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RESEARCH

Environmental Biofilms from an Urban Community in Salvador, Brazil, Shelter Previously Uncharacterized Saprophytic *Leptospira*

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Abstract
Biofilms are complex microecosystems with valuable ecological roles that can shelter a variety of microorganisms. Spirochetes from the genus *Leptospira* have been observed to form biofilms in vitro, in rural environments, and in the kidneys of reservoir rats. The genus *Leptospira* is composed of pathogenic and non-pathogenic species, and the description of new species is ongoing due to the advent of whole genome sequencing. Leptospires have increasingly been isolated from water and soil samples. To investigate the presence of *Leptospira* in environmental biofilms, we collected three distinct samples of biofilms formed in an urban setting with poor sanitation: Pau da Lima, in Salvador, Bahia, Brazil. All biofilm samples were negative for the presence of pathogenic leptospires via conventional PCR, but cultures containing saprophytic *Leptospira* were identified. Whole genomes were generated and analyzed for twenty isolates obtained from these biofilms. For species identification, we used digital DNA-DNA hybridization (dDDH) and average nucleotide identity (ANI) analysis. The obtained isolates were classified into seven presumptive species from the saprophytic S1 clade. ANI and dDDH analysis suggest that three of those seven species were new. Classical phenotypic tests confirmed the novel isolated bacteria as saprophytic *Leptospira*. The isolates presented typical morphology and ultrastructure according to scanning electron microscopy and formed biofilms under in vitro conditions. Our data indicate that a diversity of saprophytic *Leptospira* species survive in the Brazilian poorly sanitized urban environment, in a biofilm lifestyle. We believe our results contribute to a better understanding of *Leptospira* biology and ecology, considering biofilms as natural environmental reservoirs for leptospires.

Keywords Periphyton · Whole genome sequencing · Phylogenomic · Ecology · Leptospirosis

Introduction

Biofilms are complex communities of microorganisms embedded in an exopolymeric matrix [1, 2]. Biofilms are the main lifestyle of prokaryotes, providing protection against environmentally unfavorable conditions, such as ultraviolet light, disinfectants, antibiotics, and the hosts' immune systems [3, 4]. Natural biofilms formed in the environment may shelter autotrophic and heterotrophic organisms, pathogenic or nonpathogenic [2, 5, 6].

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Leptospires are gram-negative spirochete bacteria. The genus *Leptospira* is currently comprised of 68 pathogenic and saprophytic species [7–9]. Knowledge about this genus is expanding, due in large part to the application of whole genome sequencing [7, 8, 10, 11]. Several studies have isolated *Leptospira* species, pathogenic and saprophytic, from sewage, soil, water, streams, etc. [7–9, 12, 13]. Studies on the presence and abundance of *Leptospira* in natural environments and their ecological interactions are important to help prevent leptospirosis and improve control strategies [7].

Leptospires form biofilms in vitro [14–16]. These spirochetes also integrate mixed biofilms formed in dental unit water systems [17] and primary-treated wastewater fed system [18]. The pathogenic species *Leptospira interrogans* have been isolated from biofilms formed in a flooded rice paddy in India [19, 20]. Also, *Leptospira* form biofilms

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inside the renal tubules of naturally infected *Rattus norvegicus* captured at an epidemic region in Brazil [21]. The mechanisms of *Leptospira* survival (pathogenic or saprophytic) in the environment are still poorly understood. So far and to our knowledge, there are no studies in the literature that have isolated and identified *Leptospira* in environmental biofilms formed in urban settings with poor sanitary conditions.

In this study, we genomically and phenotypically characterized twenty isolates of *Leptospira* obtained from environmental biofilms formed at Pau da Lima, an urban community with poor sanitary conditions located in the city of Salvador, Brazil. This community has been previously used to study leptospirosis in urban environments [22–24]. The results presented here bring novel perspectives to *Leptospira* biology, suggesting that biofilms may shelter a variety of non-pathogenic *Leptospira* species, helping the maintenance of the bacteria in the environment for long periods.

Methods

Study Site and Environmental Biofilm Collection

We conducted the present study in the community of Pau da Lima, a neighborhood in Salvador, Bahia, Brazil, endemic for leptospirosis [22], with inadequate basic sanitary conditions, deficient water drainage systems, and contaminated streams (hereafter referred to as "open sewage") (Fig. 1A and B). We opportunistically collected environmental biofilms from two different sources: (1) rocks' surface and (2) discarded polyvinyl chloride (PVC) pipes (found at the field site). We additionally had a third experimental source: (3) glass slides used as abiotic support (four slides were coupled to PVC pipes and left submerged in an open sewage) (Fig. 1C and D). We did this last approach to investigate biofilm formation on the same abiotic substrate we use in the laboratory for this purpose [14, 16].

PCR of Environmental Biofilms

We extracted the total gDNA from the biofilm samples and tested for the presence of the *lipL32* gene, which is present only in pathogenic leptospires. For that, we used the Power Soil™ DNA Isolation Kit (Qiagen). We used TopTaq Master Mix Kit (Qiagen) for the amplification of the *lipL32* gene, with the following primers: forward-48F (5′-AAGCATTAC CGCTTGTGGTG-3′), reverse-286R (5′-GAACTCCCATTT CAGCGATT-3′) [25]. We added 1 µL of each primer, 12.5 µL of the Master Mix, 8.5 µL DNase/RNase free water, and 2 µL of target DNA, to a final volume of 25 µL. We performed one cycle of initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 60 s, extension at 68 °C for 2 min, and a final extension cycle at 68 °C for 7 min. We used *Leptospira interrogans* serovar Copenhageni Fiocruz L1-130 as a positive control and DNase/RNase-free water as a negative control. We performed the reaction in a GeneAmp PCR System 9700 (Applied Biosystem) and the electrophoresis of the PCR products on 0.8% agarose gels.

Environmental Biofilm Processing

All biofilms were scrapped using sterile spatulas, washed with distilled sterile water to remove non-adherent material, and transported to the laboratory at room temperature (RT). We scraped approximately 3 g of the biofilm adhered to the rock's surface, inoculated it in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) (Difco™—BD) media, and transported it to the laboratory. For the biofilm

Fig. 1 **A** and **B** Study site at Pau da Lima urban community (Salvador, Bahia, Brazil). **C** Schematic of the four glass slides inserted into the PVC pipe provided by the research team. **D** The support with the glass slides was inserted into the PVC pipe after three consecutive days submerged in open sewage. Scale bar: 1 cm

formed on the PVC pipe, we transported the pipe to the laboratory, washed it, scraped approximately 1 g of the adhered biofilm, and inoculated it in liquid EMJH. For the biofilms that formed on the surface of the glass slides, we carefully washed the slides in the field, scraped the biofilms, inoculated in liquid EMJH media, and transported to the laboratory. In the laboratory, we vortexed all the samples at maximum speed and left decanting for at least 15 min at RT.

