Journal of Drug Delivery Science and Technology 94 (2024) 105522

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

In-vitro antibiofilm activity of polycaprolactone- poly (lactic-co-glycolic acid) nanofibers loaded amphotericin B, antimicrobial peptide LfcinB $(21–25)_{Pal}$ and zinc oxide for local treatment of periodontitis associated with obstructive sleep apnea

Mayra A. Téllez Corral [a,b,e], Jean C. Villamil Poveda [a], Nelly S. Roa Molina [a], Liliana Otero [a], Zuly Jenny Rivera Monroy [c], Javier García Castañeda [c], Claudia M. Parra Giraldo [b], Maria E. Cortés [d,e,*]

[a] Centro de Investigaciones Odontológicas, Facultad de Odontología, Pontificia Universidad Javeriana, Bogotá D.C., Colombia
[b] Unidad de Investigación en Proteómica y Micosis Humanas, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá D.C., Colombia
[c] Grupo de Síntesis y Aplicación de Moléculas Peptídicas, Facultad de Ciencias, Universidad Nacional de Colombia, Bogotá D.C., Colombia
[d] Laboratório de Biomateriais e Microbiologia, Faculty of Dentistry, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
[e] Innovation Technology Graduate Program, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

ARTICLE INFO

Keywords:
Periodontitis
OSA
Polymicrobial biofilm
Nanofibers
PCL
PLGA
Amphotericin B
Antimicrobial peptide LfcinB $(21–25)_{Pal}$
Zinc oxide
Anti-Biofilm activity

ABSTRACT

Periodontitis has been linked to obstructive sleep apnea (OSA). The stage III periodontitis is more prevalent in OSA severe and the oral microbiome changes due to an increase in periodontal pathogenic bacteria and *C. albicans*. Nanofiber drug delivery systems may be used for local antimicrobial therapy against microorganisms related to periodontitis and OSA. The aim of the present study was manufacture PCL/PLGA nanofibers incorporating an antifungal, Amphotericin B (AMB); an antimicrobial peptide, LfcinB $(21–25)_{Pal}$ (P61); and zinc oxide (ZnO), for the inhibition of *in vitro* polymicrobial biofilms. Nanofibers (Nf) were produced by electrospinning method: PCL/PLGA Nf loaded with: AMB (Nf 1), AMB-ZnO (Nf 2), P61 (Nf 3), P61–ZnO (Nf 4), AMB-P61 (Nf 5), AMB-P61-ZnO (Nf 6) and ZnO (Nf 7). Different methods were used to physically and chemically characterize the nanofibers. The cell viability of human periodontal ligament fibroblasts (HPdLF) treated with the nanofibers was higher than 50%, demonstrating the biocompatibility of the nanofibers obtained. The antimicrobial and antibiofilm activities of the nanofibers were assessed using qualitative and quantitative analyses. Nine bacteria viability, *Candida albicans* viability, and biofilm formation were significantly inhibited by the Nf 6, and this effect increased with exposure time (72 h). The AMB-P61-ZnO-loaded PCL/PLGA nanofiber (Nf 6) may offer a promising strategy for treating localized cases of periodontitis in those with obstructive sleep apnea.

1. Introduction

Periodontitis is a multifactorial chronic infectious and inflammatory disease that is caused by the presence of pathogenic microbial communities, called dysbiotic microbiota, organized in stratified polymicrobial biofilms [1,2], that cause a chronic inflammatory response of the periodontal tissue, resulting in the formation of periodontal pockets, progressive loss of periodontal insertion with destruction of the soft and hard tissues of periodontal support, and subsequent loss of teeth [3]. In recent years, systemic diseases and conditions like the obstructive sleep apnea (OSA) have been linked to periodontitis [4,5]. The OSA is a respiratory sleep disorder, caused by the interaction of genetic factors with obstructive anatomical, neuromuscular or inflammatory factors that obstruct the airway, blocking the passage of air [6]. This disorder occurs when the soft tissues around the upper airway collapse, partially (hypopnea) or completely (apnea), obstructing airflow despite increased ventilatory effort [7].

Systematic studies and meta-analyses have proposed various plausible causes for the relationship between OSA and periodontitis: 1) Intermittent hypoxia may provide the conditions for systemic

* Corresponding author. Universidad Federal de Minas Gerais, Brazil.
E-mail addresses: mecortes@ufmg.br, tellezm@javeriana.edu.co (M.E. Cortés).

https://doi.org/10.1016/j.jddst.2024.105522
Received 10 August 2023; Received in revised form 17 February 2024; Accepted 28 February 2024
Available online 29 February 2024
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inflammation and oxidative stress. 2) Drying of the mouth brought on by prolonged open breathing might impede the oral mucosa from being cleaned by saliva flow, which may accelerate the development of periodontitis. 3) The pH is dropping, which can be encouraging microbial colonization and, consequently, the development and buildup of dental plaque. 4) The composition and ecology of the oral microbiota may be influenced by OSA-associated inflammation and intermittent declines in oxygenation, which may affect the development of periodontitis. And 5) Both OSA and periodontitis are associated with systemic inflammation, which may help to explain the connection between these two conditions [4,5].

Different microbiological techniques, including qPCR, 16S rRNA gene sequencing, and MALDI-TOF, were used to characterize the oral microbiota of individuals with periodontitis and OSA. The findings indicated that the oral microbiota's species richness and diversity had changed, accompanied by a rise in periodontal pathogens including *Fusobacterium* sp., *Tanarella* sp., and *Prevotella* sp. and yeasts like *Candida* sp [8–10]. Additionally, *Candida* sp. was closely linked to the bacteria of the orange and red Socransky's microbial complexes, as well as the severity of OSA, which was more prevalent in individuals with stage III periodontitis [8].

In cases of patients with severe periodontitis, scaling and root planing (SRP), the standard approach of non-surgical periodontal therapy (NSPT), may not be enough to restore periodontal health [11]. It is therefore necessary to use additional antiseptics like chlorhexidine (CHX); however, this lowers salivary pH, which increases the risk of dental demineralization, parotid gland inflammation, tooth and oral soft tissue pigmentation, type 1 hypersensitivity reactions, taste perversion, and burning sensations [12]. Amoxicillin and metronidazole are two other supplementary treatments to SRP that have substantial scientific support for their usage in daily clinical practice [13], however, patients may not respond well to standard NSPT because it cannot modify the dysbiotic infectious process to a homeostatic or commensal equilibrium. This is likely because the periodontal pocket still contains subgingival biofilm after treatment and/or periodontopathogenic microorganisms have invaded the tissue. Moreover, the use of antibiotics must be restricted to specific periodontal patients, with defined and limited dosages, taking into account both systemic side effects and negative microbiological effects, such as the emergence and spread of antibiotic resistance [12]. This is related to the fact that the likelihood of antibiotic treatment success is decreased since the administration of antibiotics is not directed towards preventing infection by polymicrobial biofilms linked to yeasts [14]. All of these factors may limit effective therapy, allowing periodontitis to progress.

