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Chitosan mouthwash: Toxicity and *in vivo* validation

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ABSTRACT

A previous study showed that a chitosan mouthwash would be a valid alternative to current mouthwashes as it demonstrated, *in vitro*, significantly higher antibiofilm activity than two commercial mouthwashes. As such, the aim of this work was to verify the safety of the developed product and to validate, *in vivo*, the biological activity ascertained *in vitro*. Chitosan mouthwash safety was evaluated through Ames, MTT and V79 chromosomal aberration assay while antimicrobial activity was evaluated through *in vivo* assays. The results showed that the chitosan mouthwash was safe, presenting lower cytotoxicity than a commercial mouthwash, and that it effectively reduced viable counts of *Streptococcus* spp. and *Enterococcus* spp. by ca. 5.5 log of CFU. Furthermore, in direct comparison with a commercial mouthwash the chitosan mouthwash possessed significantly higher antimicrobial activity. The conjunction of these results proves that the chitosan mouthwash is a safe, effective, natural alternative to the existent chemical mouthwashes.

1. Introduction

Mouthwashes formulations have evolved over the years from containing any one of a whole range of different antimicrobial agents, like chlorhexidine, to the incorporation of more natural components such as plant extracts or polysaccharides (Ahmadi, 2012; Verkaik et al., 2011).

Chitosan, a derivative of chitin (obtained by deacetylation), is a high molecular weight polysaccharide with active amino groups that grant it several biological properties While its biocompatibility, lack of allergenicity and biodegradability have led to the development of a large body of knowledge on the use of chitosan as a biomaterial, particularly in the biomedical and biotechnological fields, it was another set of properties, among which are immunostimulation, macrophages activation and antimicrobial activity that enhanced chitosan's versatility and potential (Balan & Verestiuc, 2014; Dash, Chiellini, Ottenbrite, & Chiellini, 2011; Muzzarelli, 2009, 2010; Muzzarelli et al., 1990; Raafat & Sahl, 2009; Sandri et al., 2004). However, there are some drawbacks as chitosan biological performance depends not only on the degree of acetylation, but also on the acetylation pattern, the degree of crystallinity, the average molecular weight and its polydispersity, among other parameters (Costa, Silva, Pina, Tavaria, & Pintado, 2012; Fernandes et al., 2008; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Muzzarelli, 2010; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003).

In later years, various authors (Bae, Jun, Lee, Paik, & Kim, 2006; Choi et al., 2001; Costa et al., 2012; Ji et al., 2009; Tarsi, Muzzarelli, Guzman, & Pruzzo, 1997) showed that chitosan inhibited, *in vitro*, oral disease related microorganisms growth and biofilms (Busscher, Engels, Dijkstra, & van der Mei, 2008; Carlson, Taffs, Davison, & Stewart, 2008; Costa, Silva, Tavaria, & Pintado, 2013). Incorporation of chitosan into oral care products has produced mixed results. On the one hand works (Liu, Chen, Mao, & Gao, 2007; Verkaik et al., 2011; Yadav & Mohire, 2010) describing the development of a chitosan based toothpaste reported the development of formulations with better properties, particularly higher and longer antimicrobial activity, than traditional toothpastes. On the other hand early studies regarding the incorporation of chitosan in mouthwashes (Sano, Shibasaki, Matsukubo, & Takaesu, 2001, 2003; Uraz et al., 2012; Vieira, 2005) reported contradicting results regarding the antimicrobial efficiency of the chitosan mouthwash, initial work performed by this group showed, *in vitro*, that a chitosan-based mouthwash possessed significant antimicrobial and antibiofilm efficiency against oral microorganisms (Costa et al., 2014). As such the aim of this study was to validate, *in vivo*, the results previously obtained *in vitro*, upon two groups of microorganisms associated with dental caries–lactobacilli and streptococci (Tanner et al., 2006), and to ensure the safety of the developed product through genotoxicity and cytotoxicity assays.

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Table 1
Tested commercial mouthwash full composition.

Mouthwash	Composition
Com	Water, alcohol, propylene glycol, sodium benzoate, lactic acid, poloxamero 338, sodium citrate, CI42051, allantoin, sweetener, chlorhexidine digluconate, hydroxyethyl cellulose, limonene, PEG-12 dimethicone, sodium bicarbonate, sodium saccharin, sodium borate

2. Materials and methods

2.1. Source of chitosan and mouthwash formulations

The commercial mouthwash (Table 1) tested had as active principle chlorhexidine (**Com**). Chitosan mouthwash formulation (**Chi**) was prepared using High Molecular Weight (DD > 75%; MW 624 kDa) and Low Molecular Weight chitosan (75% < DD< 85%; MW 107 kDa) (Sigma–Aldrich, St. Louis, USA) with the final concentration of either chitosan being 0.4% (v/v). The chitosan based mouthwash, prepared for a final pH of 5, contained 0.5% (w/v) salt (NaCl), 1% (w/v) stabilizer (arabic gum), 5% (w/v) sweetener (mannitol). After complete dissolution food grade flavouring and colouring agents were added at 0.1% (v/v) each.

2.2. Genotoxicity assays

Biocompatibility of mint (**Chi1**) and strawberry (**Chi2**) flavoured chitosan mouthwash was assessed through the following assays. In the MTT assay, the commercial mouthwash **Com** was used as a commercial control for comparison purposes.