Leptospira Isolation from Environmental Biofilms

We processed the samples according to the protocol from (Chakraborty et al. 2011) with modifications [26]. Briefly, we filtered 2 mL of the supernatant of each sample with a 0.45 µm membrane and transferred the filtrate to a tube containing 2.5 mL of liquid EMJH 1 × and 500 µL of STAFF 10 × (400 µg/mL sulfamethoxazole, 200 µg/mL trimethoprim, 50 µg/mL amphotericin, 4000 µg/mL phosphomycin, and 1000 µg/mL 5-fluoracyl). We incubated the filtrate and a 1:1,000 dilution at 30 °C for 72 h and observed the samples using dark field microscopy. When we observed contamination, we filtered 2 mL of the sample in 0.45 µm membrane and incubated the filtrate and a 1:1,000 dilution at 30 °C. We observed the samples every day, for 10 days, using dark-field microscopy. When we observed bacteria with morphology and motility typical of *Leptospira*, we counted cells using a Petroff-Hausser chamber, plated 50 cells on solid EMJH agar media using sterile glass beads, and incubated them at 30 °C. We observed the plates daily, from day 3 after inoculation until day 13. Per the biofilm sample, we collected individual white, with distinct border, subsurface colonies. We confirmed the morphology and motility characteristic of *Leptospira* by dark field microscopy, followed by clonal subculture in liquid EMJH media [12, 26]. We froze the pure subcultures in liquid nitrogen using DMSO (1:40 v/v).

DNA Extraction from Isolates

We extracted total DNA from the obtained fresh subcultures. For that, we centrifuged 5 mL of each subculture at 12,000 × g, at 25 °C for 20 min, resuspended the pellet into 500 µL CTAB buffer (100 mM Tris–HCl pH 8, 20 mM sodium EDTA pH 8, 1.4 M NaCl, 2% CTAB), added 10 µL of proteinase K, and incubated at 65 °C for 1 h. We centrifuged the samples at 12,000 × g, at 25 °C for 10 min, added 500 µL of chloroform/isoamyl alcohol (24:1 vol/vol) solution, vortexed, and left the tubes separating phases for 5 min at RT. We centrifuged at 6000 × g, at 25 °C for 10 min, added 0.65 × of cold isopropanol PA, incubated the samples at – 20 °C overnight (ON), and centrifuged at 12,000 × g, for 30 min at 25 °C. We washed the pellets with 500 µL of cold 70% ethanol, centrifuged at 12,000 × g, for 10 min at 25 °C, and let the tube dry for 20 min. Finally, we eluted the DNA in 30 µL of autoclaved MiliQ water and incubated the samples at 4 °C ON. We maintained the DNA in – 80 °C until further analysis.

Whole Genome Sequencing, Assembly, and Annotation

We sequenced the DNA from the twenty subcultures using Illumina Hiseq 2500 platform, with paired-end sequencing (2 × 150 bp) and library fragments of 450 bp. We used FastQC (version 0.11.9) for quality check of the genomes. For genome assembling, we used the assembly pipelines Spades (version: 3.15.0), Edena (version: 3.131028), Newbler (Software Release: 2.9), and Unicycler (version: 0.4.5) [27–29]. To select the best assemblies, we used the QUAST tool (version: 5.0.2) [30] and observed the values of N50, the total number of contigs and base pairs, and the percentage of GC content (Supplementary Table 1). We used Prokka [31] to annotate the genomes. To evaluate the number of single-copy orthologues present in the genomes, we used the BUSCO tool [32].

Species Delineation Analysis

Average nucleotide identity (ANI) was performed using Pyani version 0.2.10 and the ANIb method [33]. For this, we used the 20 newly sequenced genomes and 68 representative genomes of *Leptospira* species from the NCBI genome server (Supplementary Table 2). To confirm the ANI cutoff within the *Leptospira* genus, we performed this analysis for each *Leptospira* species and calculated the median and interquartile range. Additionally, we performed an online calculation of digital DNA:DNA hybridization (dDDH) using a type (strain) genome server (TYGS), a platform for genome-based taxonomy [34]. We uploaded the 20 new isolates genomes to the platform and considered a dDDH value < 70% as new species [35].

Phylogenomic Tree Analysis

We used the OrthoFinder program (version 2.5.4) [36], adding the species *Leptonema illini* and *Turneriella parva* as external groups. We used Mafft (version: 7.310) [37] for the alignment of the core genome using the single-copy orthologues sequences output from OrthoFinder. To obtain a more accurate tree, we trimmed the dataset using BMGE (version: 1.12) [38]. To concatenate the files, we used AMAS [39]. We performed a maximum likelihood analysis using the IQTree version: 1.6.12 algorithm, with an ultrafast bootstrap of 10,000 and an approximate likelihood-ratio test of 1000 [7, 40]. For tree visualization, we used iTOL [41].

Data Availability

The sequence data reported in this paper was deposited in the NCBI server (Supplementary Table 3).

Cultivation Protocols and Cell Counting

Phenotypic characterization was performed only with the isolates genetically identified as new *Leptospira* species. From this part of the methodology on, *Leptospira* was cultivated in liquid EMJH at 30 °C with no agitation, unless when specified in the text. Cell counting was performed using a Petroff-Hausser chamber under dark-field microscopy.

Cell Growth Curves and 8-Azaguanine Test

We standardized an initial inoculum of 5E + 06 *Leptospira*/mL from the exponential phase and grew at 30 °C, 37 °C, and 14 °C. We counted cells after 1, 3, 7, and 10 days of incubation. We also grew *Leptospira* in the presence of 225 µg/mL of 8-azaguanine (Hybri-Max 50x, Sigma—A5284) and counted cells after 3, 10, and 14 days of cultivation. Experiments were repeated two times independently.

