Development of a chlorhexidine delivery system based on dental reline acrylic resins

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ABSTRACT

The high recurrence rate of common denture stomatitis after antifungal treatment is still concerning. This condition is caused by low patient compliance and incomplete local elimination of the main etiological factor — *Candida albicans*, often associated with other microorganisms, such as *Streptococcus* species. Impregnating denture materials with antimicrobials for local delivery is a strategy that can overcome the side effects and improve the efficacy of conventional treatments (topical and/or systemic). In this work, we describe the development of three hard autopolymerizing reline acrylic resins (Kooliner, Ufi Gel Hard, and Probase Cold) loaded with different percentages of chlorhexidine (CHX). The novel formulations were characterized based on their antimicrobial activity, mechanical, morphological and surface properties, *in-vitro* drug release profiles, and cytotoxicity. The addition of CHX in all resins did not change their chemical and mechanical structure. Among all the tested formulations, Probase Cold loaded with 5 wt% CHX showed the most promising results in terms of antimicrobial activity and lack of serious detrimental mechanical, morphological, surface, and biological properties.

1. Introduction

Denture stomatitis is a common condition among people who wear removable dentures. Treatment of this condition remains challenging since it is associated with a high recurrence rate after conventional therapy based on topical and/or systemic antifungal drugs (Ohshima et al., 2018). Factors such as low patient compliance and incomplete elimination of the main etiological factor, *Candida* species, can explain these phenomena (Chandra et al., 2001). Furthermore, other pathogens, namely *Streptococcus* species, may have an important role in the formation of the complex *Candida* biofilms that often colonize the denture-bearing mucosa (Garaicoa et al., 2018; Lamfon et al., 2005).

Impregnating denture materials with antimicrobials for localized delivery near the infection site is an advanced strategy that can deter the adherence of microorganisms and consecutively the onset of the disease

(Gendreau and Loewy, 2011). This approach also overcomes the side effects of a systemic drug and guarantees the drug availability in the target area at a therapeutic dosage for an extended time (Bertolini et al., 2014; Ryalat et al., 2011; Salim et al., 2013a). The monitoring is minimal, and the patient compliance dependency is reduced (Salim et al., 2012b; Salim et al., 2013c).

Numerous previous studies investigated the addition of antimicrobial agents into denture soft lining materials with promising results (Bertolini et al., 2014; Chopde et al., 2012; Sanchez-Aliaga et al., 2016). However, since their core base material is easily degradable by the oral environment, they present a short life cycle (one to two weeks) and are considered temporary. Moreover, due to their porous structure, they are prone to intense microbial colonization and need cleaning procedures like brushing and cleaning solutions, which also degrade the material (Lima et al., 2016a). To overcome these disadvantages (Taylor et al.,

Table 1
Dental materials under evaluation.

Material	Manufacturer	P/L ratio (g/mL)	Composition	Curing cycle
K	GC America Inc., Alsip, Illinois, USA	1.4/1	P: PEMA L: IBMA	10 min 37 °C
UFI	VOCO GmbH, Cuxhaven, Germany	1.77/1	P: PEMA L: HDMA	7 min 37 °C
PC	Ivoclar Vivadent AG, Liechtenstein	1.5/1	P: PMMA L: MMA	15 min 40 °C 4 bar

K - Kooliner; UFI - Ufi Gel Hard; PC - Probase Cold; P – Powder; L – Liquid; PEMA – poly(ethyl methacrylate);IBMA – isobutyl methacrylate;HDMA - hexanediol dimethacrylate; PMMA – poly(methyl methacrylate); MMA - methyl methacrylate.

2008), alternative drug delivery systems based on hard reline materials have been proposed (Bacali and Baldea, 2020; Salim et al., 2013a; Salim et al., 2012b).

Among the various antimicrobials proposed, chlorhexidine (CHX), commonly used as an antiseptic mouthwash in dentistry, has been suggested as a therapeutic supplement for denture stomatitis due to its antimicrobial activity against a broad spectrum of organisms, including *Candida* (Bertolini et al., 2014; Salim et al., 2013a). In previous studies, the immersion of acrylic dentures into a CHX solution suppressed the adhesion of *Candida albicans* to the buccal epithelial cells and acrylic denture surfaces (Redding et al., 2009; Ryalat et al., 2011). Compared to antifungals, CHX's *Candida* inhibition lasts longer than amphotericin B and nystatin (Radnai et al., 2010; Ryalat et al., 2011). CHX also showed better results than fluconazole, both on releasing and microbiological tests (Bertolini et al., 2014; Gong et al., 2007; Salim et al., 2013b). In the literature, rare allergic reactions associated with CHX have been described (Brookes et al., 2020). The most common side effect associated with the use of CHX is brownish discoloration of teeth, restorations, and tongue. There is some evidence that regular and frequent application of CHX mouthwashes may temporarily impair the taste sensation. Membrane irritation and desquamative lesions in the oral mucosa were observed in a small number of individuals with prolonged use of CHX but these symptoms are transient and disappear following termination of therapy (Genovesi, et al., 2017). Nevertheless, with minimal and transitory local and systemic side effects, CHX is still considered a safe compound for usage in the oral cavity (Varoni, et al., 2012).

