 doi:10.20944/preprints202101.0437.v1

Inhibition of *Candida albicans* and Mixed Salivary Bacterial Biofilms on Antimicrobial Loaded Phosphated Poly(methyl methacrylate)

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ABSTRACT

Biofilms play a crucial role in the development of Candida-associated denture stomatitis. Inhibition of microbial adhesion to PMMA and phosphate containing PMMA has been examined in this work. *C. albicans* and mixed salivary microbial biofilms were compared on naked and salivary pre-conditioned PMMA surfaces in the presence or absence of antimicrobials (cetyl pyridinium chloride [CPC], KSL-W, histatin 5 [his 5]). Polymers with varying amounts of phosphate (0-25%) were tested using four *C. albicans* oral isolates as well as mixed salivary bacteria and 24 h biofilms were assessed for metabolic activity and confirmed using Live/Dead staining and confocal microscopy. Biofilm metabolism was reduced as phosphate density increased (15%: P=0.004; 25%: P=0.001). Loading of CPC on 15% phosphated disks showed a substantial decrease (P=0.001) in biofilm metabolism in the presence or absence of a salivary pellicle. Salivary pellicle on uncharged PMMA enhanced the antimicrobial activity of CPC only. CPC also demonstrated remarkable antimicrobial activity on mixed salivary bacterial biofilms under different conditions displaying the potent efficacy of CPC (350 µg/ml) when combined with an artificial protein pellicle (Biotene half strength).

Keywords: *Candida albicans* biofilm, Mixed salivary bacterial biofilm, Phosphated PMMA, Antimicrobials, Salivary pellicle

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1. INTRODUCTION

The formation of biofilms on implants and devices used in dentistry and medicine has been known to cause significant morbidity [1-3]. On poly(methyl methacrylate) (PMMA) materials in particular, the adhesion of *Candida albicans* has been recognized as a problem for decades [4-6], and along with poor denture hygiene is thought to play a central role in the development of Candida-associated denture stomatitis (CADS) [7-12].

Prevalence rates of CADS vary widely (18-65%) depending on the population studied, with higher rates often seen in the institutionalized elderly [4, 13]. Current treatments for CADS include the use of topical antifungal agents repeatedly applied to both the mucosa and the denture surface, but the re-infection rate is extremely high. Regular professional oral care, including simply removing dentures at night and leaving them to dry in the air, results in a decrease in the number of positive Candida cultures as well as a decrease in oral mucosal inflammation [14]. Moreover, incorporating professional oral care, including denture hygiene, resulted in a lower incidence of pneumonia, and a decrease in the number of febrile days in this frail population [15].

Such observations have spurred an increase in research on material alterations in an attempt to reduce candidal adhesion and subsequent biofilm formation on PMMA and non-PMMA based surfaces [16-22].

Poly(methyl methacrylate) (PMMA) is the most commonly used material for the fabrication of dentures. However, it has been speculated that PMMA has a limitation in terms of its surface properties, in particular, the absence of ionic charge that may be required for selective adsorption of salivary antimicrobials such as defensins and histatins [23, 24]. Since salivary antimicrobials are cationic peptides, they are strongly adsorbed onto the tooth surface by electrostatic interaction. On the other hand, absence of negative charge on PMMA surface not only minimizes the adsorption of defense molecules, but the attractive London-van der Waals forces actually facilitates the adherence of *C. albicans* on the denture surface, leading to denture stomatitis [25, 26].

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It has been demonstrated that carboxylated PMMA derivatives provide a negative charge and inhibit *C. albicans* adhesion in vitro and enhance adsorption of cationic salivary antimicrobial peptides onto the surface [16, 17, 27].

We postulated that addition of phosphate would provide the required surface properties to PMMA polymers that would yield a phosphate density-dependent decrease in *Candida albicans* adherence to the negatively charged denture base material compared to the normal uncharged PMMA. We further examined the surface charge effects on the adhesion and biofilm formation of mixed fresh salivary bacteria, as well as the effects of several antimicrobials exposed to the various PMMA polymers.