Scanning Electron Microscopy (SEM)

We centrifuged approximately 1E + 08 cell/mL of the cultures at 2,630 × g for 10 min at 4 °C and washed the pellets in sterile phosphate-buffered saline (PBS) solution (NaCl 140 mM; 2.7 mM KCl; 10 mM Na2HPO4; 1.8 mM KH2PO4; pH 7.4). Then, we resuspended the pellet in a fixation buffer (2.5% glutaraldehyde, 4% paraformaldehyde, and 0.1 M sodium cacodylate buffer, pH 7.4), washed the pellets with 0.1 M sodium cacodylate buffer, and post-fixated in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 30 min. We washed and dehydrated through a series of ethanol concentrations (from 30 to 100%, for 10 min each), performed the critical point dry (Leica EM CPD030, Austria), and metalized in gold (25 nm). We observed and examined the samples using a SEM (JSM6394LV; JEOL, Japan) operated at 10 kV. We measured the cells in 10,000 × magnification and captured 20 images of individual leptospires per species. We measured (i) length, (ii) diameter, (iii) wavelength, and (iv) helical amplitude [42]. For each captured image, we obtained one measure for cell length and five measures for each of the other parameters. We used ImageJ (https://imagej.nih.gov/ij/download.html) software for the analysis.

Biofilm Formation Assay and Biomass Quantification

We inoculated 5E + 06 cell/mL from the exponential phase in 5 mL of liquid EMJH media in glass tubes and incubated at 30 °C [14]. We analyzed biofilm biomass using crystal violet (CV) assay [14, 15, 43] for seven consecutive days and on the 9th incubation day. We gently removed the culture medium from the glass tubes, air-dried under hot light for 20 min, and fixed the biofilms in 4 ml of 2% (g/v) sodium acetate solution for 20 min. Then, we removed the fixing solution, air-dried the tubes for 20 min, and stained the biofilms with 4 mL of 1% (g/v) CV for 20 min. Gently, we removed the CV solution and washed the biofilms 2 × with distilled sterile water at RT. We solubilized the reminiscent CV solution in 30% acetic acid solution (v/v) and quantified at 600 nm. We used non-inoculated EMJH liquid media as negative controls. We performed this methodology at least three times, independently, and the average and standard errors were calculated in excel.

Immunofluorescence (IF) and Confocal Laser Scanning Microscopy (CLSM)

We cultivated *Leptospira* biofilms on glass slides used as abiotic support. When biofilms reached the higher measure for CV staining, we gently removed the glass slide from the support, washed the biofilms with sterile water at RT, and fixated biofilms in paraformaldehyde (PFA) 2%. We then washed the biofilms with PBS, incubated in bovine serum albumin (BSA) 1%/PBS (wt/vol) for 30 min, and incubated in primary antibody anti-*Leptospira interrogans* serovar Copenhageni strain RGA (1:500) for 1 h. We then washed the biofilms with PBS, incubated in FITC (Zymed) secondary antibody (1:500) added with 2 µg/ml propidium iodide (PI) (Invitrogen) for 1 h, washed twice with PBS 1 ×, added the anti-fading solution (Invitrogen), and covered with a coverslip. We used CLSM (model TCS SP8, Leica Microsystems, Wetzlar, Germany) to visualize the biofilms, Leica software to acquire de 2D and 3D images, and COMSTAT 2 software [44] to analyze the biomass and thickness for both FITC (leptospires; green) and PI (nucleic acids; red) channels. We also obtained the biofilm thickness using the 3D image data. We used a 63 × lens and acquired FITC fluorescence signal with 450–490 nm/500–550 nm laser excitation/emission and PI fluorescence signal with 541–551 nm/565–605 nm laser excitation/emission. We repeated this experiment twice, independently, obtaining six images per species in each experiment. We calculated the average and standard error of the parameters using Excel software. Because we used a primary antibody anti-*Leptospira interrogans* serovar Copenhageni strain RGA, which is a pathogenic species, we firstly tested its use with the species analyzed in the present work, observing that it worked properly with this species (Supplementary Fig. 1).

Results

Isolation of *Leptospira* Species from Environmental Biofilms Formed in an Urban Community

We observed biofilms adhered to different biotic and abiotic surfaces, such as rocks, plants' roots, solid garbage, and pieces of disposed PVC pipes left in the open sewage, in Pau da Lima, Brazil. We collected three types of biofilms samples (Fig. 2): biofilm formed on the surface of a rock, biofilm formed on the inside wall of a PVC pipe piece found partially submerged in the open sewage, and biofilm formed on glass slides placed by our research team and immersed in the open sewage for three consecutive days. All collected biofilms were firmly adhered to the substrates. However, they were macroscopically diverse and presented in different colors: the biofilm from the rock was green, the PVC pipe biofilm was brown (Fig. 2A to D), and the glass slide biofilms were white/beige (Fig. 2G). The biofilms recovered from the rock and PVC pipe were not immersed in water. However, the day we collected the biofilms followed three consecutive days of rain (a total of 13 mm of rain).

To investigate the presence of pathogenic *Leptospira* in the collected biofilms, we performed a PCR test targeting the *lipl32* gene, which was negative, suggesting the absence of pathogenic leptospires in the biofilm samples (data not shown). After 3 days of incubation of the biofilm samples in liquid EMJH media, we observed microorganisms with typical leptospiral morphology and motility (helical, thin, highly motile cells, with one or both ends hooked, or unhooked ends). We plated the cultures in EMJH solid media, and the colonies started to appear within 4 days of incubation. Between the 4th to the 13th day of incubation, we collected 20 isolated colonies (five from the rock; five from the PVC tube; and ten from the glass slides). The colonies we obtained were translucid, grew fast, and spread horizontally in solid EMJH. The short period (13 days) for the collection of the colonies was due to their fast growth, which led to the complete spread of colonies on the plate.