Nevertheless, antimicrobial agents can be administered *in-situ* using devices known as Controlled Drug Delivery Systems (CDDS). These systems can encapsulate therapeutics, enhance their biopharmaceutical properties and efficacy, adapt to different administration routes, shield drugs from the environment, and enable controlled and directed release, among other benefits [15]. CDDS offer continuous drug administration at higher concentrations and directly to the infection site, with sustained release over prolonged periods; this is an advantage over systemic therapy (multiple dosage administered over longer periods). Better patient compliance with their administration, a decrease in toxic effects, and systemic complications such side effects of systemically administered antibiotics are additional advantages of these systems [16,17]. Nanofibers, a form of CDDS, offer great drug loading efficiency and have been used in the field of dentistry to deliver sufficient drug concentrations right at the target tissue (pocket/peridontal tissue). Due to their biodegradable, biocompatible, low-toxicity qualities, and FDA-approved, Poly (ε-caprolactone) (PCL) and Poly (lactic-co-glycolic acid) (PLGA) are two polymers that are frequently utilized as CDDS (nanofibers) [18–21]. PCL is a semi-crystalline aliphatic polyester that degrades slowly, is hydrophobic, biodegradable, and non-toxic [22]. Due to its mechanical characteristics, it is combined with other copolymerization polymers to increase its utility [23]. One example of this is PLGA, which is a highly biodegradable, biocompatible, and low-toxicity polymer that is ideal for use in biological environments as delivery systems. It is made up of two different ratios of lactic acid and glycolic acid monomers. However, its weak mechanical qualities are enhanced with copolymerization with PCL. Therefore, PCL and PLGA combined, result in controlled release of drugs biomaterials with higher efficacy because of their complementing mechanical qualities and biodegradability [18]. Both polymers are employed in the synthesis of nanofibers aimed for the treatment of oral diseases [24].

The Amphotericin B (AMB) is fungicidal antifungal that usually is administered parenterally and has a broad spectrum of action against fungi that cause systemic mycoses [25]. Even, it is used to treat oral candidiasis, and it is further applied topically for 14 days at a dose of 100 mg/mL as a cream, ointment, or lotion [26]. It works by attaching to the cell membrane's ergosterol and altering its permeability, resulting in the loss of cytoplasmic material and cell lysis. *In vitro* antifungal activity of AMB has been reported against most *Candida* species associated with oral infections, including *C. albicans* [27] and also, AMB has been shown to be effective against *C. albicans* biofilms [28].

Bovine lactoferricin, also known as Lactoferricin B (LfcinB), is a peptide with 25 amino acids that exhibits antibacterial, antifungal, antiparasitic, antiviral, and anticancer activity. The RRWQWR sequence has been described as the minimal motif of LfcinB that has shown antimicrobial and anticancer activity [23–25,29]. The palindromic LfcinB $(21–25)_{Pal}$: RWQWRWQWR has been shown to have significant antimicrobial activity, suggesting it a prosing peptide for the development of novel medicinal agents [30]. On the other hand, zinc oxide (ZnO) has the power to remove bacteria linked to caries [31]. *A. actinomycetemcomitans, P. gingivalis, P. intermedia,* and *F. nucleatum* are among the bacteria that are successfully stopped from growing by ZnO [32] and similarly, ZnO has shown antifungal action against *C. albicans* biofilms [33]. The viability and osteodifferentiation of periodontal ligament stem cells were supported by even ZnO nanoparticles in nanofibers, which showed antibacterial action against reference strains of *P. gingivalis* and *F. nucleatum* with low cytotoxic activity [34,35]. A controlled and sustained release of oxytetracycline hydrochloride was also made possible by ZnO nanoparticles present PCL nanofibers, with a release rate of 3.7 g/mL from 1 to 4 h, higher than the rates attained by systemic administration of the antibiotic [16].

Incorporating AMB, Lfcin $(21–25)_{Pal}$, and ZnO all at once into PCL/PLGA nanofibers may provide a prolonged release of these compounds, as anti-biofilm agents for use in treatment periodontitis in OSA patients. The current study's objective was to assess the antimicrobial activities of PCL/PLGA nanofibers incorporating AMB, LfcinB $(21–25)_{Pal}$, and ZnO for the inhibition of *in vitro* polymicrobial biofilms related to periodontitis associated with OSA.

2. Materials and methods

2.1. Nanofibers materials

The polymers, poly ε-caprolactone (PCL) (Mw = 43,000–50,000 g/mol; inherent viscosity = 1.7 dL/g) and Poly (lactic-co-glycolic acid, 75:25) (PLGA) (Mw = 66,000–107,000 g/mol; inherent viscosity = 0.65 dL/g) were purchased from Polysciences, Inc., (USA) and Sigma-Aldrich, Brazil Ltda, respectively. Chloroform ($CHCl_3$) and methanol (CH_4O) were acquired from Vetec (Brazil). Amphotericin B (AMB) (Mw = 924.08 g/mol) was purchased from Sigma-Aldrich, Brazil Ltda. The antimicrobial palindromic peptide (P61: LfcinB $(21–25)_{Pal}$) was supplied by SAMP-UNAL; peptide was synthesized by Solid Phase Peptide Synthesis and purified by RP-SPE chromatography and was analyzed by RP-HPLC and MS. Zinc oxide (ZnO) nanoparticles were supplied by LEMB-UFMG (Laboratory of Molecular Encapsulation and Biomaterials at Universidade Federal de Minas Gerais).

2.2. Microorganisms and cellular lines

The microorganisms were obtained from the collection of clinical isolates collected from oral samples cultured from individuals with OSA and with periodontitis [8], stored at *Centro de Investigaciones Odontológicas (CIO)* of the Faculty of Dentistry at the *Pontificia Universidad Javeriana-PUJ*, Bogotá, D.C., Colombia. Microbiological tests were performed using nine bacteria and one yeast isolated: *S. salivarius, S. gordonii, A. odontolyticus, V. parvula, F. nucleatum, P. melaninogenica, P. gingivalis, C. albicans, L. paracasei* and *C. granulosum.* Prior to the experiments, all microorganisms were pre-cultivated in modified Brain-Heart Infusion (BHI) (Millipore Corp.) and on Trypticase™ Soy Agar with 5% Sheep Blood (Becton Dickinson). This modified BHI was supplemented with 2.5 g/L mucin, 1.0 g/L yeast extract, 0.1 g/L cysteine, 2.0 g/L sodium bicarbonate, 5.0 mg/mL hemin, 1.0 mg/mL menadione and 0.25% (v/v) glutamic acid [30]. XTT ((2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfo-phenyl)-2H-Tetrazolium-5-Carboxanilide) were purchased from Sigma-Aldrich®.

The cytotoxicity tests were performed using Human Periodontal Ligament Fibroblasts (HPdLF), which was supplied by CIO-PUJ. The HPdLF was cultured in Dulbecco's Modified Eagle Medium – DMEM (Gibco®). The medium was supplemented with 5% Fetal Bovine Serum (FBS) (Gibco®), and antibiotic and antimycotic solution (Gibco®). MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma-Aldrich®.

2.3. Preparations of nanofibers solutions and electrospinning process

The PCL/PLGA (90:10) nanofibers were fabricated by conventional electrospinning method. Seven nanofibers were obtained: PCL/PLGA loaded with AMB (Nf 1), PCL/PLGA loaded with AMB and ZnO (Nf 2), PCL/PLGA loaded with P61 (Nf 3), PCL/PLGA loaded with P61 and ZnO (Nf 4), PCL/PLGA loaded with AMB and P61 (Nf 5), PCL/PLGA loaded with AMB, P61 and ZnO (Nf 6) and PCL/PLGA loaded with ZnO (Nf 7). To prepare all the nanofibers, 900 mg of PCL and 100 mg of PLGA were dissolved in 10 mL of a mixture of chloroform and methanol - $CHCL_3$: CH_3OH (1:1). For each nanofiber preparation, 15 mg AMB, 3 mg P61 and 40 mg ZnO were added in the polymeric solution, respectively. The solutions were stirred for 24 h at room temperature before the electrospinning process. Then, using a syringe pump (Harvard Apparatus PHD 2000), each solution was dispensed into a 10 mL standard syringe with a 27 G blunted stainless-steel needle. The electrospinning process was carried out at a voltage of 20 kV, a solution flow rate of 4 mL/h, and a distance of 15 cm from the needle tip. Nanofibers were collected and preserved for physicochemical characterization, cytotoxicity assays, and microbiological tests.

