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ORIGINAL PAPER

Fatty acid profiles in *Leishmania* spp. isolates with natural resistance to nitric oxide and trivalent antimony

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Abstract Fatty acids, especially those from phospholipids (PLFA), are essential membrane components that are present in relatively constant proportions in biological membranes under natural conditions. However, under harmful growth conditions, such as diseases, environmental changes, and chemical exposure, the fatty acid proportions might vary. If such changes could be identified and revealed to be specific for adverse situations, they could be used as biomarkers. Such biomarkers could facilitate the identification of virulence and resistance mechanisms to particular chemotherapeutic agents. Therefore, specific biomarkers could lead to better therapeutic decisions that would, in turn, enhance treatment effectiveness. The objective of this study was to compare the fatty acid profiles of trivalent antimony and nitric oxide (NO)-resistant and -sensitive *Leishmania chagasi* and *Leishmania amazonensis* isolates. Fatty acid methyl esters (FAMEs) were obtained from total lipids (MIDI), ester-linked lipids (ELFA), and ester-linked phospholipids (PLFA). FAMEs were analyzed by chromatography and mass spectrometry. Species- or resistance-associated differences in FAME profiles were assessed by nonmetric multidimensional scaling, multiresponse permutation procedures, and indicator species analyses. The isolate groups had different MIDI-FAME profiles. However, neither the ELFA nor PLFA profiles differed between the sensitive and resistant isolates. Levels of the fatty acid 18:1 Δ9c were increased in sensitive isolates ($p < 0{,}001$), whereas the fatty acid 20:4 Δ5,8, 11,14 showed the opposite trend ($p < 0.01$). We conclude that these two fatty acids are potential biomarkers for NO and antimony resistance in *L. chagasi* and *L. amazonensis* and that they could be helpful in therapeutic diagnoses.

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Introduction

Leishmaniasis represents a spectrum of diseases that affect over 12 million people worldwide according to the World Health Organization (WHO) (2010). Although it has a major impact on public health, especially in developing countries, effective treatments for this "neglected" disease are still lacking (Hotez et al. 2007). In addition, the first-choice drugs—the pentavalent antimonials—require cautious administration under clinical and laboratory monitoring due to toxic side effects such as nephrotoxicity, cardiotoxicity, hepatotoxicity, and fatal arrhythmias (Rath et al. 2003; Ouelette et al. 2004).

Leishmaniasis is caused by *Leishmania* spp., a kinetoplastide protozoan that lives as a flagellate form (promastigote) in the midgut of its sandfly vector. After transmission to a vertebrate host, typically a human or dog, *Leishmania* are converted into

amastigote forms. Depending on the *Leishmania* species, the disease can be presented in various clinical forms such as cutaneous, mucocutaneous, and visceral.

Nitric oxide (NO), a free radical generated in macrophages by the nitric oxide synthase enzyme (iNOS), is a microbicidal molecule produced to control infections. Several studies have demonstrated the antiparasitic effects of NO against *Leishmania* (Mauel and Ransjin 1997). Nonetheless, *Leishmania* were observed to reduce endogenous NO production, and some isolates were found to be naturally resistant to nitric oxide (Giudice et al. 2007; Santos et al. 2012). Isolates resistant to available leishmaniasis treatment drugs such as antimony (Croft et al. 2006) have also been reported. Additionally, there are isolates that are resistant to both antimonies and NO, suggesting a common resistance pathway (Holzmuller et al. 2005).

Studies that employed a metabolomics approach as a tool to analyze the diversity of *Leishmania* isolates are emerging (Scheltema et al. 2010). T'Kindt et al. (2010) characterized clinical *Leishmania donovani* isolates that were resistant or not to sodium stibogluconate. The authors found that lipids comprised the largest class of identified metabolites and that the differences in the phospholipid and sphingolipid contents were the main differences observed between the phenotypes. According to Zhang and Beverley (2010), these two lipid classes are both abundant and critical to virulence and viability in *Leishmania*.

Fatty acid analysis is a method of studying lipids. Piotrowska-Seget and Mrozik (2003) reported that polyunsaturated fatty acids are rare in bacterial membranes and might be common in eukaryotes. According to Adosraku et al. (1993), polyunsaturated fatty acids represent a large proportion of the total lipids in *Leishmania*. Moreover, Alloatti and Uttaro (2011) emphasized the role of polyunsaturated fatty acids in cellular maintenance, especially in trypanosomatid membranes.

Interestingly, in *L. donovani*, the fatty acids from phosphatidylcholine and phosphatidylethanolamine were found to differ in drug-resistant strains compared to sensitive strains. Additionally, polyunsaturated fatty acids from phosphatidylcholine were shown to be present in higher amounts in antimony-resistant isolates compared to sensitive isolates (T'Kindt et al. 2010).

