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Enriched PUFA environment of *Leishmania infantum* promastigotes promotes the accumulation of lipid mediators and favors parasite infectivity towards J774 murine macrophages

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Abstract

Leishmania parasites are the causative agents of visceral or cutaneous leishmaniasis in humans and of canine leishmaniasis. The macrophage is the predicted host cell of *Leishmania* in which the promastigote stage is transformed into amastigote. We previously showed changes in the fatty acid composition (FA) of lipids in two strains of *Leishmania donovani* upon differentiation of promastigote to amastigote, including increased proportions of arachidonic acid (AA) and to a less extent of docosahexaenoic acid (DHA). Here, we carried out supplementation with AA or DHA on two *Leishmania infantum* strains, a visceral (MON-1) and a cutaneous (MON-24), to evaluate the role of these FA in parasite/macrophage interactions. The proportions of AA or DHA in total lipids were significantly increased in promastigotes cultured in AA- or DHA-supplemented media compared to controls. The content of FA-derived oxygenated metabolites was enhanced in supplemented strains, generating especially epoxyeicosatrienoic acids (11,12- and 14,15-EET) and hydroxyeicosatetraenoic acids (5- and 8- HETE) from AA, and hydroxydocosahexaenoic acids (14- and 17-HDoHE) from DHA. For both MON-1 and MON-24, AA-supplemented promastigotes showed higher infectivity towards J774 macrophages as evidenced by higher intracellular amastigote numbers. Higher infectivity was observed after DHA supplementation for MON-24 but not MON-1 strain. ROS production by macrophages increased upon parasite infection, but only minor change was observed between control and supplemented parasites. We

Abbreviations: AA, arachidonic acid; CL, *Cutaneous leishmaniasis*; DHA, docosahexaenoic acid; FA, fatty acid; HDoHE, hydroxydocosahexaenoic acids; HETE, hydroxyeicosatetraenoic acids; LA, linoleic acid; MUFA, monounsaturated fatty acids; PG, prostaglandins; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; SFA, saturated fatty acids; VL, visceral leishmaniasis.

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propose that under high AA or DHA environment that is associated with AA or DHA enrichment of promastigote lipids, FA derivatives can accumulate in the parasite, thereby modulating parasite infectivity towards host macrophages.

KEYWORDS

arachidonic acid, docosahexaenoic acid, *Leishmania infantum*, lipid mediators, macrophages, reactive oxygen species

INTRODUCTION

Leishmaniasis are a complex of tropical and subtropical human diseases caused by unicellular parasites of the genus *Leishmania* that belongs to the family of trypanosomatids. Leishmaniasis is transmitted by a sand-fly vector and displays various clinical expressions (i.e., cutaneous, muco-cutaneous and visceral). The *Leishmania* life cycle involves two different stages: the promastigote inside the insect vector and the amastigote in the vertebrate's macrophages. The promastigotes inoculated by the vector are internalized by host macrophages via phagocytosis and undergo a transformation into the amastigote stage within a parasitophorous vacuole of phagolysosomal origin. Entry of intracellular pathogens and cell differentiation are generally associated with the reorganization of plasma membrane where lipids, essentially phospholipids (PL), glycolipids and sterols, are the main components. The fatty acid (FA) composition of plasma membrane PL was shown to modulate parasite/host cells interactions and antileishmanial drug sensitivity/resistance through regulation of membrane fluidity. Besides, FA are components of membrane anchors for a large diversity of surface glycoproteins in trypanosomatids including *Leishmania*, which have essential roles in host-parasite interaction (Aquino et al., 2021). We recently highlighted some FA specificities in lipids from *L. infantum* and *L. donovani* promastigotes, especially regarding polyunsaturated FA (PUFA), with a relative abundance of docosahexaenoic acid (DHA, 22:6 n-3) compared to very low proportion of arachidonic acid (AA, 20:4 n-6), which is the opposite of what is usually observed in mammalian cells. Noteworthy, we showed that AA content, and also to a lower extent DHA content, were specifically increased in amastigotes isolated from infected macrophages compared to promastigotes, supporting a functional role of these FA during parasite infection and/or intracellular survival (Bouazizi-Ben Messaoud et al., 2017).

The survival of the parasite in the host, in these two forms, is closely related to the immune status of the host and the ability of the parasite to escape to the host defenses. Macrophages play a primary role in the innate inflammatory response against the parasite, involving what is defined as the concept of classical and alternative activation, orchestrated respectively by

the so-called M1 and M2 macrophages. The M1 phenotype is characterized by the secretion of pro-inflammatory cytokines and microbicidal molecules (Covarrubias et al., 2013; Martinez & Gordon, 2014) whereas the M2 phenotype exhibits anti-inflammatory properties (Martinez & Gordon, 2014; Parisi et al., 2018). During *Leishmania* infection, host macrophages can undergo both M1 and M2 activation, leading respectively to killing or survival of the parasite (Tomiotto-Pellissier et al., 2018). The role of lipids in macrophage polarization during infection with trypanosomatids has recently aroused growing interest. It was reported that lipid extracts from *Trypanosoma cruzi* amastigotes induced a pro-inflammatory response in murine peritoneal macrophages, as evidenced by increased formation of lipid bodies, COX2 expression, TNF-alpha secretion, and NO release (Bott et al., 2018). Similarly, lipid extracts from *L. braziliensis* amastigotes were shown to favor M1 macrophage polarization relative to the M2 phenotype whereas promastigotes conversely favored the M2 phenotype with increased arginase activity, IL10 secretion and M2 mRNA markers (Carfagna et al., 2020). It is well-known that PUFA play a key role in inflammatory processes and their resolution through the production of lipid mediators, the interaction with cell surface receptors or the regulation of inflammatory gene expression. In mammalian cells, AA is well known as the precursor of proinflammatory mediators while DHA is conversely converted into proresolving derivatives (Dyall et al., 2022; Marion-Letellier et al., 2015). PUFA derivatives have been involved in host response to infection with *Trypanosoma* or *Leishmania* parasites (López-Muñoz et al., 2018). It was also demonstrated that these parasites were able to produce both proinflammatory and proresolving mediators, thereby modulating macrophage responses to parasite infection (Colas et al., 2018; López-Muñoz et al., 2018; Machado et al., 2011; Paloque et al., 2019).

Besides, PUFA such as AA and DHA are also known to be highly sensitive to oxidative stress due to their unsaturation level generating lipid peroxides, that are themselves a source of reactive oxygen species (ROS) and can initiate cell death (Su et al., 2019). Several studies have reported an increase of ROS production in macrophages during early stage of *Leishmania* infection most likely as a defense mechanism by

inducing parasite killing (Carneiro et al., 2016; Deschacht et al., 2012; Gantt et al., 2001).