2.4. Physicochemical characterization

2.4.1. Nanofiber morphology by scanning electron microscopy (SEM)

The morphology and average diameter of nanofibers were determined using the scanning electron microscope FEG- Quanta 200 FEI, at an accelerating voltage of 20 kV. Applying a sputter coater, samples of nanofibers were coated with a layer of carbon that was 5 nm thick (Bal-Tec, MD20 instrument). Using Image J software and at least 100 observations, average nanofiber sizes were determined. To further establish the existence of the various elements in the nanofibers, energy dispersive X-ray spectrometry (EDS) and chemical histogram were carried out using EDS coupled in FEG-Quanta 200 (UFMG).

2.4.2. Thermal analysis (TA)

The TGAQ5000 equipment from TA Instruments was used to perform the thermogravimetry analysis (TGA). The nanofibers loss weight during heating was determined by Thermogravimetry (TG) and Derivate thermogravimetry (DTG). Nanofiber samples were heated under nitrogen gas at a flow rate of 50 mL/min from 25 °C to 900 °C at a rate of 10 °C/min. The software TA instrument universal analysis 200 was used to examine the data.

2.4.3. Contact angle measurement

The wettability of the nanofibers was evaluated using the Contact Angle Measuring System, model SEO Phoenix 300 Touch contact angle. Water was dropped from a sample stand onto the surface of the nanofibers (size 10 mm^2) to determine the contact angles. After a camera took an image of the drop, Surfaceware9 software was used to calculate the average contact angle. Each measurement was carried out once per nanofiber.

2.5. Cytotoxicity test

2.5.1. Preparation of eluates from nanofibers

Eluates from the nanofiber's supernatants were produced to test the cytotoxicity of the nanofibers. Nanofibers (Nfs 1–7) samples were incubated with 1 mL of Dulbecco's Modified Eagle Medium (DMEM) for 24, 48 and 72 h at 37 °C, then the nanofiber samples were removed and eluates were used for the cytotoxicity tests.

2.5.2. Cell cultures and cell toxicity assay

Human Periodontal Ligament Fibroblasts (HPdLF) (Lonza Bioscience) were cultured in DMEM supplemented with 10% FBS, and antibiotics (0.1 mg/mL streptomycin and 100 U/mL penicillin) and incubated in a humidified environment of 5% CO_2 kept 37 °C. After being subcultured to 80% confluence, the HPdLF were seeded at a density of 10.000 cells per well into 96-well plates. Cells were exposed to nanofiber eluates for 24 h and the untreated control, HPdLF were not exposed to the eluted nanofibers. After that, cell viability was evaluated using trypan blue exclusion assay and photograph three random fields per well. Also, the 96-well plates were covered with 30 μL of MTT solution (1 mg/mL in PBS), and incubated at 37 °C for 4 h in the dark in a humidified environment of 5% CO_2. The formazan crystals were then dissolved using 100 μL DMSO. Using a BioTek ELx800TM microplate reader and the Gen5TM Data Analysis Software, optical densities (OD) at a wavelength of 492 nm were recorded. Each experiment was carried out in triplicate three times independently.

2.6. Microbiological test

2.6.1. Disk diffusion method

The disk diffusion method was used to assess the initial antibacterial activity of the nanofibers against a culture of *Candida albicans* and a culture of mixed microorganisms. In order to conduct the test, 5 mm diameter nanofiber disks were attached to the surface of BHI agar and Sabouraud agar, which had previously been spread with 100 μL of *C. albicans* and mixed microorganism culture solutions (OD 600 nm, 0.8–1.0), respectively. The mixed microorganism plates were incubated anaerobically (with 90% nitrogen and 10% CO_2) for 72 h at 37 °C, while the *C. albicans* plates were incubated aerobically for 48 h. The diameters of the clear areas within the inhibition zone were measured in millimeters after incubation, and the results were expressed as the mean value ± standard deviation (SD).

2.6.2. Broth microdilution method

The antimicrobial activity of nanofiber against a mixed microbe culture was evaluated using the broth microdilution method. The mixed microorganisms culture suspensions (OD 600 nm, 0.8–1.0) and nanofiber disks having a diameter of 5 mm were used. The mixed microorganism culture suspension and the modified BHI broth were utilized as the positive and negative controls, respectively. On 96-well plates, the nanofiber disks were placed. Following that, 50 μL of mixed microorganism culture and 50 μL of culture broth were added to each well. The plates were subsequently incubated in an anaerobic atmosphere with 90% nitrogen and 10% CO_2 for 24, 48, and 72 h at 37 °C, respectively.

The nanofiber disks were carefully taken out of the 96-well plates after the incubation period. One hundred μL of XTT solution (1 mg/mL in PBS) was added to each well, and the mixture was incubated for 2 h at 37 °C in the dark and an anaerobic atmosphere with 90% nitrogen and 10% CO_2. Each test was performed six times, and a Lambda 25 UV–vis spectrophotometer (PerkinElmer, USA) was used to measure the tested solutions at 490 nm.

2.6.3. *In vitro biofilm experiments*

Using nanofibers with known antimicrobial action, the polymicrobial biofilms' susceptibility was examined. On CELLviewTM cell culture slides (Greiner Bio-one), the ten microorganisms that were chosen were utilized to develop *in vitro* biofilms. Using spectrophotometry, the concentration of the microorganisms was adjusted to produce an inoculum in modified BHI (OD 600 nm, 0.8–1.0). One hundred μL of prepared inoculum was added to each well, and the mixture was incubated for 72 h at 37 °C in an anaerobic atmosphere with 90% nitrogen and 10% CO_2 [36]. To get removal of planktonic microorganisms after the incubation, the biofilms were gently rinsed with sterile PBS. Then 100 μL of fresh modified BHI was added per well and nanofiber disks were gently immersed on each well. Biofilms that weren't nanofiber-treated were used as the negative control. Then, the treated and untreated biofilms were incubated in an anaerobic environment (90% nitrogen, 10% CO_2) at 37 °C for 24, 48 and 72 h. Subsequently, the biofilms viability was determined by Confocal Laser Scanning Microscopy (CLSM).

Before the CLSM analysis, the nanofibers were gently removed from wells and the biofilms were rinsed in 100 μL of sterile PBS, in order to eliminate nonadherent microorganisms. Then, biofilms were stained with LIVE/DEAD® BactLight™ Bacterial Viability Kit (Invitrogen) and