Besides immersion in solutions (Gong et al., 2007; Salim et al., 2013a), drugs can also be added to a coating system (Cochis et al., 2012; Feng et al., 2017; Garaicoa et al., 2018; Namangkalakul et al., 2020) or be loaded to an acrylic polymeric matrix during manufacture (Bacali and Baldea, 2020; Gad and Al-Thobity, 2020; Sodagar et al., 2013). Loading acrylic resins with a 10 wt% CHX concentration was considered effective and feasible in drug release and microbiological tests against *C. albicans* (Amin et al., 2009; Salim et al., 2012a). However, loading a drug into the polymeric matrix can alter its structure (Gad and Al-Thobity, 2020; Sodagar et al., 2013). Adding CHX can affect the porosity and water sorption of temporary soft liners (Lima et al., 2016a; Lima et al., 2016b), and the incorporation of antimicrobial nanoparticles may influence the mechanical and surface properties of acrylic resins, changing their flexural strength, roughness, and hardness (Chladek et al., 2019; Gad and Al-Thobity, 2020). Therefore, the efficacy of this drug delivery system using lower concentrations of CHX should be investigated.

The objective of this study was to develop a novel drug delivery system to prevent and treat denture stomatitis based on loading hard reline acrylic resins with the lowest CHX percentage that has an antimicrobial effect and maintains mechanical, structural, surface, and biological properties. The drug release profile in artificial saliva is also described.

2. Materials and methods

2.1. Fabrication of chlorhexidine delivery system

Three autopolymerizing hard reline acrylic resins, in the powder-liquid form, with different chemical compositions and physical structures were selected: the direct reline resins Kooliner (K) and Ufi Gel Hard (UFI), and the indirect reline resin Probase Cold (PC) (Table 1) (Arima et al., 1996). CHX diacetate monohydrate was obtained from Panreac Applichem (Darmstad, Germany).

A control (pristine sample) and experimental groups of specimens loaded with different wt% of CHX were settled for each material and according to the type of the assays. In the experimental groups, CHX was mixed with the corresponding reline resin powder, using a mortar and a pestle for homogenization. Then, liquid monomer was added to the resulting powder and the final mixture was placed into a specific mold (the specimen's final shape varied with the test and it is described ahead).

Specifically, for the antimicrobial assays, six groups were settled for each material: a pristine group, represented as the control group (C), and five experimental groups, with 1, 2.5, 5, 7.5, and 10 wt% of CHX. For the subsequent experimental tests, only the samples with the lowest wt% of CHX and antimicrobial activity against *C. albicans* and *S. oralis* were tested, namely 2.5 wt% CHX-loaded K, 5 wt% CHX-loaded UFI and 5 wt % CHX-loaded PC.

For all the samples, the mold was maintained under compression at 37 °C during the recommended polymerization time to simulate the intraoral polymerization of the direct reline resins (Table 1). Polymerization of the indirect reline resin was carried out in a pressure device (Ivomat, Ivoclar Vivadent, Liechtenstein) at the recommended time, temperature, and pressure (Table 1). The edges of each specimen were polished with a 600-grit silicon carbide paper (Carbimet Paper Discs, Buehler Ltd., Lake Bluff, IL., USA) on a polisher with constant water cooling (DAP-U, Struers, Denmark) to remove irregularities. The specimens were stored in distilled water at 37 °C for 24 h in an oven (Memmert, Schwabach, Germany) before testing.

2.2. Antimicrobial testing

The microorganisms selected for the Kirby-Bauer diffusion test (Severo et al., 2021) were *C. albicans* (ATCC® 10231TM) and *S. oralis* (ATCC® 3507) from the American Type Culture Collection (ATCC). *C. albicans* was cultured on a Glucose-Peptone-Yeast extract agar medium (20 gL^{-1} glucose, 5 gL^{-1} peptone, 5 gL^{-1} yeast extract, 15 gL^{-1} agar; Biokar Diagnostics, France) and *S. oralis* on a Brain Heart Infusion (Biolab, Budapest, Hungary) agar supplemented with 1% sucrose for 24 h at 37 °C. The inoculum was prepared from 24 h-cultured plates by direct colony suspension in appropriate medium. Final inoculum was adjusted to 0.5 McFarland units using a spectrophotometer ($\lambda = 600$ nm) in Mueller-Hinton broth (i.e. $\cong 1 \times 10^6$ cells/mL for yeast and $\cong 1 \times 10^8$ cells/mL for bacteria) and then swabbed on Mueller-Hinton agar (MHA, Biokar Diagnostics, France) or modified MHA (CLSI, 2015). Disk-shaped (5×1 mm) samples were placed on top of the swabbed plates and consisted of biomaterials control (pristine sample) and five experimental groups (i.e. 1, 2.5, 5, 7.5, and 10 wt% of CHX) for each of the three resins, totalizing the fabrication of 54 specimens (Marques et al., 2019). Paper disks with 10 µg of CHX were used as the positive control. After 24 h for bacteria and 48 h for yeast at 35 °C, the presence of inhibition zones was observed and measured using a Vernier caliper (Mitutoyo Digimatic, MFG.Co., Ltd). Assays were performed in three independent experiments. (CLSI, 2018; CLSI, 2009)

2.3. Mechanical, surface, morphological and chemical analysis

2.3.1. Microhardness and flexural strength
Mechanical properties were determined in 64 × 10 × 3.3 mm

Table 2
Results of the Kirby-Bauer diffusion assays. Data are expressed as the mean (±standard deviation) of at least three independent experiments.