Currently, fatty acids are used as biomarkers for different groups of microorganisms such as fungi, bacteria, and actinomyces, among others. The fatty acids 11Me19:1 and 18:2ω6, 9 can be used as biomarkers of *Vibrio cholera* (Guckert et al. 1986). Fatty acid biomarkers can also indicate starvation or stationary growth phase in bacteria (Guckert et al. 1986; Piotrowska-Seget and Mrozik 2003). Furthermore, fatty acids are key molecules that can indicate clinical conditions such as drug resistance in infectious parasites (Mohanty et al. 2012).

Therefore, clinically useful biomarkers can help to identify pathogenic organisms that are resistant to particular chemotherapeutic agents. To make treatments more effective, specific biomarkers could lead to better therapeutic decisions with regard to candidate drugs and their indications. Thus, our hypothesis is that antimony and NO-resistant and -sensitive strains of *Leishmania chagasi* and *Leishmania amazonensis* will present with different fatty acid profiles that could lead to the identification of a fatty acid-resistance biomarker in these organisms. In this study, fatty acid profiling of antimony (Trivalent antimony®) and NO-resistant and -sensitive *L. chagasi* and *L. amazonensis* isolates was performed according to ELFAME, phospholipid fatty acids (PLFA), and MIDI procedures.

Material and methods

Parasites

Isolates of nitric oxide and trivalent antimony-resistant and -sensitive *L. chagasi* ($n=10$) and *L. amazonensis* ($n=2$) were selected from the *Leishmania* bank of the Molecular Biology Laboratory of the Universidade Federal de Sergipe (UFS) Medicine Department (Table 1). *L. chagasi* were isolated from patients with visceral leishmaniasis at the UFS hospital. The local ethics committee approved all procedures used in this study (CAAE 0151.0.107.000–07). *L. amazonensis* isolates were obtained from a *Leishmania* bank at the Immunology Laboratory of HUPES, Universidade Federal da Bahia. The viability of *L. amazonensis* and *L. chagasi* in response to NO was previously determined by Giudice et al. (2007) and Santos et al. (2012), respectively. The viability of *L. chagasi* in response to antimony was evaluated by direct cell counting. Briefly, exponentially growing parasites (1×10^6 mL) were seeded in 96-well microplate (100 μL or 10^5 parasites/well) in the presence of SbIII (Sigma Chemical Co., St. Louis, MO, USA), in concentrations varying of 250–3,

Table 1 Characterization of *L. chagasi* e *L. amazonensis* isolates for resistance/sensitivity to NO and trivalent antimony

Isolates		NO	Antimony
L. chagasi	HU UFS 02	−	−
	HU UFS 07	−	−
	HU UFS 09	+	+
	HU UFS 14	+	+
	HU UFS 15	+	+
	HU UFS 17	−	−
	HU UFS 23	+	+
	HU UFS 49	+	+
L. amazonensis	LTCP 9667	−	+
	LTCP 10432	+	+

+ resistant, − sensitive

000 μM for 48 h. Wells without SBIII was used as control and considered 100 % of viability.

Parasite growth conditions and sample preparation

L. chagasi and *L. amazonensis* promastigotes were thawed and cultured in biphasic Novy–MacNeal–Nicolle (NNN) blood agar. Next, cultures were initiated by inoculating 1×10^5 promastigotes into Schneider's insect medium, supplemented with heat-inactivated fetal bovine serum FBS (20 %) and penicillin (1 %). The cultures were kept in an incubator at 24±1 °C and until they reached the stationary growth phase.

Fatty acids extraction

The stationary growth phase promastigotes (5×10^8 cells mL^{-1} in 50 mL) were centrifuged at 2,608×*g*, 4 °C for 10 min. The supernatant was discarded, and the pellet was washed twice with phosphate-buffered saline (PBS). Subsequently, the samples were lyophilized and used in cell fatty acid extractions. All growth experiments and analyses were performed in triplicate.

MIDI commercial procedure for the extraction of total fatty acids (MIDIFA)

The MIDI procedure followed the steps described by Sasser (1990). Briefly, lyophilized samples were treated with a 3.75-M NaOH solution, in equal parts, water and methanol and vortexed and heated for 30 min in a water bath. After incubation, 2 mL of a mixture of 6 M acetic acid and methanol (1:0.85) and 3 mL of hexane were added to the tubes, followed by homogenization and centrifugation (2,608×*g*, 10 °C for 10 min). The organic phase was aspirated and 1 mL of 0.3 M NaOH was added to this phase with subsequent vortexing. The samples were dried with ultrapure nitrogen and resuspended in 270 μL hexane.

