Antimicrobial activity of experimental chitosan solutions on acrylic resin and cobalt-chromium surfaces

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Abstract

Purpose: To evaluate the application of chitosan as a cleanser in the control of biofilm formation on cobalt-chromium (Co-Cr) alloy and acrylic resin surfaces. Materials and
**Methods:** In total, 172 Co-Cr discs and 172 acrylic resin discs (14 mm x 3 mm) were contaminated with *Streptococcus mutans*, *Staphylococcus aureus*, *Candida albicans*, or *Candida glabrata* and incubated for 48 hours. Then, specimens were randomly divided into groups and immersed in the following solutions for 15 minutes: WC = solution without chitosan (control); CH = chitosan solution (5 mg/mL); CN = chitosan nanoparticle solution (3.8 mg/mL); and ET = effervescent tablet. Biofilm recovery rates (n = 9) were evaluated by counting the colony-forming units (CFU/mL). Biofilm morphology was evaluated using scanning electron microscopy. Data were compared with Kruskal-Wallis or analysis of variance followed by Tukey post hoc tests. **Results:** For acrylic resin, ET showed the lowest number of CFU for *S. aureus* and *S. mutans* (P < .001). CH exhibited intermediate values for *S. mutans*, *S. aureus*, and *C. albicans*, and CN exhibited intermediate values for *S. mutans* and *S. aureus*. For *C. glabrata*, there was no statistical difference between the solutions (P = .264). For Co-Cr, ET showed the highest level of antimicrobial action against all microorganisms (P < .001), and CH showed an intermediate level of action against *S. mutans* and *S. aureus*. Against *C. albicans* and *C. glabrata*, there was no significant difference among CH, CN, and WC. **Conclusion:** Although ET had a broader spectrum of antimicrobial action, the chitosan solution showed promise as a denture cleanser. *Int J Prosthodont 2021. doi: 10.11607/ijp.7264*

**Introduction**

Prosthetic rehabilitation with partial and/or complete dentures makes it necessary for patients to have meticulous daily hygiene habits. After the dental protheses has been inserted into the mouth, the number of microorganisms rises significantly\(^1\) due to the presence of surfaces that favor the adhesion of microorganisms\(^2\), therefore, intensifying the need for specific and targeted care.
Prostheses can be cleaned using mechanical methods such as brushing - the most frequently used method, considered effective, simple and low cost; and by chemical methods that allow access to the most retentive areas that tend to accumulate biofilm\textsuperscript{3,4}. The association of methods has been reported to be effective\textsuperscript{4}. However, a wide variety of materials are used for manufacturing dental prostheses, such as polymers, metals and acrylic resin, and this makes it difficult to select the most efficient chemical cleanser for use, since there are no reports of a product that has been developed considering effective antimicrobial activity associated with the absence of deleterious effects on different prosthetic materials\textsuperscript{3}.

Commercial effervescent tablets have been the cleansers most recommended by dentists\textsuperscript{5} because they do not cause adverse effects on the metal surface\textsuperscript{6,7}. However, their effectiveness in removing biofilm is still controversial\textsuperscript{8}. In the absence of a consensus on the ideal product to complement the mechanical cleaning method, especially in areas that are difficult to access by means of brushing, in general, denture hygiene is still poor, leaving the oral cavity susceptible to imbalance of microbial flora and colonization. Consequently, diseases such as prosthetic stomatitis (chronic atrophic candidiasis) related to the presence of \textit{Candida albicans} and \textit{Candida glabrata} may develop\textsuperscript{9}. In addition to \textit{Candida} spp., other microorganisms such as \textit{Pseudomonas aeruginosa}, \textit{Escherichia coli}, \textit{Staphylococcus aureus}\textsuperscript{10} and \textit{Streptococcus mutans}\textsuperscript{11} also adhere easily to both acrylic resin and cobalt-chromium (Co-Cr) surfaces, after contact with human saliva\textsuperscript{12}. These microorganisms are closely related to the development of caries, periodontal disease and aspiration pneumonia, among other systemic diseases\textsuperscript{10,11}.