2.2.1. Ames assay

The assay was performed with the TA98 and TA 100 tester strains of *Salmonella typhimurium*, according to the method described by Ames (Ames, Mccann, & Yamasaki, 1975; Maron & Ames, 1983; Mortelmans & Zeiger, 2000). Different volumes of mouthwash were tested in the plate assay, with and without metabolic activation (S9 mix). Benzo(a)pyrene (BaP) at 5 μg/plate and quercetin at 10 μg/plate, dissolved in dimethyl sulfoxide (DMSO), were used as positive controls for strain TA98, in the presence and absence of metabolic activation, respectively and 2-aminoanthracene (2-AA) at 1 μg/plate and quercetin at 10 μg/plate, dissolved in DMSO, for strain TA100, in the presence and absence of metabolic activation, respectively. Negative controls were also performed, according to the solvent used to prepare extracts and standards. The assays were carried out in duplicate.

2.2.2. V79 cell culture

The wild-type V79 Chinese hamster cells (MZ) used in this study were kindly provided by Prof. H.R. Glatt (German Institute of Human Nutrition, Nuthetal, Germany). The cells were routinely maintained in 175 cm^2 culture flasks (Sarstedt, AG&CO, Nümbrecht, Germany) using Ham's F-10 medium, supplemented with 1% antibiotic solution (penicillin–streptomycin) and 10% newborn calf serum (Sigma–Aldrich, St. Louis, MO, USA). The cells were kept at 37 °C, under an atmosphere containing 5% CO_2.

2.2.3. MTT assay

The MTT assay was performed as previously described (Martins, Doran, Laires, Rueff, & Rodrigues, 2011). Briefly, cells at a density of 5×10^3 cells per well were seeded into 96-well plates and after 24 h were exposed to different concentrations of mouthwashes, for a 24-h period. Cells were then washed, MTT solution was added (0.5 mg/ml) and the plate was incubated for 3 h. Following that the plate was washed with PBS and DMSO was added to each well. Hydrogen peroxide was used as a positive control (data not shown). Absorbance was read at 595 nm using a microplate reader. Absorbance values presented by control cultures correspond to 100% cell viability. Two independent assays were conducted, each one of them in quadruplicate.

2.2.4. Chromosomal aberration assay

The results obtained in the Ames assay and the MTT assay enabled the calculation of appropriate doses, without being cytotoxic. Since there was no alteration in the presence of an exogenous metabolic activation system, and no indication of metabolization of chitosan, this assay was performed in the absence of exogenous metabolic activation. The chromosomal aberration assay was performed according to established protocols (Maralhas et al., 2006). Briefly, V79 cells were exposed to the test mouthwashes, without metabolic activation, for 18 h. After this period, cells were treated with a metaphase inhibitor (colchicine) for 2.5 h, harvested, stained and posteriorly analyzed under a microscope. One hundred metaphases were totalled for each dose, in duplicate. Mitomycin c (MMC), a potent inhibitor of the mitotic spindle and genotoxic agent was used as a positive control, at 1.5 μM. The mitotic index (MI) was also assessed and is given using the following formula:

$$MI = \left[\frac{\text{No. of metaphases}}{\text{Total no. of cells } (1000)}\right] \times 100$$

2.3. In vivo studies

This randomized *in vivo* study was conducted as a parallel group design. Each volunteer had to fulfil the following inclusion criteria: (i) be in good general health; (ii) have an age between 18 and 65; (iii) be in good oral health; (iv) be taking no regular antibiotics; (v) be a mouthwash user; (vi) have no fixed or removable prostheses, or orthodontic appliances. Those reported to be: (i) hypersensible to oral care products; (ii) allergic to shellfish; (iii) be a smoker; (iv) be pregnant; were not allowed to participate. All volunteers included in the assay were fully informed and their informed consent was obtained.

The study protocol was the following: before the experimental phase each participant thoroughly brushed its teeth, without toothpaste, to remove all non-adhered microorganisms and to standardize the initial oral cavity conditions between participants.

2.3.1. Rinsing volume optimization

In order to optimize chitosan mouthwash rinsing volume participants were separated into two groups: Group 1 used chitosan mouthwash; Group 2 used a placebo mouthwash without chitosan. Mouthrinsing was performed with 10 and 20 ml aliquots of mint flavoured chitosan mouthwash during 30 s by 12 volunteers. Oral microflora was assayed in three different sites (cheek, teeth and tongue) before and after (immediately and 30 min) rinsing using a swab which was then placed in 10 ml of the appropriate media and serially diluted. Samples were plated in WCA, for total anaerobic bacteria, Mitis Salivarius Agar (MSA) (Fluka, St. Louis, USA) with tellurite (Merck, Darmstadt, Germany) for total streptococci and in Kanamycin Esculin Azide Agar (KEA) (Merck, Darmstadt, Germany) for total enterococci, using the spread plate technique. Incubation for WCA and MSA was performed at 37 °C in anaerobic conditions during 48 h while for KEA incubation was done in aerobic conditions at 37 °C during 24 h. Results were given in terms of viable counts versus the sampling time for total anaerobic bacteria, enterococci and streptococci.

2.3.2. Antimicrobial activity assessment

In order to evaluate chitosan mouthwash antimicrobial efficiency 25 participants were separated in three groups: Group 1

used chitosan mouthwash; Group 2 used a commercial mouthwash; Group 3 used a placebo mouthwash without chitosan. Mouthrinsing was performed with optimized volume during 30 s and oral microflora was assayed in three different sites (cheek, teeth and tongue) before and after (immediately and 30 min) rinsing using a swab which was then placed in 10 ml of the appropriate media and serially diluted. Samples were plated using the spread plate technique in selective media for assessment of total anaerobic bacteria, total streptococci and total enterococci, as previously described. Incubation was performed at 37 °C during 24 h for enterococci and 48 h for streptococci and total anaerobic bacteria. Results were given in terms of viable counts versus the sampling time for total anaerobic bacteria, enterococci and streptococci.