Ester-linked fatty acids

This method followed the steps described by Schutter and Dick (2000) and resulted in the formation of fatty acid methyl esters (FAMEs) from all ester-linked fatty acids (ELFA) present in the cells. For this procedure, 3.3 mL of a methanolic KOH solution (0.2 M) were added to the cell pellet. Samples were incubated in a water bath (37 °C) for 60 min, with a vortexing step of approximately 15 s every 15 min throughout the incubation period. Subsequently, 2 mL of 1 M acetic acid were added to each sample, and the contents were homogenized by vortexing for 5 min. After the addition of 2.2 mL of hexane, the vortexing step was repeated, followed by an interval of 10 min for phase separation. The samples were centrifuged (480×*g*, 10 °C for 10 min), and the lipid fractions were removed and dried under ultrapure nitrogen atmosphere. Additional hexane (250 μL) was added, and the samples were again homogenized.

Phospholipid fatty acids

This procedure followed the method described by Bligh and Dyer (1959) for total lipid extraction. In this procedure, the samples were first prepared for total lipid extraction. First, the cell pellets were dissolved in 2 mL of 100 mM phosphate buffer, 5 mL of methanol, and 2.5 mL of chloroform, agitated in a shaker for 2 h and centrifuged (805×*g*, 10 °C for 12 min). Next, the samples were filtered through Whatman #1 paper over glass funnels. Next, 5 mL of methanol and 2.5 mL of chloroform were added again, and the samples were homogenized on a vortex; this step was repeated once more. Then, 7.5 mL of 2 M NaCl were added, and the chloroform phase was transferred and dried with ultrapure nitrogen.

Second, the lipid separation was performed with silica columns, according to Buttler et al. (2003). The dried lipids were dissolved in 1 mL of chloroform and loaded onto the columns. The neutral phase was removed with chloroform, the glycolipids with acetone and the phospholipids, our fraction of interest, with methanol. The drying procedure was repeated.

Finally, the dried phospholipids were dissolved in 1 mL of a 1:1 methanol: toluene solution while vortexing. Next, 1 mL of 0.2 M KOH was added to each sample, and the samples were heated in a water bath (38–48 °C) for 15 min. Then, 2 mL of deionized water, 0.3 mL of 1 M acetic acid, and 0.5 mL of hexane were added, and the samples were homogenized by vortexing. After phase separation, the hexane fraction was removed, and the same amount of hexane was added and removed twice. The hexane extract was dried under ultrapure nitrogen and the methyl esters were redissolved in hexane (270 μL).

Fatty acid methyl esters analysis

FAME analysis was performed in a Perkin Elmer Clarus 500 Gas Chromatograph that was programmed to run the following conditions for all experiments: a run temperature of 120–270 °C with an increase of 4 °C per minute, an injector temperature of 220 °C, and a detector temperature of 280 °C. Furthermore, the Perkin Elmer Elite-5 column (crossbond 5 % diphenyl/95 % dimethyl polysiloxane; cat #N9316076; serial #862923) was used.

Data processing and statistical analysis

The fatty acid analysis was performed with the available gas chromatograph program, the TotalChrom navigator—Clarus 500. The fatty acid profile of each sample was inferred with the multivariate ordination technique. Prior to the ordination,

the molar masses of each fatty acid (FAMES) in a sample (shown as peak chromatograms) were determined, and the samples were relativized by the sum of the weights of all present fatty acids. Thus, the FAMES were converted to molar percentages of total peaks (mol%). The fatty acid profiles of *Leishmania* isolates were next analyzed by nonmetric multidimensional scaling (NMS) (McCune and Grace 2002), using the PC-ORD program (MJM Software Design, Gleneden Beach, OR, USA). The *T* test was used to evaluate the hypothesis that the fatty acid compositions differed among the species and isolates of NO and antimony-sensitive or -resistant *Leishmania*, and a multivariate hypothesis test of the multivariate nondifferences between treatments (multiresponse permutation procedure (MRPP)) (Mielke and Berry 2000) was used. Finally, to identify the specific fatty acid to be used as a biomarker of resistant or sensitive isolates or to identify a species biomarker, the indicator species analysis (ISA) was used (Dufrene and Legendre 1997). The ISA provides an indicator value (IV), ranging from 0–100, that expresses the degree of relationship between the peak (fatty acid) and each group (species or resistance/sensitivity). This value is the product of the FAME relative abundance (amount of FAME in a given group relative to its amount across all groups) and relative frequency (number of samples in which the FAME was detected relative to the number of total samples within a given group). IVs were tested for statistical significance in a randomization procedure (Monte Carlo Test).