Products derived from natural sources have been increasingly tested in order to improve their clinical applications\textsuperscript{7}, since they represent lower cost alternatives that are
easily accessible to the general population, and have shown activity against several microorganisms present in the oral cavity. Among these products, chitosan, a polycationic biopolymer, obtained by means of alkaline deacetylation of chitin - the main component of the crustacean exoskeleton, has been studied in several areas of knowledge. Chitosan, easily found in nature, is low cost and has low toxicity to tissues, in addition to being biodegradable and biocompatible.

Chitosan has potent antimicrobial and antifungal activity. Its action is influenced by several factors, among them, biofilm composition, degree of deacetylation and its molecular weight, making it difficult to understand the mechanism of antimicrobial action. However, studies have described that the negative charge of fungi and bacteria potentially begins a reaction with the positive charge of chitosan, leading to the permeabilization of the cell surface, causing extravasation of the intracellular material and consequent cell death. It has also been reported that chitosan can bind to nucleic acids, affecting RNA expression. In dentistry, chitosan has been widely used and satisfactory results have been observed when a chitosan-based mouthwash was able to inhibit microbial adhesion and biofilm formation, in addition to promoting the dissolution of biofilms that had already formed.

Considering that it has characteristics that allow several applications, various presentations and modifications of chitosan have been studied. The special importance attached to chitosan nanoparticles has been attributed to its capacity for extensive diffusion through biofilm structures. Moreover, chitosan nanoparticles incorporated into the prosthesis-based acrylic resin has been found to promote an inhibitory effect on the biofilm formation of species of Candida albicans, Candida glabrata, Candida tropicalis and Candida Krusei. However, there are no studies about the action of
chitosan nanoparticles relative to their denture cleansing effect on bacteria and fungi present on metal and acrylic resin surfaces.

Therefore, considering the benefits of chitosan and the need for an adequate cleanser with satisfactory antimicrobial activity on a variety of different types of surfaces, it is relevant to evaluate the application of chitosan as a cleanser, for the control of biofilm formation. The null hypothesis of this study was that there would be no difference in the antimicrobial action between the solutions tested against all microorganisms.

Materials and methods

Sample preparation

One hundred and seventy-two wax patterns measuring (14 mm in diameter x 3 mm in thickness) were obtained. The metal specimens were cast in cobalt-chromium alloy (Co-Cr) (DeguDent®, Dentsply Ind. and Com. Ltda, São Paulo, Brazil) using the lost wax casting technique. Metal disks were polished in a horizontal polishing machine (AROPOL-E, Arotec, Cotia, SP, Brazil) using 220, 400, 600 and 1200-grade Al₂O₃ abrasive paper (Norton Abrasivos Brasil, Saint-Gobain, France). Afterwards, the disks were polished by using a lathe (Kota, Cotia, SP, Brazil) and diamond paste with granulation of 2 to 3 microns (Kota, Cotia, SP, Brazil). The roughness of the metal specimens was standardized between 0.15 to 0.20 µm, considering the clinically acceptable roughness²⁵.

Another 172 disks made of heat-polymerized acrylic resin were obtained (Clássico, Artigos Odontológicos Clássico Ltda., São Paulo, Brazil) using plaster molds (Durone, Dentsply Ind. Com., Rio de Janeiro, Brazil) in conventional metal flasks (Jon, Jon Indústria Brasileira, São Paulo, Brazil). The resin was manipulated and polymerized according to the manufacturer's instructions, and then polished with 150-grade Al₂O₃.
paper (Norton Indústria Brasileira, São Paulo, Brazil). In an endeavor to reproduce the surface of a dental prosthesis, as found in a clinical situation, roughness was standardized from 2.70 to 3.70 µm, corresponding to the roughness of an internal surface of a prosthesis.26

Preparation of chitosan solutions

The chitosan solutions were prepared according to the protocol described by Costa et al.17, using low molecular weight chitosan (75-85% deacetylation) (Sigma-Aldrich, Saint Louis, MO, USA) dissolved in 1.0% acetic acid (v/v) (Sigma-Aldrich, Saint Louis, MO, USA). After complete dissolution, the pH was adjusted to values ranging between 4.6 and 5.0, using a 10 N sodium hydroxide solution (Sigma-Aldrich, Saint Louis, MO, USA). The resulting solution contained 5 mg/mL by weight of chitosan.