The goals of this study were to assess the effects of phosphate addition to PMMA on *C. albicans* and mixed salivary bacterial biofilms and to further examine biofilm metabolism on PMMA surfaces after loading one of three antimicrobials (histatin 5, KSL-W and cetylpyridinium chloride) on the naked as well as salivary pre-conditioned PMMA surfaces prior to microbial adherence.

2. EXPERIMENTAL PROCEDURE

2.1. Polymer synthesis and fabrication of phosphated PMMA disks

Four polymers with varying amounts of phosphate (0%, 5%, 15%, 25%) were synthesized by monomer substitution using Lucitone 199 denture base polymer beads and mixtures of methyl methacrylate (MMA) and ethylene glycol methacrylate phosphate (EGMP) monomers. The ratios of MMA/EGMP were 100:0 (PMMA control), 95:5 (5% EGMP), 85:15 (15% EGMP), and 75:25 (25% EGMP), respectively. Disks (diameter: 15 mm, thickness: 3 mm) were heat-processed according to the manufacturer's instructions and polished to 2 mm thickness with 600 grit silicon carbide metallographic grinding paper (PACE Techonologies, USA). The mean surface roughness (Ra) of the disks was 0.40 µm. Disks were stored in sterile distilled water until used.

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2.2. Candida adhesion and biofilm metabolism on phosphated PMMA surfaces

Four clinical isolates of *C. albicans* (A1, A3, AD1, 1.1) were obtained from various sources and identified through germ tube formation. Strain A1 was a sputum sample. Strain A3 was obtained from a bronchial lavage sample. Strains AD1 and 1.1 were oral isolates from denture stomatitis patients. *C. albicans* cells were grown in YPD growth medium (yeast extract 10 g, peptone 20 g, dextrose 20 g in 1000 mL water) at 37°C overnight. Cells were harvested, washed with phosphate-buffered saline, and standardized to 1 x 10^7cells/mL.

Six disks from each phosphate density group (0%, 5%, 15%, 25%) were selected as samples. The polished side of each disk was covered with 60µL of standardized *C. albicans* suspension and all the samples were incubated at 37°C for 90 min to allow the Candida cells to adhere to the disk surfaces. Following this inoculation procedure, the disks were gently rinsed with sterile distilled water to remove non-adherent cells and placed in wells of a 12-well tissue culture plate containing SD media (yeast nitrogen base 1.7 g, ammonium sulfate 5.0g , dextrose 20 g in 1000 mL water). The plates were incubated at 37°C for 24 h. The disks were then transferred to the wells of a 12-well tissue culture plate containing 2mL fresh 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-5-[(phenyl amino) carbonyl]-2H-tetrazolium hydroxide (XTT). The plates were incubated for 3 h at 37°C. The entire contents of the well were transferred into a 2mL tube and centrifuged (5 min, 6000 g). From each tube, 200 µL XTT formazan in the supernatant was transferred to one of the wells of the 96-well microtiter plate for XTT assay (Chandra, 2005). Metabolic activity of Candida biofilm on each disk was estimated using a microtiter plate reader (PowerWave™ XS, BioTek Instruments, Inc. VT, USA) at 492 nm. Negative controls included no inoculation as well as heat-killed *C. albicans*. Candida biofilm on polystyrene disks served as positive control. Statistical significance was analyzed using a one-way ANOVA at the 95% confidence level to determine if the means of the biofilm metabolic activity were significantly different between the disks with different phosphate density.

2.3. Microbicidal activity of antimicrobials on oral microorganisms

The microbicidal effects of histatin 5, KSL-W, and cetylpyridinium chloride (CPC) on *C. albicans* as well as mixed salivary bacterial biofilms were assessed. Twenty four 0% phosphated PMMA

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disks and twenty four 15% phosphated PMMA disks were pre-conditioned with filtered clarified whole human saliva for 60 min. Non-preconditioned disks served as control. The disks were then either loaded or unloaded with histatin 5, KSL-W or CPC (100 µg/ml) for 60 min at room temperature. Subsequently, *C. albicans* (1 x 10^7 cells/mL) or mixed salivary bacteria (1 x 10^7 cells/mL) was inoculated on the disks and incubated at 37°C for 90 min. Afterward, the disks were gently rinsed with sterile distilled water to remove non-adherent cells. Subsequently, the disks were placed in 12-well tissue culture plates containing SD media (for *C. albicans*) or Todd-Hewitt Broth (for mixed salivary bacteria) and then incubated at 37°C for 24 h. The biofilms developed on the disks were subjected to an XTT assay to evaluate their metabolic activity. Statistical analysis using a multifactorial ANOVA was performed on the data to determine if there was a significant difference between the different groups.