| | | Inhibitory zone diameter (mm) | | | | | |
| | | CHX (wt%) | | | | | |
		0	1	2.5	5	7.5	10
C. albicans	K	–	–	9.0 ± 0.97	8.1 ± 0.91	9.8 ± 1.55	10.8 ± 0.57
	UFI	–	–	–	10.0 ± 3.29	9.9 ± 0.81	9.8 ± 1.02
	PC	–	–	–	7.3 ± 1.18	8.3 ± 1.06	9.1 ± 0.86
S. oralis	K	–	13.6 ± 0.19	17.4 ± 0.69	13.8 ± 1.04	17.4 ± 1.02	20.3 ± 0.88
	UFI	–	11.6 ± 0.38	13.4 ± 1.35	16.6 ± 1.26	14.9 ± 2.41	16.7 ± 0.33
	PC		8.6 ± 2.14	9.8 ± 1.68	10.3 ± 0.67	13.6 ± 4.09	14.1 ± 0.38

CHX – chlorhexidine; K - Kooliner; UFI - Ufi Gel Hard; PC - Probase Cold.

rectangular-shaped specimens ($n = 8$), obtained from stainless-steel molds as recommended by ISO 20795-1: 2013. The Knoop microhardness test was performed (Duramin, Struers DK 2750, Ballerup, Denmark) with a 98.12 mN load for 30 s. The mean of 12 equidistant measurements in each specimen was used as the reference value. Immediately after microhardness testing, the specimens were submitted to a three-point flexural test using a universal testing machine (Instron model 4502, Instron Ltd, Bucks, England), with a 1 kN load cell at a crosshead speed of 5 mm/min and 50 mm between rods (Rijo et al., 2018; Neves et al. 2019).

2.3.2. Surface energy

The surface free energy was measured in $16 \times 25 \times 1$ mm specimens ($n = 5$) obtained from rectangular-shaped metallic strips. Assays were made with Tensiometer K12 (Kruss, Hamburg, Germany) using the Wilhelmy plate method by immersing plates into deionized water and 1,2-propanediol (Merck, Germany) at a speed of 20 mm/s and 25 °C. Advancing contact angles were used to estimate the specimens' surface free energy (γ) based on the harmonic mean method. Equations for surface free energy estimation were solved using the equation-handling KRUSS software program: K121 contact angle measuring system (version 2.05) (Bettencourt et al., 2001; Matos et al., 2015).

2.3.3. X-ray microtomography (micro-CT)

Cylinder-shaped specimens were prepared from stainless-steel molds (12×6 mm) to obtain qualitative data regarding the reline resins' morphological structure. The 3D microarchitecture morphology was evaluated by morphometric analysis and 3D model images in a micro-CT device (SkyScan 1174; Bruker, Kontich, Belgium). The specimen was mounted on a stub and scanned with the following parameters: 30.1 μm image pixel size; 50 kV; 800 mA; 5500 ms exposure time; 0.9° rotation step; and no aluminum filter. Data were reconstructed with the NRecon software (Bruker, microCT, Kontich, Belgium) (Chiang et al., 2010).

2.3.4. Scanning electron microscopy (SEM)

The surface morphology of (5×1)mm disk-shaped specimens was examined by SEM using the Vega3 Tescan equipment (Tescan, Brno, Czechia). Before the examination, samples were coated with a gold/palladium (Au/Pd) thin film by sputtering using the sputter coater equipment (Quorum Technologies).

2.3.5. Fourier-transform infrared spectroscopy (FTIR) analysis

FTIR-ATR spectroscopy was used for the specimens' chemical analysis with an Alpha-P spectrometer (Bruker, Brussel, Belgium). The tests were performed at room temperature in a spectral range of 400–4000 cm^{-1} at a resolution of 4.00 cm^{-1}. The obtained spectra were averaged
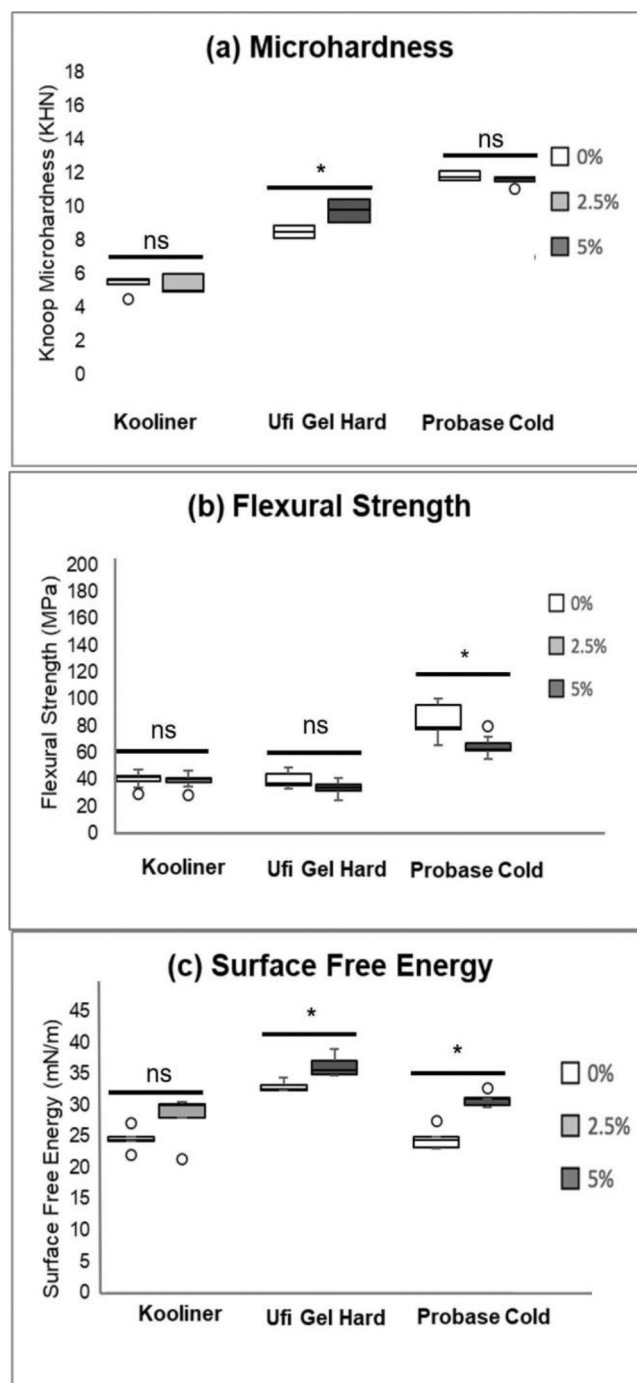