The chitosan nanoparticles were prepared in the same manner as described above, however, with the addition of the ionic gelation methodology described by Calvo et al.27. In this method, the nanoparticles were formed by adding 0.25% tripolyphosphate (Sigma-Aldrich, Saint Louis, MO, USA) (ratio TPP solution: Chitosan solution - 1:3), under constant agitation. Thus, chitosan nanoparticles with a final concentration of 3.8 mg/mL were obtained.

Determination of the minimum inhibitory concentration

Prior to performing the analysis, the minimum inhibitory concentration (MIC), fungicidal (MFC) and bactericidal concentrations (MBC) of the chitosan and chitosan nanoparticle solutions were determined (Table 1).

MIC values were established based on the microdilution method carried out in 96-well cell culture plates and in duplicate. The initial chitosan solutions were obtained at a concentration of 10 mg/mL, and decreasing concentrations of the antimicrobial
agent were placed in each well. For each type of microorganism, 100 µl of selective culture medium was added to each well, consisting of: *S. aureus*-Brain Heart Infusion broth (HiMedia Laboratories Pvt. Ltda. Mumbai, MH, India); *S. mutans*-Mitis Salivarius broth with 20% glucose (HiMedia Laboratories Pvt. Ltda. Mumbai, MH, India); *C. albicans* and *C. glabrata*-Sabouraud Dextrose broth (HiMedia Laboratories Pvt. Ltda. Mumbai, MH, India). Inocula (~ 10^7 CFU / mL) were obtained from cultures in the exponential growth phase, in phosphate buffered saline (PBS). For bacteria, the optical density reading was performed in a spectrophotometer (Thermo Scientific, MA, USA), at a wavelength of 625 nm. For yeasts, due to the variable morphology of the genus, counting was performed in a Neubauer chamber (HBG, Gießen, Germany), using an optical microscope (Axio Observer A1, Carl Zeiss, Jena, Germany). Each well was inoculated with the microorganism (bacteria ~ 10^5 CFU/mL and yeast ~ 10^4 CFU/mL) to be tested and the plates were incubated at 37°C for 24 hours. The results were analyzed based on the turbidity of the medium and the fungicidal and bactericidal concentrations, by seeding the suspension in Petri dishes. The culture media used for each microorganism were: *S. aureus* - Tryptic soy agar (HiMedia Laboratories Pvt. Ltda. Mumbai, MH, India); *S. mutans* - Brain Heart infusion agar (HiMedia Laboratories Pvt. Ltda. Mumbai, MH, India); *C. albicans* and *C. glabrata* - Sabouraud Dextrose agar (HiMedia Laboratories Pvt. Ltda. Mumbai, MH, India).

**Immersion in cleaning solutions**

Initially, for biofilm formation of the biofilm, the sterile specimens were individually distributed in 24-well cell culture plates (TPP, Trasadingen, Switzerland), containing 2 mL of culture medium for each microorganism, as previously described in the MIC analysis. To evaluate the antimicrobial action by means of CFU (n = 9) and SEM (n = 1) a simple–biofilm model composed of *C. albicans* (ATCC 9028), *C.
*glabrata* (ATCC 2001), *S. mutans* (ATCC 25175) and *S. aureus* (ATCC 6538) was used. Each well received an inoculum of 10⁶ CFU/mL. In order to test the sterility of the experiment, three specimens did not receive microorganism inocula. The plates were maintained at 37°C for 90 minutes, under agitation at 75 rpm (adhesion period). Then, the specimens were washed twice with PBS, in order to remove non-adhering microorganisms. Then, 2 mL of the sterile culture medium were added, and the plates were incubated again at 37°C for 24 hours. After this period, half of the culture medium was removed and replaced with sterile culture medium. The plates were incubated for another 24 hours, a period necessary for biofilm maturation. The metal and acrylic resin specimens were randomly distributed, according to the following experimental groups: 1. Solution without chitosan (WC - control, distilled water, pH 5.0); 2. Chitosan solution at 5 mg/mL (CH); 3. Chitosan nanoparticle solution at 3.8 mg/mL (CN); 4. Effervescent tablet (ET, Nitradine®-BonifAG, Vaduz, Liechtenstein) and negative control group (NC, n=3, without contamination and immersion) (Figure 1).

Subsequently, the specimens were submitted to immersion procedures for 15 minutes; the ET group was immersed according to the manufacturer's recommendations and the other groups were immersed at room temperature, according to a similar protocol. For the negative control group, the specimens remained in PBS.