2.4. Image analysis

A confocal microscope (Nikon Eclipse Ti, Nikon Instruments Inc., NY, USA) with a 40X lens (pinhole size 69.0 µm) was used to record confocal image stacks of Candida biofims and mixed salivary bacteiral biofilms in five random locations near the center of the each disk.

3. RESULTS

The mean XTT values of the Candida biofilms (AD1) on 0, 5, 15, and 25% phosphated PMMA surfaces were 0.195 ± 0.009, 0.189 ± 0.008, 0.176 ± 0.004, and 0.173 ± 0.007, respectively. The one-way ANOVA indicated that there was a phosphate dependent decrease in Candida biofilm metabolic activity with 15 and 25% phosphated PMMA groups reaching statistical significance (15%: P=0.004 ; 25% P=0.001) (**Figure 1**).

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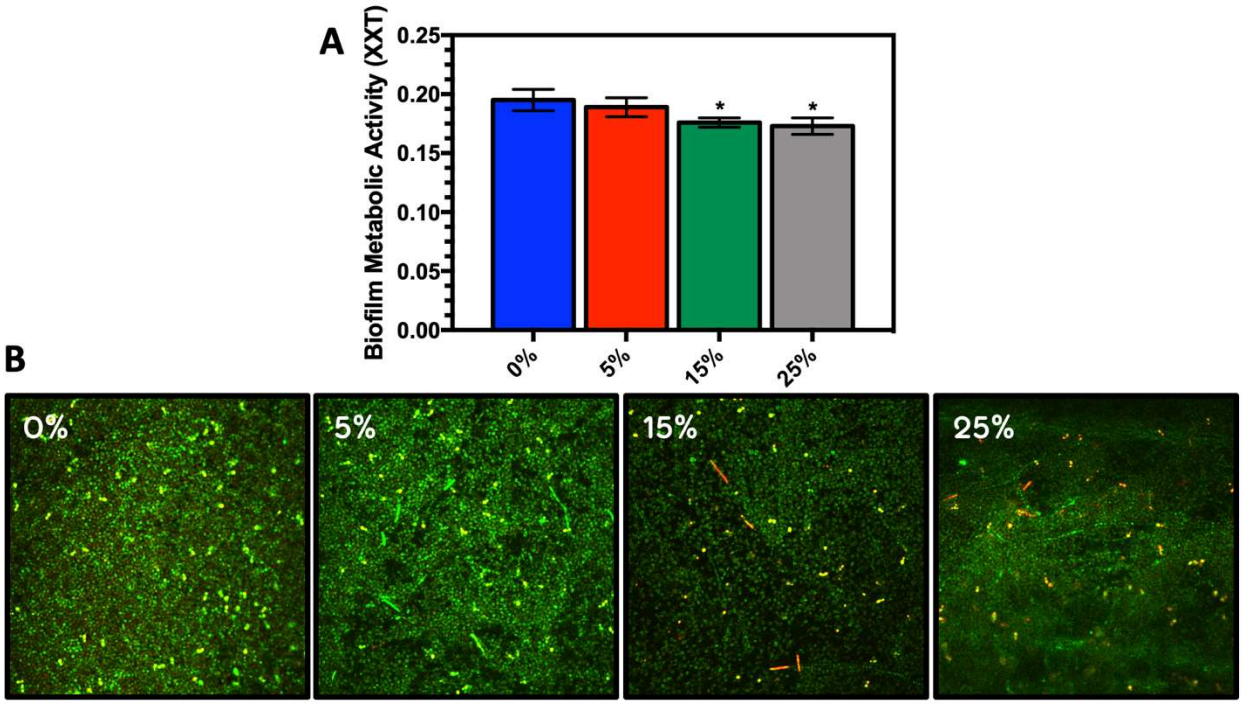

Figure 1. (A) Metabolic activity and (B) Confocal microscopic images of Candida (AD1) biofilm of on 0, 5, 15, and 25% phosphated PMMA

Loading 100 µg/ml CPC on 15% phosphated PMMA disks resulted in a significant decrease in *Candida* biofilm as well as mixed salivary bacterial biofilm metabolic activity, whereas the level of inhibition by the two peptide antimicrobials, histatin 5 and KSL-W, was unimpressive compared to CPC on a charged surface (**Figure 2a**).