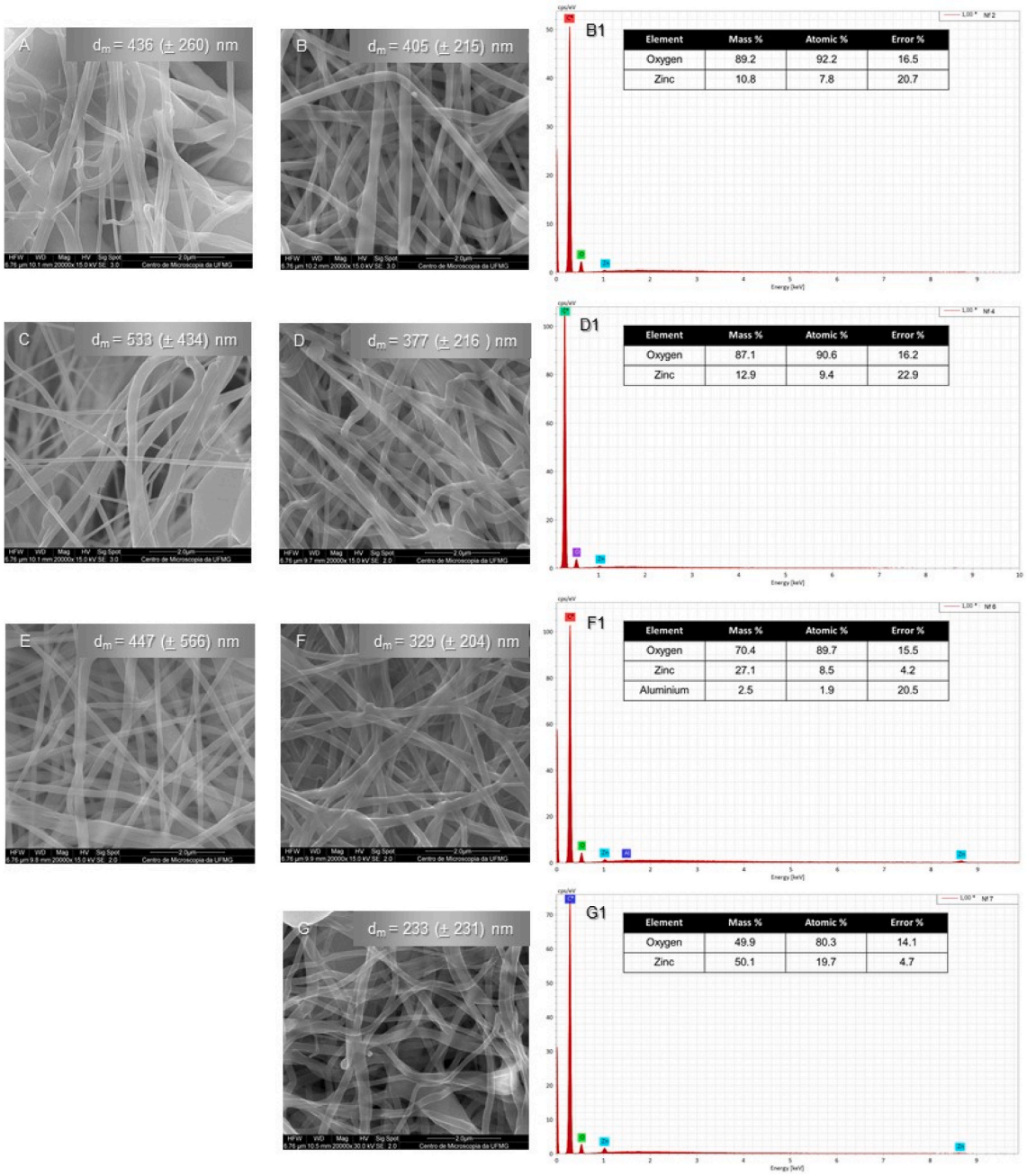


Fig. 1. SEM micrographs: the morphology and mean diameter (d_m) and standard deviation (±SD) of PCL/PLGA nanofibers: (A) Nf 1: AMB, (B) Nf 2: AMB-ZnO, (C) Nf 3: P61, (D) Nf 4: P61–ZnO, (E) Nf 5: AMB-P61, (F) Nf 6: AMB-P61-ZnO, (G) Nf 7: ZnO. EDS analysis of Nf 2, 4, 6 and 7 (B1, D1, F1 and G1, respectively.

LIVE/DEAD™ Yeast Viability Kit (Invitrogen), according to the manufacturer's instructions. Total fluorescence staining of the micrographs taken by the Olympus FV1000 confocal microscope. The fluorescence staining was analyzed using the image analysis software ImageJ (NIH, USA), to determine the cell viability inside the treated and untreated biofilms.

Additionally, in order to observe the anti-biofilm activity, the nanofibers were exposed to 500 μL of microorganisms' inoculum adjusted (OD 600 nm, 0.8–1.0), and incubated in an anaerobic environment (90% nitrogen, 10% CO_2) at 37 °C for 24, 48 and 72 h. Then, the nanofibers were washed with PBS and placed in 2.5% glutaraldehyde (Sigma-Aldrich) overnight. After, they were washed with PBS to remove the glutaraldehyde and dehydrated with 70, 90 and 100% of ethanol for 30 min, three times. The nanofibers were coated with carbon (thickness: 5 nm) and imaged with a Scanning Electron Microscope (SEM) (JEOL, model JSM 6490-LV) in high-vacuum mode at 15 kV.

2.7. Statistical analysis

Tukey's multiple comparison text and a two-way analysis of variance (ANOVA) were employed to assess the differences between microorganisms or biofilm treated and the microorganism and biofilm untreated. The level of significance was set to $p < 0.05$. Software GraphPad Prism 9.0.2 (GraphPad Software, California, USA) was used.

3. Results and discussion

3.1. Physicochemical characterization

Similar characteristics included the smooth texture, strong mechanical stability, and ease of manipulation of the nanofibers. The diameter and morphology of the nanofibers were assessed using SEM imaging. The randomly distributed nanometer-sized fibers that formed up the PCL/PLGA nanofibers charged with AMB, P61, and/or ZnO (Fig. 1). The distribution of the nanofibers' fiber diameters, which ranged from 233 to 533 nm, was uniform. The existence of beads in Nanofibers 1, 3, and 5 may have resulted from the AMB and P61 being in solution during the electrospinning process. However, the beads' presence diminished when ZnO was present in the nanofibers (Nf 2, 4 and 6), resulting in a lower average fiber diameter than those that were ZnO-free. Other authors have reported similar results, showing that adding ZnO particles reduced the diameter of the nanofibers [16], while adding LfcinB $(21–25)_{Pal}$ increased the formation of beads in the nanofibers [26], which could be further enhanced by adding ZnO (Fig. 1 - B, D, F and G). Additionally, the formation of beads may have resulted from the electrospinning solution's higher viscosity, which was caused by PCL's higher viscosity (1.7 dL/g) and proportion compared to PLGA's (0.65 dL/g). ZnO particles were successfully integrated into nanofibers, based on the EDS analysis (Fig. 1 – B1, D1, F1 and G1).

The thermogravimetric analysis described the chemical and physical changes that took place while nanofibers were heated. The analysis of the TG and DTG curves was used to determine the thermal stability of nanofibers. Fig. 2 shows the thermal degradation of the PCL/PLGA