*CFU determination*

After cleaning with the respective test solutions, specimens were rinsed 5 times in PBS and transferred to test tubes containing 10 mL of Letheen broth culture medium (HiMedia Laboratories Pvt. Ltda. Mumbai, MH, India). To release the microorganisms resistant to the cleaning procedure, the tubes were sonicated (200W, 40KHz) (Altsonic, Clean 9CA, Ribeirão Preto, São Paulo, Brazil), for 20 minutes. Then, serial dilutions were performed (10⁻¹ to 10⁻³) and the suspension was seeded in Petri dishes containing
culture media for each of the microorganisms previously described. After incubation (37°C for 24 h), the number of colony forming units was calculated and the values transformed into Log10(CFU/mL+1).

**Scanning Electron Microscopy (SEM)**

In order to observe the characteristics of the biofilm after the cleaning procedure, one specimen from each group was fixed with 2.5% glutaraldehyde for 24 hours. Samples were then dehydrated in a graded series of ethanol 30%, 50%, 70%, 90% and 100%, for 30 minutes in each, dried, mounted on aluminum specimen holders and sputter-coated with gold. The images were captured at 5000x magnification under high vacuum with a Scanning Electron Microscope (EVO 10, CARL ZEISS, Cambridge, England).

**Statistical analysis**

All tests were carried out with the use of the IBM SPSS Statistics 21.0 software (IBM Corp Armonk, NY, USA). The data were tested for normality (Shapiro-Wilk) and homocedasticity (Levene). Kruskal-Wallis and Dunn post tests were used to compare groups in terms of biofilm recovery rates of *S. mutans* and *C. glabrata*, on Co-Cr surfaces, and *C. albicans* on acrylic resin surfaces. One-way ANOVA and Tukey post test were used to compare groups in terms of biofilm recovery rates of *S. aureus* and *C. albicans*, on Co-Cr surfaces, and *S. mutans*, *S. aureus* and *C. glabrata* on acrylic resin surfaces. All comparisons were made with Bonferroni adjustment, and a significance level of 5% was adopted. The photomicrographs obtained by SEM were analyzed using a descriptive method.

**Results**

The MIC, MFC and MBC values are presented in Table 1. *C. albicans* was considered the microorganism most susceptible to CH and CN, while the most resistant
to these solutions was *S. mutans*. When compared, MIC, MFC and MBC values were higher for bacteria than for yeasts. The lowest MIC, MFC and MBC values were found for CH solution. CN solution showed values 3 times higher.

**CFU**

As regards acrylic resin (Table 2), ET was the most effective solution for reducing biofilm, as it reduced the *S. mutans* count to zero and decreased the CFU for *S. aureus* (p<0.001) and *C. albicans* (p<0.001). CH and CN significantly reduced the CFU count for *S. mutans* (CH: p <0.001; CN: p <0.001) and *S. aureus* (CH: p <0.001; CN: p = 0.008), with no significant difference between them. CH showed moderate action against *C. albicans* since it had an intermediate count value between ET and WC. No solution demonstrated activity against *C. glabrata*.

Relative to Co-Cr surfaces (Table 3), ET was the most effective solution for reducing biofilm, as it had an effect on all the microorganisms tested. CH and CN significantly reduced the CFU count only against *S. aureus*. CH showed moderate action on *S. mutans*, as it had an intermediate count value between ET and WC.

**SEM**

The representative SEM micrographs of biofilms grown on acrylic resin surfaces are shown in Figure 2. Observing *S. mutans* and *S. aureus* biofilms, a significant reduction in areas covered with cells could be visualized after immersion in ET solution. The reduction in cells caused by immersion in CH and CN cleansing solutions was considered moderate. *C. albicans* biofilms showed the same feature observed in the CFU counts. Immersion in ET produced the best result, followed by immersion in CH. As regards *C. glabrata* biofilm, a high density of yeast cells was observed after immersion in all cleansing solutions. The CFU results obtained suggested the absence of antimicrobial effect of ET, CH and CN against this microorganism. On all surfaces
immersed in CH and CN, a type of impregnation was observed, indicated by arrows in the micrographs.