CPC showed a significant antimicrobial effect on Candida as well as mixed salivary bacterial biofilm metabolism (**Figures. 2b** and **3**). CPC efficacy was minimal when loaded onto a naked PMMA surface, but in the presence of a salivary pellicle, the antimicrobial efficacy of CPC was enhanced in a manner that was similar to the CPC-loaded phosphated PMMA surface.

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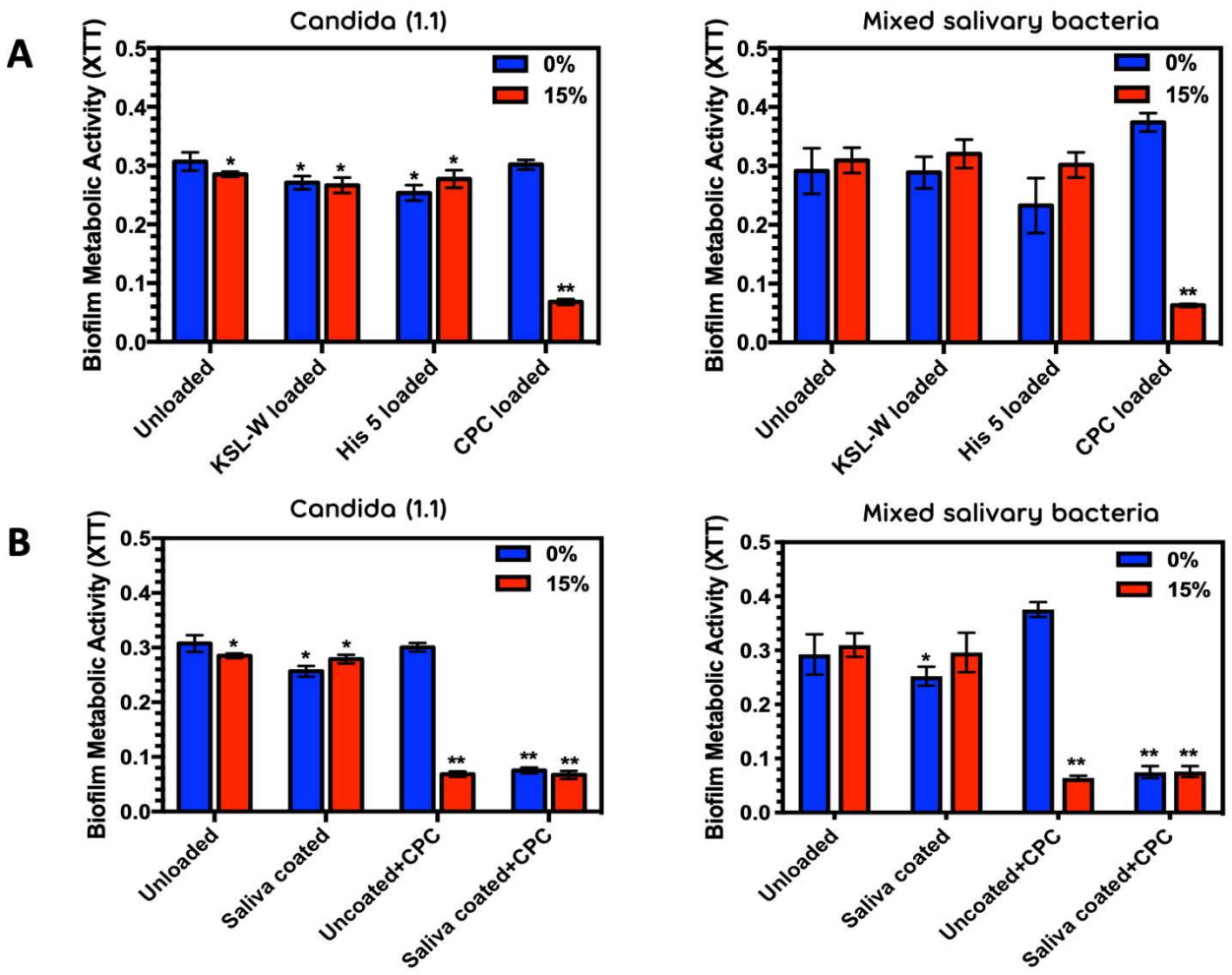