2.4. Statistical analysis

The statistical differences in the methods were evaluated using PASW Statistics v. 18.0.0 (New York, USA). The normality of the results distribution was evaluated through Shapiro–Wilk's test. The differences were evaluated using one way ANOVA test associated with Scheffe's test (for normal distributions) or the non-parametric Mann–Whitney test for the rest. The differences were considered significant at a 0.05 level.

3. Results

3.1. Toxicity assays

The results obtained with the Ames assay, both without and with an exogenous metabolic system (S9 Mix) were negative for the chitosan mouthwash tested (Table 2). The number of spontaneous revertants per plate did not double a necessary condition to consider the results as positive. Thus, we can consider the chitosan mouthwash as non genotoxic in the Ames assay, in the conditions tested. The positive control quercetin, a genotoxic compound in the Ames test in the absence of S9 Mix, both for TA98 and TA100, induced 133 and 198 revertants per 10 μg, respectively, which are in accordance with historical data in the laboratory. In the presence of S9 Mix, the positive controls used were BaP at 5 μg/plate for TA98 and 2-AA for TA100, which resulted in 265 and 203 revertants, respectively, also in accordance with historical data.

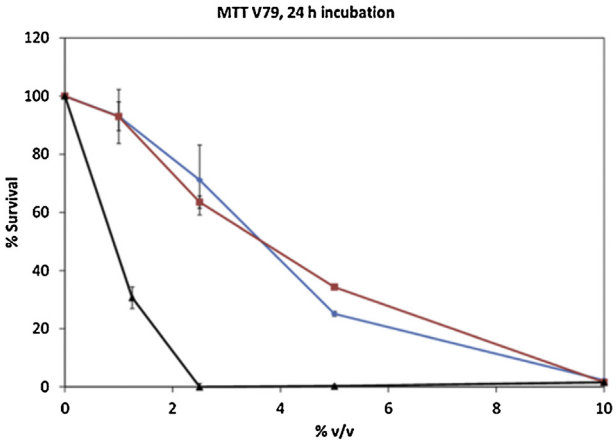

Fig. 1. Cytotoxicity of the chitosan mouthwash (**Chi1** and **Chi2**) in the MTT assay. A commercial mouthwash (**Com**) was also tested. Two independent assays were conducted, each one of them in quadruplicate. ♦ – **Chi1**; ■ – **Chi2**; ▲ – **Com**.

Chitosan mouthwash (**Chi1** and **Chi2**) presented a dose dependent increase in toxicity towards V79 cells, as expected (Fig. 1). Above 2% (v/v) toxicity increased significantly. A commercial mouthwash (**Com**) was more toxic towards V79 cells in the MTT assay. The toxicity observed is expectable since all mouthwashes present a significant antibacterial effect.

The results obtained in the chromosomal aberrations assay in V79 cells are presented in Table 3. The chitosan mouthwash tested did not induce chromosomal aberrations in these cells, under the conditions tested, and therefore were considered negative for this assay.

3.2. In vivo studies

The main hurdle in the validation of most pharmaceutical products is the transition between *in vitro* and *in vivo* tests with a loss of efficiency and secondary effects being the main concerns. With that in mind an *in vivo* study was performed with the objectives of optimizing the chitosan mouthwash rinsing volume and validating, *in vivo*, the mouthwash antimicrobial action. The results obtained (Fig. 2) in the rinsing volume optimization assay showed that both tested volumes were capable of reducing total

Table 2
Results of the Ames assay for the mint (**Chi1**) and strawberry (**Chi2**) flavoured chitosan mouthwash. Strains TA 98 and TA 100 were tested both in the absence and presence of S9 Mix. All assays performed in duplicate.

	TA98		TA100	
	−S9	+S9	−S9	+S9
Chi1				
0 μl/plate	13 ± 1	15 ± 2	126 ± 3	126 ± 16
5 μl/plate	11 ± 4	15 ± 1	150 ± 24	160 ± 12
10 μl/plate	14 ± 6	13 ± 1	159 ± 23	154 ± 31
25 μl/plate	16 ± 3	14 ± 0	160 ± 15	164 ± 2
50 μl/plate	19 ± 5	15 ± 1	165 ± 13	
100 μl/plate	13 ± 8	14 ± 0	182 ± 21	
Chi2				
0 μl/plate	13 ± 1	15 ± 2	126 ± 3	126 ± 16
5 μl/plate	11 ± 1	18 ± 1	165 ± 4	138 ± 15
10 μl/plate	15 ± 1	14 ± 6	153 ± 6	151 ± 9
25 μl/plate	15 ± 7	17 ± 3	168 ± 22	141 ± 4
50 μl/plate	14 ± 3	20 ± 8	178 ± 4	
100 μl/plate	16 ± 1	16 ± 1	184 ± 24	
Positive control, Quercetin				
10 μg/plate	133 ± 6		198 ± 33	
Positive control, Benzo[a]pyrene				
5 μg/plate		265 ± 13		
Positive control, 2-aminoanthracene				
1 μg/plate				203 ± 4

Table 3
Chromosomal aberrations induced by the mint (**Chi1**) and strawberry flavoured (**Chi2**) chitosan mouthwashes in V79 cells. CTG – chromatid gap; CSG – chromosome gap; CTB – chromatid break; CSB – chromosome break; ACENT – acentric fragment; DIC – dicentric chromosome; TRI – triradial chromosome; TETRA – tetraradial chromosome; OTHER – other aberration; RING – ring chromosome; MA – multiaberrant metaphase; %AC – % of aberrant cells, excluding CTG and CSG; MI – mitotic index; MMC – mitomycin C.