Representative SEM micrographs of biofilms grown on Co-Cr surfaces are shown in Figure 3. For all microorganisms, a substantial reduction in areas covered with cells was observed when samples were immersed in ET cleansing solution. In agreement with CFU counts, immersion in CH solution promoted a reduction in areas covered with *S. mutans* and *S. aureus* biofilms. Immersion in CH and NH did not appear to have promoted massive changes in *C. albicans* and *C. glabrata* biofilms. In the same way as occurred on acrylic resin surfaces, a type of impregnation was also observed on all Co-Cr surfaces immersed in CH and CN; this is indicated by arrows in the micrographs.

**Discussion**

The null hypothesis was partially accepted since there were no significant differences among all solutions for *Candida glabrata* on acrylic resin surfaces.

ET promoted the best antimicrobial action on acrylic resin surfaces, except for *C. glabrata*, against which there was no action from any of the tested cleansers. These data were consistent with the trend of results reported by Coimbra et al.\(^8\), in which antimicrobial action of the denture cleanser tablets against the same microorganisms was reported, however, only on acrylic resin specimens.

The composition of the effervescent tablets includes components such as sodium lauryl sulfate, for which antimicrobial action has previously been verified\(^8,28\). Furthermore, studies have proposed that the mechanical action resulting from the oxygen bubbles released by its effervescence\(^{29}\), could contribute to biofilm removal. In the SEM images, the reduced amount of microorganisms covering both types of surfaces could be visualized (Figures 2 and 3, A-D, ET). Although the literature has
shown that immersion in ET is an adjuvant to control prosthetic biofilm, new cleaning solutions should be evaluated, since effervescent tablets indicated for daily use are expensive, and therefore, unavailable to a large part of the population.

Thus, in an attempt to find a more economically accessible solution, with comparable antimicrobial action, the CH solution was tested and showed results equivalent to those of ET on acrylic resin surfaces. This result suggested that *C. albicans* responded to the two cleansing solutions in a similar. In other studies, on the action of chitosan against *C. albicans* and prosthetic stomatitis, chitosan mouthwash has been shown to significantly reduce the erythematous area and the burning sensation; and the incorporation of chitosan into tissue conditioner has been proved to be a safe and alternative therapy to treat prosthetic stomatitis. The antimicrobial effect of chitosan may be related to positive charges of the chitosan molecule that interact with the cell membranes of negatively charged microorganisms, inducing leakage of intracellular constituents, consequently reducing metabolism and resulting in cell death.

Interestingly, in this study, *C. glabrata* and *C. albicans* were the microorganisms least susceptible to the action of chitosan, which may probably have been related to the reduced prevalence of negative charges on their cell surfaces, when compared with other yeasts. Another aspect that must be considered is the difference in antimicrobial susceptibility between microorganisms in their planktonic form and when they are organized in biofilm, since the phenotypes associated with the biofilm, are infinitely less susceptible to antimicrobial agents. Biofilms are a complex and well-structured aggregate of microorganisms surrounded by an extracellular polymeric matrix that protects and makes them tolerant to antimicrobial agents. This may explain the discrepant difference between the results of CFU counts and those
found in the MIC analysis. Therefore, the restricted antimicrobial effect of chitosan on *C. albicans* biofilm found on the metal surface, and on *C. glabrata* biofilm found on both metal and acrylic resin surfaces, could be related to antimicrobial susceptibility of microorganisms in states associated with biofilm, and in states not associated with biofilm.

Therefore, in order to improve the antimicrobial effect of chitosan, the use of CN has been proposed\(^{34}\). Ionic gelation is one of the methods that can be used to obtain chitosan nanoparticles\(^{27}\). Basically, crosslinking agents such as sodium tripolyphosphate or sodium sulfate interact with the positively charged chitosan chains, and disrupt the integrity of the cell membrane by means of structural changes in the molecule\(^{34}\), in homogeneous manner throughout the biofilm\(^{35}\). This feature has been attributed to the particle size, since it would be easier for smaller particles to infiltrate deeper into the layers of biofilm. In our study, however, modification of the biomaterial did not show satisfactory anti-biofilm activity. Contrary to existing reports in the literature\(^{23,34}\), the action of the CN was inferior to that of CH. Gondim et al.\(^{19}\) evaluated the antimicrobial activity of a chitosan nanoparticle solution (3.8 mg/mL), against a multispecies biofilm of *Candida* spp formed on acrylic resin specimens. Broad-spectrum anti-biofilm activity against *Candida* spp was reported for mature biofilms. However, the biofilms remained in direct contact with the solution for eight hours, whereas in the present study the immersion period established was only 15 minutes.