Figure 2. (A) Effects of histatin 5, KSL-W, and CPC on Candida (1.1) biofilm metabolism and mixed salivary bacteria biofilm metabolism on 0 and 15% phosphated PMMA and (B) Antimicrobial effect of CPC on Candida (1.1) biofilm metabolism and mixed salivary bacterial biofilm metabolism in the presence or absence of a salivary pellicle on 0 and 15% phosphated PMMA

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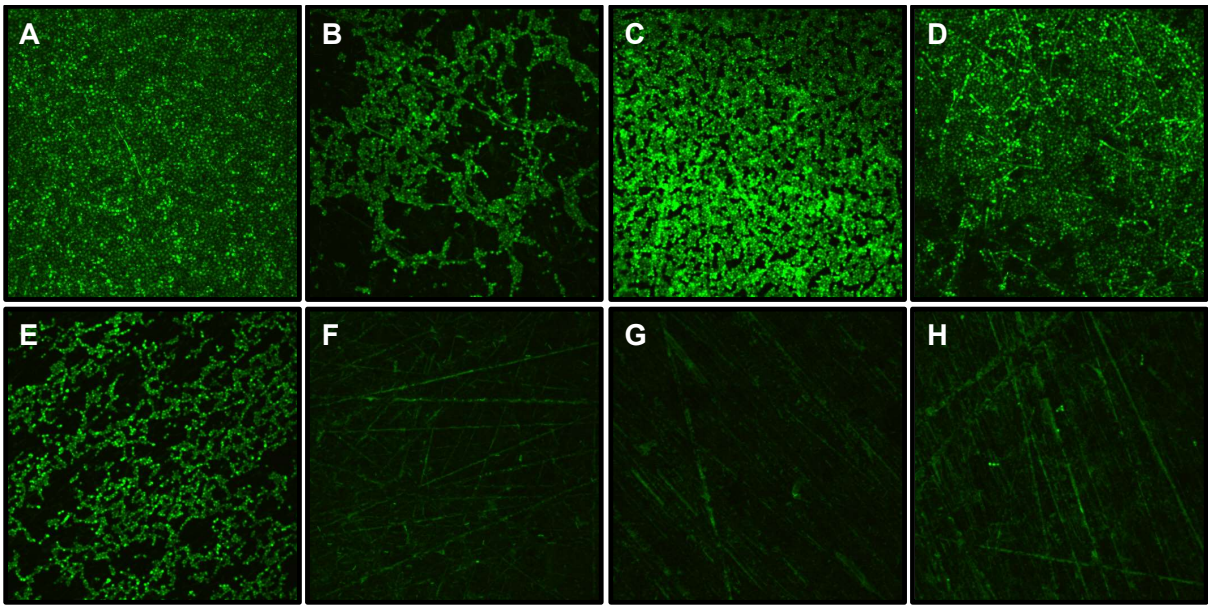

Figure 3. Confocal microscopic images of Candida (AD1) biofilms on naked or saliva-coated 0 and 15% phosphated PMMA (24h, 40X); (a) 0%, naked, (b) 15%, naked, (c) 0% saliva-coated, (d) 15% saliva-coated, (e) 0% CPC-loaded, (f) 15% CPC-loaded, (g) 0% saliva+CPC and (h) 15% saliva+CPC

CPC showed significant antimicrobial activity on Candia biofilm when it was combined with an artificial pellicle (**Figure 4a** and **b**).

