Evaluation of Two Novel Alcohol-Free Oral Rinses Containing a Biguanide and Botanicals against Ventilator-Associated Pneumonia Pathogens Using an *in vitro* Biofilm Model

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Abstract

The objective of this study was to evaluate the efficacy of two novel alcohol-free oral rinses: polyhexamethylene biguanide - botanical oral rinse (PBOR) and chlorhexidine gluconate - botanical oral rinse (CBOR), against common ventilator-associated pneumonia (VAP) pathogens using in vitro methods and compare their antimicrobial activity to commercially available oral care products viz. Listerine, Scope and Gum. PBOR and CBOR were highly effective in rapid-kill (15s exposure) tests against VAP pathogens. PBOR, CBOR and Gum displayed equivalent prophylactic antimicrobial activity. In vitro artificial-teeth model showed that PBOR and CBOR were significantly effective against S. aureus and P. aeruginosa compared to others (P <0.05). SEM images revealed that PBOR and CBOR show efficacy in inhibiting biofilm forming P. aeruginosa on artificial-teeth surfaces. In conclusion, our in vitro studies demonstrated that PBOR and CBOR are more effective against VAP pathogens compared to commercial mouth rinses and they can also inhibit biofilm formation.

Keywords: Polyhexamethylene biguanide, Chlorhexidine gluconate, Botanical blend, Ventilator-associated pneumonia, Microorganism, Biofilm, Oral rinse, Scanning electron microscopy.

1. Introduction

Ventilator-associated pneumonia (VAP) is one of the major causes of mortality in patients admitted into the intensive care unit (ICU) and occurs 48-72 h after receiving mechanical ventilation or following endotracheal intubation (American Thoracic Society 2005; Kalanuria et al. 2014). Microorganisms generally involved in the pathogenesis of VAP are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Acinetobacter baumannii*, Methicillin-resistant *Staphylococcus aureus*, *Streptococcus* sp., *Klebsiella pneumonia*, *Haemophilus influenza* and *Candida* sp. (Park 2005; Koenig and Truwit 2006). These microbes can colonize in the oral cavity and induce biofilm formation on the teeth of immuno-compromised patients. It has been shown that there is a correlation between microbial colonization in the oropharynx and dental plaque with VAP (Amaral et al. 2009). VAP pathogens are able to replace the microflora of oropharyngeal mucosa during ventilator intubation of ICU patients and enter into the pulmonary epithelium causing inflammation and pneumonia. Thus, the control and prevention of VAP pathogens are crucial for the care of intubated patients.

Endotracheal tubes coated with chlorhexidine and silver salt have shown to prevent microbial colonization of VAP pathogens on their surface in a study using an *in vitro* airway model (Pacheco-Fowler et al. 2004). In fact, frequent cleaning of the oral cavity of intubated patients with 0.2% chlorhexidine gluconate (CHX) containing mouth rinse has been shown to reduce oropharyngeal bacterial colonization. However, it has not been shown to reduce fungal colonization in ICU patients (Postma et al. 2012). Furthermore, as a prophylactic measure for VAP, treatment with 0.12% or 0.2% CHX containing oral care products have been reported to reduce the risk of acquiring VAP (Koeman et al. 2006; Chan et al. 2007; Senol et al. 2007; Labeau et al. 2011; Nicolosi et al. 2014). Despite its documented antimicrobial efficacy, the use of CHX (0.12% or 0.2%) oral rinses has been associated with various adverse effects such as dental calculus formation (Zanatta et al., 2010), irritation of the oral mucosa, changes in sensitivity of the tongue, extrinsic staining of teeth and altered taste perception (Gürgan et al., 2006). Oral rinse containing 0.05% CHX and 0.05% cetylpyridinium chloride (CPC) has been found to be effective in preventing dental plaque formation with reduced side effects (Quirynen et al., 2005). A mouthwash containing 0.12% polyhexamethylene biguanide has also been shown to reduce bacterial counts on the tooth surface and oral mucosa (Rosin et al., 2002). The use of antimicrobial botanical agents as an alternative to CHX containing mouth rinses has increased over recent years.

