA) method, where a loading plot was calculated to visualize the relationship between covariance and correlation within the PLS-DA data. Discriminating variables were selected according to variable importance in projection values (VIP). An independent Kruskal Wallis test (>2 groups) or non-parametric Wilcoxon

rank-sum test (2 groups) with a 0.05 *p*-value cutoff was also used as a variable selection. All *p*-values and false discovery rate (FDR), a *p*-value correction, are displayed in Tables S6–S8 (ESI†). For easier visualization of class changes the data were displayed as bar graphics, where the sum and standard deviation of all significant lipids from each class were displayed.

Results and discussion

Leishmania parasites have a dimorphic life cycle in which they reside as extracellular promastigotes in the vector and as intracellular amastigotes in mammalian host mononuclear phagocytes such as macrophages (Fig. 1).[16] Several mechanisms are used for protozoa to successfully spread and infect the host, but these mechanisms differ according to clinical manifestations.

We believe that the mechanisms of cell infection at early stages should differ between *L. amazonensis* and *L. infantum*.[17] They were analyzed by LC-MS/MS to evaluate the composition of the lipid extracts from promastigotes, macrophages, and *in vitro* infected macrophages. Statistical analyses were also used to reveal differences between *L. infantum* (viscerotropic) *vs.* *L. amazonensis* (cutaneous) promastigotes (Fig. 2); and macrophage *vs.* infected macrophages (Fig. 3). To assess the quality of the lipid MS data, we first performed an unsupervised multivariate method (PCA), since it could show sample outliers and/or reveal hidden biases. PCA showed good distinction between promastigote forms of *L. infantum* and *L. amazonensis* with 84.8% explained variance at the first two principal components (Fig. S1, ESI†). In addition, 91.4% and 99.3% explained variances were obtained for infection experiments of *L. infantum* and *L. amazonensis*, respectively (Fig. S3 and S5, ESI†). To better access the major changes in lipid composition between two *Leishmania* species, we also applied a supervised statistical method; PLS-DA (Fig. 2 and 3). Both the protocols showed great robustness as indicated by their Q2 and R2 values and permutation tests (Fig. S2, S4 and S6, ESI†). Some lipid classes were shown as important biomolecules able to differentiate the two *Leishmania* species, in promastigote form or infected macrophages. They are discussed separately in the following sections.

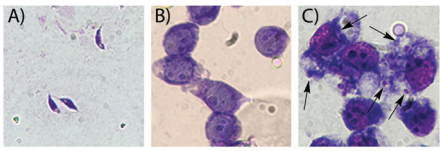

Fig. 1 Microscopic view of (A) promastigotes of *Leishmania amazonensis*, (B) control group of J774 macrophages and (C) J774 macrophage infection (Giemsa staining, magnification 100×). Arrows show amastigotes inside macrophages vacuoles.

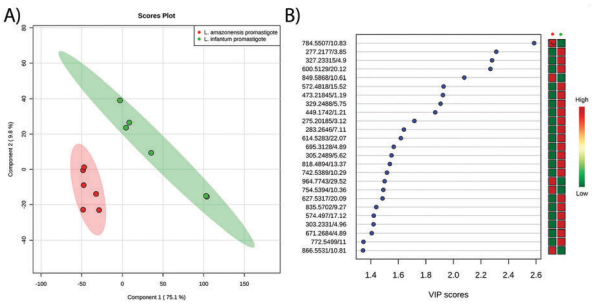

Fig. 2 (A) PLS-DA score plot of lipid profile data for the discriminations of *L. amazonensis* promastigotes (red) and *L. infantum* promastigotes (green). (B) Compounds with higher variable importance in projection values (VIP). Additional information is displayed in the ESI.†

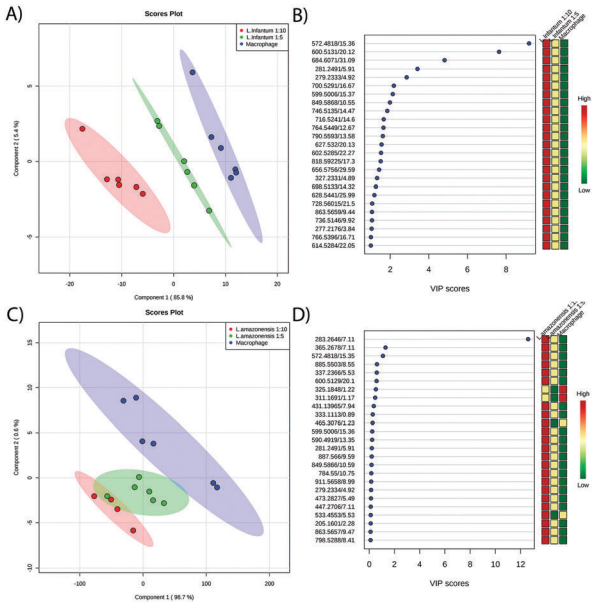

Fig. 3 PLS-DA score plot of lipid profile data for the discriminations of uninfected macrophages (blue), and *in vitro* macrophage infection 1 : 5 (green) and 1 : 10 (red) of (A) *L. infantum* and (C) *L. amazonensis*. In addition, their corresponded compounds with higher variable importance in projection values (VIP) are shown. Additional information is displayed in the ESI.†