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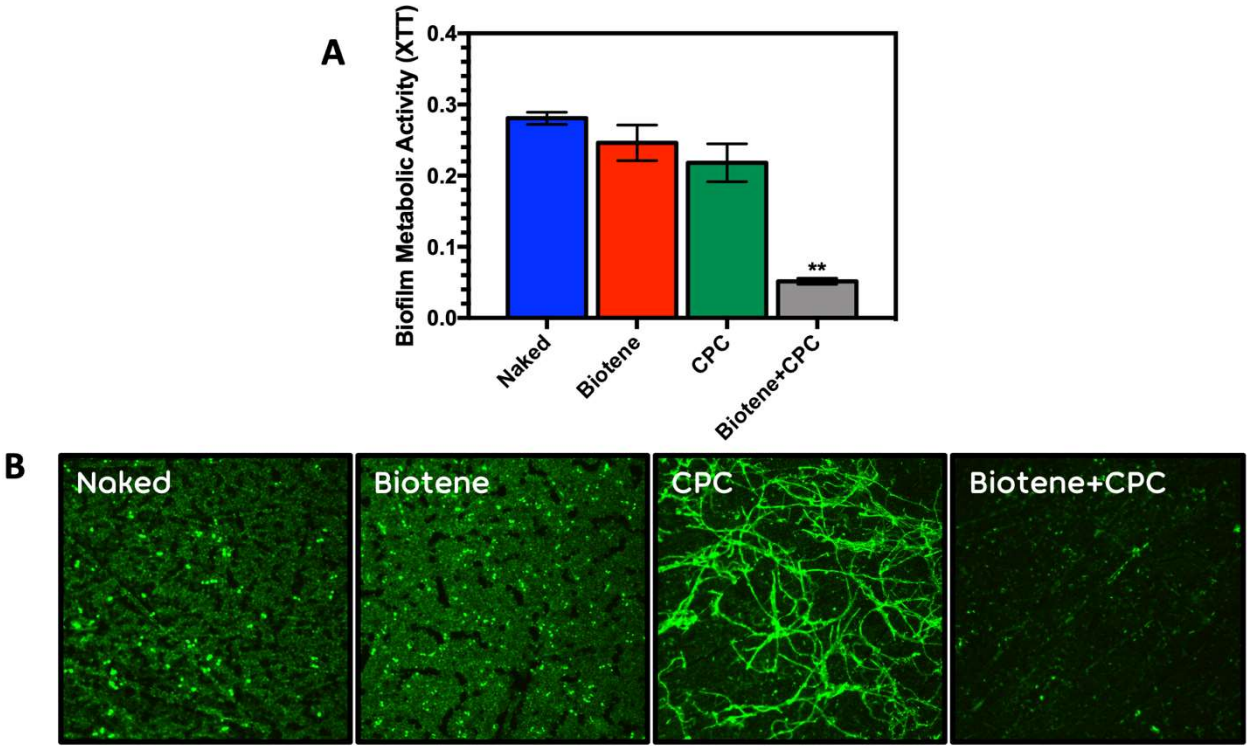

Figure 4. (A) Metabolic activity and (B) Confocal microscopic images of Candida (AD1) biofilm metabolism on PMMA surfaces in the presence of an artificial pellicle; naked PMMA, Biotene preconditioned, CPC preconditioned and Biotene + CPC

CPC also showed significant antimicrobial activity on mixed salivary bacterial biofilms under different conditions showing the potent efficacy of CPC (350 µg/ml) when combined with an artificial protein pellicle (Biotene half strength) (**Figure 5a** and **b**).