For *S. aureus*, both CH and CN solutions showed moderate antimicrobial activity on acrylic resin, whereas on Co-Cr surfaces, CH was more effective. This result was considered satisfactory since this microorganism is associated with serious opportunistic infections.\(^{10}\) Chitosan has an increased inhibitory effect in comparison
with *S. aureus* when the molecular mass (MW) varies from 5 to 305 kDa\textsuperscript{16}, which was confirmed in the present study, since chitosan MW varied from 50 to 190 kDa.

As regards *S. mutans*, both CH and CN showed a similar antimicrobial action on acrylic resin. On Co-Cr surfaces, CH was equivalent to ET. These results suggested that the antimicrobial action of CH against *S. mutans* was satisfactory. It is worth emphasizing that *S. mutans* is one of the largest producers of extracellular polysaccharides (EPS) in dental biofilms\textsuperscript{37}. This fact, in addition to the increased exposure of teeth to bacterial acids, influences the action of antimicrobial agents, because EPS act as a barrier to diffusion of chemical cleaners into the deeper layers of biofilm\textsuperscript{33}. Furthermore, with reference to partially edentulous individuals, *S. mutans* is one of the microorganisms most frequently present in the oral cavity\textsuperscript{1}. Costa et al.\textsuperscript{17} demonstrated a significant antibacterial effect of chitosan against *S. mutans*, as well as the ability to inhibit biofilm formation.

On the Co-Cr surfaces, CN showed no antimicrobial activity. Biofilm formation is controlled by several factors that include environmental aspects and surface characteristics, such as roughness, porosity, surface free energy, hydrophobicity and other surface conditions inherent to each material\textsuperscript{38}.

Therefore, the reduction in antimicrobial activity found on the metal surface may be related not only to the form of chitosan presentation, but also to the surface characteristics of the substrate\textsuperscript{12,39}, influencing all the groups of microorganisms. Moreover, the metal surface tended to favor a higher degree of microbial adhesion than the acrylic resin surface\textsuperscript{12}. Corrosion of cobalt alloys frequently occurs, and this process leads to greater surface roughness, facilitating the subsequent accumulation of biofilm\textsuperscript{40}. These factors can influence the characteristics of biofilms, including biomass accumulation and susceptibility to antimicrobial treatments, since the same biofilm
species formed on different surfaces have significant differences in sensitivity to antimicrobial treatment\textsuperscript{39}. These findings may explain the difference in the results found in the present study and indicate the need to develop a suitable cleaner, capable of acting effectively on different surfaces.

Analysis of the SEM images showed chitosan covering aggregated microorganisms, with the appearance of impregnation of the biomaterial (Figures 2 and 3). A previous report described the elimination of an important bacterium related to severe gastric infections in the stomach, by means of its adherence to chitosan microspheres\textsuperscript{15}. Chitosan acted as a facilitating agent for eliminating \textit{Helicobacter pylori}. The advantages or disadvantages of chitosan adherence to biofilm as a measure for developing a prosthesis cleaning solution was not assessed. Subsequent removal of the biofilm from the prosthetic surface by means of brushing would probably be easier since the microorganisms would be agglomerated and surrounded by chitosan. However, further studies to investigate this possibility are still needed.

Considering that the chitosan solution showed antimicrobial activity against some microorganisms, and showed moderate action against others, when compared with the ET, it was possible to infer that chitosan could be an option for use as a dental prostheses cleaner, since it is a natural alternative that is simple to prepare and low cost.

Future studies should be conducted in order to assess the difference in the effectiveness of cleanser solutions on metal and acrylic resin surfaces, considering the structural characteristics of the biofilm formed. Furthermore, the association of a cleanser solution with a mechanical cleaning method, as well as simulating the conditions of the oral cavity, could be analyzed.

\textbf{Conclusion}

The effervescent tablet promoted a higher level of antimicrobial activity against
all microorganisms, except for *C. glabrata*, on acrylic resin surfaces. The CH solution showed a similar result to that of the tablet against *S. mutans* on the metal surface, and against *C. albicans* on acrylic resin, therefore it showed intermediate antimicrobial activity and potential application as a cleanser.