Fig. 1. Boxplot of the mechanical (a and b) and surface (c) characterization of materials among the experimental groups. Note: * corresponds to $P < 0.05$ and ns corresponds to "not significant".

over 64 scans. After scanning, characteristic infrared absorption frequencies of organic functional groups were analyzed using the Opus software 6.5 version (Cherchali et al., 2020).

2.4. Drug release assays

In-vitro CHX release from cylinder-shaped (12×6 mm) specimens of the experimental groups ($n = 3$) was assessed with each sample incubated in an adequate volume of artificial saliva prepared at pH $= 7$, in a ratio of 1 g/5 mL, in a shaking water bath at 37 °C for 28 days (Bettencourt et al., 2016). Specifically, the components of the artificial saliva
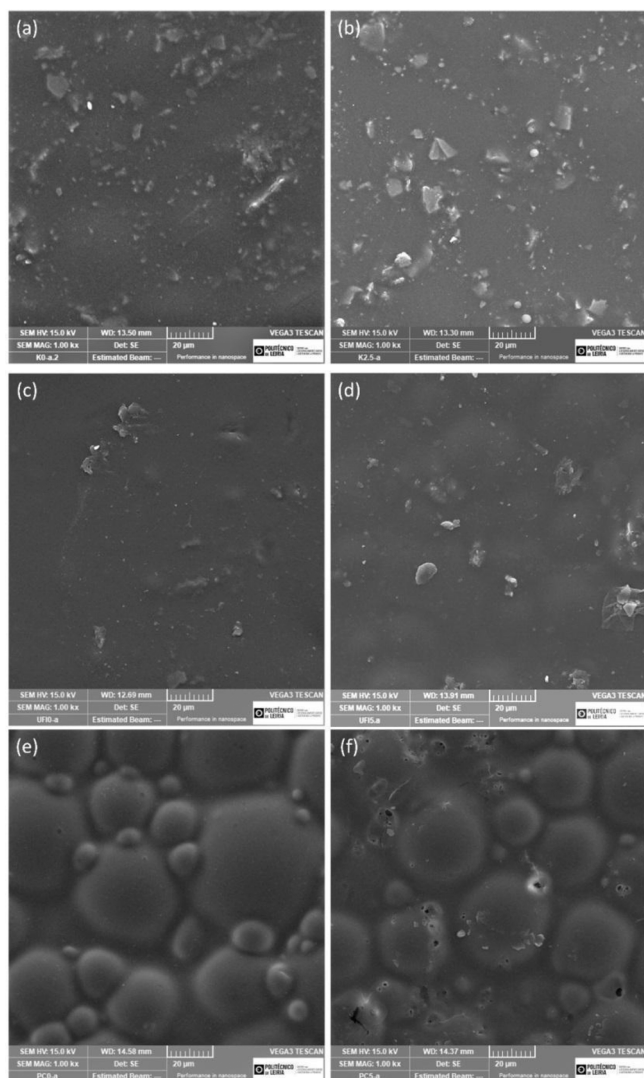
Fig 2. Scanning electron microscopy images representative of resins and CHX-loaded resins, (a) K; (b) CHX-loaded K; (c) UFI; (d) CHX-loaded UFI; (e) PC; (f) CHX-loaded PC.

were: phosphate buffer pH = 7.0 (Sigma-Aldrich), xanthan gum (Sigma-Aldrich), 1.2-propanediol (Emplura-Merck), sodium chloride (Fluka), potassium and calcium chloride dihydrate from Chem-lab NV. At pre-determined intervals, aliquots of the supernatant were collected and analyzed in triplicate. The withdrawn aliquots were then replaced with equal volumes of fresh release solution to simulate the constant salivary renovation. CHX content was determined based on a linear calibration methodology by UV spectrophotometry (255 nm) using a microplate reader (FLUOstar Omega, BMGLabtech, Germany).