Mass spectrometry

The GC/MS analyses were performed on a GC-MS/FID (GC-2010 Plus; GCMS-QP2010 Ultra, Shimadzu Corp., Kyoto, Japan) equipped with an AOC-20i autosampler (Shimadzu). The separations were performed on an Rtx ®-5MS Restek fused silica capillary column (5 % diphenyl/95 % dimethyl polysiloxane) with a 30 m×00.25 mm internal diameter (d.i.) and a 0.25-μm-thick film with a constant helium (99.999 %) flow rate of 1.2 mL min^{-1}. An injection volume of 0.5 μL (5 mg mL^{-1}) was used with a split ratio of 1:10. The oven temperature was programmed at 50 °C (isotherm for 1.5 min) with an increase of 4 °C/min up to 200 °C, another increase of 10 °C/min up to 250 °C, and a final 5 min isotherm at 250 ° C.

The MS and FID data were acquired simultaneously with a detector separation system, and the flow split ratio was 4:1 (MS:FID). A restrictor tube of 0.62 m×0.15 mm d.i. (capillary column) was used to connect the splitter to the detector MS; a restrictor tube of 0.74 m×00.22 mm d.i. was used to connect the splitter to the FID detector. The injector temperature was 250 °C, and the ion source temperature was 200 °C. Mass spectra were generated at 70 eV with a scan rate of 0.3 s scan, and the fragments were detected in the range of 40–350 Da. The FID temperature was adjusted to 250 °C, and the gasses supplied to the FID were synthetic air, hydrogen, and helium at flow rates of 30, 300, and 30 mL min^{-1}, respectively. Each constituent quantification was estimated by normalizing the peak area generated in the FID (percentage). The compound concentrations were calculated from the GC peak areas and arranged in the order of elution from the GC.

Component identification was performed on the basis of mass spectroscopy (MS) fragmentation pattern and retention index analyses in comparison with bacterial acid methyl ester mix (BAME) patterns, SUP 37 from Supelco®, and polyunsaturated fatty acids (PUFA) from Sigma®. Three equipment libraries, WILEY8, NIST107, and NIST21, that permitted comparisons of spectral data with those contained in the libraries at a similarity index of 80 % were also used.

Results

The experiments were conducted with eight *Leishmania chagasi* and two *L. amazonensis* isolates, classified in accordance with Table 1. Isolate viability experiments in response to NO and antimony were performed to confirm the report by Holzmuller et al. (2005) that indicated a strong correlation between NO and antimony resistance. Other studies also reported the existence of isolates resistant to both NO and antimonial drugs (Croft et al. 2006; Holzmuller et al. 2006; Giudice et al. 2007; T'Kindt et al. 2010; Santos et al. 2012). Santos et al. (2012) further observed that *L. chagasi* isolates that were refractory to antimony treatment showed higher levels of NO resistance. Common pathways for the actions of NO and antimonials have been reported. Sudhandiran and Shaha (2003) suggested that antimonial activity could be directly related to the induction of compounds such as NO. Holzmuller et al. (2005) stated that some biochemical targets such as thiol groups are common to the mechanisms of antimony and NO resistance.

The isolates were grown until reaching the stationary growth phase to determine the fatty acid profiles. The three methods used to produce the FAMEs differed in the extraction procedures. ELFA is based on a direct mild alkaline methanolysis of the samples (Schutter and Dick 2000). In PLFA, total lipids are first extracted, after which the extract is fractionated into three lipid moieties and, finally, the FAMEs are directly produced from the phospholipid fraction. MIDIFA differs because it instead uses saponification/acid methanolysis (Sasser 1990; Cavigelli et al. 1995). In the first two methods, the obtained FAMEs are derived from fatty acids with ester bonds, while the last method also extracts other types of fatty acids such as specimens linked by ether bonds (Sasser 1990; Cavigelli et al. 1995).

Fatty acid conversion in more volatile FAMES is necessary for the identification of fatty acids in gas chromatography. Before NMS ordination, chromatogram peak analysis was performed by relating the area of each peak to the peak area

of the fatty acid, 16:0 (palmitic acid). Palmitic acid was identified with standards such as PUFA and BAME. The analyzed peaks had minimum integration values equivalent to 4 % of the area of 16:0. Only peaks eluted between the retention times of 10.6 and 33 min were included in the multivariate analysis.

Identification of the other peaks was performed with MS because there is no current standard or well-defined commercial method of *Leishmania* fatty acid identification. Figure 1 shows the fatty acid methyl esters chromatogram from the gas chromatography associated with mass spectrometry (GC-MS) analysis of all MIDI samples. Table 2 lists the peak identification.