Our earlier studies showed that synergistic combinations of botanicals at low concentration exhibit a broad spectrum of antimicrobial activity (Baiju and Modak 2007; Baiju et al. 2008). Therefore, we have developed two novel alcohol-free oral rinses comprising of low concentrations of essential oils (thymol and menthol), botanical extract (grapefruit-seed extract) and polyhexamethylene biguanide (PHMB, 0.05%) or chlorhexidine gluconate (CHX, 0.05%). The antimicrobial efficacy of newly developed PHMB-botanical oral rinse (PBOR) and CHX-botanical oral rinse (CBOR) was evaluated by various *in vitro* methods. Additionally, these oral rinses were tested against *Streptococcus mutans*, one of the prevalent bacteria causing periodontal diseases (Contardo et al. 2011). It has also been reported that oral biofilm formation can be prevented by the use of antimicrobial oral care products (Marsh, 2010). Therefore, the antimicrobial potency of PBOR and CBOR in preventing *in vitro* biofilm formation was tested. Thus, the aim of the present study was to evaluate the efficacy of PBOR and CBOR against common VAP pathogens and compare their antimicrobial activity with commercially available oral care products, namely Listerine Cool Mint (Johnson & Johnson), Scope Classic Mouthwash – Original Mint (Procter & Gamble) and Gum (Sunstar Americas, Inc.). Furthermore, efficacy of the mouth rinses to inhibit biofilm formation by *P. aeruginosa* was determined by scanning electron microscopy (SEM) analysis using an *in vitro* artificial tooth model.

2. Materials and methods

2.1 Antimicrobials

Polyhexamethylene biguanide (PHMB) and chlorhexidine gluconate (CHX) were obtained from Lonza Inc. (Allendale, NJ, USA) and Ruger Chemical Co. Inc. (Irvington, NJ, USA) respectively.

2.2 Essential oils and botanical extract

Thymol (extracted from *Thymus vulgaris*) and menthol (extracted from *Mentha arvensis*) were obtained from Symrise Inc. (Teterboro, NJ, USA) and Sigma-Aldrich (St. Louis, MO, USA) respectively. Grapefruit-seed (*Citrus paradisi*) extract was obtained from Natural Sourcing (Oxford, CT, USA).

2.3 Microorganisms and growth conditions

Staphylococcus aureus (ATCC 6538), *Acinetobacter baumannii* (ATCC 19606), *Enterobacter aerogenes* (ATCC 13048), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATTC 11651) and *Streptococcus mutans* (ATCC 25175) were obtained from the American Type Culture Collection (ATCC). Clinical isolate of Methicillin-resistant *Staphylococcus aureus* (MRSA) was obtained from New York-Presbyterian Hospital, New York. Bacterial and *C. albicans* cultures were prepared from stock agar slants in trypticase soy broth (TSB) [BD, Sparks, MD] and Sabouraud dextrose broth (SDB) [BD, Sparks, MD] respectively. *S. mutans* culture was prepared in brain heart infusion broth (BHIB) [BD, Sparks, MD]. After 24 h of incubation at 37°C, all cultures were centrifuged, washed twice with phosphate-buffered saline (PBS) and re-suspended in PBS to a final concentration of 10^8 CFU ml^{-1} for each microorganism.

2.4 Dilution fluid with neutralizers (DFN)

DFN was prepared by addition of 34 g of KH_2PO_4, 3 g of lecithin (Fisher Scientific, Fair Lawn, NJ) and 10 ml of Tween 80 (Sigma-Aldrich, St. Louis, MO) followed by adjusting volume to 1 L with deionized water (pH adjusted to 7.2 prior to autoclaving at 121°C). The effectiveness of using neutralizers in the dilution fluid was validated in our previous studies (Geraldo *et al.* 2008) following the method described by ASTM International (ASTM E1054-08, 2013).