Dose	CTG	CSG	CTB	CSB	ACENT	DIC	TRI	TETRA	OTHER	RING	MA	MI	% AC
DMSO	0	0	0	0	0	0	0	0	0	0	0	3.4	0
MMC1.5 μM	8	4	28	14	0	0	16	2	0	4	0	0.4	42
Chi1, % (v/v)													
0.5	0	0	0	0	0	0	0	0	0	0	0	3.5	0
1	1	0	0	0	0	0	0	0	0	0	0	3.4	0
2	0	0	0	0	0	0	0	0	0	0	0	7.8	0
Chi2, % (v/v)													
0.5	0	0	0	0	0	0	0	0	0	0	0	4.0	0
1	0	0	0	0	0	0	0	0	0	0	0	3.1	0
2	0	0	0	0	0	0	0	0	0	0	0	4.6	0

microbial counts, by approximately 2 log for 20 ml and 1 log for 10 ml (Fig. 2A), immediately after and up to 30 min after usage. Furthermore, at both volumes tested, chitosan mouthwash was capable of effectively reduce the two selected pathogenic markers by 3 (20 ml) and 1.5 (10 ml) log of CFU for streptococci (Fig. 2B) and by 2.2 (20 ml) and 0.7 (10 ml) log of CFU for enterococci (Fig. 2C). When considering the effect of the rising volume on the antimicrobial activity of the mouthwash, the statistical analysis of the results showed that the increase of volume (from 10 to 20 ml) had a significant ($p \leq 0.01$) correlation with the increase of the reduction of viable counts of streptococci and enterococci. This can be seen in Fig. 2B and C, where rising with 20 ml presented significantly higher ($p \leq 0.05$) viable counts reduction, when in comparison with rising with 10 ml; additionally, the 20 ml was the only, of the tested rising volumes, maintaining activity up to 30 min. However, as volunteer's expressed that rinsing with 20 ml was uncomfortable, a 15 ml rinsing volume was selected for the remaining assays.

As can be seen from Fig. 3, the results obtained when rinsing with chitosan mouthwash under optimized conditions, showed a decrease of total counts of the selected bacterial markers with statistical differences ($p < 0.05$) being found for both pathogenicity markers streptococci and enterococci, but not for the total anaerobes. On a closer look one can see that for total streptococci and enterococci there were viable counts reductions in both sampling times after rinsing, with a 2.3 log of CFU reduction immediately after rising and a reduction superior to 3 log of CFU after 30 min, which represents a bactericidal effect (bactericidal activity being defined as a reduction superior to 3 log of CFU as described by Fernandes, Eaton, Gomes, Pintado, and Xavier Malcata (2009)), for both pathogenic markers. These values are particularly significant if one considers that they represent a reduction approximately 50% in viable counts for both microbial markers in a short-time span (30 min). On the other hand, the values obtained for the total anaerobic counts showed that rinsing did not had a statistical significant impact ($p > 0.05$). Considering that, total anaerobic counts were

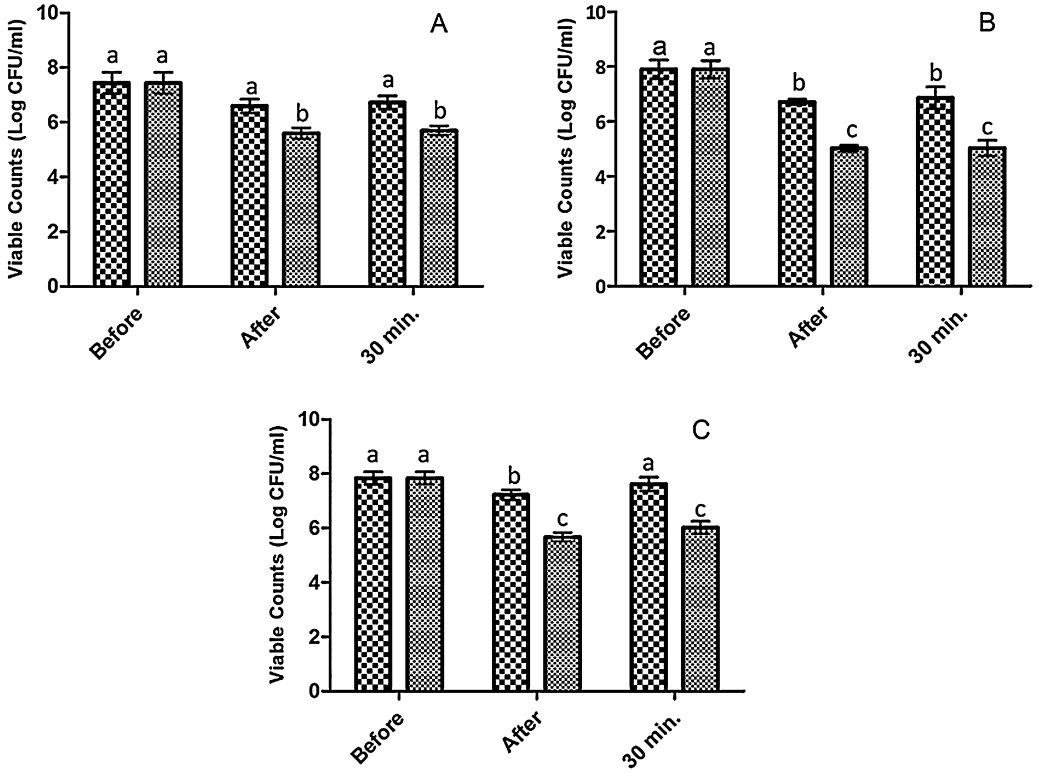

Fig. 2. Comparison of the total viable counts (for the total anaerobic viable cells (A); *Streptococcus* spp. (B); *Enterococcus* spp. (C)) at different sampling times before and after the administration of the rinsing solution. Different letters represent statistically different results ($p < 0.05$). All assays were performed in duplicate. ▒ – 10 ml; ▓ – 20 ml.