MIDIFA

For MIDIFA analyses, each isolate was tested in quadruplicate (samples A, B, C, and D), except for LVH SE 17, for which sample A was lost during the procedures, and the isolate LTCP 9667, for which sample A was an outlier. Thus, the analyses were performed on a total of 38 samples, and 44 fatty acid methyl esters were obtained.

An NMS analysis of MIDIFA profile was performed. Each point (score) in the ordination represents all the fatty acids of each isolate. Pearson's correlations were used between the variable values and the isolate scores (determined in a multivariate analysis) to characterize the main variations along the axes. The variable scores express the central tendency and the range of values for those variables among the samples.

Figure 2 shows the graphic that resulted from the NMS of the MIDI analysis, grouping the isolates according to NO/antimony susceptibility. Although the isolates seem to have overlapped, there were statistically significant differences in the fatty acid compositions between the NO/antimony-sensitive and -resistant isolates when *L. amazonensis* and *L. chagasi* were analyzed together. This was clearly observed in the MRPP statistics that showed a significance value below 0.04 %. Regarding the total data variability, 86 % were explained by ordination, with 38.8 and 47.2 % of the fatty acid diversity represented along axes 1 and 2, respectively.

A comparison of the fatty acid profiles also reveals variability in the fatty acid profiles of the *L. chagasi* and *L. amazonensis* species (Fig. 3). There is a concentration of scores, thus indicating a distinction between the FAME profiles.

Notwithstanding, there is greater variability when the data are organized according to the two variables of NO/ antimony susceptibility and species, as shown in Fig. 4. A superior distinction of the profiles, considered significant by the MRPP test, could also be observed.

Because the MIDI fatty acid profiles differed significantly between *L. amazonensis and L. chagasi* and between the isolates that were resistant or not to NO/trivalent antimony, an indicator species analysis was performed for each variable (NO/antimony susceptibility and species). This analysis was used to identify the fatty acids responsible for greater variability between the phenotypes. The most expressive fatty acid differences are listed in Table 3.

The fatty acid 18:1 Δ9c, oleic acid, was increased in the sensitive isolates ($p<0.001$), whereas 20:4 Δ5,8,11,14 showed the opposite trend ($p<0.01$). The average relative abundance of the fatty acid 18:1 Δ9c in the four sensitive isolates was 0.12 %, while in the six resistant isolates, it was 0.06 %. However, the fatty acid 20:4 Δ5,8,11,14 was increased in the resistant isolates, with an average value of 0.20 vs. 0.12 % in the sensitive isolates. According to the indicator values for species differentiation, two as yet unidentified fatty acids were increased in *L. amazonensis* species ($p<0.001$).

In the ELFA analyses, 30 samples were used (each isolate was analyzed in triplicate), and 49 fatty acid methyl esters were obtained. In the PLFA analyses, 40 samples were used (each isolate was analyzed in quadruplicate), and 40 fatty acid methyl esters were obtained. The fatty acid profile analyses performed with these two techniques showed that there were no differences in the fatty acid profiles of *L. amazonensis* and *L. chagasi* or between NO/antimony-resistant and -sensitive

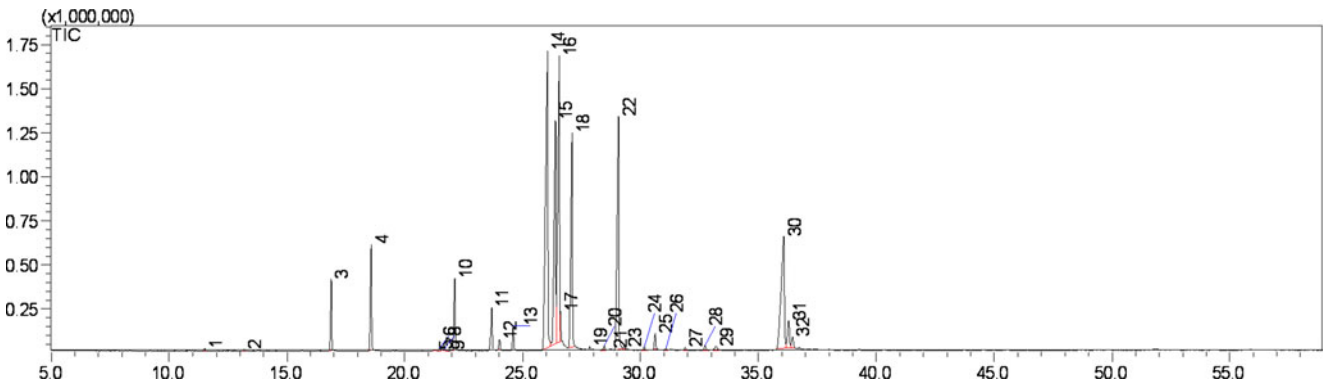

Fig. 1 GC-MS chromatogram representative from all the MIDI samples

Table 2 Fatty acid methyl esters resulting from the GC-MS MIDI analysis from all isolates biomass