2.5 Oral care products

The active ingredients in PBOR consist of low concentration of essential oils (0.1% thymol and 0.06% menthol) and botanical extract (0.8% grapefruit-seed extract) and PHMB (0.05%). These active ingredients were incorporated in an aqueous oral care base containing natural surfactant, fruit acid and flavouring agent. CBOR contained a similar formulation to PBOR, except PHMB was replaced by CHX (0.05%). The concentrations of other ingredients in PBOR and CBOR are proprietary information. Both of the oral rinses did not contain alcohol in their formulation. In this study, the combination of essential oils and botanical extract is referred to as botanical blend. The commercial oral care products *viz.* Listerine Cool Mint (essential oil containing oral rinse); Scope Classic Mouthwash – Original Mint (0.045% CPC containing oral rinse) and Gum (0.12% CHX containing oral rinse) were obtained from the local pharmacy. Alcohol concentrations in Listerine and Scope are 21.6% and 15% respectively, whereas Gum is alcohol free.

2.6 Antimicrobial activity of biguanide and botanical blend combination

Oral rinses containing botanical blend, PHMB, CHX as well as PHMB or CHX in combination with botanical blend were prepared and evaluated for their rapid antimicrobial activity against *S. aureus* using a rapid-kill method (ASTM E2783-11, 2011). Briefly, in a sterile culture tube, 0.1 ml of 10^8 CFU ml^{-1} bacterial cultures was treated with 0.9 ml of oral rinse for 15 s. The reaction was stopped with the addition of 9 ml of DFN followed by serial dilution with DFN. Appropriate aliquots were then spread on trypticase soy agar (TSA) plates (Fisherbrand, 100X15 mm) and kept overnight at 37°C incubator. The colonies were counted and the log_{10} reduction values were determined with respect to control growth. The aqueous oral care base without synthetic antimicrobials and botanical blend was used as control. The samples were tested in triplicate for each experiment and all experiments were performed three times.

2.7 *In vitro* rapid-kill test (suspension test)

In vitro rapid-kill test was carried out according to ASTM E2783-11 with a minor modification; 10^8 CFU ml^{-1} microbial cultures were prepared in a media containing 50% of bovine serum albumin in order to determine the antimicrobial activity of PBOR, CBOR and other commercial oral rinses in a proteinaceous surrounding. The rest of the procedure was same as described earlier. PBS was used as a control. In this study, an oral care product is considered effective, if the log_{10} reduction exhibits 3.0 or higher.

2.8 Prophylactic activity of oral rinses

In order to determine the prophylactic activity, 0.5 ml of oral rinses or PBS (control) were spread on TSA plate and incubated for 1 h at 37°C. Then, 0.3 ml of 10^4 CFU ml^{-1} microbial cultures was spread on the same plate and incubated overnight at 37°C. The colony counts were determined.

2.9 Antimicrobial activity of oral care products against microbes colonized on agar plate surface

In this experiment, 0.3 ml of 10^5 CFU ml^{-1} microbial cultures were seeded on TSA plates and incubated for 4 h at 37°C in order for microbes to colonize.

Then, 0.5 ml of oral rinse or PBS (control) was added and spread on the surface of the plate. After 1 min, 2.5 ml of DFN was added to inactivate the active ingredients, swirled to cover the whole plate with a glass spreader and collected in a culture tube. Then 2 ml of DFN was added again on the agar plate and the process was repeated once. Finally, the fluid was transferred into the same culture tube and mixed well. After serial dilution with DFN, 0.5 ml of aliquot was spread on TSA plate (brain heart infusion agar plate was used for *S. mutans*) and incubated at 37°C for 24 h. The colony counts on the plates were determined.