Complete genome sequencing of the isolates showed an average genome size of 4,087,492 ± 91,095 bp, with an average of 37.7% GC content (Supplementary Table 4). The largest genome was from the isolate VSF10 (4,278,525 bp, 38.26% GC content), while the smallest genome was from the isolate VSF8 (3,913,765 bp and 37.53% GC content), both originating from the PVC pipe's biofilm (Supplementary Table 4). Using BUSCO, we observed more than 95% completeness of the leptospiral genomes isolated from

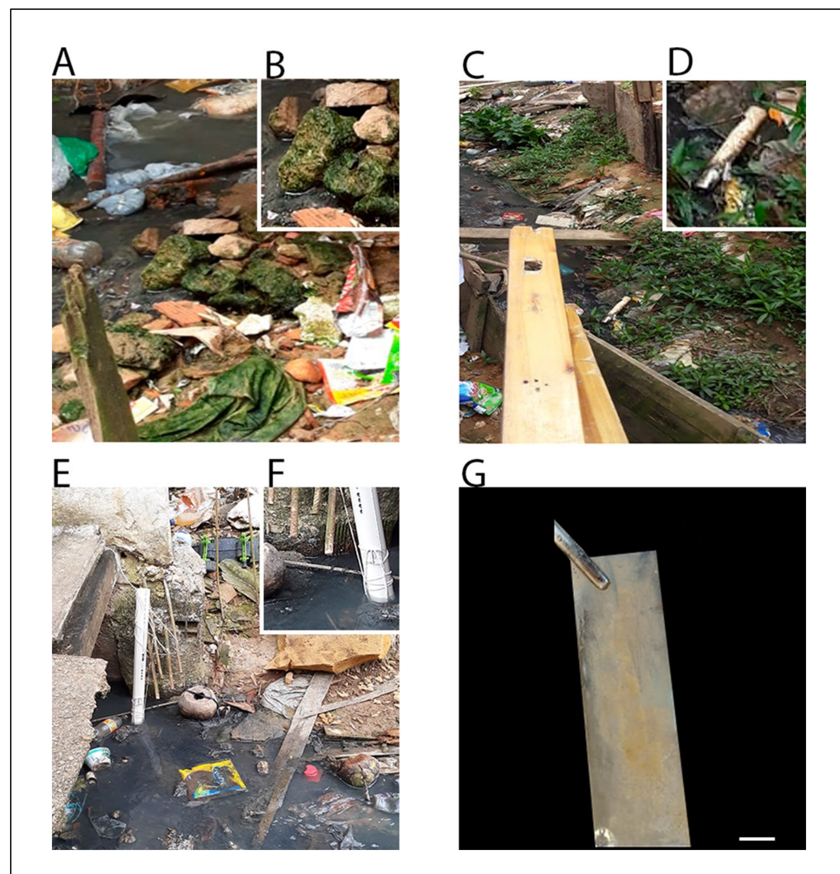

Fig. 2 Biofilm samples collected at an urban endemic area for leptospirosis. **A** and **B** Biofilm adhered to the surface of a rock. **C** and **D** PVC pipe found in the open sewage. **E** and **F** PVC support containing glass slides submerged in the open sewage for three consecutive days. **G** Biofilm formed on the glass slide that was submerged in open sewage, after the slide was washed with distilled water. Scale bar: 1 cm

biofilm, when compared to the reference genomes of *Leptospira biflexa* and *Leptospira levettii* (Supplementary Fig. 2).

We compared the newly twenty sequenced genomes with the 68 *Leptospira* reference genomes available at the NCBI genome server (Supplementary Table 2). We considered as the same *Leptospira* species when ANIb was > 0.95 [7] (Supplementary Table 5). Thirteen biofilm isolates identified in this work belonged to four previously described species (*L. kanakyensis*, *L. levettii*, *L. bandrabouensis*, and *L. meyeri*), while the other seven were grouped within three species proposed here as new ones (Fig. 3, Table 1). Similarly, TYGS (type (strain) genome server) analysis predicted those seven isolates as three novel species as they did not belong to any species from the TYGS database and had dDDH values < 70% compared with other species (Supplementary Table 6). Altogether, these results suggest that the strains VSF14, VSF16 to VSF20, and VSF25 represent three new species of saprophytic *Leptospira*.

To assess the phylogenomic classification of the isolates, we used Orthofinder single copy orthologue sequences (738 genes), with *Leptonema illini* and *Turneriella parva* as