Plasmalogens as extreme environment survival lipids

We have therefore observed both the increase of some phosphatidylethanolamine plasmalogens (PEPs) which were present in the promastigote forms of *L. infantum* and *L. amazonensis* and the formation of other PEPs probably due to promastigote–amastigote transformation (Fig. 4 and Table S1, ESI†), showing the importance of these lipids at different protozoa life-cycles. In contrast to mammalian cells, *Leishmania* contains high levels of ether lipids, for example, most of the *Leishmania* phosphatidylethanolamines carry an ether double bond at position sn-1 and it is esterified with a fatty acid at position sn-2. Zufferey *et al.* showed that the ether phospholipids are not essential for virulence,[18] but they later reported that the initial step of dihydroxyacetone phosphate acylation (precursor of ether and glycerolipid metabolism in *Leishmania*) was essential for growth, survival, and parasite virulence.[19] Plasmalogens are more susceptible to oxidative reactions than their fatty acid ester analogues, due to the reactivity of their enol-ether function,[20] hence ether lipids may serve as endogenous antioxidants becoming an

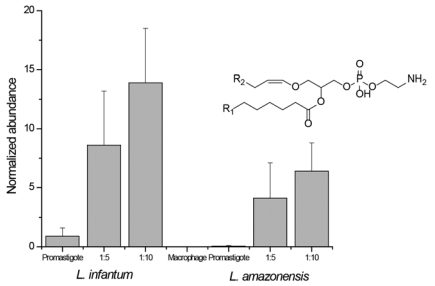

Fig. 4 Normalized phosphatidylethanolamine plasmalogen (PEP) total abundance from macrophage, promastigote and *in vitro* infection (*L. infantum* and *L. amazonensis*) lipid extract cells. Representative PEP chemical structure.

interesting approach to protozoan survival in the phagosome environment increasing their oxidative resistance.[21] Despite the presence of PEPs in *L. amazonensis* during macrophage infection, the relative amount of this lipid class was lower than in *L. infantum* implying that these compounds may be related to the increased oxidative stress environment in VL compared to CL. VL species need to be much more adapted to survive and proliferate in visceral organs than the cutaneous species. For instance, the dermis temperature varies between 28 and 32 °C, whereas fever in VL exceeds 40 °C, increasing the production of oxidant compounds by phagocytic cells.[4] None of the PEPs were observed in macrophages and few are observed in the promastigote form (Fig. 4), hence our results corroborate that these compounds are essential to the intracellular form of the parasite.

Phosphatidylserine plasmalogens (PSPs) as a natural spread of infection control

We also observed a greater amount of PSPs in *L. amazonensis* promastigotes as compared with *L. infantum* promastigotes (ESI,† Table S2 and Fig. 5). PSPs are another plasmalogen lipid class and may also act as antioxidants. Surface exposure of phosphatidylserine (PS) by viable pathogenic organisms is a well-known infection mechanism used by several organisms, named apoptotic mimicry.[22] Rochael *et al.* showed that PS exposure of *L. amazonensis* has a strong procoagulant effect in the host.[22] Consequently, this higher PSP production by *L. amazonensis* promastigotes promotes a stronger local immune response, which may limit the parasite spread and prevent dissemination to the visceral organs. Warburg *et al.* observed similar results,[23] which showed that the saliva of sandflies from cutaneous leishmaniasis endemic areas causes low levels of vasodilatation, whereas the saliva of sandflies from visceral leishmaniasis endemic areas causes higher vasodilatation, suggesting that vasodilatation can facilitate the access to visceral organs by the parasite. During *in vitro* infection, the total amount of PSPs is lower and it remains practically unchanged for both species (Fig. 5) indicating that PS is more important for promastigotes in early stages of infection.

Sphingolipids as a driving force for the spread of infection

Sphingolipids (SL), ceramides, and sphingoid bases (SBs) play crucial roles in several membrane functions such as regulation of cell growth and differentiation, apoptosis, raft formation, and modulation of the immune response.[24,25] Unlike mammalian cells, which synthesize sphingomyelin (SM) and glycosylsphingolipids in high abundance, the majority of SLs in *Leishmania* are inositol phosphorylceramide (IPC or PI-Cer).[26] It has been reported that a main function of SL synthesis and degradation is to generate ethanolamine (EtN), a metabolite essential for promastigote survival and metacyclogenesis in *Leishmania* (plasmalogen synthesis).[27] We found a great amount of ceramides and IPC especially in *L. infantum* (Fig. 6 and Table S3, ESI†), probably due to their need to synthetize PEPs (Fig. 4). Some ceramides, absent in promastigotes, increased with infection, corroborating with Zhang *et al.*[26] who showed the amastigote ability to salvage these lipids from the host by hydrolyzing their SM. Additionally, the degradation of host SLs by amastigotes could also disrupt SL-dependent signaling pathways in macrophages, suppressing nitric oxide generation and aiding intracellular parasitic survival.[28]