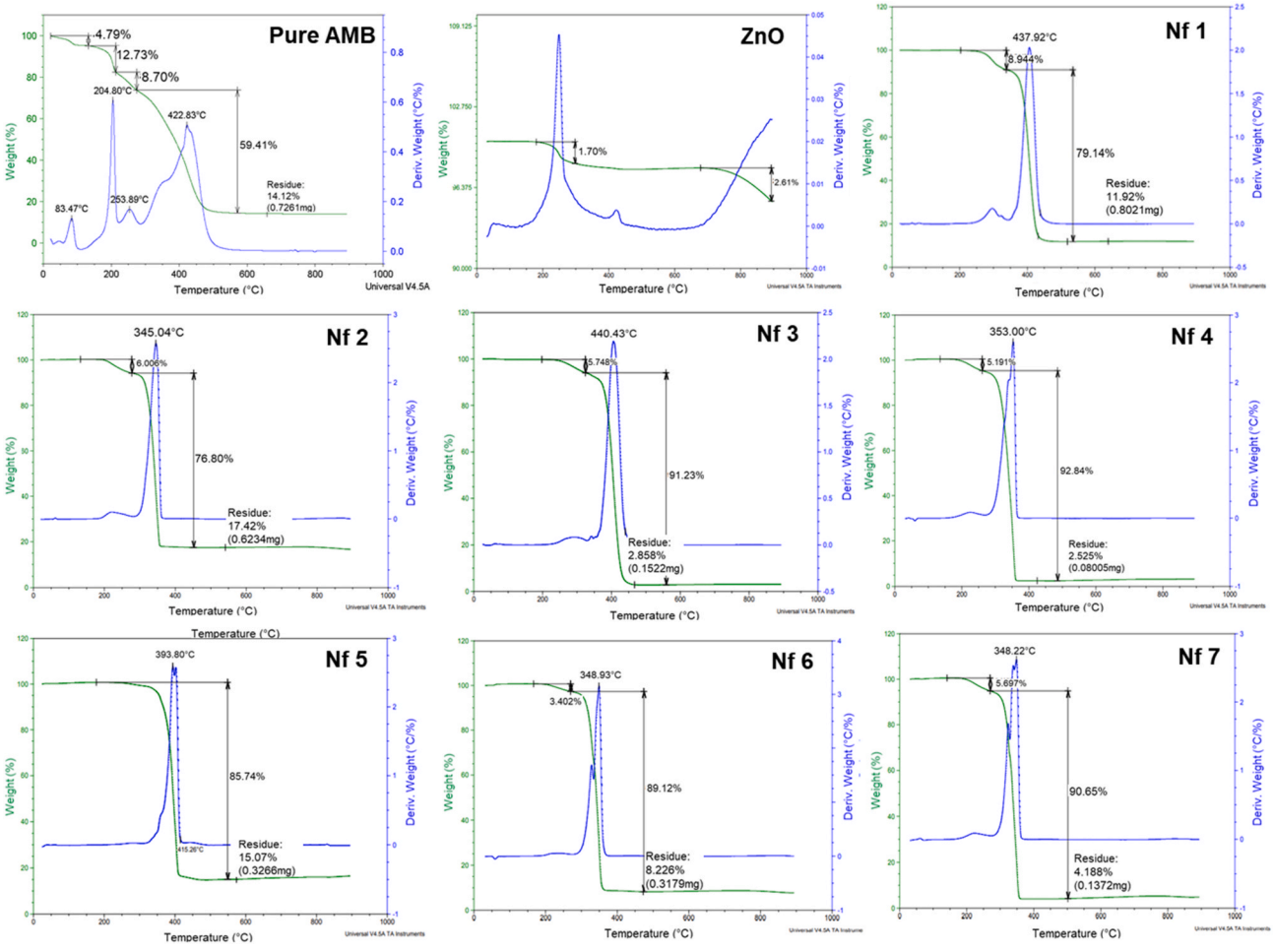


Fig. 2. TG and DTG curves of PCL/PLGA nanofibers, pure AMB and ZnO.

nanofibers as well as pure AMB and ZnO particles. P61 was not evaluated for the amount available. Pure AMB had a gradual degradation from 200 °C, while the ZnO particles exhibited high thermal stability until 900 °C, with a weight loss of 4.3%. This result is consistent with what Magalhães et al. reported in 2019 [16]. The curves showed that the nanofibers did not significantly degrade as a function of temperature up to 350 °C and that the addition of the AMB or ZnO did not significantly alter this behavior, showing the PCL/PLGA nanofibers exhibited high thermal stability [16,37].

To evaluate the hydrophilicity of the nanofibers, the contact angles of their surfaces were evaluated. The contact angles for the PCL/PLGA nanofibers are shown in Fig. 3. All nanofibers, except the nanofibers 4 and 6, presented a hydrophobic surface (contact angle higher than 90°), showing poor wettability, as consequence of hydrophobic nature of PCL and PLGA polymers.

Despite the amphipathic characteristics of both amphotericin B and P61 (LfcinB), Nf1 and Nf3 exhibited a hydrophobic surface. This is because amphotericin B is composed of a hydrophobic domain, namely the polyene hydrocarbon chain, and tryptophan and phenylalanine are two hydrophobic aromatic amino acids, are present in P61. This appears to be the cause of the hydrophobic properties of the Nf1 and Nf3, respectively. The nanofiber 4 showed hydrophilic character, it could be due that ZnO nanoparticles have a larger surface area, which could result in more H-linkages being formed with P61. In addition, these ZnO nanoparticles make amphotericin B more hydrophilic, which reduces the angle contact of Nf6. The nanofiber 4 image could not be displayed, due to its extremely hydrophilic nature.

More studies on this interaction's compounds are necessary to comprehend how the molecules of the AMB, P61 and ZnO can interact to make the nanofibers more hydrophilic when combined.

3.2. Cytotoxicity test

The Human Periodontal Ligament Fibroblasts (HPdLF) cell line was used to perform an indirect contact MTT assay to determine the cytotoxicity of nanofibers. The cytotoxicity was calculated based on cell viability relative to control (cells untreated), adjusted to 100% of viability (Fig. 4). Grades of cytotoxicity were assigned, with non-cytotoxic being defined as viability values greater than 90%, slightly cytotoxic being defined as viability values between 60% and 90%, moderately cytotoxic being defined as viability values between 30% and 59%, and severely cytotoxic being defined as viability values lower than 30% [16]. Moreover, the cytocompatibility of nanofibers is considered when the cell viability is higher than 50% [38].

Although the nanofibers were slightly cytotoxic, all of them presented a cell viability higher than 50%. The least cytotoxic of the complete group of nanofibers were nanofibers 3 and 4, which were loaded with P61. Surprisingly, the cell viability improved with the exposure time. This result might be explained by the presence of AMB, P61, and ZnO, which might stimulate cellular growth. The viability and osteodifferentiation of periodontal ligament stem cells have been reported to be supported by zinc oxide particles, without adversely impacting their biocompatibility traits [39]. Other authors reported that ZnO has a great potential used in guided tissue/bone regeneration strategies in the periodontal treatment [16,34]. It is important to verify these findings using other methods, such as Annexin V Apoptosis Assay and to find out whether AMB and P61 can stimulate *in vitro* cell growth.

3.3. Antimicrobial activity

The oral microbiota of individuals with periodontitis and OSA was characterized and defined by a rise in periodontal pathogens including *Fusobacterium* sp., *Tanarella* sp., and *Prevotella* sp. and yeasts like *Candida albicans*, [8,10]. The severity of OSA, which was more prevalent in individuals with stage III periodontitis, and the bacteria in the orange and red Socransky's microbial complexes were both closely related to *Candida albicans* [8]. These microorganisms have the capacity to build polymicrobial biofilms that are challenging to eradicate or prevent, given their complex physical and biological characteristics [40].

The antimicrobial activity of PCL/PLGA nanofibers loaded AMB (Nf's 1, 2, 5 and 6), P61 (Nf's 3, 4, 5 and 6) and ZnO (Nf's 2, 4, 6 and 7) was tested against *C. albicans* alone and mixed microorganisms' culture, which is made up of nine bacteria and *C. albicans* (Fig. 5). Studies on the use of PCL nanofibers as possible drug delivery systems for the treatment of periodontitis have mainly concentrated on the antibacterial properties of metronidazole, amoxicillin, ciprofloxacin, and tetracycline [16,33,34,38,39]. These antibiotics does not have activity against yeasts. Being the first study to describe this activity, this work used PCL/PLGA nanofibers loaded with AMB, which showed an antimicrobial effect against the mixed microbial culture. The AMB and P61-loaded