The present study was undertaken to better understand the role of AA and DHA during parasite infection with a focus on metabolic aspects. We therefore examined the effects of a specific supplementation with AA or DHA on lipid FA composition and FA oxygenated metabolism in *Leishmania* promastigotes, as well as the impact in terms of parasite infectivity and ROS production in infected macrophages. We used two strains of *L. infantum*, one isolated from a patient with visceral leishmaniasis (VL) and one isolated from a lesion of a patient with cutaneous leishmaniasis (CL).

MATERIALS AND METHODS

Parasite strains and culture

Two isolates of *L. infantum* human strains, causing VL (MON-1) or CL (MON-24) were used in this study. The strains were initially isolated as part of the routine diagnostic (culture) procedure recommended by WHO for VL and CL samples. They were anonymized, archived and cryopreserved in the biobank of the Parasitology department of the Pasteur Institute at Tunis. No personal data was used. The VL strains were isolated from the bone marrow or blood leukocytes and the CL strains were isolated from dermal serosity as described (Chouihi et al., 2009). Primocultures of promastigotes were performed in biphasic Novy-MacNeal-Nicolle (NNN) medium at 26°C with subculture every week for 5 consecutive weeks. Identification of isolates was performed by isoenzyme electrophoresis (Rioux et al., 1990).

Promastigotes were cultured at 26°C in TC-199 medium (GE Healthcare, Pasching, Austria) supplemented with 20% fetal bovine serum and 100 µg/ml Penicillin/Streptomycin (Dutscher, Issy-les-Moulineaux, France). The parasites were cultured in 75-cm² culture flasks and were harvested during their exponential growth phase by centrifugation at 1300g for 15 min. The parasite pellet was washed three times with 10 ml of PBS and before lipids analysis a total protein assay was performed with the Bradford method.

Macrophage culture

The BALB/c mice macrophage cell line J774A.1 (European Collection of Cell Culture) was maintained by successive passages in RPMI-1640 culture medium (Dutscher) containing 10% (v/v) foetal bovine serum (FBS) (Sigma, St-Quentin Fallavier, France), 25 µg/ml nanomycopolitine and 100 µg/ml Penicillin/Streptomycin (Dutscher). Cultures were performed at 37°C with 5% CO₂.

Supplementation of promastigotes with FA

Stock ethanolic solutions of AA and DHA were prepared and diluted in RPMI without FBS at a final concentration of 1 mM. Promastigotes (MON-1 and MON-24 strains) were cultured in medium containing 10% FBS with addition (supplemented) or without addition (control) of AA or DHA at a final concentration of 50 or 100 µM with a final ethanol concentration not exceeding 0.1%. FBS was lowered to 10% to reduce the quantity of FA supplied by serum. The amounts of free FA (unesterified) and total FA (unesterified + esterified in glycerolipids) provided by the serum are detailed in Supplementary Table 1. In control conditions, free AA and DHA were thus supplied at only 1.2 and 0.1 µM, respectively, which is negligible compared to supplemented conditions. After 24 h, control or supplemented promastigotes were collected by centrifugation (15 min, 1300 g) and the supernatant containing FA was removed. The pellet containing parasites was either stored at -20°C (for lipid analyses) or resuspended in control culture medium for macrophage infection. Promastigote viability was determined before infection using (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt)/phenazine methosulfate (MTS/PMS) solution (CellTiter 96®; Aqueous Non-radioactive cell Proliferation Assay, Promega, Madison, USA). The absorbance of the formazan product was measured at 490 nm with a plate reader (Thermo Fisher Scientific 51118177 Multiskan EX Microplate Photometer, Villebon sur Yvette, France). None of the added FA induced toxicity as verified by viability ≥95% for both strains (data not shown). Furthermore, optical microscopic observation of supplemented promastigotes showed normal mobility and morphology compared to control parasites.

Fatty acid analysis of total promastigote lipids

FA composition of total lipids was determined as previously described (Bouazizi-Ben Messaoud et al., 2017). Briefly, promastigotes were lysed in 1 ml water containing 0.1% Triton X-100. Ten microliter of butylated hydroxytoluene in ethanol (50 µM final) was added as an antioxidant. Total lipids were extracted by the modified Bligh and Dyer method (Bligh & Dyer, 1959) with chloroform/methanol at a final ratio of 2:2:1.8 (chloroform/methanol/water, (v/v)). Lipid extracts were dried under nitrogen and total lipids were transmethylated for 90 min at 100°C using boron trifluoride (Sigma, St-Quentin Fallavier, France). The resulting FA methyl esters (FAME) were extracted by isooctane and analyzed by GC using a Hewlett-Packard system equipped with a Supelco SP2380 capillary column (60 m × 0.22 mm) with helium as a carrier gas.

FAME were identified by comparison with commercial standards. FA profiles were expressed as molar percentage of each individual fatty acid calculated from the sum of all detected FA taken as 100%.

Analysis of fatty acid-derived oxygenated metabolites in promastigotes

Promastigotes were cultured in TC-199 medium (GE Healthcare, Pasching, Austria) supplemented with 10% fetal bovine serum and 100 $\mu\text{g}/\text{mL}$ Penicillin/Streptomycin (Dutscher) in the absence or presence of AA and DHA at a final concentration of 100 μM . After 24 h, the parasites were collected by centrifugation (15 min, 1300g), washed 3 times with PBS, and stored at -20°C for analysis. For extraction, parasites were crushed with a FastPrep[®]-24 Instrument (MP Biomedical, Santa Ana, CA) in 0.5 ml of HBSS (Invitrogen, Carlsbad, CA, USA). 300 μl of cold methanol and 5 μl of internal standard (Deuterium labeled compounds) were added. After centrifugation at 900g for 15 min at 4°C , supernatants were transferred into 2 ml 96-well deep plates and diluted in H_2O to 2 ml. Samples were then submitted to solid phase extraction (SPE) using OASIS HLB 96-well plate (30 mg/well, Waters, St Quentin en Yvelines, France) pretreated with MeOH (1 ml) and equilibrated with 10% MeOH (1 ml). After sample application, extraction plate was washed with 10% MeOH (1 ml). After drying under aspiration, lipids mediators were eluted with 1 ml of MeOH. Prior to LC-MS/MS analysis, samples were evaporated under nitrogen gas and reconstituted in 10 μl on MeOH.

LC-MS/MS analyses of FA derivatives were performed as described (Le Faouder et al., 2013). Briefly, lipid mediators were separated on a ZorbAX SB-C18 column (2.1 mm, 50 mm, and 1.8 μm) (Agilent Technologies) using Agilent 1290 Infinity HPLC system coupled to an ESI-triple quadrupole G6460 mass spectrometer (Agilent Technologies Les Ulis, France). Data were acquired in Multiple Reaction Monitoring (MRM) mode with optimized conditions (ion optics and collision energy). Peak detection, integration and quantitative analysis were done using Mass Hunter Quantitative analysis software (Agilent Technologies) based on calibration lines built with commercially available eicosanoids standards (Cayman Chemicals, Ann Arbor, MI, USA). All detected metabolites were quantified in $\text{ng}/10^8$ parasites.