Peak	Retention time	Compound—fatty acid	GC-MS (%)	GC/FID (%)
1	11.515	Methyl dodecanoate (C12:0)	0.08	0.0792
2	13.175	Methyl tridecanoate (C13:0)	0.07	0.0638
3	16.880	Methyl tetradecanoate (C14:0)	2.41	2.3182
4	18.570	Methyl pentadecanoate (C15:0)	3.92	3.7191
7	21.570	Cis-9-methyl hexadecanoate (C16:1 Δ9c)	0.11	0,1607
10	22.115	Methyl hexadecanoate (C16:0)	2.56	2.3601
11	23.680	15-methyl methyl hexadecanoate (C16:0i)	1.83	1.7519
12	24.00	Methyl heptadecenoate (C17:1)	0.4	0.4417
13	24.595	Methyl heptadecenoate (C17:1)	0.87	0.7812
14	26.005	6,9,12-methyl octadecatrienoate (C18:3 Δ6c,9c,12c)	20.34	21.4975
15	26.370	Cis-9,12-methyl octadecadienoate (C18:2 Δ9c, 12c)	12.44	12.4524
16	26.525	Cis-9-methyl octadecatrienoate (C18:1 Δ9c)	15.14	16.1968
17	26.605	Trans-9-methyl octadecatrienoate (C18:1 Δ9 t)	0.62	–
18	27.075	Methyl octadecanoate (C18:0)	10.51	10.3550
22	29.000	Cis-9,10-methylene methyl octadienoate (C19:0 cy9c)	12.94	11.5237
23	28.460	Methyl nonadecanoate (C19:0)	0.09	0.0319
24	30.145	Cis-5,8,11,14-methyl eicosatetranoate (C20:4 Δ5c,8c,11c,14c)	0.10	0.0733
25	30.605	8,11,14-methyl eicosatrienoate (C20:3 Δ8,11,14)	0.64	0.6714
26	31.085	11,14-methyl eicosadienoate (C20:3 Δ5,8,11c,14c)	0.10	0.0733
27	31.895	Methyl eicosanoate (C20:0)	0.15	0.1377
28	32.725	5,8,11,14-methyl eicosatetranoate (C20:4 Δ5,8,11,14)	0.29	0.2751
31	36.425	Cis-5,8,11,14,17-methyl eicosapentaenoate (C20:5 Δ5c,8c, 11c,14c,17c) – EPA	1.66	1.8845

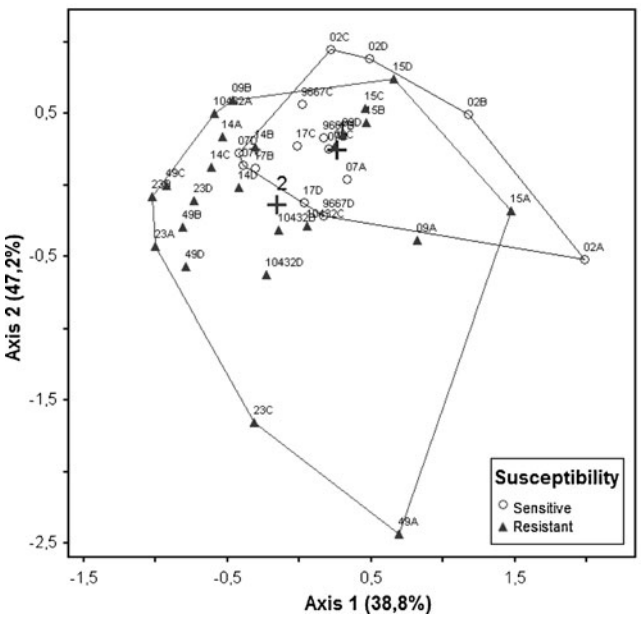

Fig. 2 NMS representation of the sample distance according to the MIDIFA composition separating the antimony/NO-resistant isolates and the sensitive ones. *Continuous line* encloses the study groups. The proportion of variance explained by each axis is based on the correlation (*R* between distance in the reduced NMS space and the original space; it is reported after each axis heading). MRPP test: $p = 0.00036787$; $A = 0.05103330$