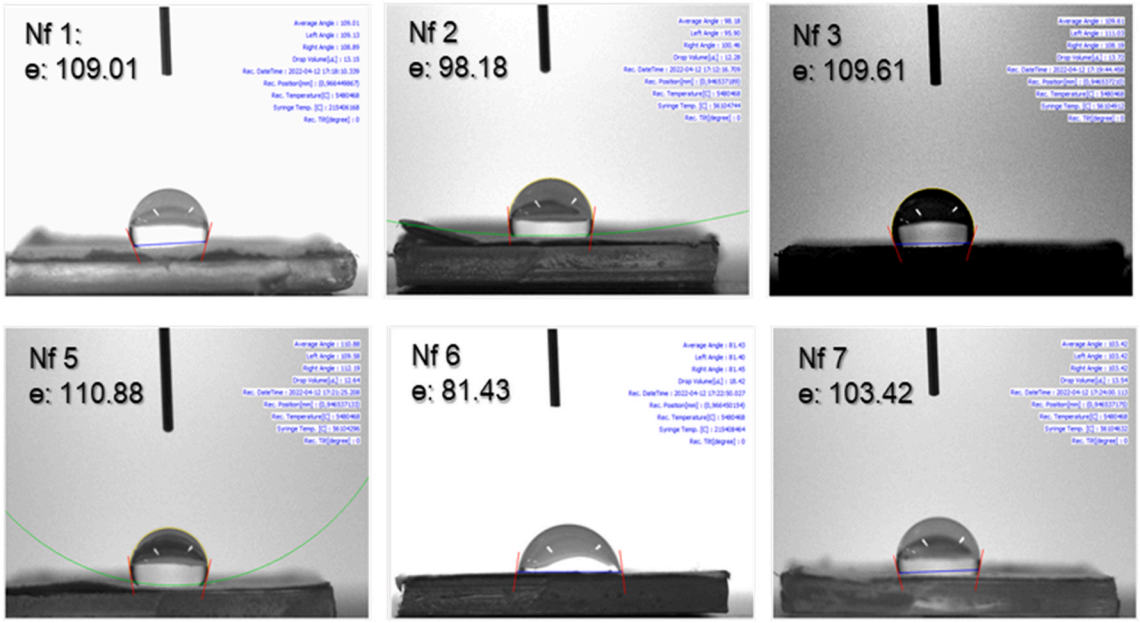


Fig. 3. Images of the contact angle (Θ) formed between water and PCL/PLGA nanofibers.

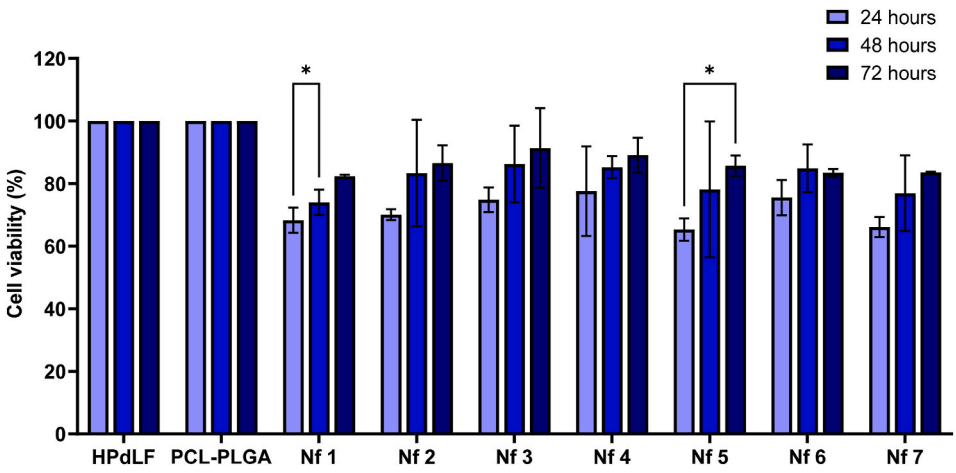


Fig. 4. Cell viability of HPdLF in presence of nanofibers' eluates measured by the MTT (DO_{492nm}) method.

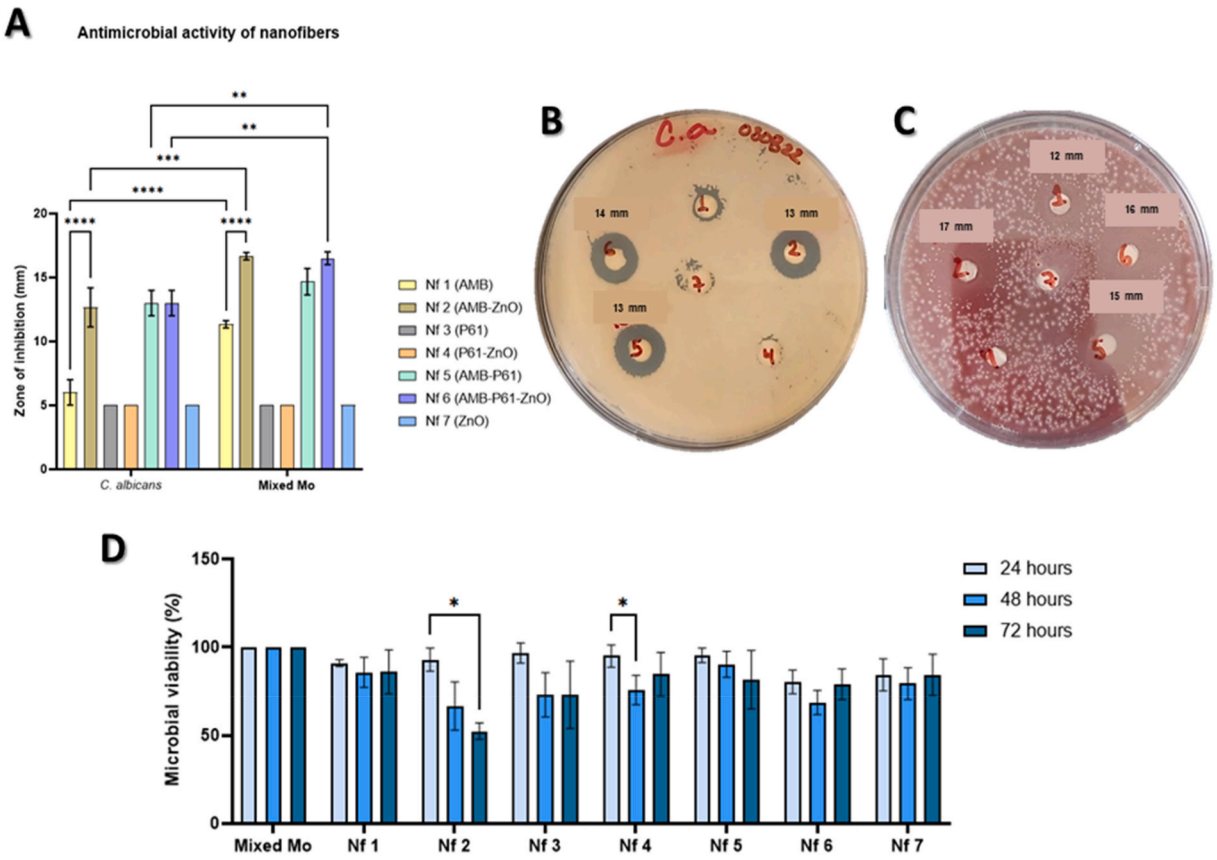


Fig. 5. Antimicrobial activity of nanofibers' discs. (A) Growth inhibition of *C. albicans* and mixed microorganisms. (B) Representative macrophotograph showing growth inhibition of *C. albicans.* (C) Representative macrophotograph showing growth inhibition of mixed microorganisms. (D) Viability (%) of mixed microorganisms for 24, 48, and 72 h in the presence of nanofiber discs. Data was generated from a 2Way-ANOVA, followed by Tukey's multiple comparisons ($p < 0.05$).

nanofibers demonstrated an increase in antimicrobial activity, suggesting a possible synergy between the two drugs, even if the P61-loaded nanofibers did not show any antimicrobial activity. The lowest antimicrobial activity of P61-loaded nanofibers could be attributed to the peptide's accessible concentration in the nanofiber. Compared to unloaded ZnO nanofibers, the ZnO-loaded nanofibers showed more activity.

The antimicrobial action of PCL/PLGA nanofibers is time-dependent due to their ability to release drugs that last for a long period [39,41]. Nevertheless, the activity of each nanofiber differed. (Fig. 5D). The antimicrobial activity of Nf 2 (AMB-ZnO) against the mixed microbial culture was verified by the microdilution method, and it increases with exposure duration. Furthermore, Nf 3 (P61) demonstrated increased antimicrobial activity after 48 and 72 h; however, activity did not increase in the presence of ZnO (Nf 4), and similar results were seen for Nf 3 and Nf 4. The inclusion of AMB (Nf 5) did not improve the results obtained from Nf 4, but ZnO (Nf 6) showed better antimicrobial activity after 24 h when compared to the other nanofibers at the same exposure time. After 48 h, Nf 6 produced results that were similar to Nf 2's.