2.10 Biofilm formation on artificial tooth surface

Artificial teeth (first molar teeth of same size) collected from Columbia University Dental School, New York, were sterilized with 70% ethanol. They were immersed individually in 2 ml of *P. aeruginosa* (10^7 CFU ml^{-1}) and *S. aureus* (10^7 CFU ml^{-1}) cultures overnight at 37°C incubator. Similarly, teeth were dipped in *C. albicans* culture (10^6 CFU ml^{-1}) for 3 days at 37°C incubator. After incubation, all teeth samples were rinsed twice with sterile normal saline and blotted to dry. The artificial teeth were then treated with 2 ml of oral rinse or PBS (control) for 30 s followed by placement on a filter paper for 1 min to drain out the residual liquid from artificial teeth. Each tooth sample was transferred in a culture tube containing 5 ml of DNF and sonicated in a water bath at 37°C for 30 min. After appropriate dilution, 0.5 ml of aliquot was spread on TSA plate and incubated overnight at 37°C.

2.11 Detection of bacterial biofilm on artificial teeth by scanning electron microscopy (SEM)

Artificial teeth recovered after incubation in *P. aeruginosa* culture (10^7 CFU ml^{-1}) for 24 h were rinsed twice with sterile normal saline. The teeth were then treated with oral care products for 30 s and blotted to dry for 1 min on a filter paper. They were rinsed immediately with PBS twice and immersed into a fixing solution (Karnovsky) at pH 7.2 (Electron Microscopy Sciences, Hatfield, PA) and kept for 24 h at 4°C. Teeth treated with PBS were used as control. After fixing, the teeth samples were washed three times with sodium phosphate buffer (SPB, pH 7.2) for 10 min and post-fixed with a solution of 1% osmium tetroxide for 1 h at 4°C. The teeth were washed three times with SPB and dehydrated in a gradient series of ethanol solutions (10%, 30%, 50%, 70%, 90% and 100%). The dehydration time for each gradient series treatment was 20 min except the 100% ethanol step, which was dehydrated for 30 min. The teeth were then dried using Critical Point Dehydration (Bal-Tec CPD 030) and sputter coated with Au/Pd for 20 s and imaged using FE-SEM (Hitachi S-4700).

2.12 Statistical analysis

Differences between data sets corresponding to each oral care product were analyzed statistically using GraphPad InStat 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Kruskal-Wallis non-parametric test was performed to determine if a significant difference existed between the groups followed by Dunn's multiple comparison tests between groups to compare and establish significance with a threshold set at $P < 0.05$.

3. Results

3.1 Antimicrobial potency of biguanide and botanical blend combination

Oral rinses containing PHMB (0.05%) or CHX (0.05%) were tested individually and in combination with low concentration of essential oils (0.1% thymol and 0.06% menthol) and botanical extract (0.8% grapefruit-seed extract) to determine their rapid antimicrobial efficacy against *S. aureus*. Our results showed that PHMB or CHX or botanical blend (essential oils and botanical extract) individually did not exhibit significant antimicrobial activity against *S. aureus*. However, addition of botanical blend to PHMB or CHX enhanced the antimicrobial potency of both of these agents (Figure 1, $P < 0.05$).

3.2 Rapid antimicrobial efficacy of oral rinses against VAP pathogens

PBOR and CBOR showed rapid antimicrobial efficacy against all bacterial species tested yielding $\log_{10}$ reductions ranging from 4.36 ± 0.28 to 5.36 ± 0.13 and 3.29 ± 0.24 to 5.42 ± 0.87 respectively (Table 1). PBOR was significantly more effective against *S. aureus*, MRSA and *P. aeruginosa* compared to all other commercial oral rinses *viz.* Listerine, Scope and Gum ($P < 0.05$). On the other hand, the antimicrobial efficacy of CBOR was significantly higher against *S. aureus* and *A. baumannii* compared to Gum mouth rinse ($P < 0.05$). Listerine and Scope were effective against all the bacteria except MRSA, while Gum was effective only against *P. aeruginosa* and *E. aerogenes*. In case of *C. albicans*, CBOR exhibited highest antimicrobial efficacy among all other groups.

3.3 Prophylactic effect of oral rinses

The agar plates were treated with oral care products and then challenged with VAP pathogens.