2.5. Cytotoxicity evaluation

The cytotoxicity was assessed using extracts from disk-shaped (10 × 1 mm) specimens of each group (n = 3) obtained by immersing the specimens at 37 °C for 48 h in an adequate volume of cell culture medium in a ratio of 1 g/5 mL (ISO 10993-5, 2009). The extracts' cytotoxicity was assessed using the general cell viability endpoint MTT (3-(4,5- dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) on L929 (mouse fibroblast cell line, ATCC® CCL-1™) (Ferreira et al., 2015; Martin et al., 2019). Cells were seeded at 2×10^4 per well on a 96-well plate (Greiner, Germany) in RPMI 1640 culture medium (Gibco, Thermo Fisher Scientific, USA), supplemented with 10% (V/V) fetal

bovine serum, 100 units of penicillin G (sodium salt) (Invitrogen, UK), 100 mg of streptomycin sulfate (Invitrogen, UK), and 2 mM of L-gluta-mine (Invitrogen, UK), at a concentration that allowed cells to grow exponentially during the time of the assay. All samples to be tested and sodium dodecyl sulfate (SDS) (positive control) were diluted in the cell culture medium. After 24 h, the cell media was removed and replaced with a fresh medium, and the cell viability was assessed. The MTT dye solution was then added to each well (stock solution 5 mg/mL in 10 mM phosphate buffer solution at pH 7.4). After 3 h of incubation, the media was completely removed, and the intracellular formazan crystals were solubilized and extracted with 100 mL dimethylsulfoxide (DMSO). After 15 min at room temperature, the absorbance was measured at 570 nm in a microplate reader (FLUOstar Omega, BMGLabtech, Germany). The relative cell viability (%) compared to control cells was calculated by the formula: $(Abs_{sample})/(Abs_{control}) \times 100$. Assays were performed in three independent experim

2.6. Statistical analysis

Since normality and homogeneity of variance were not verified in scale variables (Shapiro-Wilk and Levene tests, $P < 0.05$), data were submitted to Kruskal-Wallis (followed by Bonferroni correction of post-hoc tests) and Mann-Whitney non-parametric tests ($\alpha = 0.05$).

3. Results and discussion

The present study investigated the development of a drug-releasing device for denture stomatitis' therapeutic management by loading CHX in reline acrylic resins. Previous studies found that loading soft or hard acrylic resins with 10 wt% CHX has an inhibitory effect on *C. albicans* (Amin et al., 2009; Salim et al., 2012a). In the clinical context, a continuous CHX release into the surrounding fluid helps saturate the salivary film that bathes the tissue surface of a denture base (Ryalat et al., 2011). This interesting finding should encourage the use of antimicrobials adjusted to the lowest drug concentration, maintaining antimicrobial potential but reducing the chance of developing local side effects (such as allergic reactions). No studies evaluating the addition of a CHX percentage lower than 10 wt% into long-term reline resins to prevent or treat denture stomatitis caused by *C. albicans* were found in the literature. Thus, our first aim was to select the lowest percentage of CHX to be loaded into the acrylic resins retaining its antimicrobial properties against *C. albicans* and *S. oralis* (a bacteria often associated with *C. albicans* oral infections) (Garaicoa et al., 2018; Lamfon et al., 2005; Thein et al., 2006).

3.1. Antimicrobial testing

The Kirby-Bauer diffusion assay was selected to establish the minimal antimicrobial concentrations of CHX required to be loaded into the acrylic resins. When testing biomaterials antimicrobial activity, the Kirby-Bauer test can be pointed out as a gold standard method and it is often recommended for comparison purposes (Salina et al., 2019). Results showed that the lowest concentration of CHX that promoted antimicrobial activity against both tested strains (*C. albicans* and *S. oralis*) was 2.5 wt% for K and 5 wt% for UFI and PC (Table 2). The assay also indicated that the loading method for CHX incorporation onto the tested resins had not compromised the drugs antimicrobial activity.