ZnO in Nf 4 may increase P61's antimicrobial action, which is

effective against both Gram-positive and Gram-negative bacteria [34]. By using the microdilution method, ZnO-loaded nanofiber (Nf 7) showed antimicrobial activity, reducing the viability of mixed microorganisms by 15%. However, this nanofiber did not demonstrate antimicrobial activity using the disk-diffusion test. Similar results showed antimicrobial efficacy against bacteria that are Gram-negative [16]. Based on these results, it may be concluded that nanofibers 1, 2, 5, and 6 might have some antimicrobial effect.

Using the CLSM technique, nanofibers 1, 2, 5 and 6 were selected based on the previous results to assess the anti-biofilm activity (Fig. 6). The Nf 6 had better activity when comparing the anti-biofilm activity between nanofibers and between exposure times, and this activity grew stronger with exposure time. The Nf 6 decreased a 40% the biofilm viability at 72 h. The number of dead cells (in red color) at 72 h revealed that the combination of AMB, P61 and ZnO in Nf 6, has stronger anti-biofilm activity (Fig. 6C). The Nf 2 continues to present antimicrobial activity, even much better than that presented by the Nf 1, this may be due to the presence of ZnO, improving the activity of the AMB. The Nf 5 has better results with a longer exposure time (72h).

On the other hand, it was determined how well AMB, P61 and ZnO loaded in the nanofibers could prevent microorganisms' adherence to them. The four nanofibers limited the formation of a biofilm, despite evidence that microorganisms, primarily coccoid bacteria, are adherent to the nanofibers. This inhibition is correlated with exposure time; a longer the exposure time, the stronger the inhibition. There was no evidence of even the presence of yeasts or hyphae (Fig. 7). These findings must be confirmed by measuring each microorganism exposed to the nanofibers using molecular methods.

The hydrophobic properties of PCL, which were used to manufacture the nanofibers, restrict the release of the drugs tested; as a result, other polymers, such as PLGA, must be used to increase PCL's bioavailability. The majority of the studies discuss an ongoing, prolonged controlled release of antimicrobial agents that is enhanced by other polymers [42, 43]. Disk Diffusion method was the primary methodology employed in the majority of research to evaluate the antibacterial activity of nanofibers [16,38,44–47]. This approach is helpful, but it also requires the use of other tools and quantifiable microbiological methods to complete the data on the ability to inhibit periodontitis-related microorganisms.

Despite, the antimicrobial activity of the ZnO-loaded nanofiber (Nf 7) was not shown by the disk diffusion test; the microdilution method showed antimicrobial activity in this nanofiber, which decreased the viability of mixed microorganisms. Comparable results illustrated the antimicrobial efficacy of nanofibers loaded with ZnO particles against *A. actinomycetemcomitans, F. nucleatum, P. gingivalis*, and *P. intermedia* through the use of the Broth Microdilution test [16] and the disk diffusion test for *P. gingivalis* and *F. nucleatum* [38].

There is limited evidence about the anti-biofilm activity against polymicrobial biofilms, which include periodontal pathogenic bacteria and yeast. The majority of research that has been reported on the use of nanofibers for periodontal therapy, evaluated the antimicrobial activity

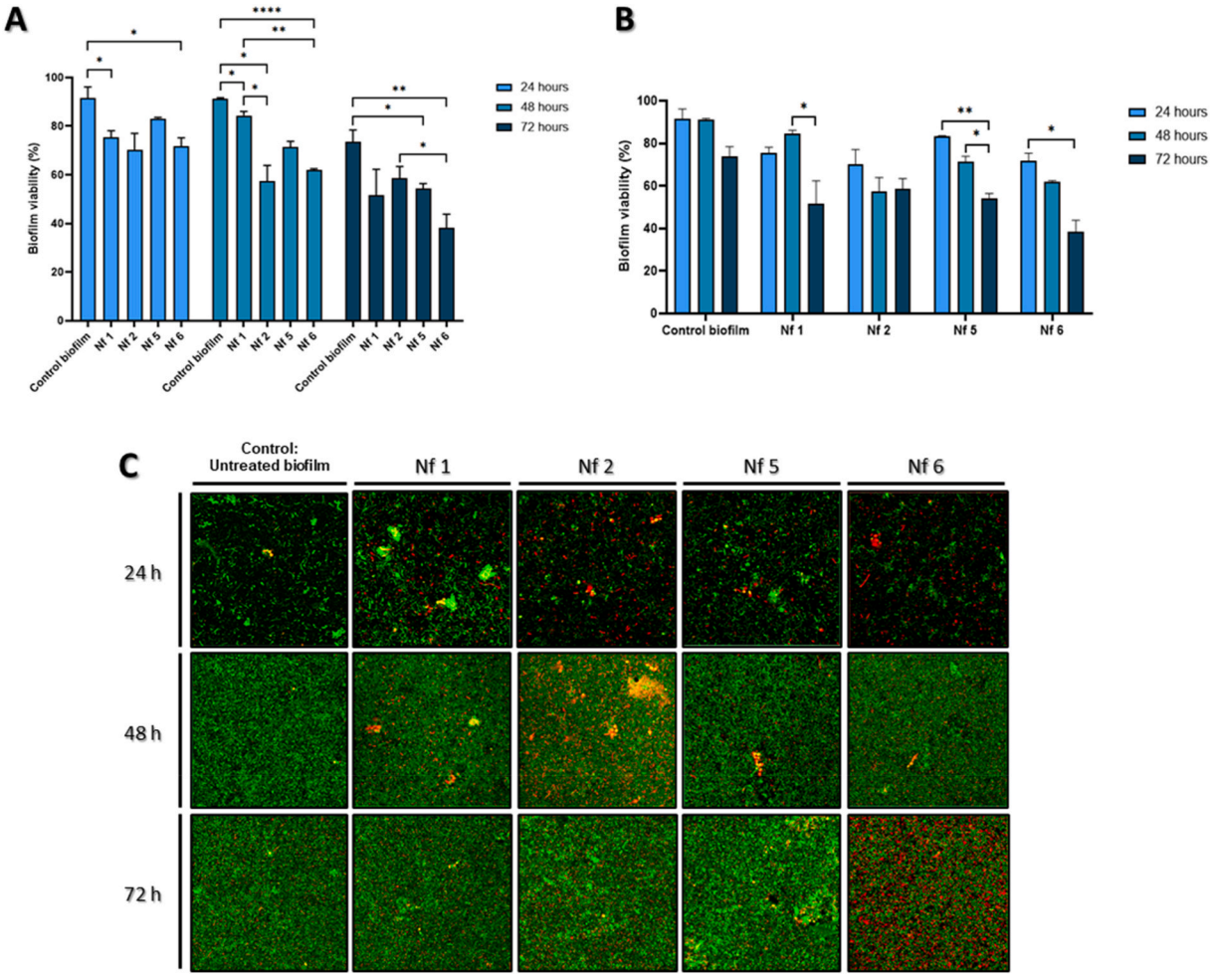


Fig. 6. Anti-biofilm activity analyzed by CLSM. (A) Biofilm viability (%) in the presence of nanofiber discs: comparison of the anti-biofilm activity of the different nanofibers. (B) Biofilm viability (%) in the presence of nanofiber discs: analysis of anti-biofilm activity during different treatment times. Data was generated from a Mixed effects analysis, followed by Tukey's multiple comparisons ($p < 0.05$). (C) Confocal micrographs of biofilms treated with nanofiber discs during different times: Green color: live cells; Red color: dead cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