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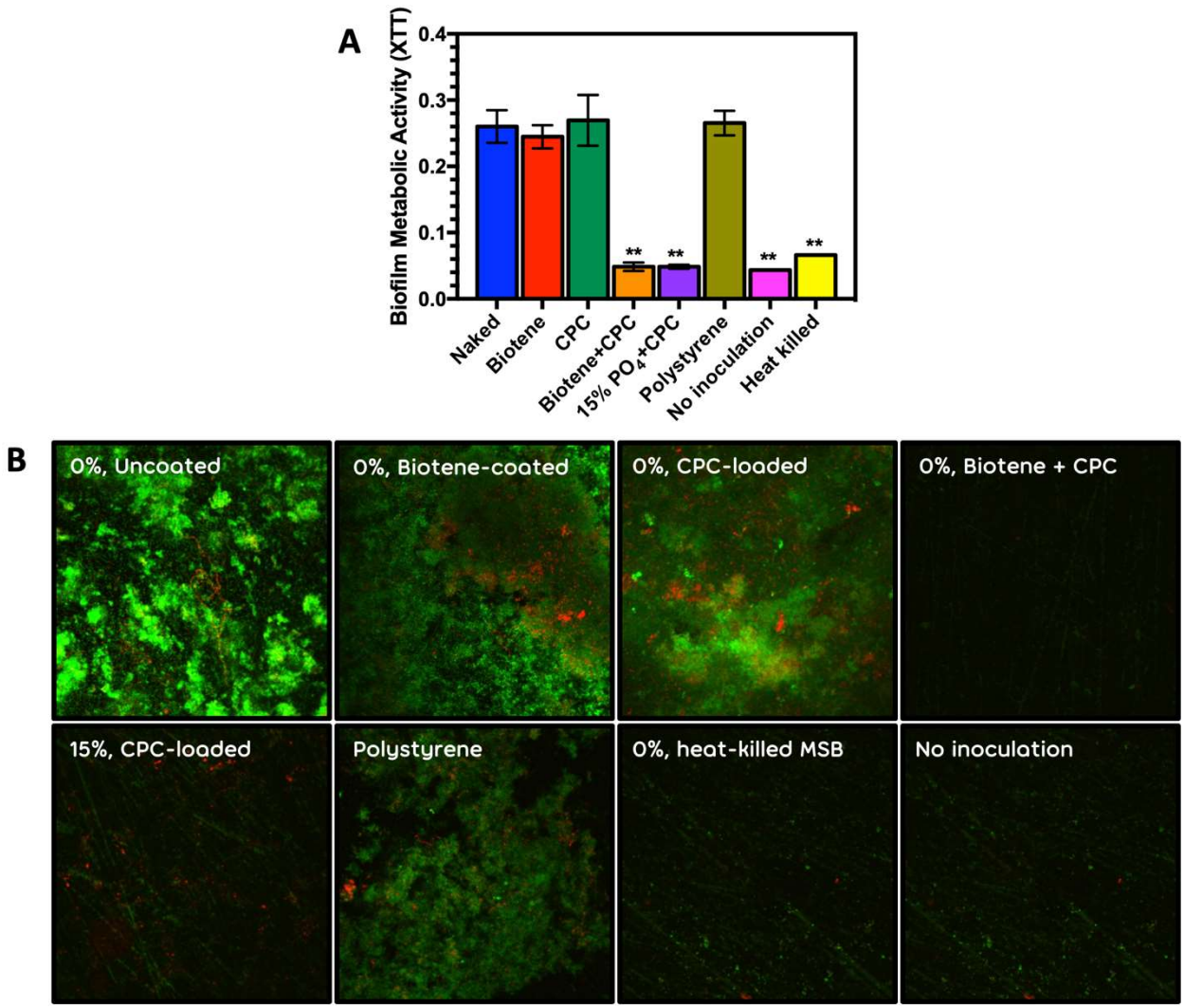

Figure 5. (A) Metabolic activity and (B) Confocal microscopic images of mixed salivary bacterial biofilm metabolism (XTT) on PMMA; 0%, Uncoated, 0%, Biotene-coated, 0%, CPC-loaded, 0%, Biotene + CPC, 15%, CPC-loaded, Polystyrene, 0%, heat-killed MSB and No inoculation

4. DISCUSSION

Creating surfaces that resist microbial fouling is of great interest. PMMA is a perfect material for denture base production because of its attractive properties [28-30]. Be that as it may, this material is powerless to colonization by different microbial species, including C. albicans, C. glabrata and gram positive/negative organisms. Various endeavors have been made to beat this drawback of denture base resins [31]. In this study, we examined phosphate-containing PMMA polymers for their ability to inhibit the adherence of *Candida albicans* and mixed salivary

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bacteria, and to adsorb cationic antimicrobials such as histatin 5 and KSL-W and CPC under different conditions.

The effects of phosphate addition to PMMA were examined by assessing the metabolic activity of *C. albicans* or mixed salivary bacterial biofilms on salivary pre-conditioned, antimicrobial loaded phosphated PMMA surfaces.