Evaluation of parasite infectivity

Macrophages were seeded in RPMI-1640 on glass slides in 24 well plates at a density of 70,000 cells per well and cultured at 37°C for 24 h. They were then

incubated at a ratio of 30 parasites per cell with control parasites or parasites supplemented with AA or DHA at 50 μM . The ratio parasite: cell 30:1 has been chosen as it yields an infection rate about 50% for both strains in control conditions. After 24 h, cells were washed three times with PBS, fixed in paraformaldehyde 8% and stained with DAPI (4', 6-diamidino-2-phenylindole) (Life Technologies, Paisley, UK). The slides were observed by fluorescence microscopy to count infected and non-infected cells as well as intracellular amastigotes. For each slide, a total of 500 cells were observed in two randomly selected fields. Parasite infectivity was evaluated by the percentage of infected cells to total counted cells (infection rate) and by the total number of intracellular amastigotes (parasite load) following macrophage exposure to control or supplemented promastigotes.

Measurement of ROS production

Macrophages were seeded in RPMI-1640 in 24 well plates at a density of 70,000 cells per well and cultured at 37°C for 24 h. Conditions of macrophage infection with *Leishmania* promastigotes were optimized by varying parasite: cell ratio from 5:1 to 30:1 and incubation time from 1 h to 6 h to induce ROS production. In the following experiments, macrophages were incubated for 4 h at a ratio of 30 parasites per cells with control or supplemented parasites. Incubation with 50 μM menadione that induces intracellular ROS generation through redox cycling was done as positive controls. Cells were then washed three times with PBS. Intracellular ROS production by macrophages was revealed with the intracellular probe H2DCFDA (Molecular probes, Life Technologies) at 5 μM at 37°C with 5% CO_2 . After 30 min incubation with the probe, cells were washed twice with PBS and the fluorescence intensity was analyzed on BD LSRII flow cytometer and data processed with BD FACSDiva (BD Biosciences, San Diego, CA, USA) and FlowJo software (Tree Star, Ashland, OR, USA). Fluorescence was also measured in not infected macrophages in absence and in presence of the probe. Data are expressed as mean intensity fluorescence (MFI).

Data presentation and statistical analyses

Results of FA profile are expressed as molar percentage of each individual FA calculated from the sum of all detected FA taken as 100%. Results of FA derivatives are expressed as $\text{ng}/10^8$ parasites. For both set of data, we identified the number of comparisons we had to perform using unpaired Student's *t*-test. We computed an adjusted *p*-value for multiple comparison using Bonferroni correction to maintain a family-wise error rate of 0.05.

TABLE 1 FA composition of total lipids from control and FA-supplemented promastigotes

	Control		AA-supplemented		DHA-supplemented	
	MON-1	MON-24	MON-1	MON-24	MON-1	MON-24
14:0	1.01 ± 0.20	1.58 ± 0.20	1.15 ± 0.32	1.2 ± 0.20	3.06 ± 1.14	1.38 ± 0.35
16:0	11.73 ± 2.89	15.93 ± 3.48	12.89 ± 3.04	13.34 ± 3.76	16.87 ± 0.95	15.02 ± 0.35
18:0	18.02 ± 2.64	11.97 ± 0.25	12.44 ± 2.16	11.27 ± 0.70	9.27 ± 0.31 ^a	10.8 ± 0.13 ^c
20:0	0.79 ± 0.24	0.2 ± 0.05	0.5 ± 0.07	0.2 ± 0.17	0.19 ± 0.03 ^{a,b}	0.16 ± 0.01
Σ SFA	31.50	29.68	26.98	26.01	29.39	27.36
16:1 n-9	0.86 ± 0.10	0.73 ± 0.24	0.78 ± 0.21	0.99 ± 0.13	0.66 ± 0.06	0.81 ± 0.02
16:1 n-7	1.12 ± 0.20	0.92 ± 0.15	1.09 ± 0.24	1.14 ± 0.07	1.12 ± 0.06	1.03 ± 0.03
18:1 n-9	33.4 ± 1.43	29.95 ± 0.11	28.61 ± 1.25 ^a	29.3 ± 0.86	25.57 ± 0.43 ^a	28 ± 0.15 ^{a,c}
18:1 n-7	3.76 ± 0.32	4.71 ± 0.29	3.27 ± 0.32	4.87 ± 1.01	4.47 ± 0.14 ^b	4.4 ± 0.18
Σ MUFA	39.14	36.31	33.75	36.3	31.82	34.24
18:2 n-6	3.84 ± 0.31	2.39 ± 0.39	4.24 ± 0.53	2.63 ± 0.23 ^c	2.78 ± 0.12 ^{a,b}	2.58 ± 0.03
18:3 n-6	12.51 ± 1.48	14.04 ± 0.33	10.46 ± 3.28	12.98 ± 1.38	13.1 ± 0.67	12.92 ± 0.25
20:2 n-6	ND	0.37 ± 0.02	0.11 ± 0.05 ^a	0.17 ± 0.18	0.32 ± 0.01 ^a	0.32 ± 0.04
20:3 n-6	4.21 ± 0.55	3.33 ± 0.99	4.66 ± 1.42	3.9 ± 0.20	1.95 ± 0.19 ^a	3.75 ± 0.05 ^c
20:4 n-6 (AA)	0.65 ± 0.11	0.89 ± 0.04	10.38 ± 1.77 ^a	5.77 ± 1.66 ^a	0.77 ± 0.04 ^b	0.92 ± 0.03 ^b
22:4 n-6	0.61 ± 0.11	0.7 ± 0.02	0.39 ± 0.09	0.63 ± 0.03	0.72 ± 0.03	0.66 ± 0.02
Σ n-6 PUFA	21.82	21.71	30.24	26.08	19.64	21.15
18:3 n-3	0.47 ± 0.09	0.01 ± 0.02	0.22 ± 0.08	0.05 ± 0.03	0.12 ± 0.10 ^a	0.02 ± 0.10
20:3 n-3	0.08 ± 0.17	0.05 ± 0.01	0.65 ± 0.53	0.06 ± 0.00	0.06 ± 0.01	0.06 ± 0.00
20:5 n-3	ND	0.03 ± 0.00	0.13 ± 0.05 ^a	0.1 ± 0.06	0.04 ± 0.00 ^a	0.03 ± 0.01
22:5 n-3	3.47 ± 0.52	5.95 ± 0.98	3.79 ± 0.43	5.64 ± 0.84	6.1 ± 0.41 ^{a,b}	6.22 ± 0.06
22:6 n-3 (DHA)	3.49 ± 0.62	6.22 ± 0.78	4.26 ± 0.50	5.77 ± 0.71	12.91 ± 1.17 ^{a,b}	10.91 ± 0.54 ^{a,b}
Σ n-3 PUFA	7.51	12.26	9.05	11.57	19.14	17.22