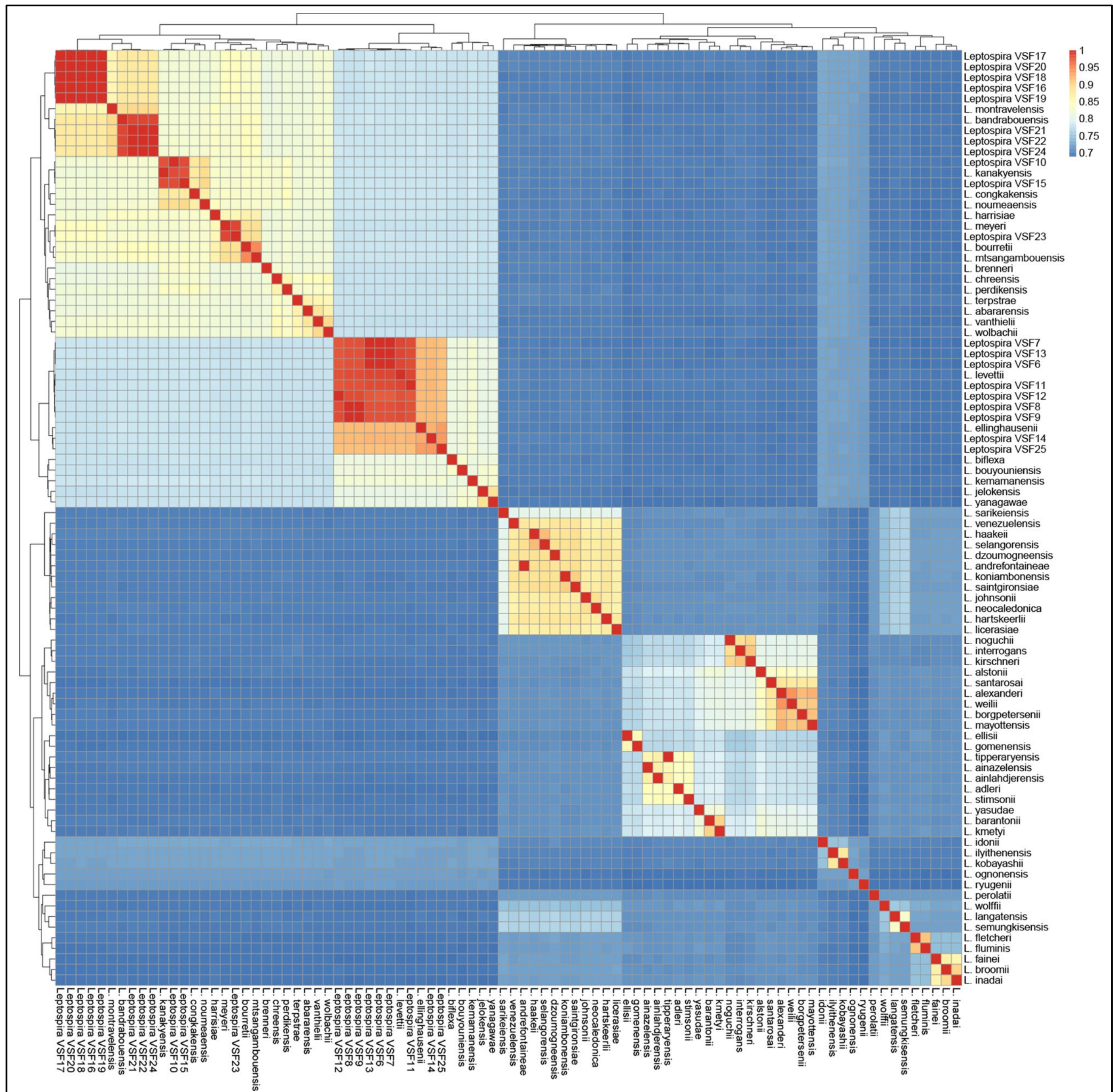

Fig. 3 Matrix representing the ANIb values calculated using Pyani for the classification of *Leptospira* species identified in this study. The legend indicates the average nucleotide identity values. We considered species with ANI > 95% as belonging to the same species

Table 1 Characterization of the *Leptospira* species isolated from environmental biofilms

Characterization of the isolates according to the biofilm source							
Species	Isolates	Rock's biofilm		PVC pipe's biofilm	Glass slides' biofilm		
Leptospira sp. 1	1	-		-	VSF14		
Leptospira sp. 2	5	-		-	VSF16 to VSF20		
Leptospira sp. 3	1	VSF25		-	-		
L. kanakyensis	2	-		VSF10	VSF15		
L. levettii	7	-		VSF6 to VSF9	VSF11 to VSF13		
L. bandrabouensis	3	VSF21, VSF22, VSF24		-	-		
L. meyeri	1	VSF23		-	-		
Genomic characterization of the proposed new species							
Species	Total sequences	Contigs	Total length (bp)	N50	GC (%)	CDS	tRNA
Leptospira sp. 1 VSF14	2,460,000	4	4,060,000	3,670,000	37.42	3,747	36
Leptospira sp. 2 VSF16	3,250,000	110	4,130,000	279,000	37.70	3,888	36
Leptospira sp. 2 VSF17	3,350,000	98	4,160,000	390,000	37.71	3,904	36
Leptospira sp. 2 VSF18	3,350,000	101	4,140,000	391,000	37.69	3,897	36
Leptospira sp. 2 VSF19	2,470,000	101	4,140,000	391,000	37.69	3,896	36
Leptospira sp. 2 VSF20	3,050,000	99	4,160,000	390,000	37.71	3,905	36
Leptospira sp. 3 VSF25	2,670,000	17	3,930,000	2,890,000	37.54	3,658	35

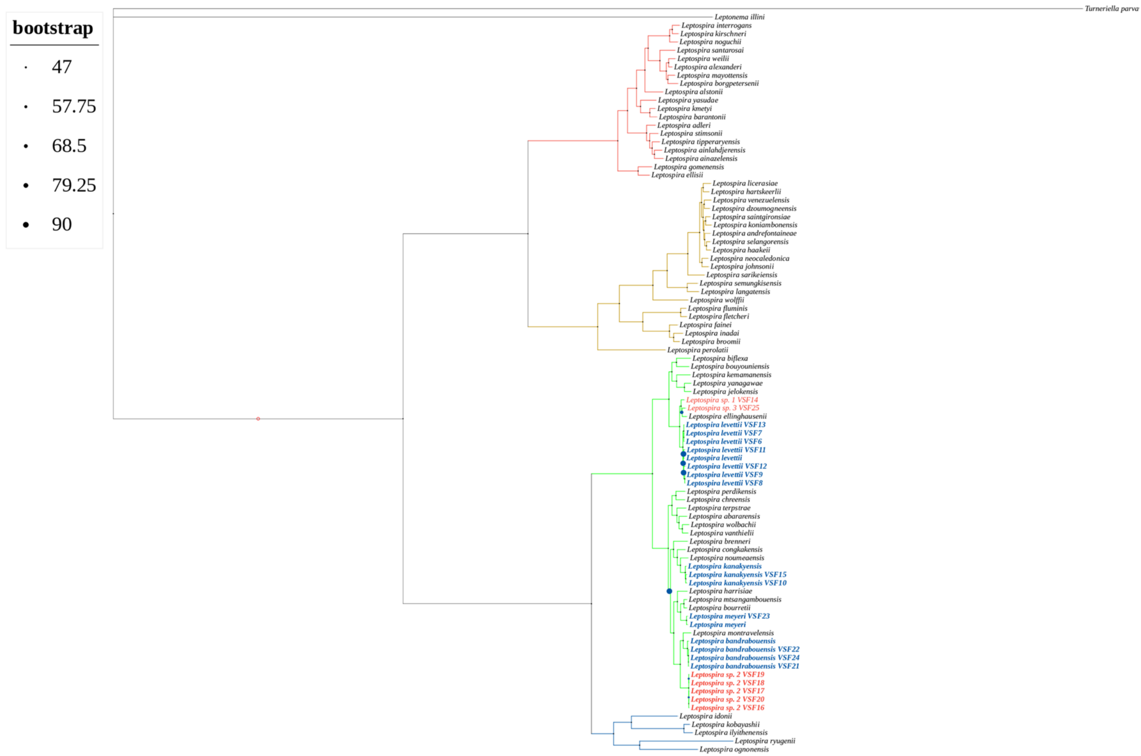

Fig. 4 Phylogenetic tree reconstruction based on the 738 single copy orthologues genes. The bootstrap values < 95% are represented. Branches: pathogenic P1 (red); pathogenic P2 (mustard); Saprophytic S1 (green); and Saprophytic S2 (blue). Nodes: blue (thirteen environmental biofilm isolates belonging to four previously described species); red (environmental biofilm isolates that belong to the proposed new three *Leptospira* species)

outgroups (Supplementary Table 4). The phylogenomic analysis corroborated the ANIb results (Figs. 3 and 4). The 20 isolates were placed into the Saprophytic S1 clade. Closely related isolates that belonged to the same species were placed into the same clusters. The thirteen isolates belonging to four previously described species grouped within their correspondent species clades. The isolates that we propose as new species were grouped into individual clades. *Leptospira* sp. 1 VSF14 and *Leptospira* sp. 3 VSF25 were closely grouped, while *Leptospira* sp. 2 VSF16—VSF20 belong to a clade distant from the others (Fig. 4). *Leptospira* sp. 3 VSF25 was grouped with *Leptospira ellinghauseniii* with an ultrafast bootstrap value of 63%, which was below the 95% expected value [40, 45]. Hence, to support the proposed classification of *Leptospira* sp. 3 VSF25 as a new species, we performed an in-depth investigation of the ANIb values within each *Leptospira* species. We observed that *Leptospira* species classified as the same species had an ANIb ranging from 0.95 to 0.99, with an average of 0.987 ± 0.006 (Supplementary Table 5; Supplementary Fig. 3). These results corroborate the suggested classification of *Leptospira* sp. 3 VSF25 as a new species, since its ANIb is less than 0.95 when compared with the other species of the genus [7].