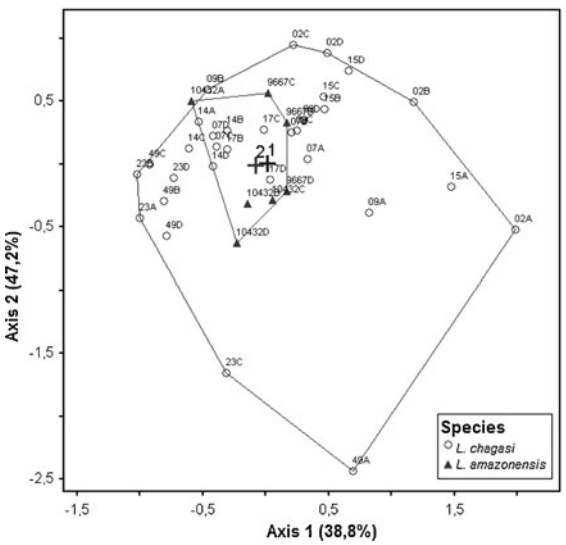

Fig. 3 *L. amazonensis* vs *L. chagasi* NMS graphic representation of the sample distance according to the MIDIFA composition grouping the isolates according to species. *Continuous lines* enclose the study groups. The proportion of variance explained by each axis is based on the correlation (*R* between distance in the reduced NMS space and the original space; it is reported after each axis heading). MRPP test: $p = 0.00489206$; $A = 0.03432629$

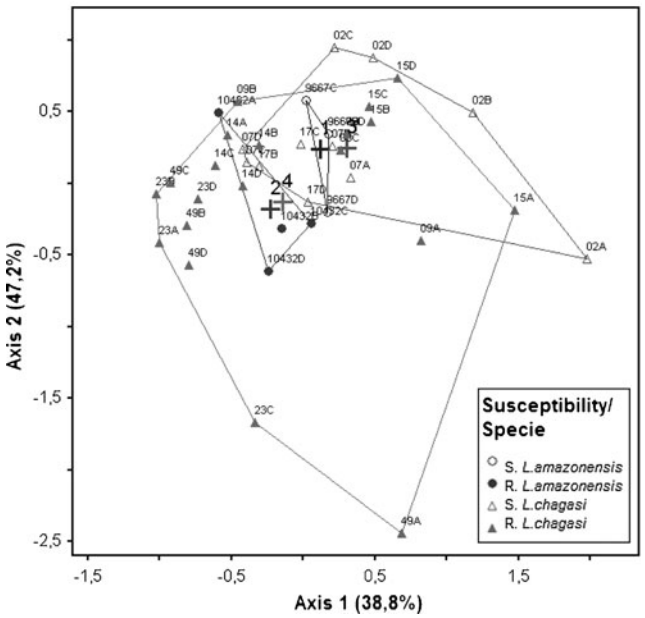

Fig. 4 NMS graphic representation of the sample distance according to the MIDIFA composition grouping the isolates according to species and also to NO/antimony susceptibility. The proportion of variance explained by each axis is based on the correlation (*R* between distance in the reduced NMS space and the original space; it is reported after each axis heading). MRPP test: $p = 0.00009271$; $A = 0.07796919$

isolates. The MRPP results from the NMS ordination of the ELFA and PLFA analysis yielded no statistically significant results, as shown in Table 4.

Discussion

Fatty acids are commonly used as biomarkers to profile microbial communities or for bacterial identification (Sasser 1990; Schutter and Dick 2000; Buttler et al. 2003; Lanoiselet et al. 2005). Fatty acid methyl ester (FAMEs) profiles from isolates of trivalent antimony and nitric oxide-resistant and -sensitive *L. amazonensis* and *L. chagasi* were produced by three different methods of fatty acid extraction: ELFA, PLFA, and MIDI. Hence, the presented results showed that MIDI-FAME profiles differed between *L. amazonensis and L. chagasi* and between isolates resistant or not to NO/trivalent antimony. The MIDI procedure was originally developed to produce FAMEs from bacterial cells to identify

Table 3 MIDI Indicator values as the NO/antimony susceptibility

Fatty acid	Indicator value (IV)		Expressive group	p[a]
18:1 Δ9c	63	34	NO/Antimony Sensitive	0.0002
20:4 Δ5,8,11,14	24	63	NO/Antimony Resistant	0.0038

[a] Monte Carlo test of significance

Table 4 MRPP results of NMS ordination from ELFA and PLFA analysis of the *L. chagasi* and L. amazonensis isolates relating the resistance and sensibility to NO/antimony, the species and both characteristics

	ELFA		PLFA	
	P	*A*	*P*	*A*
Sensibility	0.26007990	0.00482760	0.30770480	0.00172086
Specie	0.01837509	0.04369502	0.31499664	0.00184066
Sensibility/ Specie	0.08801758	0.04781894	0.34227467	0.00637848

strains based on their lipid profiles. This method also seems useful when differentiating *Leishmania* species and isolates with different phenotypes. Differences in phospholipid-derived fatty acids in antimonial drug-sensitive and -resistant *L. donovani* isolates were also reported by T'kindt et al. (2010). Previously, Rakotomanga et al. (2005) had already reported changes in the lipid composition of miltefosine-resistant *L. donovani* promastigotes when compared to sensitive promastigotes.