Note: The FA composition was determined by GC analysis as described in materials and methods. Data, expressed as mole percent, are means ± SD of four independent determinations. Statistical analyses were performed using unpaired Student's *t*-test with Bonferroni correction for multiple comparison. Letters indicate statistical difference ($p < 0.05$): (a), AA or DHA versus respective control; (b), AA versus DHA; (c), MON-1 versus MON-24. Abbreviations: MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Results of parasite load are presented as the ratio of the number of parasites in macrophages infected with supplemented promastigotes to the number of parasites in macrophages infected with control promastigotes. We compared AA- or DHA-supplemented parasites versus respective controls (from values of parasite number, paired data) using paired Student's *t*-test. *p*-Values adjusted for these four comparisons was computed using Bonferroni method. For ratio comparisons between strain and acid, we performed an one-way ANOVA with ratio as dependent variable and an independent variable at four levels obtained by crossing strain and acid levels (mon1:AA, mon1:DHA, mon24:AA, and mon24DHA). Tukey-HSD post-hoc test for multiple comparison was performed a posteriori as appropriate.

Results of ROS production are expressed as mean intensity fluorescence (MFI). The ROS concentration

according to the ratio of parasite (0, 10, 20, and 30) was analyzed for each strain independently. A mixed-effects model was performed with MFI as dependent variable, parasite ratio as independent variable, and a random intercept for the samples.

The ROS concentration according to time (0, 1, 2, 3, 4, and 6 h) was analyzed by taking time as a variable factor to compare the ROS concentration at any time to time 0. A mixed-effects model was performed with MFI as dependent variable, strain and time as independent variables and a random intercept for the samples.

The ROS concentration according to the supplemented FA was analyzed independently per strain by modeling the MFI in macrophages against the variable FA defined with 5 levels (0; 50:AA; 100:AA; 50 DHA; and 100:DHA). A mixed-effects model was fitted for each strain with MFI as dependent variable, FA as

independent variables and a random intercept for the samples.

For all models, interactions were tested as appropriate. Models were simplified to keep variables with significant effect. Model assumptions were checked. For all test a p -value less than 0.05 was considered statistically significant.

RESULTS

FA profile in total lipids of control and fatty acid supplemented promastigotes

The whole FA compositions of total lipids from the two *Leishmania* strains are reported in Table 1. Statistical analyses show no significant differences between MON1 and MON24 in control conditions. Both strains contained about 30% saturated FA (SFA), 40% mono-unsaturated FA (MUFA) and 30% polyunsaturated FA (PUFA), with the n -6 FA being more abundant than the n -3 FA. Thereby, AA (20:4 n -6) proportion was very low representing less than 1% of total FA in both strains compared to DHA (22:6 n -3) proportion accounting for 3 and 6% of total FA in MON-1 and MON-24, respectively.

After supplementation with 50 μ M AA, the proportion of AA was significantly increased in both MON-1 ($p = 0.0009$ vs. control) and MON-24 ($p = 0.0099$ vs. control). The supplementation with 50 μ M DHA led to a highly significant increase of DHA proportion in MON-1 ($p = 0.0001$ vs. control) and in MON24 ($p = 0.0081$ vs. controls). For both strains, statistical analyses indicate that AA proportions in AA-supplemented strains were significantly different from those of DHA supplemented strains, and inversely for DHA proportions, indicating specificity for the added FA. Statistical analyses further show no significant differences of AA proportion in MON1 and MON24 after AA supplementation, as well as of DHA proportion after DHA supplementation, indicating no strain specificity towards AA or DHA.

Regarding other FA, only minor changes were observed. AA supplementation did not change the proportions of other n -6 FA whatever the strain. After DHA supplementation, some n -3 PUFA namely 22:5 n -3 and 20:5 n -3 were slightly increased in MON-1 compared to respective controls, and not changed in MON-24. The proportions of C-18 FA, especially 18:0 and 18:1 n -9 that are among major FA in non-supplemented parasites, were decreased in MON1 after DHA supplementation, which was not the case for MON-24.

Altogether, these data indicate that the FA composition of both strains was specifically modified according to the FA added to the culture medium and that AA and DHA proportions were increased up to similar values in both strains.

Fatty acid-derived oxygenated metabolites content in control and supplemented promastigotes

We also evaluated the consequences of AA or DHA supplementation on the levels of FA oxygenated derivatives in promastigotes, especially those originating from PUFA metabolism. In control conditions, mainly linoleic acid (LA) derivatives were recovered in both MON-1 and MON-24 promastigotes grown in control conditions, whereas AA and DHA derivatives were barely detected (Figure 1 and Table 2). After AA supplementation, the level of AA derivatives was markedly increased in MON-1 and MON-24, to a similar extent in both strains. DHA supplementation was associated with a significant increase of DHA derivatives in both strains, slightly higher in MON1 than in MON24 without reaching statistically significant difference between the strains ($p = 0.11$). The distributions of major metabolites are detailed in Table 2. In promastigotes supplemented with AA, significant increase was observed especially for epoxyeicosatrienoic acids (EET) and hydroxyeicosatetraenoic acids (HETE) compared to respective controls and DHA supplemented promastigotes. In addition, the levels of each derivative were very close in the two strains (no statistical differences between MON1 and MON24, $p > 0.8$ for all AA derivatives). Promastigotes supplemented with DHA showed significant increased levels of 17-hydroxydocosahexaenoic acids (HDoHE) and 14-HDoHE in both strains compared to respective controls and AA-supplemented promastigotes. Note that levels of 17-HDoHE was about 2.5-fold higher in MON1 than in MON 24 although not reaching statistical significance ($p = 0.08$). Overall, these data show that AA and DHA derivatives are specifically increased in promastigotes respectively supplemented with AA and DHA, with no difference between the strains except for a trend to higher content of the DHA derivative 17-HDoHE in MON1 than in MON24.

We also determined the profiles of hydroxyderivatives derived from saturated FA (Table 2). AA supplementation has no significant impact of SFA hydroxyderivatives levels in both strains. After DHA supplementation, SFA hydroxyderivatives are significantly increased in MON1 versus control and AA supplemented whereas no change was observed in MON24.