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Both of the PBOR and CBOR showed significantly lower colony counts in the case of *S. aureus*, MRSA and *A. baumannii* compared to Listerine oral rinse (Figure 2A-C, $P < 0.05$). PBOR and CBOR were also more effective against *E. aerogenes* and *P. aeruginosa* compared to Listerine and Scope (Figure 2D and E, $P < 0.05$). All of the oral care products displayed prophylactic activity against *C. albicans* (Figure 2F, $P < 0.05$). However, Gum exhibited equivalent prophylactic activity to PBOR and CBOR against all VAP pathogens tested.

3.4 Antimicrobial activity of oral rinses against microbes colonized on the surface of agar plate

The agar plates seeded with microbes were incubated for 4 h followed by exposure to oral care products for 1 min. This experiment was designed to evaluate the activity of mouth rinses against VAP pathogens that colonize on the teeth and gum surfaces. PBOR and CBOR exhibited higher antimicrobial activity against *S. aureus* and *A. baumannii* compared to Listerine and Gum (Figure 3A and C, $P < 0.05$). They showed higher efficacy against MRSA compared to Listerine (Figure 3B, $P < 0.05$). In case of *E. aerogenes,* PBOR and CBOR significantly inhibited microbial growth compared to all other commercial oral rinses tested (Figure 3D, $P < 0.05$). Listerine and Scope displayed lower activity than PBOR and CBOR against *P. aeruginosa* (Figure 3E, $P < 0.05$). CBOR was found to be more effective than Gum when tested against *C. albicans* (Figure 3F, $P < 0.05$). The five oral rinses were also tested for their antimicrobial activity against periodontal pathogen *S. mutans*. The results showed that PBOR and CBOR displayed a mean $\log_{10}$ growth of 1.64 ± 0.38 and 0.59 ± 0.75 respectively, which was significantly lower than that of Listerine, Scope and Gum mouth rinses (Figure 4, $P < 0.05$).

3.5 Efficacy of oral rinses to prevent biofilm formation on artificial teeth surfaces

The antimicrobial activity of the oral care products on biofilm developed *in vitro* on the surfaces of artificial teeth was determined. PBOR and CBOR were significantly effective in lowering the number of biofilm forming bacteria (*S. aureus* and *P. aeruginosa*) compared to Listerine, Gum and Scope (Fig 5A and B, $P < 0.05$). In case of *C. albicans*, CBOR displayed higher antimicrobial potency than all other commercial oral rinses, whereas PBOR significantly inhibited microbial growth compared to Scope and Gum (Fig 5C, $P < 0.05$)

3.6 Detection of biofilm on artificial teeth by SEM

SEM images revealed that PBOR and CBOR (Figure 6E and F) were highly effective in inhibiting biofilm formation by *P. aeruginosa* on the surfaces of artificial teeth compared to the other commercial oral care products. Gum showed superior activity (Figure 6D) in preventing biofilm formation than Listerine (Figure 6B). Scope was found to be least effective among all other oral rinses tested (Figure 6C).

4. Discussion

Oral rinses containing PHMB or CHX have been demonstrated to have antimicrobial activity against oral pathogens (Rosin et al. 2002; Herrera et al. 2003; Welk et al. 2005; Haffajee et al. 2008). In our study, we evaluated the antimicrobial efficacy of oral rinses containing PHMB or CHX in combination with botanical blend. The results of our *in vitro* study show that PBOR and CBOR oral rinses exhibit superior and sustained antimicrobial activity against VAP pathogens, when compared to the commercially available oral care products *viz.* Listerine, Scope and Gum. Additionally, both oral rinses exhibit antimicrobial potency against the periodontal pathogen *S. mutans*. Antimicrobial composition comprising of CHX and essential oil has been reported to have greater efficacy against biofilm forming cultures of both *Streptococcus mutans* and *Lactobacillus plantarum* than planktonic cultures (Filoche et al. 2005). The rapid-kill test carried out in this study was intended to determine the effect of rinsing the oral cavity with an oral rinse for 15 s and its ability to eradicate VAP pathogens present in oral mucosa. The antimicrobial activity of PBOR and CBOR appears to result from different modes of action of its active ingredients.