3.2. Mechanical, surface, morphological and chemical analysis

In drug delivery systems' development, it is essential to determine whether the addition of the drug influences the mechanical and surface properties of the biomaterial. Some studies reported that loading resins with antimicrobial agents caused detrimental mechanical properties, including decreased flexural strength (Gad and Al-Thobity, 2020; Rijo et al., 2018; Sodagar et al., 2013). In the present study, the addition of
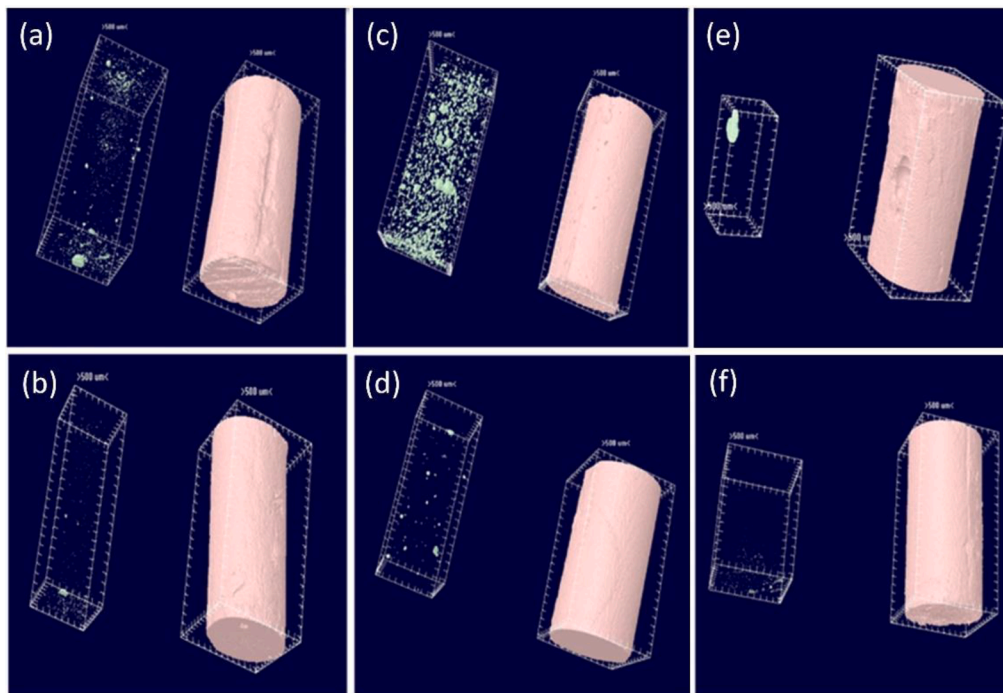
Fig. 3. Representative three-dimensional reconstruction models indicating different porosity of reline resins' specimens by micro-CT. The green color is related to pores in the samples. (a) K sample; (b) 2.5 wt% CHX-loaded K; (c) UFI; (d) 5 wt% CHX-loaded UFI; (e) PC; (f) 5 wt% CHX-loaded PC. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CHX did not decrease the microhardness of the three reline resins (Fig. 1 (a)). The flexural strength of the K ($P = 0.382$) and UFI ($P = 0.105$) specimens was not significantly affected by the CHX incorporation. On the other hand, the 5 wt% CHX-loaded Probase Cold specimens showed significantly ($P = 0.005$) lower flexural strength than the control group (Fig. 1(b)). Since CHX is mixed with the powder of poly(methyl methacrylate) (PMMA) pre-polymerized particles (Arima et al., 1996), it can hamper the polymerization process by preventing interchain bonds and increase distances among polymers chains (Chladek et al., 2019). The polymerization process change also results in a higher amount of residual monomer, which will have adverse consequences on the resins' mechanical properties due to having a plasticizing effect (Feng et al., 2017; Neppelenbroek et al., 2008). Even though the results showed decreased flexural strength when CHX was added to PC specimens, the values obtained were still above the limit of 60 MPa defined by ISO requirements for an adequate function of this material (ISO 20795-1, 2013).

Consistent with a previous study (Costa et al., 2019) 5 wt% CHX-loaded UFI and 5 wt% CHX-loaded PC specimens showed significantly ($P = 0.008$) higher surface free energy values than their control (Fig. 1 (c)). In general, the increase in the total surface free energy is directly related to a lower contact angle measurement (Matos et al., 2015). Thus, it should improve materials' surface wettability, providing the necessary conditions for saliva to spread more easily over the denture surfaces and facilitating their retention (Zissis et al., 2001; Costa et al., 2016).

In terms of surface morphology, SEM images demonstrated that PC samples presented a more spherical nature than the other two reline resins (Fig. 2). Specimens loaded with CHX present a similar morphology compared to the control in K and UFI samples. However, 5 wt% CHX-loaded PC showed smaller and more irregular spherical particles with higher interparticle distance. Since CHX is incorporated in the powder of poly(methyl methacrylate) (PMMA) pre-polymerized particles (Arima et al., 1996) it can hamper the polymerization process that prevents interchain bonds and growth of PMMA particles (Chladek et al., 2019). Also, the impairment of the polymerization process can produce higher amounts of residual monomer with

plasticizing effect to the polymer matrix (Feng et al., 2017), that can be related to lower flexural strength of CHX-loaded PC specimens shown in the present study.

Selected 3D models reconstructed by micro-CT allowed a qualitative evaluation of the specimens' total porosity (Fig. 3 (a-f)). Although there were some differences between the resins tested, with UFI exhibiting the most porous structure and Probase Cold showing almost no porosity, micro-CT images suggest that CHX loading decreased the total porosity of all resins. CHX particles seem to fill the resin's pores (Chiang et al., 2010).

Moreover, FTIR-ATR analysis was conducted to get further insight into the interaction between the drug and the biomaterial (Fig. 4) with the IR spectrum of the CHX provided for reference. The CHX spectrum reveals characteristic absorption bands at 3323 cm^{-1} and 3119 cm^{-1} were due to stretching vibration N–H, bands at 1610 cm^{-1} and 1489 cm^{-1} were due to C = C aromatic bending. Peaks were also observed at 1650, 1600, 1550, and 1500 cm^{-1}, which can be attributed to the C = C stretching of the aromatic moiety of CHX (Huynh et al., 2010; Rema et al., 2014). In samples with 2.5 wt% CHX-loaded K, 5 wt% CHX-loaded UFI and 5 wt% CHX-loaded PC, at 1610 cm^{-1}, it is possible to observe intensity changes compared to the unloaded K, UFI and PC spectrum, which are associated to the contribution of C = C aromatic bending from CHX, confirming the incorporation of CHX into the dental reline acrylic resins (Fig. 4 (a-d)).

Moreover, FTIR-ATR revealed that adding CHX did not change the polymers' main absorption bands, suggesting that CHX did not chemically react to the polymer chains and remained apart from the chemical structure of the resins, filling the pores of the biomaterial (Cherchali et al., 2020).