Supplementary Table 2 shows the levels of FA derivatives recovered in the culture medium in control, AA-supplemented and DHA-supplemented conditions. Compared to control medium, AA-supplemented medium contained significantly higher amounts of AA derivatives, as well as DHA derivatives in DHA supplemented medium. By contrast, no change in SFA hydroxy derivatives was observed. These data indicate that AA and DHA derivatives accumulate in supplemented media. Of interest, the relative distribution of FA

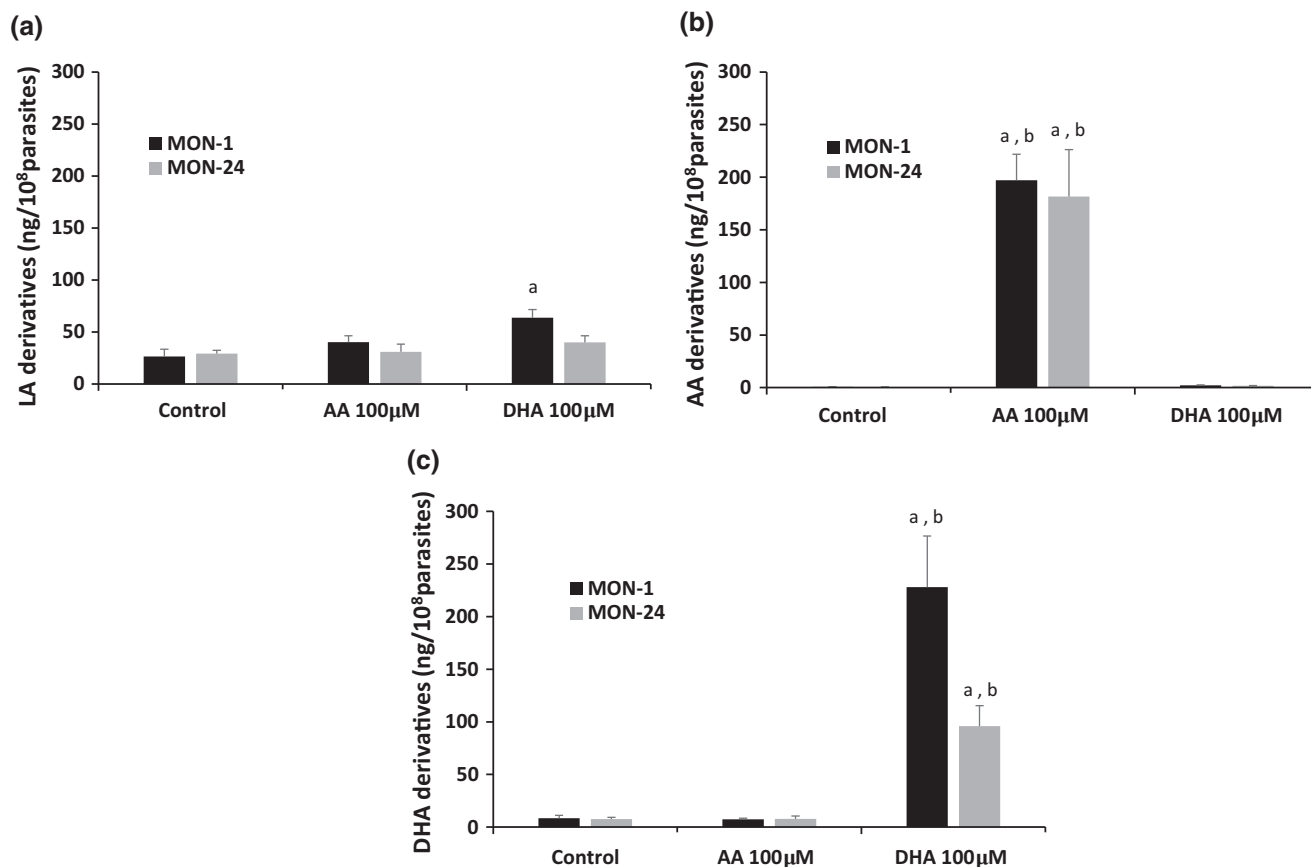


FIGURE 1 FA derivatives in control and supplemented promastigotes. FA derivatives from (a) linoleic acid (LA), (b) arachidonic acid (AA), and (c) docosahexaenoic acid (DHA) were detected by LC–MS/MS analysis in control and supplemented promastigotes (AA or DHA 100 μM). Data represent the sum of derivatives from LA, AA or DHA, expressed in ng/10⁸ parasites and are means ± SD of 3 independent experiments. Statistical analyses were performed using unpaired Student's *t*-test with Bonferroni correction for multiple comparison. Letters indicate statistical difference ($p < 0.05$): a, AA or DHA versus respective control; b, AA versus DHA

derivatives differs between media and parasites. These data therefore suggest that while FA derivatives recovered in promastigotes could be taken up from the culture media, they also likely originate from FA metabolism in the parasites.

Effect of fatty acid supplementation on promastigote infectivity to cultured macrophages

The functional impact of AA or DHA supplementation was then evaluated on promastigote infectivity towards J774 macrophages as indexed by infection rate (% of infected cells over total cells) and parasite load (number of intracellular amastigotes). As an illustration, Figure 2a shows DAPI staining of macrophages infected with *Leishmania* promastigotes with small blue dots representing amastigote nuclei, from which infection rate and parasite load are determined.

For both strains, the infection rate was about 40%–60% of total macrophages after exposure with control promastigotes. No significant change was observed with AA-

or DHA- supplemented promastigotes (data not shown). Figure 2b shows the parasite load ratios for the supplemented parasites on the respective controls whose value was set to 1. Parasite load in macrophages infected with AA-supplemented parasites was significantly increased in both MON-1 and MON-24 when compared to macrophages infected with control parasites (Bonferroni adjusted p -value = 0.026 and 0.021, respectively). Parasite load was increased after infection with DHA-supplemented MON-24 parasites (adjusted p -value = 0.023 vs. control) while no significant difference was observed with DHA-supplemented MON-1 parasites (adjusted p -value = 1 vs. control). Tukey-HSD test after ANOVA indicated that infectivity ratios in DHA supplemented promastigotes was significantly different between MON1 and MON24 (adjusted p = 0.020) in contrast to AA-supplemented promastigotes (adjusted p -value = 0.996).