For example, PHMB and CHX have been suggested to interact with the bacterial cytoplasmic membrane and disrupt its structural integrity (McDonnell and Russell 1999; Gilbert and Moore 2005). Thymol and menthol act by altering the membrane permeability (Trombetta et al., 2005). Grapefruit-seed extract disrupts bacterial cell membrane by inhibiting cellular enzymatic activities (Heggers et al., 2002). The rapid antimicrobial activity of Listerine and Scope (Table 1) results from the combined action of alcohol and other active ingredients present in these oral care products. Alcohol kills bacteria by denaturing the membrane proteins and penetrating through the bacterial cell wall (McDonnell and Russell, 1999). Prophylactic activity of the oral rinses was evaluated *in vitro* on the surface of agar plate. Lower prophylactic activity of Listerine may be due to the evaporation of alcohol from the surface of agar plate, indicating that alcohol plays an important role in the efficacy of Listerine.

The antimicrobial activity of Scope can be attributed to the action of cetylpyridinium chloride which binds to the bacterial cells (Busscher et al., 2008). On the other hand, cationic biguanide such as CHX present in CBOR, forms a strong complex with proteins of mucous membrane and remains active for long periods after application and provides sustained antimicrobial activity (Adams and Addy, 1994; Lim and Kam, 2008). Since PBOR and CBOR do not contain alcohol, their efficacy is entirely due to the combined action of biguanide (PHMB or CHX) and botanical blend present in the oral rinses. In this study, we have described an *in vitro* model for biofilm formation by *P. aeruginosa* on artificial teeth surfaces. This model was used to assess the effectiveness of oral care products in preventing biofilm formation. PBOR and CBOR showed higher activity against biofilm forming microbes adhered on the surface of artificial tooth compared to commercial mouth rinses. It has been reported that an oral rinse containing 0.12% CHX displays activity against microbial biofilm formed on hydroxyapatite surfaces (Shapiro et al., 2002; Babu and Garcia-Godoy, 2014). Antimicrobial activity of commercially available antiseptic mouth rinses has also been compared using *in vitro* static and flow-through biofilm model systems (Pan et al., 2010). However, there has not been any report on the antimicrobial efficacy of oral rinses on artificial teeth surfaces exposed to bacterial cultures. SEM imaging was also carried out to visualize and characterize *in vitro* biofilms formed by *P. aeruginosa* on the surfaces of artificial teeth treated with mouth rinses. Biofilm inhibition efficacy of oral care products obtained from SEM imaging appears to directly correlate to the results of the microbial adherence using artificial tooth model. Therefore, the *in vitro* artificial teeth biofilm model can be used as a valuable tool for preclinical testing of antimicrobial activity of oral rinses to prevent biofilm formation.

5. Conclusions

The results of our *in vitro* study demonstrates that PBOR and CBOR mouth rinses show superior, rapid and sustained antimicrobial activity against VAP pathogens compared to the commercially available oral care products. PBOR and CBOR also displayed antimicrobial activity against oral pathogen *S. mutans*. Furthermore, these novel oral rinses inhibit *in vitro* bacterial biofilm formation by *P. aeruginosa* on the surfaces of artificial teeth. Thus, PBOR and CBOR alcohol-free oral rinses may be used as an alternative to other routinely used alcohol-containing oral care products which have been reported to influence the development of oral cancer (McCullough and Farah, 2008). Most importantly, PBOR and CBOR may be used as prophylactic oral rinses against VAP pathogens with reduced risk of adverse effects due to the use of lower amount of biguanide (0.05%) compared to 0.12% CHX containing rinse. These studies need to be performed *in vivo* before they can be used routinely and in clinical settings as a prophylaxis for VAP.