3.3. Drug release assays

The main purpose of the release study was to compare the carrier properties of the different resins in an artificial saliva with similar ionic and viscoelastic properties of the biological saliva (Pytko-Polonczyk et al., 2017). Although the *in vitro* research cannot reproduce all the
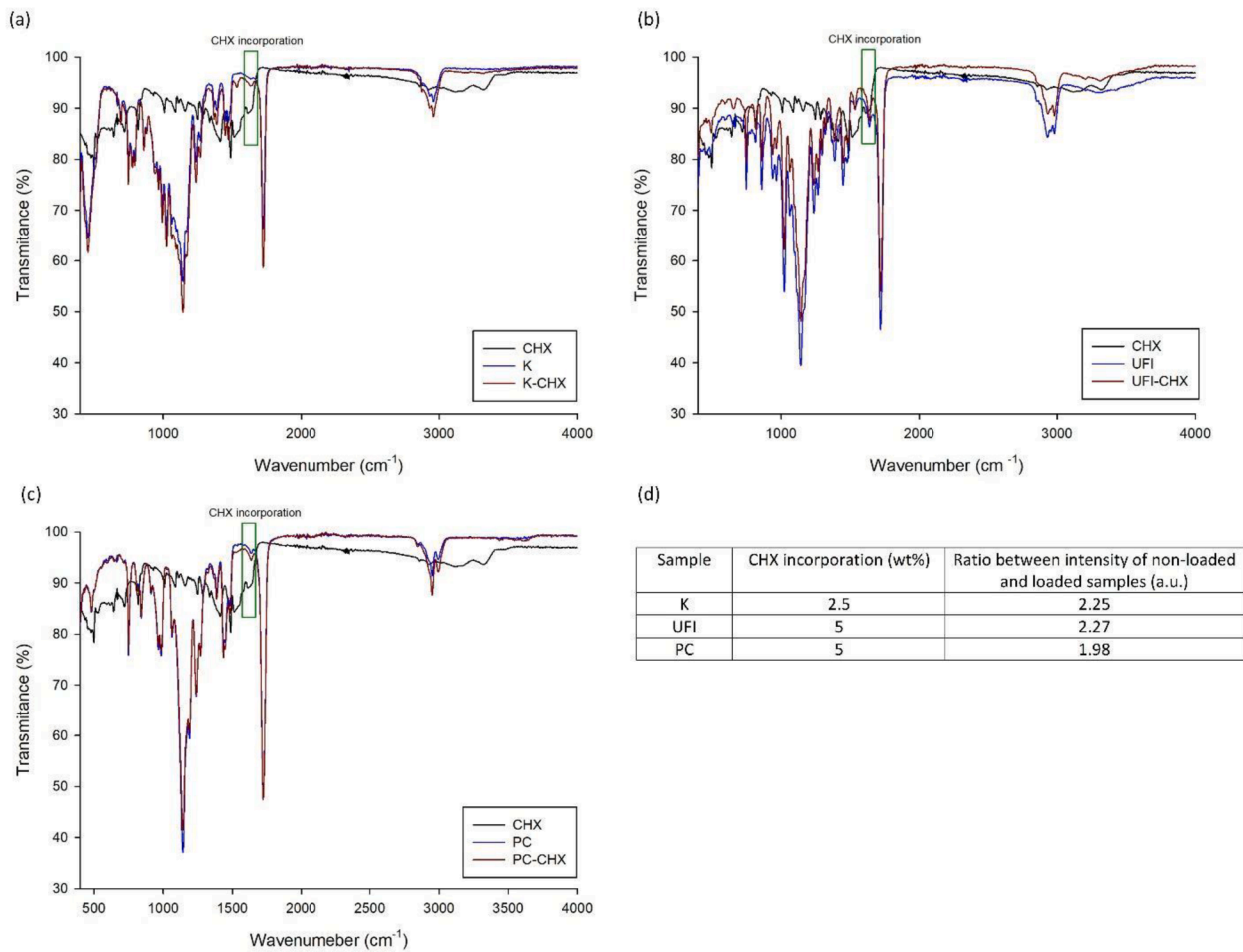
Fig. 4. FTIR-ATR spectra of dental reline acrylic resins with CHX as a control (a) K and CHX-loaded K (K-CHX); (b) UFI and CHX-loaded UFI (UFI-CHX); (c) PC and CHX-loaded PC (PC-CHX) and (d) ratio between band intensity of non-loaded and loaded samples, ratio ≥ 1 means presence of CHX.
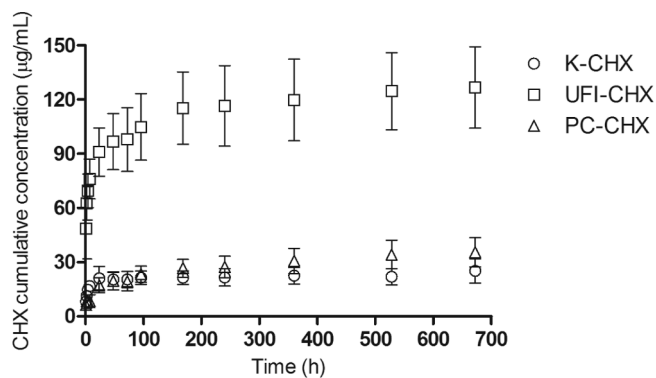

Fig. 5. Drug release profiles of CHX during 28 days. Data are expressed as mean ± standard deviation ($n = 3$).