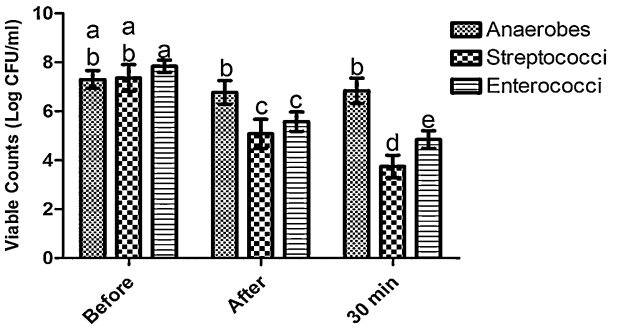

Fig. 3. Effect of rinsing with 15 ml of chitosan mouthwash upon total anaerobic, streptococci and enterococci bacterial counts at the three sampling times. Different letters represent statistically different results ($p < 0.05$).

monitored to assess the overall impact of the mouthwash, these results lead to the conclusion that the **Chi** mouthwash presents a targeted action, as rinsing produces an effective action in reducing key pathogens, but was not harmful for the general anaerobic microorganisms present in the oral microbiota.

When looking into the impact of the mouthwash upon particular sites in the oral mouth, teeth and tongue (Fig. 4), clear differences were observed. For total anaerobic bacteria counts (Fig. 4A) statistically significant differences ($p < 0.05$) on the tongue were only found immediately after rinsing, with a 12% reduction in log of CFU, in the teeth statistically significant differences ($p < 0.05$) were found in both post-rising sampling times with 9% and 13% reduction of log of CFU immediately and 30 min after rinsing, respectively. On the other hand, both streptococci and enterococci counts showed significant differences ($p < 0.05$) between sampling sites and times. For the first group (Fig. 4B) one can see clear differences ($p < 0.05$) between sampling sites, with teeth showing a reduction of ca. 6 log of CFU after 30 min, which corresponds to a reduction percentage of ca. 88%, while, in the same period, a reduction of 0.93 log of CFU, which corresponds to a reduction percentage of only 14.8%, was found for the tongue. For enterococci (Fig. 4C) clear differences ($p < 0.05$) were also found between sampling sites and times with a 4.1 log of CFU reduction, which corresponds to a reduction percentage of 63%, for the teeth and a 1.2 log of CFU reduction (17% reduction percentage) for the tongue.

When comparing these results with the ones obtained for the commercial mouthwash (Fig. 5), we can see that only **Chi** was capable of presenting bactericidal activity (reduction higher than 3 log of CFU (Fernandes et al., 2008)) for the considered pathogenic markers. As can be seen in Fig. 5B and C there are evident differences ($p < 0.05$) between **Chi** and **Com** activity upon streptococci and enterococci, particularly after 30 min with a clear loss of antimicrobial activity of the **Com** mouthwash. A proof of this is the recovery of viable counts observed for enterococci in the time between post-rinsing samples. On the other hand the **Chi** mouthwash showed a continuous reduction of bacterial counts. Considering that the **Com** active principle is chlorhexidine, the main compound used as antimicrobial in most oral care products (Jamilian, Ghasemi, Gholami, & Kaveh, 2008), these results show that chitosan may be a better, natural, alternative to most of the antimicrobials used in oral health nowadays.

Analysing the evolution of the population over the course of the study changes in CFU levels in the different microbial markers, for the studied sampling sites, can be observed (Fig. 6). With the exception of the total anaerobic bacteria counts, CFU values were observed to significantly differ in each group at the tested time intervals ($p < 0.05$). When looking into the values obtained in the tongue after 30 min it is possible to see that the lowest CFU values were obtained, for streptococci and enterococci, in the presence of the **Chi** mouthwash. In comparison the **Com** mouthwash presented statistically significant ($p < 0.05$) lower reductions of CFU levels. This pattern was also observed in the teeth but with remarkably higher differences in CFU levels between mouthwashes. In the teeth the lowest CFU value obtained, either for enterococci and streptococci, was in the presence of the **Chi** mouthwash and, when comparing these values with those obtained for the **Com** mouthwash, statistically significant differences ($p < 0.05$) were found with CFU levels for the **Chi** mouthwash being significantly lower than those registered in the presence of the **Com**. Furthermore, 100%,