ROS production

Several studies have reported that ROS production was increased in macrophages in response to

TABLE 2 Quantification of FA derivatives in control and FA-supplemented promastigotes

	Control		AA-supplemented		DHA-supplemented	
	MON1	MON24	MON1	MON24	MON1	MON24
LA derivatives						
9-HODE	4.92 ± 1.30	4.24 ± 0.96	6.55 ± 1.28	4.39 ± 1.77	18.51 ± 4.48	9.50 ± 1.79
13-HODE	13.86 ± 5.35	14.44 ± 4.27	25.43 ± 4.66	18.62 ± 4.60	35.94 ± 8.55	20.04 ± 2.63
9,10-DiHOME	6.52 ± 0.24	9.01 ± 2.28	7.07 ± 0.04	5.81 ± 0.71	6.70 ± 4.24	7.61 ± 1.37
12,13-DiHOME	1.01 ± 0.70	1.40 ± 0.16	1.20 ± 0.24	1.98 ± 0.92	2.57 ± 0.82	2.77 ± 0.70
AA derivatives						
5,6-EET	0	0	5.19 ± 1.34 ^{a,b}	5.73 ± 1.91	0	0
8,9-EET	0	0	15.40 ± 4.75 ^{a,b}	20.08 ± 2.16 ^{a,b}	0	0
11,12-EET	0	0	105.95 ± 9.93 ^{a,b}	93.92 ± 23.85	0	0
14,15-EET	0	0	27.22 ± 10.13	24.49 ± 6.57	0	0
5-HETE	0.45 ± 0.04	0.28 ± 0.09	13.48 ± 3.54 ^{a,b}	10.96 ± 2.72 ^{a,b}	1.10 ± 0.21	0.96 ± 0.20
8-HETE	0	0	11.49 ± 0.59 ^{a,b}	11.17 ± 1.99 ^{a,b}	0	0
12-HETE	0	0	7.12 ± 0.09 ^{a,b}	5.31 ± 1.32	0	0
15-HETE	0.36 ± 0.07	0.25 ± 0.21	4.75 ± 1.08 ^{a,b}	4.09 ± 1.20	0.99 ± 0.26	0.76 ± 0.11
LxA4	0	0	2.08 ± 0.34 ^{a,b}	2.00 ± 0.97	0	0
PGD2	0	0	3.61 ± 0.32 ^{a,b}	3.19 ± 1.74	0	0
PGE2	0	0	0.72 ± 0.204 ^{a,b}	0.69 ± 0.36	0	0
DHA derivatives						
14-HDoHE	2.32 ± 0.77	2.36 ± 0.59	2.35 ± 0.78	2.48 ± 0.96	47.01 ± 9.98 ^{a,b}	27.45 ± 6.47 ^{a,b}
17-HDoHE	6.14 ± 2.02	5.13 ± 1.25	5.07 ± 0.86	5.20 ± 1.91	181.1 ± 39.8 ^{a,b}	68.54 ± 13.2 ^{a,b}
Saturated FA derivatives						
C14-3OH	27.76 ± 5.70	12.53 ± 7.28	79.19 ± 22.73	15.72 ± 3.66 ^c	45.98 ± 0.13 ^a	27.41 ± 5.59 ^c
C16-2OH	6.85 ± 0.11	7.06 ± 1.13	10.31 ± 1.82	6.57 ± 1.49	99.63 ± 5.72 ^{a,b}	12.34 ± 2.48 ^c
C16-3OH	18.65 ± 7.22	7.40 ± 4.32	29.41 ± 3.24	8.70 ± 3.27 ^c	21.76 ± 0.56 ^{a,b}	15.66 ± 2.71 ^c
C18-2OH	1.64 ± 0.64	2.52 ± 0.61	1.93 ± 0.71	2.37 ± 0.50	46.08 ± 0.58	3.42 ± 0.92

Note: FA derivatives were detected by LC-MS/MS analysis as described in materials and methods. Data are expressed as ng/10³ parasites and are means ± SD of three independent determinations. Statistical analyses were performed using unpaired Student's *t*-test with Bonferroni correction for multiple comparison. Letters indicate statistical difference ($p < 0.05$): (a), AA or DHA vs respective control; (b), AA versus DHA; (c), MON-1 versus MON-24.

Leishmania infection. It is commonly accepted that due to their high level of unsaturation, AA and DHA are very sensitive to peroxidation thus promoting the production of ROS (Su et al., 2019). We therefore investigated whether AA or DHA enrichment of promastigotes following supplementation could modify their ability to trigger ROS production in infected macrophages.

ROS production was evaluated with the fluorescent probe H2DCFDA. In average, MFI ranged to 170–190 in macrophages without H2DCFDA and to 180–220 after incubation with H2DCFDA, indicating that fluorescence background is low. MFI were markedly increased (range to 300–700) after incubation with the positive control menadione. As shown in Figure 3, MFI was consistently increased after infection with parasite (range of 300–400), indicating ROS production. Infection with both MON1 and MON24 promastigotes elicited significant ROS production in macrophages at a ratio of 30:1 (number of parasites per cell, 3A) ($p = 0.0018$ and $p = 0.014$, respectively). ROS production was

significantly increased at any time compared to time 0 ($p < 0.05$) with no strain difference (3B). The impact of FA supplementation of parasites was then examined at a ratio 30:1 after 4 h infection (3C). ROS levels were slightly higher in macrophages infected with MON-24 promastigotes supplemented with AA at 50 μ M ($p = 0.0021$) and 100 μ M ($p = 0.0026$) and with DHA 100 μ M ($p = 0.028$) compared to controls. No difference was observed with AA- or DHA-supplemented MON-1. Together these data show only minor change of ROS production in macrophages infected with supplemented vs. control parasites.

DISCUSSION

Our present data show that the infectivity of two *L. infantum* strains, one cutaneous and one visceral, can be modulated by FA available in their environment. Both MON-1 and MON-24 promastigotes supplemented with

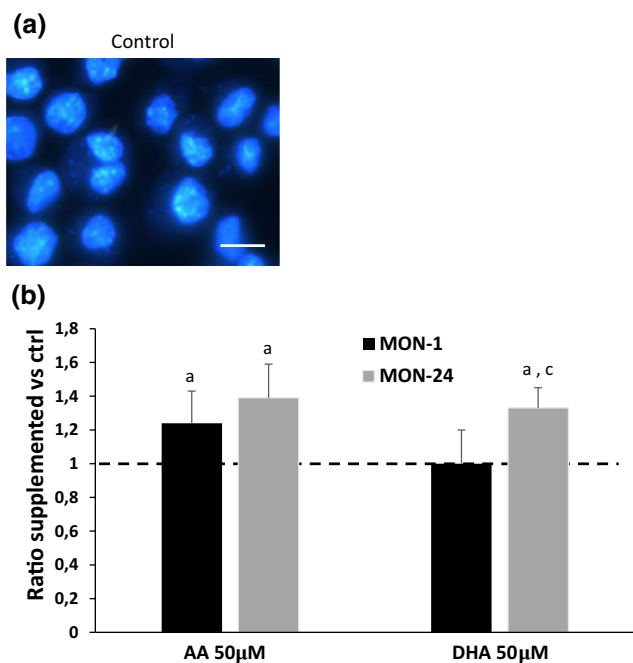


FIGURE 2 Parasite load in control and supplemented promastigotes. J774 macrophages were infected with control or supplemented promastigotes (AA or DHA 50 µM) for 24 h at ratio 30:1 parasite/cell. (a) Representative DAPI staining of J774 macrophages infected with control MON-24 promastigote. Small DAPI stained dots (arrow) represent nuclei of intracellular parasites localized in the cytoplasm of infected macrophages. (b) Parasite load. Data are expressed as the ratio of parasite number in macrophages infected with supplemented promastigotes over parasite number in macrophages infected with control promastigotes and are the mean \pm SD of 5 independent experiments. Statistical analyses were performed using paired Student's *t*-test with Bonferroni correction for multiple comparison or one-way ANOVA followed by Tukey-HSD post-hoc test for multiple comparison as detailed in materials and methods. Letters indicate statistical difference ($p < 0.05$): a, AA or DHA versus respective control; c, MON-1 versus MON-24