Phenotypic Characterization of the New *Leptospira* Species Isolated from Environmental Biofilms

For the phenotypic characterization of the new *Leptospira* species, we randomly selected one representative isolate per species, when there was more than on isolate (Supplementary Table 7). Under an optical dark field microscope, the three species were highly motile, with both rotational and translational motility. The three species grew in liquid EMJH media at the tested temperatures (Fig. 5A; Table 2) and in liquid EMJH media supplemented with 225 µg/mL of 8-azaguanine (Table 2). Regarding cell morphology using electron microscopy, the three species presented typical helical morphology, with typical length, wavelength, cell diameter, and helical amplitude (Fig. 5B; Table 2). We observed under dark field microscopy, microorganisms with typical characteristics of leptospires; that is, helical, thin, highly motile cells, with one or both ends hooked or unhooked ends (straight variants [46]).

Biofilm Formation In Vitro by the Proposed New *Leptospira* Species

We tested the three proposed new *Leptospira* species for their ability to form biofilms in vitro. Quantification by crystal violet solution (CV) staining showed that the three proposed new *Leptospira* species formed biofilms within 1 day of cultivation in glass tubes. The maximum biofilm biomasses were reached within 2, 3, and 4 days for VSF25, VSF14, and VSF16, respectively (Fig. 5C, Table 2). From these time points on, the biofilms persisted and adhered to the glass tubes until the ninth day of observation, although weaker and with detachment areas (Fig. 5C). Using CLSM and 3D-image analysis, we estimated the biomass and thickness of the biofilms, considering *Leptospira* cells and nucleic acid fluorescence (Table 2; Fig. 6). The three species formed biofilms that firmly adhered to the glass slides, with a typical surface coverage of the abiotic surface and bacteria embedded within a dense biofilm matrix (Fig. 6).

Description of the Proposed New Species

Leptospira sp. 1—*Leptospira paudalimensis* sp. nov.: pau.da.lim.en'sis. N. L. masc. adj. paudalimensis of the neighborhood of Pau da Lima, in Salvador, Bahia, Brazil. The type strain is VSF14. BioSample Accession is SAMN28772821, and Genome Accession is JAMQPR000000000. The DNA GC content (%) is 37.42 and it belongs to the subclade S1. Cells are aerobic; motile; grow in liquid EMJH media at 30 °C, 37 °C, and 14 ºC; and grow in liquid EMJH media supplemented with 225 µg of 8-azaguanine. Cells are 9.40 ± 0.41 µm long and 0.30 ± 0.003 µm in diameter, with a wavelength of 0.58 ± 0.008 µm and 0.43 ± 0.004 µm of helical amplitude, under a scanning electron microscope. Cells form biofilm in vitro in abiotic support.

Leptospira sp. 2—*Leptospira soteropolitanensis* sp. nov.: so.te.ro.po.li.tan.en'sis. N. L. fem. adj. soteropolitanensis from Salvador, city in Brazil where the isolate was obtained. The type strain is VSF16. BioSample Accession is SAMN28772823, and Genome Accession is JAMQPP000000000. The DNA GC content (%) is 37.7 and it belongs to the subclade S1. Cells are aerobic; motile; grow in liquid EMJH media at 30 °C, 37 °C, and 14 °C; and grow in liquid EMJH media supplemented with 225 µg of 8-azaguanine. Cells are 6.92 ± 0.40 µm long and 0.27 ± 0.002 µm in diameter, with a wavelength of 0.37 ± 0.005 µm and 0.42 ± 0.005 µm of helical amplitude, under a scanning electron microscope. Cells form biofilm in vitro in abiotic support.

Leptospira sp. 3—*Leptospira limi* sp. nov.: li'mi. M. L. masc. sing. gen. n. limi from the lime, a periphyton biofilm collected from the urban community of Pau da Lima, in Salvador, Bahia, Brazil. The type strain is VSF25. BioSample Accession is SAMN28772817, and Genome Accession is JAMQPV000000000. The DNA GC content (%) is 37.54 and it belongs to the subclade S1. Cells are aerobic; motile; grow in liquid EMJH media at 30 °C, 37 °C, and 14 °C; and grow in liquid EMJH media supplemented with 225 µg of 8-azaguanine. Cells are 8.92 ± 0.50 µm long and 0.18 ± 0.002 µm in diameter, with a wavelength of 0.44 ± 0.005 µm and 0.30 ± 0.003 µm of helical amplitude, under a scanning electron microscope. Cells form biofilm in vitro in abiotic support.