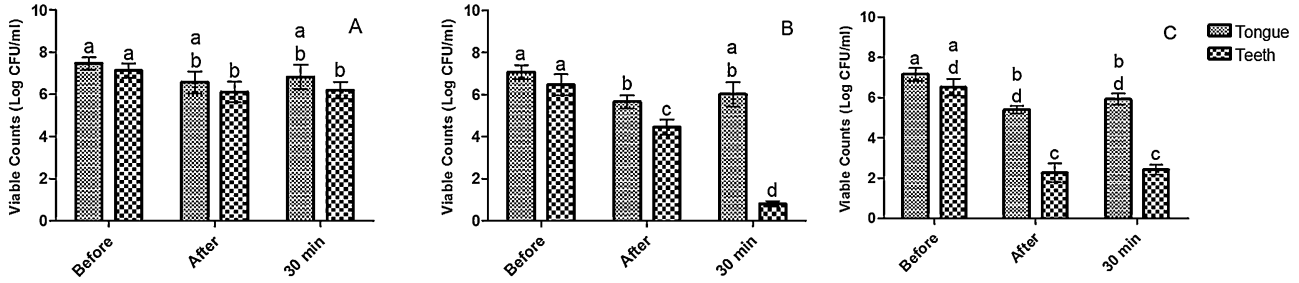

Fig. 4. Effect of rinsing with 15 ml of chitosan mouthwash upon total anaerobic (A), streptococci (B) and enterococci (C) bacterial counts in teeth and tongue in the different sampling times. Different letters represent statistically different results ($p < 0.05$).

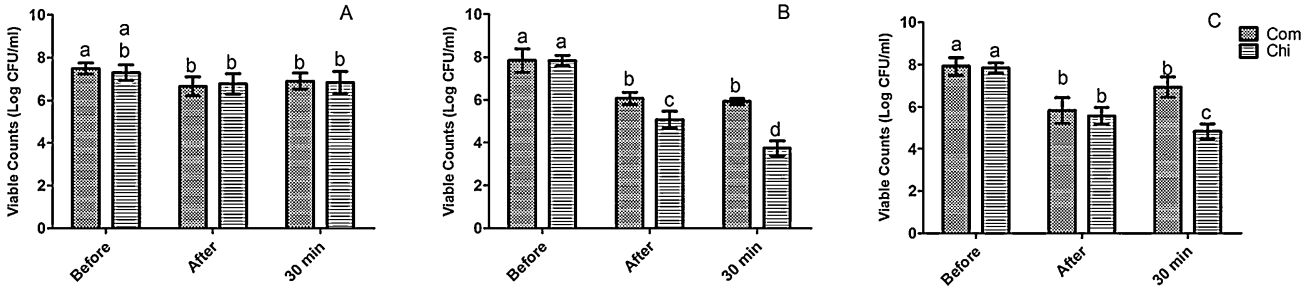

Fig. 5. Comparison of the antimicrobial activity of the chitosan mouthwash ( ) with the chlorhexidine mouthwash ( ) upon total anaerobic (A), streptococci (B) and enterococci (C) bacterial counts in the different sampling times. Different letters represent statistically different results ($p < 0.05$).