**Acknowledgments**

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The authors report no conflicts of interest.

**References**

6. Vasconcelos GLL, Curylofo PA, Raile PN, Macedo AP, Paranhos HFO, Pagnano


Table 1

Minimum concentration of chitosan solutions for inhibitory, fungicidal and bactericidal effect.

<table>
<thead>
<tr>
<th>Minimum inhibitory concentration (mg/mL)</th>
<th>Minimum fungicidal and bactericidal concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CH</td>
</tr>
<tr>
<td>S. mutans</td>
<td>0.312</td>
</tr>
<tr>
<td>S. aureus</td>
<td>0.312</td>
</tr>
<tr>
<td>C. albicans</td>
<td>-</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>-</td>
</tr>
</tbody>
</table>

(-) absence of microbial growth at all concentrations tested; CH - chitosan solution; CN – Chitosan nanoparticle solution.
Table 2

Descriptive statistics and comparisons for Log10(CFU+1) of the biofilm recovery rates from the acrylic resin surface, after immersion in the cleaning solutions.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Mean ± SD (Median)</th>
<th>95% CI (Range)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. mutans</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WC</td>
<td>6.60 ± 0.58 (6.69)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.16; 7.05 (5.30; 7.43)</td>
<td></td>
</tr>
<tr>
<td>CH</td>
<td>4.27 ± 0.54 (4.42)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.85; 4.69 (3.20; 4.76)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>CN</td>
<td>4.29 ± 0.73 (4.05)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.73; 4.85 (3.08; 5.68)</td>
<td></td>
</tr>
<tr>
<td>ET</td>
<td>0.00 (-)*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WC</td>
<td>7.74 ± 0.38 (7.87)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.45; 8.03 (7.15; 8.16)</td>
<td></td>
</tr>
<tr>
<td>CH</td>
<td>5.93 ± 0.83 (6.02)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.29; 6.57 (4.78; 7.41)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>CN</td>
<td>6.44 ± 1.17(6.59)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.53; 7.34 (4.98; 8.06)</td>
<td></td>
</tr>
<tr>
<td>ET</td>
<td>3.58 ± 0.58 (3.67)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.13; 4.03 (2.68; 4.48)</td>
<td></td>
</tr>
<tr>
<td><strong>C. albicans</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WC</td>
<td>5.76 ± 0.30 (5.61)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.54; 5.99 (5.47; 6.38)</td>
<td></td>
</tr>
<tr>
<td>CH</td>
<td>5.03 ± 0.95 (5.25)&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.30; 5.76 (3.08; 6.33)</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>CN</td>
<td>5.48 ± 0.38 (5.57)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.19; 5.77 (4.57; 5.84)</td>
<td></td>
</tr>
<tr>
<td>ET</td>
<td>2.10 ± 1.83 (2.45)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.70; 3.51 (0.00; 4.53)</td>
<td></td>
</tr>
<tr>
<td><strong>C. glabrata</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WC</td>
<td>6.99 ± 0.27 (7.10)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.78; 7.20 (6.39; 7.25)</td>
<td>0.264**</td>
</tr>
<tr>
<td>CH</td>
<td>6.79 ± 0.48 (6.86)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.43; 7.16 (5.71; 7.25)</td>
<td></td>
</tr>
<tr>
<td>CN</td>
<td>6.82 ± 0.39 (6.85)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.52; 7.12 (6.08; 7.29)</td>
<td></td>
</tr>
<tr>
<td>ET</td>
<td>7.23 ± 0.77 (7.49)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.64; 7.82 (5.61; 7.89)</td>
<td></td>
</tr>
</tbody>
</table>

SD – Standard Deviation; CI – Confidence Interval for mean; WC- Control-Solution without chitosan; CH - Chitosan; CN- Chitosan nanoparticle; ET - Effervescent tablet. * reduced the CFU count to zero; ** ANOVA and Tukey post test; *** Kruskal-Wallis and Dunn's post-test.
Table 3
Descriptive statistics and comparisons for Log10 (CFU+1) of the biofilm recovery rates from the Co-Cr surface, after immersion in the cleaning solutions.