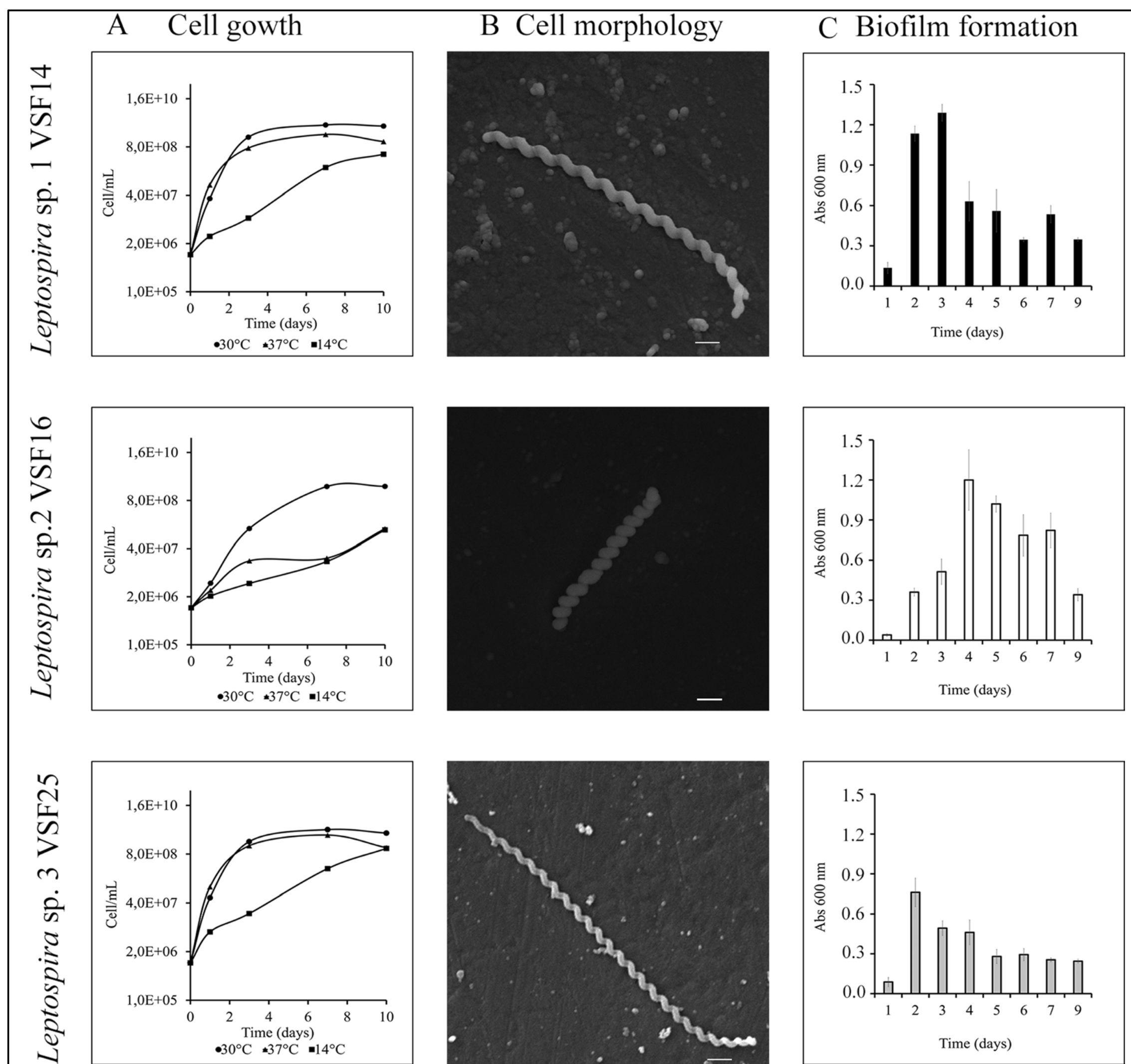

Fig. 5 Phenotypic characterization and biofilm formation by the three new *Leptospira* species. One representative isolate per *Leptospira* species was analyzed. **A** Isolates were cultivated in EMJH liquid medium (inoculum of 5E+06 cell/mL) and incubated at 30 °C, 37 °C, and 14 °C for cell growth analyses. At 1, 3, 7, and 10 days of incubation, the cells were counted using a Petroff-Hausser chamber and dark-field microscopy. The results shown are representative of two independent experiments. **B** Representative scanning electron microscope images of new *Leptospira* species showing typical helical morphology. Scale bars: 1 µm. **C** For biofilm formation assay, the *Leptospira* isolates were cultivated in glass tubes containing EMJH liquid medium (5E+06 cell/mL) and incubated at 30 °C, for 9 days. The biofilm biomass quantification was performed using crystal violet staining and the optical density was measured at 600 nm. Results are average ± standard errors derived from at least three independent experiments

Discussion

Leptospira are ubiquitous organisms whose survival in the environment is still poorly understood, even though these bacteria are transmitted to animal hosts mostly through contaminated water and soil contact [47–49]. It remains an unanswered question how leptospires survive in different environments, urban or rural, where weather conditions, nutrient availability, and toxic compounds may vary. In this study, we hypothesized that biofilms are an ecological niche for leptospires in urban environments, another strategy of *Leptospira* to survive in the environment. Hence, we investigated the presence of *Leptospira* in biofilms formed naturally in the urban community of Pau da Lima, an urban setting in the Northeast region of Brazil with a lack of adequate sanitary conditions.

In a field trip to Pau da Lima, we collected samples of biofilms that we found attached to a rock and to a PVC pipe, natural and artificial substrates for biofilm attachment, respectively. Biofilm formation on PVC pipes is commonly observed since the pipes offer a surface for cell adhesion, a dark and humid environment, and a passage of water inside of it, supplying nutrients necessary for the organisms' survival [50]. The green and slippery rock biofilm and the thin brown PVC pipe's biofilm are characteristic of "periphyton" or "periphytic biofilms" [51,

Table 2 Phenotypic and morphologic characterization of the three new *Leptospira* species isolated from environmental biofilms in Salvador, Brazil

Species	Cell growth and metabolism				Cell morphology (µm)			
	30 °C	37 °C	14 °C	30 °C in 8-azaguanine	Length	Wavelength	Diameter	Helical amplitude
Leptospira sp. 1 VSF14	Yes	Yes	Yes	Yes	9.40 ± 0.41	0.58 ± 0.008	0.30 ± 0.003	0.43 ± 0.004
Leptospira sp. 2 VSF16	Yes	Yes	Yes	Yes	6.92 ± 0.40	0.37 ± 0.005	0.27 ± 0.002	0.42 ± 0.005
Leptospira sp. 3 VSF25	Yes	Yes	Yes	Yes	8.92 ± 0.50	0.44 ± 0.005	0.18 ± 0.002	0.30 ± 0.003

Species	Biofilm maturation	Average biomass			Average thickness		
		Crystal violet OD	*Leptospira* (green)[a]	Nucleic acids (red)[a]	Biofilm (µm)	*Leptospira* (green)[a]	Nucleic acids (red)[a]
Leptospira sp. 1 VSF14	3 days	1.29 ± 0.06	3.24 ± 0.66	4.00 ± 0.57	6.46 ± 0.95	5.98 ± 1.04	6.17 ± 0.97
Leptospira sp. 2 VSF16	4 days	1.20 ± 0.23	2.22 ± 1.30	4.84 ± 1.40	10.83 ± 1.34	8.96 ± 2.15	10.09 ± 1.99
Leptospira sp. 3 VSF25	2 days	0.76 ± 0.11	2.37 ± 0.88	2.99 ± 1.02	5.21 ± 0.93	4.38 ± 0.94	4.60 ± 1.05