Acknowledgements

Conflict of interest

A patent application has been filed by Columbia University on the combination of synthetic antimicrobials and botanicals used in the oral rinses described in the present article. Shanta Modak, Arnab K. Ghosh & Jaishree Vaijanathappa are co-inventors in the patent application. According to the official policy of Columbia University, the inventors receive a percentage of the royalties.

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Table

Table 1. *In vitro* antimicrobial efficacy of oral care products by rapid-kill test (15 s exposure)

Microorganisms	Log_{10} reduction in CFU/ml ± SEM				
	Listerine Cool Mint (L)	Scope Classic (S)	Gum (G)	PHMB Botanical Oral Rinse (PBOR)	CHX Botanical Oral Rinse (CBOR)
Staphylococcus aureus	3.50 ± 0.84	3.36 ± 0.61	1.96 ± 0.49	5.36 ± 0.13a	3.45 ± 0.36b
MRSA	2.66 ± 0.41	2.19 ± 0.39	2.32 ± 0.53	5.02 ± 0.14a	3.29 ± 0.24
Pseudomonas aeruginosa	3.96 ± 0.74	3.60 ± 0.53	4.58 ± 0.59	5.23 ± 0.57a	5.08 ± 1.28c
Enterobacter aerogenes	4.40 ± 0.83	3.44 ± 0.49	4.13 ± 0.71	4.78 ± 0.45c	5.42 ± 0.87c
Acinetobacter baumannii	4.76 ± 1.31	3.86 ± 0.63	1.39 ± 0.30	4.36 ± 0.28b	5.39 ± 0.78bc
Candida albicans	2.63 ± 0.45	0.49 ± 0.08	1.33 ± 0.67	2.78 ± 0.11c	4.65 ± 1.32a

Notes:

CFU, colony forming unit; SEM, standard error of the mean

Microbial growth in control ranges from 5×10^7 to 1×10^8 CFU ml^{-1}

[a]$P \leq 0.05$ PBOR and CBOR *vs.* L, S and G ; [b]$P < 0.05$, PBOR and CBOR and *vs.* G; [c]$P < 0.05$, PBOR and CBOR *vs.* S

An oral care product is considered effective, if the log_{10} reduction exhibits 3.0 or higher.

Figure legends

Figure 1. Antimicrobial activity of biguanide and botanical blend combination against *S. aureus*.

BB, Botanical blend (essential oils + botanical extract); PHMB, polyhexamethylene biguanide; CHX, Chlorhexidine gluconate. [a]$P \leq 0.05$, PHMB + BB and CHX + BB *vs.* control (oral care base); [b]$P < 0.05$, PHMB + BB *vs.* PHMB; [c]$P < 0.05$, CHX + BB *vs.* CHX; [d]$P < 0.05$, PHMB + BB and CHX + BB *vs.* BB; ns : Not significant.