Before the experiment, we postulated that phosphated PMMA polymers, by virtue of their negative charge on the surface, would hinder microbial adherence based on previous observations regarding microbial adhesion and surface charge [5, 25, 26]. This study did show that biofilm metabolism as well as biofilm mass on naked polymer surface decreased in a phosphate dependent manner regardless of the *C. albicans* strain used, with 15 and 25% phosphate reaching statistical significance.

We noted that the effects were less dramatic as time went by and this was eventually traced to mineral deposition on the phosphate-containing polymers. An overnight soak in an EDTA solution along with a change in detergents solved this dilemma and we were able to regenerate our charged surfaces and have not had issues with reproducibility or longevity of the disks since that time.

After demonstrating a charge dependent reduction in biofilm metabolism, we began investigating the inhibitory effects of antimicrobials loaded onto the charged and uncharged polymer disks.

We limited our phosphate levels to 15% for these experiments since water sorption data suggested that this technology may have trouble meeting the water sorption ISO standard when we go to higher levels of phosphate.

Initially we hyphothesized that phosphated PMMA polymers would adsorb peptide antimicrobials such as histatin 5 and KSL-W onto the surface and show inhibitory effect on *Candida* adherence to the surface. However, the results of this study did not prove this hypothesis. In contrast, CPC showed statistically significant reductions in the adhesion/biofilm metabolism of *C. albicans* on 15% phosphated PMMA. Although histatin 5 and KSL-W did show statistically significant reductions compared to unloaded PMMA controls, the level of inhibition was unimpressive compared to CPC on a charged surface where the metabolic activity and

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confocal microscopic images showed essentially no biofilm formation. These conditions were indistinguishable from heat-killed and non-inoculated controls. Based on this repeated observation over all four *C. albicans* strains, we began to concentrate more on CPC as an active compound for phosphated PMMA surfaces.

CPC (cetylpyridinium chloride) is a cationic quaternary ammonium compound with aliphatic chain. In can interact with the bacterial cell membrane, resulting in a leakage of cellular components, disruption of cellular metabolism, inhibition of cell growth and cell death.

We also assessed pellicle effects on fungal colonization by comparing the naked 0 and 15% phosphated PMMA disk surfaces coated with fresh, mixed, clarified and filtered whole human saliva.

Again, the results showed that CPC was a strong inhibitor of *C. albicans* independent of the strain employed. Most striking was the repeated observation that CPC efficacy was minimal when loaded onto a naked PMMA surface, but in the presence of a salivary pellicle the antimicrobial efficacy of CPC was enhanced in a manner that was similar to the CPC-loaded phosphated PMMA surface. We speculate that CPC is being retained in the protein pellicle.

We felt it was worthwhile to pursue these observations further and began to use other protein pellicle sources (Biotene, bovine serum albumin) to ascertain whether this phenomenon was more generalizable using a multispecies biofilm model. We found that we could reproduce these antimicrobial effects using an artificial saliva (Biotene) and even bovine serum albumin at 700 µg/ml (data not shown).

The abundance and viability of the biofilms were anlayzed with confocal microscopy using Live/Dead stain kit (Molecular Probes/Life Technologies). The kit contains two dyes, Syto 9 and Propidium iodide. Syto 9 is a green fluorescing nucleic acid stain which readily penetrates all cells, binds to nucleic acids and fluoresces green. Propidium iodide has a larger structure which can only penetrate cells with a compromised or damaged membrane (i.e., dead cell). Once inside it binds to the nucleic acids, displacing Syto 9, and causing the cells to fluoresce red. Thus, by examining biofilms after Live/Dead staining we were able to measure attached cell abundance and determine the proportions that were alive (fluoresce green) and dead (fluoresce red) (**Figures 1b, 3, 4b** and **5b**).

5. CONCLUSIONS

New phosphate containing EGMP-PMMA co-polymers are capable of altering *C. albicans* adhesion and/or biofilm metabolism.

Pre-conditioning a negatively charged phosphated PMMA surface with CPC provides a strong anti-candidal shield whereas uncharged PMMA surface is not able to benefit from CPC exposure.

A salivary pellicle coating provides the necessary environment to establish an effective anti-candidal shield while not altering the phosphated PMMA from efficacious delivery of CPC.

6. ACKNOWLEDGMENT

This research was supported by NIDCR Grant # DE016925.

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