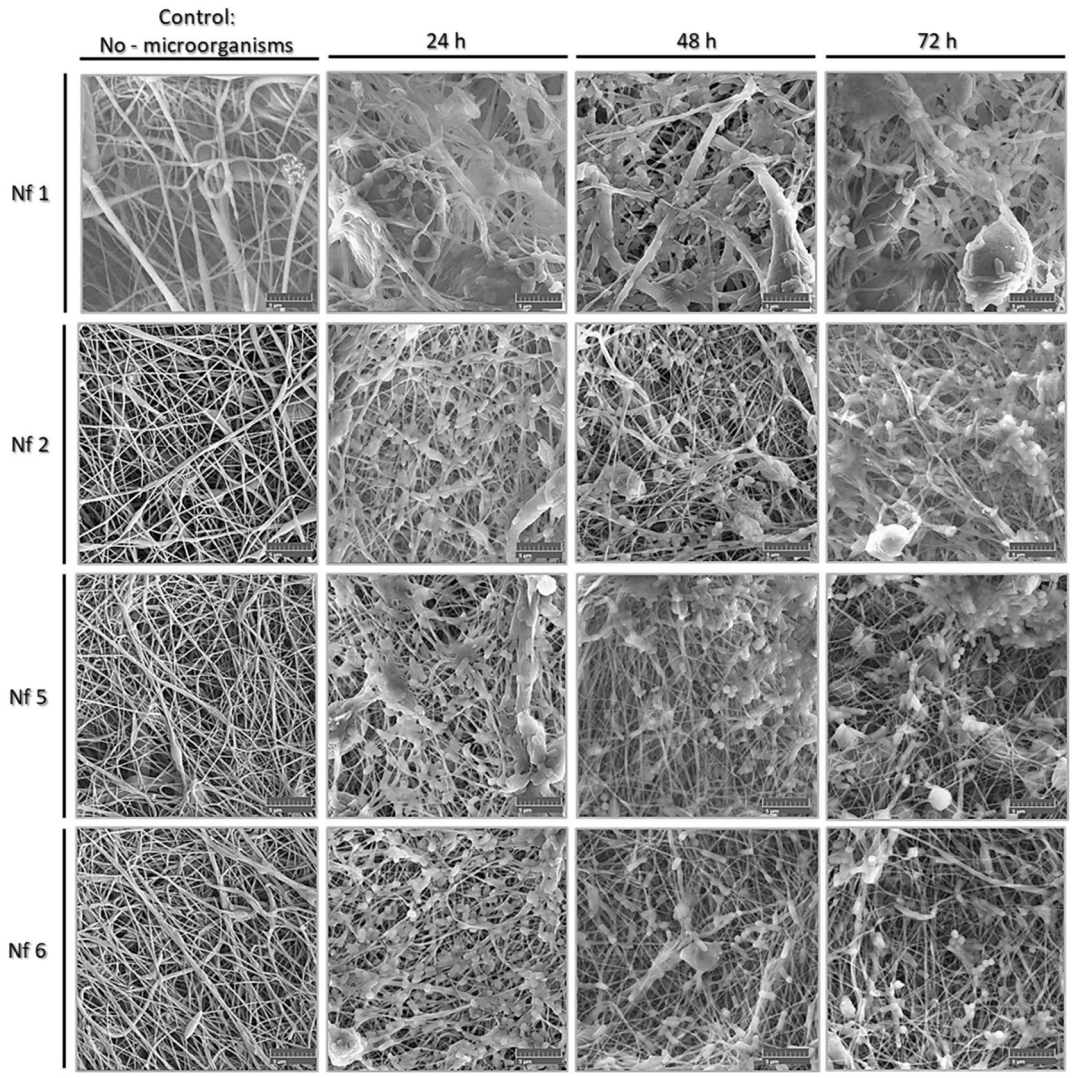


Fig. 7. Scanning electron micrograph images of the nanofibers incubated with the microorganisms for 24, 48 and 72 h. Magnification is 8.00 kx (scale bar; 5 μm).

of antibiotics-loaded nanofibers against planktonic bacteria not related to periodontitis [42,43,48]. Broad-spectrum antibiotics, including Metronidazole, Doxycycline, Tetracycline, and Ciprofloxacin, are put into nanofibers and used to inhibit planktonic bacteria, including *P. aeruginosa*, *S. aureus*, and *E. coli*, that are not often associated with periodontitis. Furthermore, without assessing the anti-biofilm activity, the last studies conclude that these nanofibers can be applied to periodontal therapies [42,43,48]. The work proposal is now significant because it suggests using an antifungal and an antimicrobial peptide (P61: Lfcin B) that can inhibit polymicrobial biofilms, which include bacteria and yeast, regard to the last evidence of yeast participation in biofilms linked to periodontitis [8,49,50].

It is important to note that broad-spectrum antibiotics are primarily used in published research on the use of nanofibers as periodontal therapy; none, however, assess antifungals or antimicrobial peptides. The current study showed antimicrobial and anti-biofilm activity of AMB, P61 and ZnO-loaded PCL/PLGA nanofibers against microorganisms associated with periodontitis and OSA, with low cytotoxicity. These nanofibers may be a potential strategy for local treatment of periodontitis in individuals with obstructive sleep apnea.

4. Conclusions

The electrospinning method was successfully used to manufacture PCL/PLGA nanofibers containing AMB, P61 and ZnO, with good mechanical stability and easy manipulation. Minimal cytotoxicity against Human Periodontal Ligament Fibroblasts (HPdLF) cells, and good antimicrobial and anti-biofilm activity were exhibited by AMB-P61-ZnO-loaded PCL/PLGA nanofibers against to mixed microbial culture and biofilms that contains nine bacteria and a yeast, *C. albicans,* related to periodontitis associated with obstructive sleep apnea. These nanofibers could be a promising approach to treating periodontitis locally in those who have obstructive sleep apnea.

CRediT authorship contribution statement

Mayra A. Téllez Corral: Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Jean C. Villamil Poveda:** Writing – review & editing, Resources. **Nelly S. Roa Molina:** Writing – review & editing. **Liliana Otero:** Writing – review & editing. **Zuly Jenny Rivera Monroy:** Writing – review & editing, Conceptualization. **Javier García Castañeda:** Writing – review & editing, Methodology. **Claudia M. Parra Giraldo:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Conceptualization. **Maria E. Cortés:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Mayra Alexandra Tellez Corral reports financial support was provided by L'Oreal fundation and UNESCO.

Data availability

Data will be made available on request.

Acknowledgments

We thank the *Pontificia Universidad Javeriana-sede Bogotá* for the financial support to the project *"Evaluación de la actividad antimicrobiana de Lactoferricina B (LfcinB) y Anfotericina B (AMB) en sistemas de liberación controlada sobre biopelículas polimicrobianas in vitro relacionadas a la Periodontitis asociada a Apnea Obstructiva del Sueño"* (project number 2054) and we greatly appreciate the support given by the Training Agreement for postgraduate studies of professors of the *Pontificia Universidad Javeriana* for their doctoral training. We also thank L'Oreal fundation and UNESCO for the support to this project by the Program "For Woman in Science 2021". We thank the Brazilian agencies CNPq (ID. 305414) and FAPEMIG (ID. APQ-03272-18), Prof. Ruben Sinisterra, Alfonso Martinez and Manuel Ramirez from Laboratório de Encapsulamento Molecular e Biomateriais, Chemistry Department, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil. Also, we thank the Microscopy Center of the UFMG (Brazil) and the μ-core, Microscopy Center of the University of the Andes (Colombia) for their support with Scanning Electron Microscopy (SEM) and Confocal Microscopy (CLSM).

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