Fig. 6. Cell viability assays after exposure to the dental reline acrylic resins. Unloaded samples (K, UFI and PC) and CHX-loaded K (K-CHX); CHX-loaded UFI (UFI-CHX) and CHX-loaded PC (PC-CHX). Negative control: cellular culture medium; positive control: sodium dodecyl sulfate (SDS). Data are expressed as mean ± standard deviation of at least three independent experiments.

complexity of an *in vivo* dynamic environment (Fagion, 2012) it enabled to perform single-variable experiments under controlled conditions. These assays provide important information about the properties of the different resins concerning their role as local drug delivery systems. Specifically, we observed that for all the tested resins, the higher CHX release in an artificial saliva occurred within the first 24–48 h of incubation (Fig. 5); this rapid elution phase indicates a surface release process. This finding agrees with a previous study that revealed a high initial 4-day CHX release (Ryalat et al., 2011). After 48 h, a slower release rate until the end of the study period (28 days) was observed
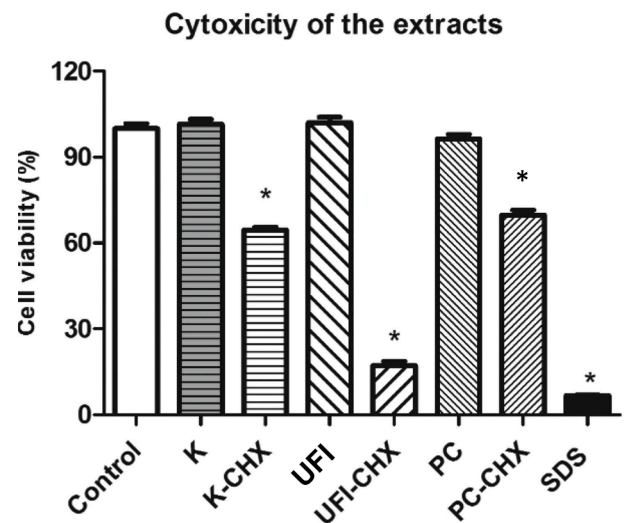
(Fig. 5). This subsequent slow phase may have been caused by the drug diffusion from the core of the polymer by complex processes involving fluid cluster formation around the CHX molecules and the interaction of these clusters with the fluid uptake process (Ryalat et al., 2011).

Comparing the three resins, UFI released the highest amount of CHX. These results are consistent with the micro-CT studies (Fig. 3) revealing that UFI had the most porous structure and support the theory that the drug elution may also be enhanced by porosity and cracks (Amin et al., 2009).

3.4. Cytotoxicity evaluation

For the cytotoxicity testing, a cell line (L929 mouse fibroblast) highly sensitive to toxics was selected (Campanha et al., 2006, Jorge et al., 2007).

Cell viability assays showed that the incorporation of the drug decreased cellular viability in the three tested resins ($P < 0.001$). 5% CHX-loaded PC was the least cytotoxic loaded resin ($70.6 \pm 6.17\%$), and 5% CHX-loaded UFI was the most cytotoxic ($16.6 \pm 5.24\%$) (Fig. 6). The results may be explained by the higher liberation of the drug from UFI samples indicated in the release assays (Fig. 5), probably associated with its higher porosity, as suggested by the micro-CT analysis (Fig. 3).

The decrease in cell viability due to the incorporation of the drug in the three tested resins was in accordance with other studies that stated that CHX was cytotoxic to fibroblast cells, having a concentration-dependent behavior (Mariotti and Rumpf, 1999). Furthermore, there is no non-cytotoxic acrylic resin available in the dental market (Chaves et al., 2012; Costa et al.,2022), and, according to the ISO standard 10993–5, only a cell viability reduction of more than 30% is considered a cytotoxicity effect (ISO 10993-5, 2009), which did not happen in 5 wt % CHX-loaded PC.

4. Conclusion

A drug delivery system based on hard reline acrylic resin loaded with chlorhexidine used to prevent and treat *Candida albicans*-associated denture stomatitis requires no patient compliance and minimal clinical monitoring, besides probably offering lower adverse risks than conventional therapy with topical and/or systemic antifungals. Moreover, it can reestablish denture stability and retention with a simple and economic clinical procedure. Our study showed that Probase Cold could be a valuable choice for that purpose. Moreover, the reline resin presented antimicrobial activity against *C. albicans* and *S. oralis*. It was also the least cytotoxic resin under evaluation. Thus, its use may be a potential approach to prevent or treat denture stomatitis. However, further studies evaluating the aging effect of the material on its antimicrobial, drug release and mechanical properties (including bond strength to denture base resin) should be performed to determine its applicability. Also, *in-vivo* work is warranted to determine its clinical relevance.

CRediT authorship contribution statement

Ana F. Bettencourt: Conceptualization, Supervision, Writing – review & editing, Project administration. **Joana Costa:** Investigation, Methodology, Writing – original draft. **Isabel A.C. Ribeiro:** Methodology, Validation. **Lídia Gonçalves:** Investigation, Validation. **Maria Teresa Arias-Moliz:** Investigation, Methodology, Writing – original draft. **Juliana R. Dias:** Investigation, Methodology. **Margarida Franco:** Investigation, Methodology, Writing – original draft. **Nuno M. Alves:** Investigation, Resources, Funding acquisition. **Jaime Portugal:** Formal analysis, Supervision, Writing – review & editing. **Cristina B. Neves:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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