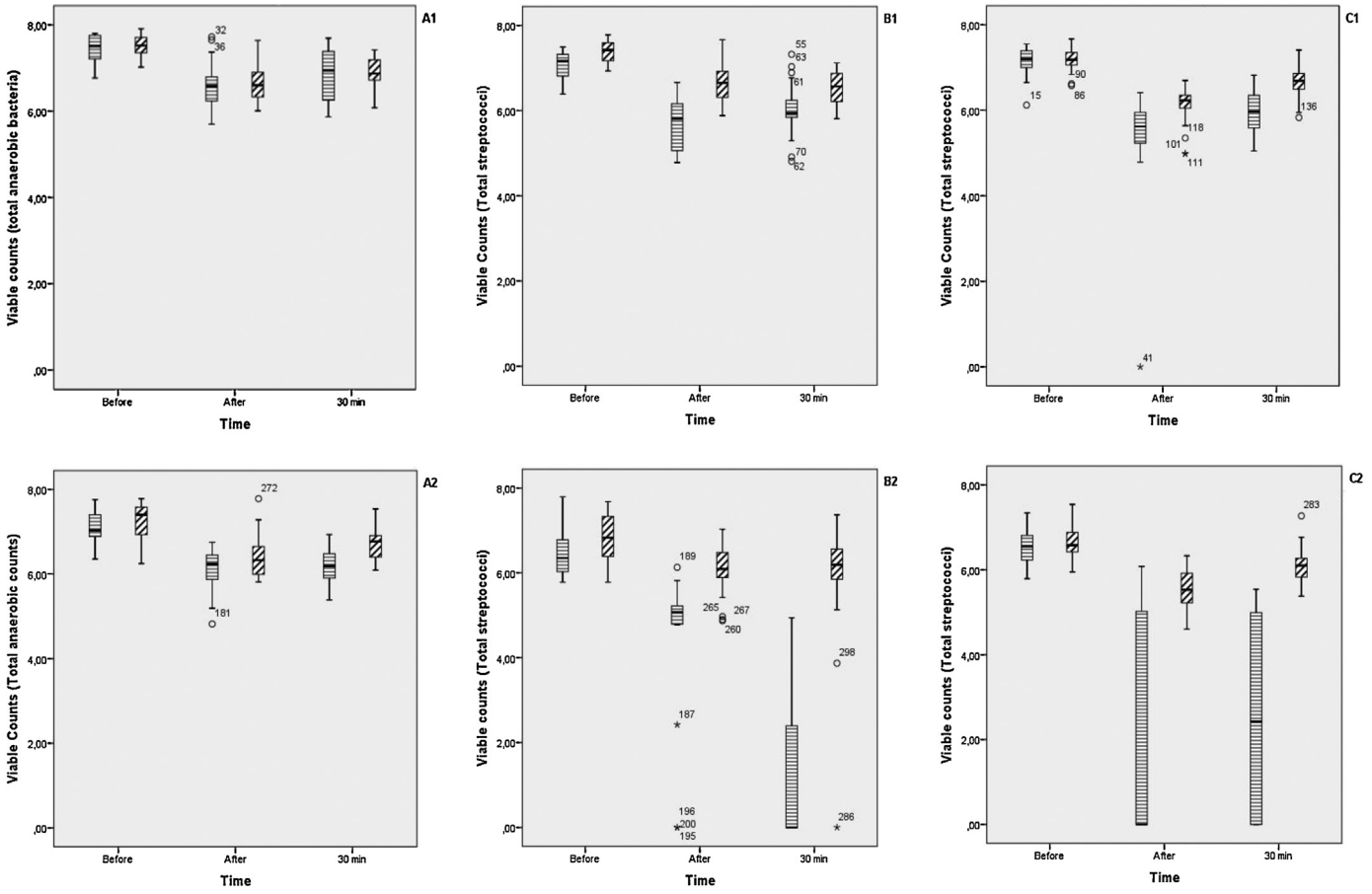

Fig. 6. Distribution of the individual markers in the target population at each sampling point for the two tested mouthwashes. Chi – chitosan mouthwash; Com – commercial mouthwash; (A) – total anaerobic bacteria; (B) total streptococci; (C) total enterococci; (1) tongue; (2) teeth.

and almost 100% for enterococci, of the CFU values obtained after 30 min for streptococci in the presence of **Chi** were under the lowest value registered for the **Com** mouthwash. These values show, once again, that in direct comparison the chitosan mouthwash (**Chi**) had greater activity upon the total anaerobic counts than the chlorhexidine mouthwash (**Com**). Moreover, it is possible to see that, in all sampling sites, CFU values obtained for the **Chi** mouthwash presented a smaller dispersion of values originating a more homogenous, and apparently with high resistance to chlorhexidine, population. On the other hand, for the **Com** mouthwash a wider distribution of results was observed, indicating the existence of distinct and clear levels of susceptibility to chitosan, within the population.

While Fig. 6 provided a better insight into the individual population fluctuations a better understanding of how both mouthwashes influence the dynamics of the oral anaerobic microflora can be gained from Fig. 7. When considering the evolution of the oral microflora in the teeth (Fig. 7A) one can see that while before rinsing there was an even distribution of total anaerobic and streptococci, immediately after rinsing (Fig. 7A2) the majority of the population is constituted by streptococci (as seen by the overlap of data points in top right quadrant of the graph), with this behaviour being observed for both mouthwashes. On the other hand, 30 min after in the presence of the chitosan mouthwash (×) there is a clear drop in total streptococci counts that it is not accompanied by a decrease in total anaerobic counts. This seems to indicate a targeted action by the chitosan mouthwash that was capable of inhibiting the pathogenic marker without causing major reductions in the oral microflora. This behaviour was not observed for the **Com** mouthwash (Δ) where the majority of the population remained to be constituted by streptococci. Regarding the effect of both mouthwashes in the tongue (Fig. 7B) a similar pattern can be observed with rinsing causing a shift in population towards predominance of streptococci. However, and contrary to what was described in the teeth, after 30 min there was no reduction in viable counts registered for either mouthwash.

4. Discussion

Chitosan by itself is a polymer with a well-known antimicrobial activity, with several authors reporting its wide range of activity against fungi and aerobic and anaerobic bacteria, with high biocompatibility and low toxicity, thus being an ideal candidate for product development (Baldrick, 2010; Costa et al., 2012; Fernandes et al., 2008, 2009; Kean & Thanou, 2010; Ong, Wu, Moochhala, Tan, & Lu, 2008; Raafat & Sahl, 2009). However, as for any new compound, one of the main concerns in the application of natural compounds in the food or medical area is its possible toxicological effects. The toxicological assays performed in this study showed that the chitosan mouthwash possessed no toxicological activity and in reality it was less aggressive to V79 cells than commercial mouthwashes. These results come in line with the previous work of Chen and Chung (2012) who reported no cytotoxicity for a water-soluble chitosan rinsing solution and with the work of Arai, Kinumaki, and Fujita (1968) who reported that chitosan has lethal dose LD50 as high as 16 g/kg (higher than for sucrose) when orally administered to mice, as amply discussed by Muzzarelli (1993). Previous work regarding any possible genotoxicological effects of chitosan showed that it