[a]($\mu m^3/\mu m^2$)

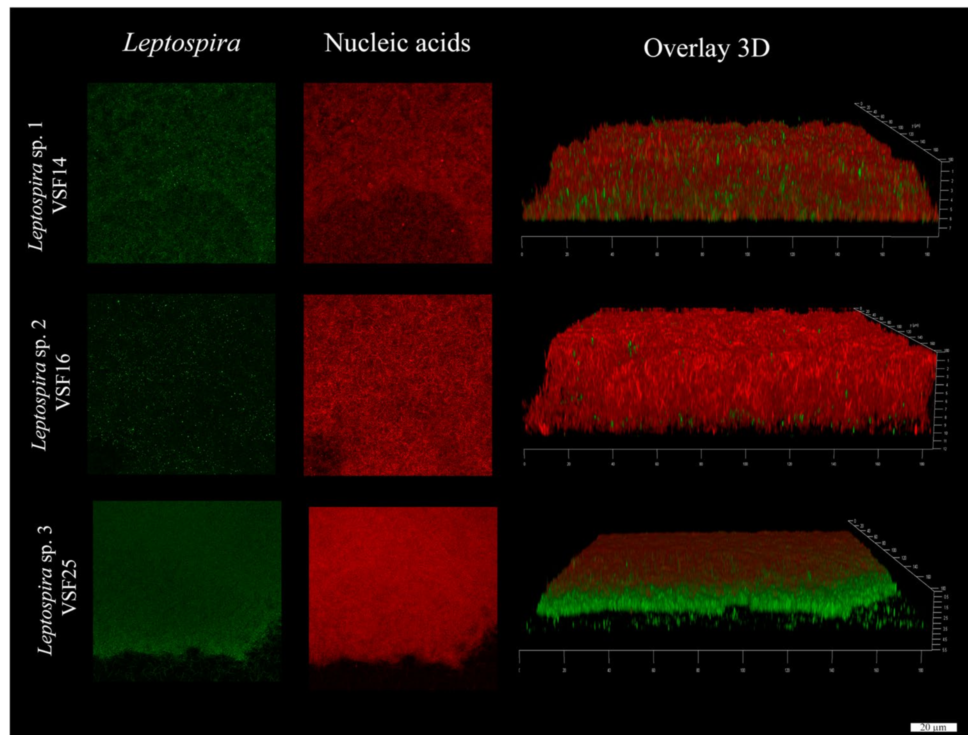

Fig. 6 CLSM images of the mature biofilms formed in vitro by the three new *Leptospira* species. One representative isolate per *Leptospira* species was analyzed. Mature biofilms were stained with anti-*Leptospira* antibody conjugated to fluorochrome ALEXA 488-FITC (green) and with propidium iodide (PI) that stains extracellular DNA (eDNA) and damaged cells DNA (red). The images were captured with a TCS SP8 Leica CLSM, using a 63 × objective. CLSM images are representatives of two independent experiments. Scale bar: 20 µm

52]. Periphytic biofilms are aquatic micro-ecosystems composed of micro- and macroscopic organisms such as bacteria, fungi, microalgae, etc., and abiotic substances such as extracellular matrix and detritus [52, 53]. We observed the presence of periphytic biofilms all over the study area, adhered to rocks, plant and plant parts, and garbage, among others. In Pau da Lima there is a non-organized rapid urbanization, overpopulation, and precarious sanitary conditions [22, 23, 52]. Consequently, the rivers are contaminated with the population's domestic waste, which can degrade the river ecosystem [52], forming what is commonly referred to as "open sewage." We suggest that the observed high production of periphytic biofilm may be a result of these environmental changes seen at Pau da Lima. Additionally, we collected biofilms formed on glass slides that our team left for 3 days submerged in the open sewage. For those biofilms, after we washed out the non-adherent material, a whitish/beige mass adhered to the slides was readily observable, a characteristic of some biofilms [3, 14, 16].

Leptospira species, including pathogenic and non-pathogenic, have been frequently isolated from environmental soil and water samples [7–9, 12, 49]. The most isolated are the saprophytic species, which are abundant in the environment [49]. Here, we identified seven species of saprophytic *Leptospira*, indicating a diversity from this genus in environmental biofilms formed at urban setting poorly sanitized. The phylogenomic tree reconstruction of the isolates resulted in a typical topology and an average genome size compatible with the *Leptospira* genus [7]. Among the seven identified *Leptospira* species, four have been previously isolated from water and soil samples [7, 13]. The remaining three species are proposed here as new saprophytic species of the genus *Leptospira* based upon phylogenomic analysis and phenotypic characterization. Those species presented typical morphology, motility, and ultrastructure, and they formed biofilms in the laboratory with complex architecture observed by CLSM 3D micrograph analysis.

In this study, although we accessed a diversity of leptospires from urban biofilms, it was not possible to identify pathogenic leptospires using PCR targeting the *lipL32* gene. One possibility is that the conventional *lipL32* PCR was not sensitive enough to identify those organisms. Further studies should be performed on order to investigate the presence of pathogenic leptospires in environmental biofilms formed at urban settings.

Previous studies have identified leptospires in biofilms found in dental unit water systems [17] and in biofilms and sludge samples formed in a system fed with primary treated wastewater obtained from a wastewater treatment plant, in a shotgun metagenomic study [18]. Furthermore, pathogenic *Leptospira* species were isolated from environmental biofilms adhered to glass rods submerged in waterlogged paddy field soils in India [19]. Taken together with our data, those results indicate a diversity of pathogenic and saprophytic *Leptospira* in natural biofilms, revealing the importance of continuing further research on *Leptospira* biofilms in the environment. Moreover, from an ecological point of view, microorganisms from environmental biofilms participate in different metabolic activities, such as the decomposition of organic matter, nutrient dynamics, and biogeochemical cycling, in addition to being indicators of water quality [54].

In conclusion, in this work, we have highlighted environmental biofilms containing a diversity of saprophytic *Leptospira* formed at an urban environment. We isolated three proposed new species based on classical phenotypic, genetic, and phylogenomic characterizations. Further studies on leptospiral biofilms in natural environments are important to better understand *Leptospira* biology and the potential roles of biofilms in the maintenance, proliferation, and evolution of *Leptospira*.

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Declarations

Conflict of Interest The authors declare no competing interests.

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