<table>
<thead>
<tr>
<th>Material</th>
<th>Mean ± SD (Median)</th>
<th>95% CI (Range)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC</td>
<td>6.30 ± 0.77 (6.30)a</td>
<td>5.71; 6.89 (4.98; 7.52)</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>CH</td>
<td>4.53 ± 0.98 (4.26)b</td>
<td>3.78; 5.29 (3.60; 6.89)</td>
<td></td>
</tr>
<tr>
<td>CN</td>
<td>5.41 ± 0.69 (5.33)a</td>
<td>4.88; 5.94 (4.38; 6.45)</td>
<td></td>
</tr>
<tr>
<td>ET</td>
<td>0.46 ± 0.91 (0.00)b</td>
<td>-0.24; 1.16 (0.00; 2.21)</td>
<td></td>
</tr>
<tr>
<td>WC</td>
<td>7.39 ± 0.48 (7.63)a</td>
<td>7.02; 0.76 (6.60; 7.86)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>CH</td>
<td>5.49 ± 0.50 (5.71)c</td>
<td>5.11; 5.88 (4.71; 6.07)</td>
<td></td>
</tr>
<tr>
<td>CN</td>
<td>6.66 ± 0.23 (6.57)b</td>
<td>6.48; 6.83 (6.38; 7.15)</td>
<td></td>
</tr>
<tr>
<td>ET</td>
<td>4.41 ± 0.55 (4.36)d</td>
<td>3.99; 4.83 (3.56; 5.24)</td>
<td></td>
</tr>
<tr>
<td>WC</td>
<td>5.62 ± 0.87 (5.74)a</td>
<td>4.95; 6.29 (4.03; 6.71)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>CH</td>
<td>5.92 ± 0.33 (5.91)a</td>
<td>5.66; 6.17 (5.26; 6.45)</td>
<td></td>
</tr>
<tr>
<td>CN</td>
<td>5.85 ± 0.60 (5.97)a</td>
<td>5.39; 6.31 (4.73; 6.74)</td>
<td></td>
</tr>
<tr>
<td>ET</td>
<td>4.19 ± 1.05 (4.12)b</td>
<td>3.38; 4.99 (2.72; 5.64)</td>
<td></td>
</tr>
<tr>
<td>WC</td>
<td>7.02 ± 0.49 (6.86)a</td>
<td>6.65; 7.40 (6.33; 7.57)</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>CH</td>
<td>6.84 ± 0.56 (6.80)a</td>
<td>6.41; 7.27 (5.88; 7.64)</td>
<td></td>
</tr>
<tr>
<td>CN</td>
<td>7.11 ± 0.63 (7.44)a</td>
<td>6.63; 7.60 (5.63; 7.62)</td>
<td></td>
</tr>
<tr>
<td>ET</td>
<td>5.38 ± 1.11 (5.64)b</td>
<td>4.52; 6.23 (3.30; 6.82)</td>
<td></td>
</tr>
</tbody>
</table>

SD – Standard Deviation; CI – Confidence Interval for mean; WC- Control-Solution without chitosan; CH - Chitosan; CN- Chitosan nanoparticle; ET - Effervescent tablet. * reduced the CFU count to zero; ** ANOVA and Tukey post test; *** Kruskal-Wallis and Dunn's post-test.
Figure Legends

Fig 1 Experimental design of the study. MIC – Minimum inhibitory concentration; WC- Control- Solution without chitosan; CH- Chitosan; CN- Chitosan nanoparticle; ET - Effervescent tablet.
Fig 2 Resin acrylic specimens contaminated with A- *Streptococcus mutans*; B- *Staphylococcus aureus*; C- *Candida albicans* and; D- *Candida glabrata* after immersion in WC- Control-Solution without chitosan; CH- Chitosan; CN- Chitosan nanoparticle; ET- Effervescent tablet. Arrows indicate microorganisms adhered to chitosan.
Fig 3 Metallic specimens contaminated with A- *Streptococcus mutans*; B- *Staphylococcus aureus*; C- *Candida albicans* and; D- *Candida glabrata* after immersion in WC- Control-Solution without chitosan; CH- Chitosan; CN- Chitosan nanoparticle; ET- Effervescent tablet. Arrows indicate microorganisms adhered to chitosan.