Phosphatidylinositol

We observed changes in two subclasses of lipids in this class: 1-alkyl,2-acylglycerophosphoinositol (PIO) and diacylglycerophosphoinositol (PI). No reports are found about the function of these PIOs in leishmaniasis, but PIs are known to be precursors of several other lipids known for acting as messengers in cells.[29] Both PI and PIO are present in the promastigote form, but in different proportions (Fig. 7 and Table S4, ESI†). In addition,

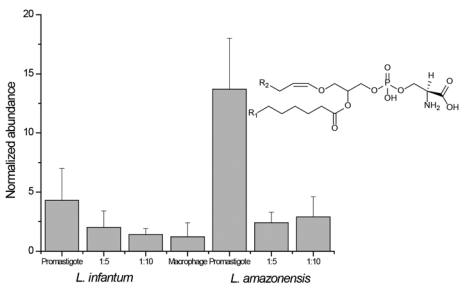

Fig. 5 Normalized phosphatidylserine plasmalogen (PSP) total abundance from macrophage, promastigote and *in vitro* infection (*L. infantum* and *L. amazonensis*) lipid extract cells. Representative PSP chemical structure.

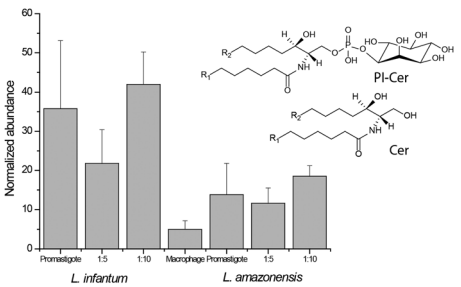

Fig. 6 Normalized Sphingolipid (SL) total abundance from macrophage, promastigote and *in vitro* infection (*L. infantum* and *L. amazonensis*) lipid extract cells. Representative SL chemical structure.

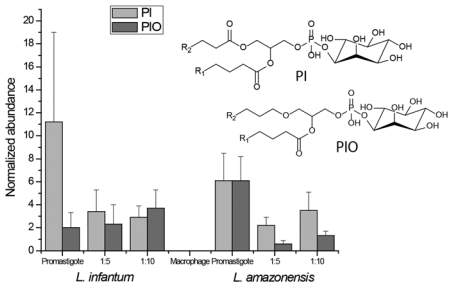

Fig. 7 Normalized phosphatidylinositol (PI and PIO) total abundance from macrophage, promastigote and *in vitro* infection (*L. infantum* and *L. amazonensis*) lipid extract cells. Representative PI and PIO chemical structures.

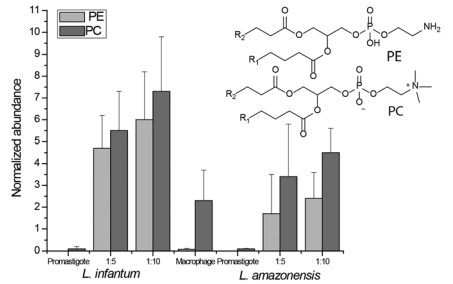

Fig. 8 Normalized phosphatidylethanolamine (PE) and phosphatidylcholine (PC) total abundance from macrophage, promastigote and *in vitro* infection (*L. infantum* and *L. amazonensis*) lipid extract cells. Representative PE and PC chemical structures.

PIs were more abundant in *L. infantum*, whereas PIOs were more abundant in *L. amazonensis* (Fig. 7 and Table S4, ESI†). Further experiments should be performed to better understand the role of PIO in *Leishmania*.

Phosphatidylcholine and phosphatidylethanolamine

Most of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) revealed by statistical analyses were detected in *in vitro* infection, but are absent in macrophages or promastigote parasite forms (Fig. 8 and Table S5, ESI†). PC is the most abundant lipid in the *Leishmania* membrane and it can be generated by a *de novo* pathway or by the threefold methylation of PE.[30] PC also serves as a reservoir of secondary messenger metabolites (*e.g.* lyso-PC and PA) and in general it seems to be an essential lipid for *Leishmania*.[31] We believe these PEs are being produced during infection due to inflammatory macrophage responses or they are involved in promastigote–amastigote transformation. If they are confirmed as a specific host response for *Leishmania* infection, they could be used as disease biomarkers.

Conclusions

The lipid composition of *L. amazonensis* has been well characterized, and found to be very different from that of *L. infantum*. Lipid changes during the life cycle of these parasites have also been demonstrated, which relates to *Leishmania* adaptation in its respective host-cells and its different clinical manifestations. Several hypotheses about involvement of PSPs in a parasite visceralization mechanism have been raised and the importance of plasmalogens in amastigotes, the intracellular form of the parasite, has been confirmed. PIO, another lipid class, was also pointed out as a possible marker able to differentiate both *Leishmania* species, but to date there are no recognized functions for these lipids in leishmaniasis. Further experiments integrating these lipidomic data with other omics approaches should enable even deeper knowledge of *Leishmania* metabolic pathways.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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