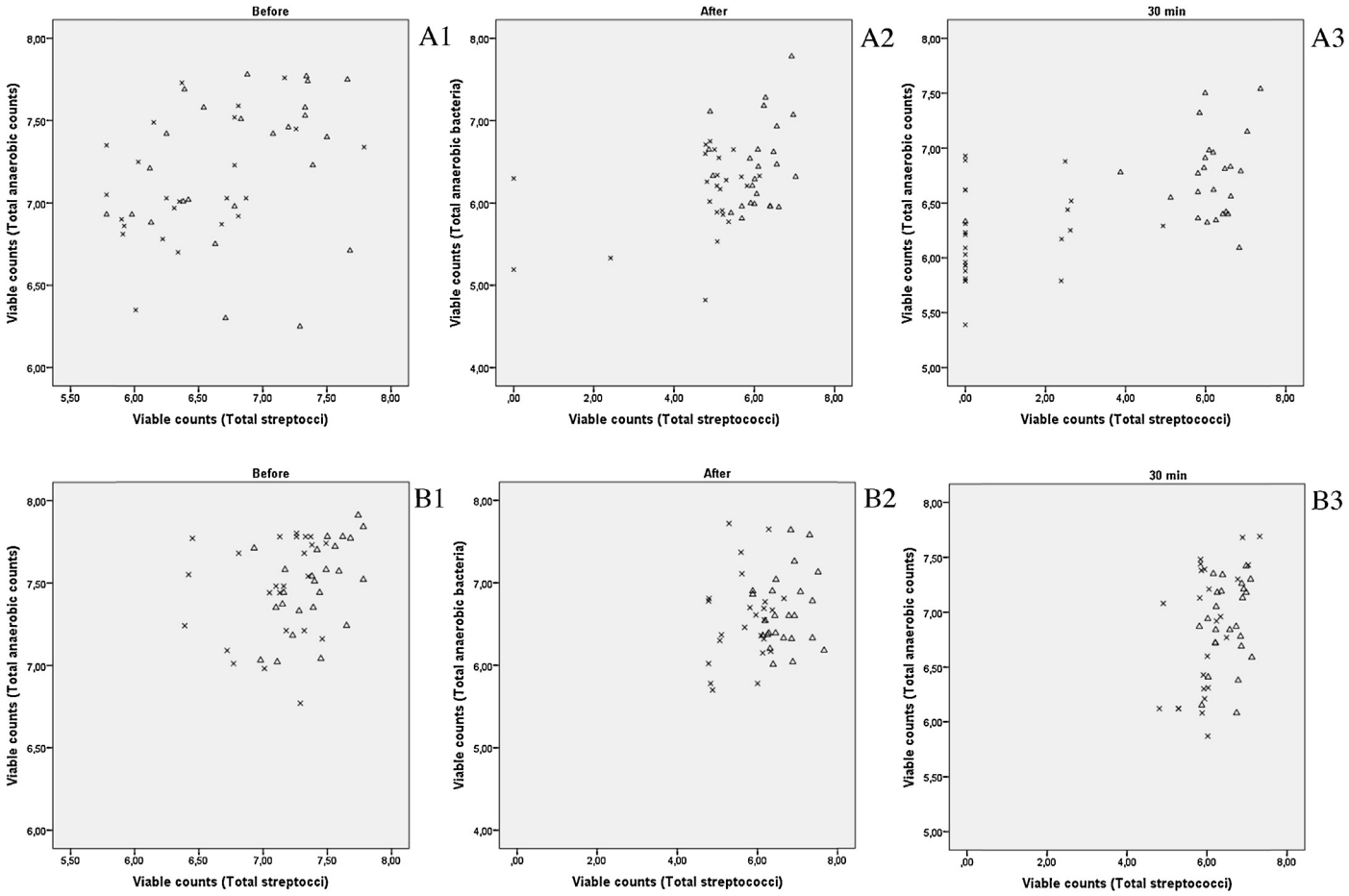

Fig. 7. Relation between total anaerobic bacteria and total streptococci. Δ – commercial mouthwash; × – chitosan mouthwash; (A) teeth; (B) tongue; (1) before rinsing; (2) after rinsing; (3) 30 min after rinsing.

possessed no genotoxic effects (Baldrick, 2010), which comes in line with the results here obtained. Furthermore, Kean and Thanou (2010) reported that any possible toxicological side effects of chitosan are Mw related and were only found in chitosan oligomers. Considering that the chitosan mouthwash developed is HMW and LMW-based those results once again confirm the safety of the developed product.

When regarding the *in vivo* assays our results are supported by those obtained by Sano et al. (2001, 2003), who showed that a phosphorylated chitosan was capable of reducing plaque index and *Streptococcus mutans* counts. Similar results were described by Uraz et al. (2012), who reported that a chitosan mouthwash had a comparable antimicrobial activity to a chlorhexidine digluconate mouthwash, with significant reduction of plaque index and of *S. mutans* and *Candida albicans* counts, and by Chen and Chung (2012), who reported that a water soluble chitosan reduced bacterial counts up to 99%. On the other hand, Vieira (2005) reported that a chitosan mouthwash was not effective on viable count reduction of *S. mutans* and *Lactobacillus* spp., however the mouthwash formulation used in that study possessed a pH value superior to 6, thus constraining one of the main conditions needed for chitosan to be active – acidic pH (Raafat & Sahl, 2009). With chitosan antimicrobial activity being related with the charge of its amino groups and these groups possessing higher positive charges at lower pH and little to none at pHs near the molecule's pK_a (6.3–6.5) (Kong, Chen, Xing, & Park, 2010), it is only natural that the chitosan mouthwash formulation described by Vieira (2005) would not display any antimicrobial activity due to its neutral pH.

When considering the relevance of the pathogenic markers selected, one has to consider that streptococci, and in particular *Streptococcus mutans*, are strongly related to dental caries (Koo, Xiao, Klein, & Jeon, 2010; Wang et al., 2012) and that enterococci are usually associated with root canal diseases (Dahlén, Samuelsson, Molander, & Reit, 2000). As such, the capability to inhibit these microorganisms is of upmost importance.

5. Conclusions

Overall the results validate a stable and active chitosan based mouthwash formulation for human usage. The chitosan mouthwash possesses lower toxicity and higher antimicrobial activity than the commercial mouthwash tested. Furthermore, while it is capable of completely inhibiting the two selected pathogenicity markers (streptococci and enterococci), contrary to the commercial mouthwash, it did not cause major reductions in the viability of the normal oral microflora. As such, the chitosan mouthwash is a safer, valid and viable alternative to the already existing mouthwashes.

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