AA showed increased infectivity towards host cells *in vitro*, whereas DHA supplementation increased infectivity only in MON-24 strain. These changes of parasite infectivity were associated with an enrichment of parasite lipids with the supplemented FA, as well as increased levels of AA- and DHA-oxygenated metabolites.

The enrichment in AA or DHA of promastigote lipids after FA supplementation supports the concept that trypanosomatids, including some *Leishmania* species are able to scavenge lipids from their host environment. Plasma lipoproteins such as low density lipoproteins have been described as an important source of FA supplied as cholesterol esters and phospholipids through internalization processes (Coppens et al., 1995; De Cicco et al., 2012). In addition, exogenous free FA are efficiently salvaged by trypanosomatids (Berman et al., 1987; Voorheis, 1980). Orthologues of human fatty acid transport protein (FATP) have been identified in the genome of *Leishmania major* and several studies have suggested the presence of fatty acid binding

proteins (FABP) and FATP that would be involved in the specific uptake of FA in *Leishmania* parasites (Aquino et al., 2021; Maache et al., 2005; Uttaro, 2014). Likewise, our present data support the hypothesis that free FA are efficiently taken up by the promastigote forms of *Leishmania*. Besides the machinery for the uptake of exogenous FA, trypanosomatids are able to synthesize FA *de novo*. The genome of *L. major* encodes FA synthase, elongases and desaturases for *de novo* synthesis of saturated, monounsaturated and polyunsaturated FA, including stearyl CoA desaturase that initiates the conversion of 18:0 into 18:1 n-9 and other desaturases for up to C-22 PUFA synthesis (Lee et al., 2007; Uttaro, 2014). In both strains, AA supplementation led to an increase of AA proportion with no significant change of other n-6 FA, in particular longer chain 22:4 n-6 or shorter chain 20:3 n-6, indicating low elongation/retroconversion rate. DHA supplementation was associated to an increased proportion of DHA in both strains, as well as a moderate retroconversion of DHA into 22:5 n-3 in MON-1 but not in MON-24. These differences suggest that although n-3 and n-6 FA share enzymatic pathways for *de novo* synthesis in *Leishmania* (Lee et al., 2007), they undergo distinct metabolism in promastigotes, with differences between parasite strains.

Long chain PUFA such as AA and DHA have been widely reported to induce cellular oxidative stress. Likewise, exogenously added AA and DHA were shown to promote ROS production in *L. donovani* promastigotes (Mathur et al., 2015). The production of ROS has been observed in macrophages infected with *Leishmania amazonensis*, *Leishmania braziliensis*, most likely as a defense mechanism to eliminate parasites (Carneiro et al., 2016; Reybier et al., 2010; Roma et al., 2016; Thomazelli et al., 2018). Our data consistently show increased ROS production in both MON-24 and MON-1 infected macrophages. No or only minor difference in ROS production was observed in macrophages infected with AA- or DHA- supplemented parasites compared to respective controls. However, since oxidative status is finely regulated through several parameters besides ROS production (i.e., activity of antioxidant enzymes, vitamin E levels), we cannot exclude that oxidative status of macrophages could be modified upon infection with AA- or DHA- supplemented promastigotes. In addition, although DHA is assumed to induce oxidative stress, some studies have conversely reported anti-oxidant activities for this FA (Lagarde et al., 2018).

We previously reported a significant increase of AA and DHA proportions in parasite lipids upon differentiation of *L. donovani* promastigotes to intramacrophagic amastigotes (Bouazizi-Ben Messaoud et al., 2017). Our present findings that AA and DHA enrichment of promastigotes increased intracellular parasite load support a role of these FA in the infection process. After AA