Interestingly, there were no significant differences among the fatty acid profiles obtained by PLFA, which extracts only phospholipid fatty acids, and ELFA, which extracts fatty acids from neutral lipids, phospholipids, and glycolipids. However, the fatty acid profiles obtained with each method of extraction were different. These differences are expected because PLFA yields FAME profiles comprised exclusively of phospholipids. Furthermore, the amounts of fatty acids extracted by MIDIFA and ELFA differ. ELFA extracts FAMEs from three lipid classes: neutral lipids, phospholipids, and glycolipids (Schtter and Dick 2000); however, MIDIFA also extracts dimethylacetals (derived from ester bonds) and other compounds that might be derived from the sphingolipid fatty acids (Kates 1986). This occurs because MIDI employs a saponification/acid methylation reaction, while ELFA is based on a mild alkaline methanolysis.

It is notable that the fatty acid profiles analyzed by NMS emphasize the differences in the relative compositions of fatty acids in the samples, rather than differences in the absolute amounts.

The MIDIFA technique employed in this study might have analyzed some of the sphingolipid fatty acid residues in the as yet unidentified molecules. The sphingolipids are ubiquitous components of eukaryotic membranes, where they have roles in membrane physiology, cellular signaling, and lipid raft formation (Zhang et al. 2009; Zhang et al. 2010). These findings suggest the importance of sphingolipids as molecular targets and suggest that SLs should be studied carefully and could possibly provide a biomarker or drug target for leishmaniasis treatment.

Once MIDI fatty acids profiles could differentiate between both *Leishmania* species and between the isolates according to their viability in the presence of NO/trivalent antimony, we performed an ISA analysis to search for fatty acids that could

be relevant in the profile differences. In fact, these analyses revealed that the fatty acid 18:1 Δ9c (oleic acid) was increased in the sensitive isolates, whereas the fatty acid 20:4 Δ5,8,11,14 showed the opposite trend. The perspective is that these fatty acids could be used as biomarkers to identify NO- and antimony-susceptible *Leishmania* isolates.

The finding that the fatty acid 20:4 Δ5,8,11,14 was increased proportionally in the resistant isolates is consistent because fatty acyl unsaturation is generally thought to decrease the ordered states of membranes and increase membrane fluidity (Hsu Chen and Feingold 1973; Mbongo et al. 1998). In fact, increased fatty acid unsaturation has already been reported in *Leishmania* parasites that were resistant to several other drugs, including miltefosine (Rakotomanga et al. 2005), amphotericin B (Mbongo et al. 1998), atovaquone (Cauchetier et al. 2002), and pentamidine (Basselin and Robert-Gero 1998). These changes in the alkyl chain compositions of plasma membrane lipids could modulate drug–membrane interactions and possibly be involved in drug resistance. Such changes in membrane lipid metabolism were also demonstrated to possibly affect interactions between drugs and the plasma membrane as well as subsequent drug uptake (Basselin and Robert-Gero 1998; Mbongo et al. 1998; Cauchetier et al. 2002; Rakotomanga et al. 2005). Thus, a higher proportion of the fatty acid 20:4 Δ5,8,11,14 in the major phospholipids of antimony-resistant *L. chagasi* isolates could have an effect upon the antimonial transport and result in modified antimonial susceptibility in these parasites, as previously proposed by T'Kindt et al. 2010.

Furthermore, two other as yet unidentified peaks, with retention times of 20.28 and 20.84 min, were found to be more highly expressed in *L. amazonensis*. For this reason, these fatty acids are potential candidate biomarkers for *L. amazonensis* identification. However, due to the small number of available *L. amazonensis* isolates, we cannot ensure that these peaks will behave similarly in a larger number of isolates. Thus, further studies are needed to confirm these findings.

In conclusion, these findings are important because, besides *Leishmania* species differentiation, they could provide biomarkers for NO and trivalent antimony resistance and sensitivity. These biomarkers should, in the future, facilitate the rapid introduction of correct therapies to patients and provide new insights for the creation of more effective leishmaniasis treatments.

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