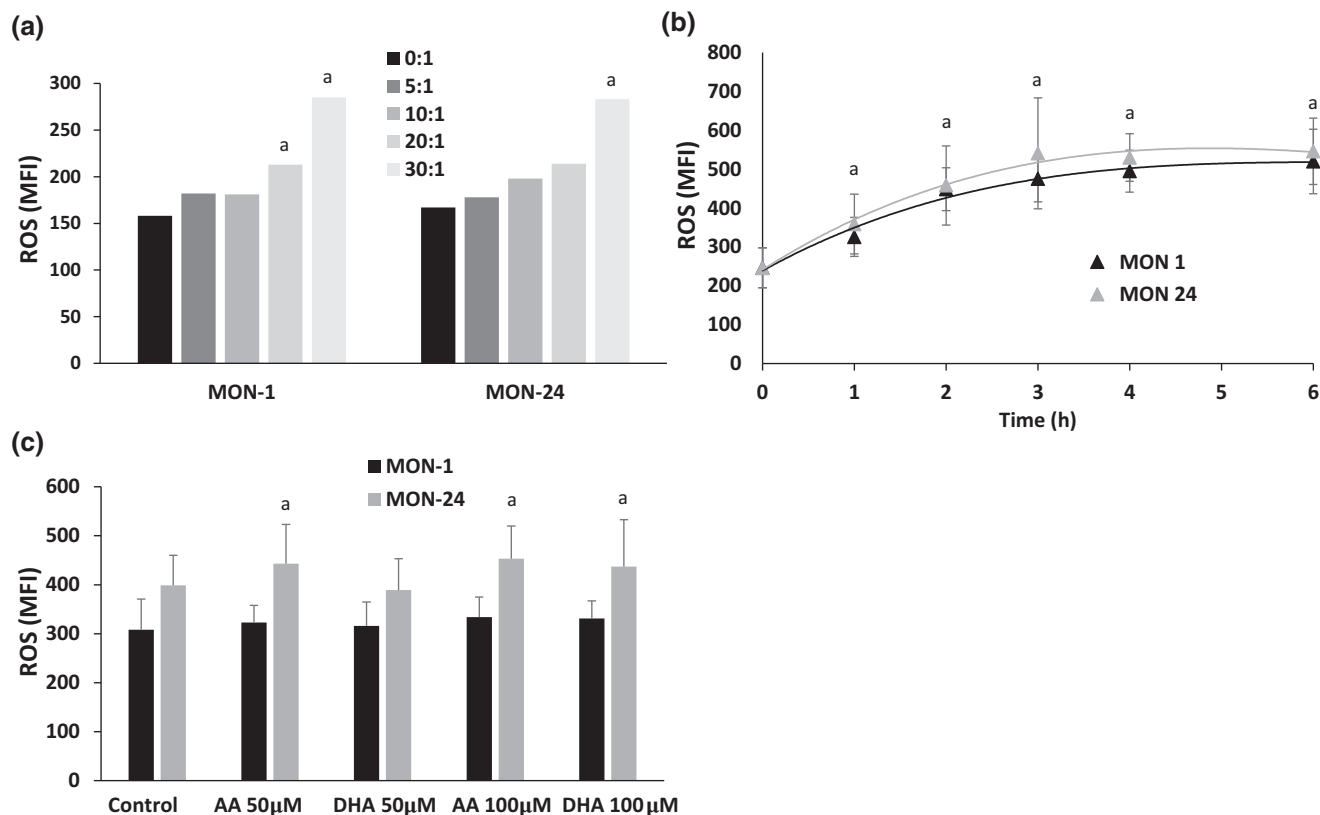


FIGURE 3 ROS production in J774 macrophages. (a) J774 macrophages were infected by MON-1 and MON-24 promastigotes at increasing parasite: cell ratios for 24 h. Non-infected macrophages correspond to ratio 0:1. Data are expressed as MFI and are the mean of duplicate values of two independent experiment. (b) J774 macrophages were infected by MON-1 and MON-24 promastigotes for increasing time at ratio 30:1 parasite: cell. Data are expressed MFI and are the mean \pm SD of 7 independent experiments in duplicate values. (c) J774 macrophages were infected by control or supplemented promastigotes (AA or DHA, at 50 and 100 μ M) at ratio 30:1 for 4 h. Data are expressed as MFI and are the mean \pm SD of 5 independent experiments in duplicate values. Statistical analyses were performed as detailed in materials et methods. 3A: letter “a” indicates statistical difference ($p < 0.05$) vs respective 0:1. 3B: letter “a” indicates statistical difference ($p < 0.05$) versus time 0; 3C: letter “a” indicates statistical difference ($p < 0.05$) versus respective control

supplementation, AA content in total lipids was increased in both strains to similar extent, as well as infectivity, suggesting a correlation between AA proportion and infectivity potency. After DHA supplementation, the proportion of DHA in total lipids increased to similar values in both strains, but infectivity increased only in MON24, suggesting that increasing infectivity with DHA is independent or only partially dependent on its proportion in the parasite lipids. Besides, AA is well known in mammalian cells as the precursor of pro-inflammatory mediators while DHA is conversely converted into pro-resolving derivatives. It has been shown that several *Leishmania* species including *L. infantum* were able to convert AA into prostaglandins PGE₂, PGD₂, and PGF_{2 α} (Araujo-Santos et al., 2014; Kabututu et al., 2003). PGF_{2 α} produced by *L. infantum* is important during its development into the infective metacyclic stage and increases the parasite burden (Araujo-Santos et al., 2014). Moreover, PGE₂ produced by host cell macrophages promotes the survival of *L. donovani* by inhibition of the host’s immune response (Saha et al., 2014). More recently, an extensive study

compared the profiles of proinflammatory and proresolving mediators in non-infectious (procyclic) and infectious (metacyclic) *Leishmania* promastigotes, revealing that specific AA and DHA metabolites were increased in the metacyclic form (Paloque et al., 2019). Noteworthy, they reported an elevated production of 8-HETE, 14- and 17-HDoHE in metacyclic promastigotes, the two latter being the precursors of proresolving mediators (i.e. maresins and resolvins). They further showed that the exposure of macrophages with the lipids extracts of metacyclic promastigotes containing both AA and DHA derivatives induced the production of proresolving mediators in macrophages such as resolving D2, protectin Dx, and maresin 1, and their polarization into M2 phenotype (M1 inflammatoire; M2 antiinflammatoire). Of interest, we found that 8-HETE and 14- and 17-HDoHE were among the major metabolites recovered in AA- or DHA- supplemented promastigotes together with 5-HETE and EET. Of interest, infectivity of AA supplemented promastigotes is positively correlated of AA derivatives in both strains. We thus propose that supplementation of promastigote with AA could

modulate parasite infectivity through the accumulation of AA derivatives in the *Leishmania* parasites, especially HET and EET. In DHA-supplemented promastigotes, the lower the levels DHA derivatives and SFA hydroxy derivatives, the higher infectivity, which may explain the difference in terms of infectivity between the two strains.

It was shown that biological effects of PUFA derivatives can vary depending on the strain. For example, while two cutaneous strains, *L. amazonensis* and *L. major*, induce the production of PGE₂ in infected macrophages, PGE₂ increases the destruction of parasites in the case of *L. amazonensis*, but conversely promotes the survival of the parasite in the case of *L. major*. Similarly, in the case of visceral strains, PGE₂ increases the killing of parasites in *L. infantum*, but reduces the immune response against the parasite in *L. donovani* (López-Muñoz et al., 2018). It can thus be hypothesized that AA and DHA derivatives, either derived from promastigotes or induced in infected macrophages could be more or less active depending on the strain of *Leishmania*.

Overall, our study improves fundamental knowledge of FA metabolism in *Leishmania* by demonstrating that AA and DHA are not only taken up by promastigotes but also incorporated into parasite lipids and converted into biologically active metabolites. Further investigations will be needed to confirm the involvement of FA oxygenated metabolites and explore other potential mechanisms including AA and DHA incorporation in specific lipids/glycerophospholipids/lipid molecular species or specific membrane domains, FA interaction with specific gene expression and/or interaction with membrane receptors (Marion-Letellier et al., 2015), and regulation of oxidative and inflammatory status in infected macrophages. As highlighted in a recent review (Aquino et al., 2021), FA play a central role in trypanosomatids, including *Leishmania*, but the mechanisms underlying their biological activities are still poorly understood. A better knowledge of peculiar metabolic pathways of different FA, in different *Leishmania* strains is therefore a key issue in leishmaniasis.

AUTHOR CONTRIBUTIONS

Marine Leroux: Investigation; Formal analysis; Visualization. **Hana Bouazizi-Ben Messaoud:** Investigation. **Céline Luquain-Costaz:** Writing-Review & Editing. **Lars P. Jordheim:** Methodology. **Pauline Le Faouder:** Methodology. **Marie-Paule Gustin:** Statistical analyses. **Karim Aoun:** Resources. **Philippe Lawton:** Writing-Review & Editing. **Samira Azzouz-Maache:** Conceptualization; Supervision. **Isabelle Delton:** Conceptualization; Supervision; Writing-Original Draft.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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