Figure 1
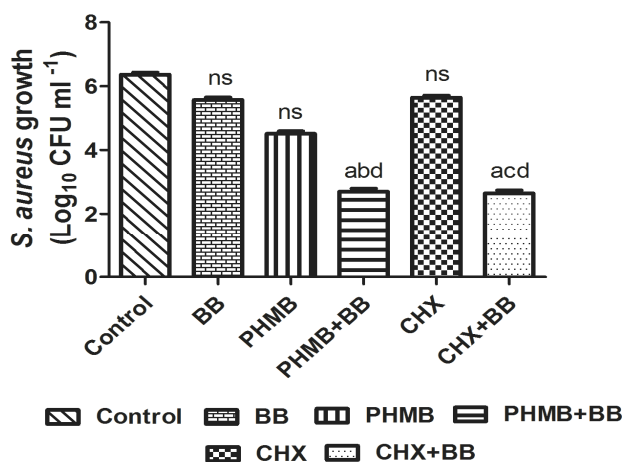
Figure 2. Prophylactic activity of oral rinses against A) *Staphylococcus aureus*, B) Methicillin resistant *Staphylococcus aureus*, C) *Acinetobacter baumannii*, D) *Enterobacter aerogenes*, E) *Pseudomonas aeruginosa* and F) *Candida albicans*. PBS, Phosphate-buffered saline; L, Listerine Cool Mint; S, Scope Classic; G, Gum; PBOR, PHMB-botanical oral rinse and CBOR, CHX-botanical oral rinse. [a]$P \leq 0.05$, L, S, G, PBOR and CBOR *vs.* PBS (control); [b]$P < 0.05$, PBOR and CBOR *vs.* L; [c]$P < 0.05$, PBOR and CBOR *vs.* S; [d]$P < 0.05$, PBOR and CBOR *vs.* G; ns : Not significant.
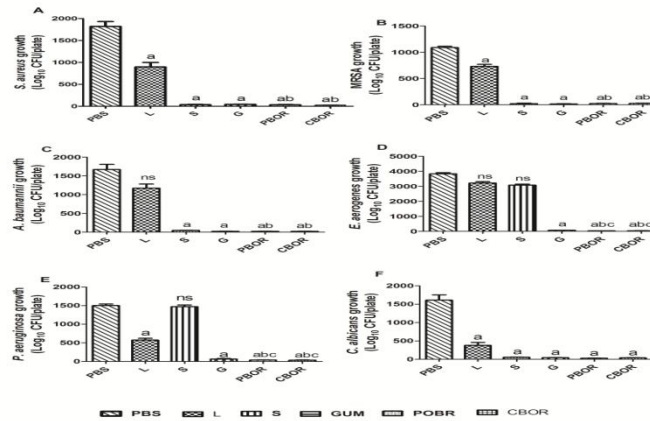
Figure 2

Figure 3. Antimicrobial activity of oral rinses against VAP pathogens colonized on the surface of agar plate: A) *Staphylococcus aureus*, B) Methycillin resistant *Staphylococcus aureus*, C) *Acinetobacter baumannii*, D) *Enterobacter aerogenes*, E) *Pseudomonas aeruginosa* and F) *Candida albicans*. PBS, Phosphate-buffered saline; L, Listerine Cool Mint; S, Scope Classic; G, Gum; PBOR, PHMB-botanical oral rinse and CBOR, CHX-botanical oral rinse. [a]$P \leq 0.05$, L, S, G, PBOR and CBOR *vs.* PBS (control); [b]$P < 0.05$, PBOR and CBOR *vs.* L; [c]$P < 0.05$, PBOR and CBOR *vs.* S; [d]$P < 0.05$, PBOR and CBOR *vs.* G; ns : Not significant.
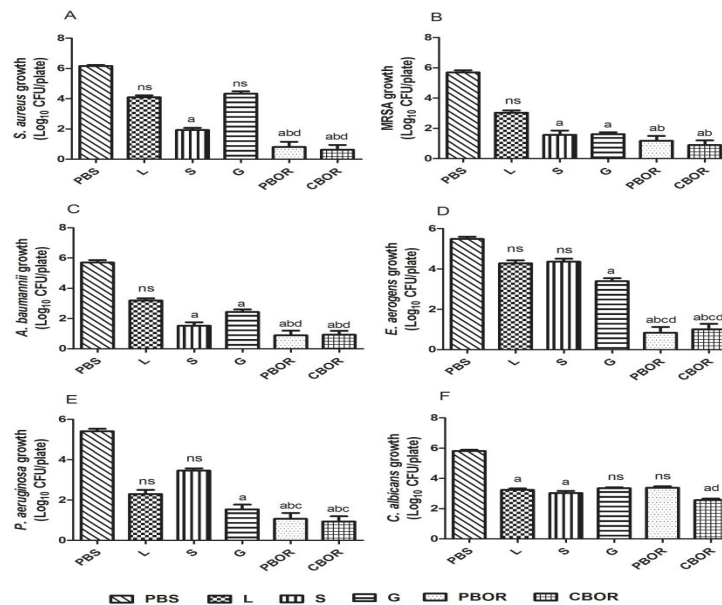
Figure 3

Figure 4. Antibacterial activity of oral rinses against oral pathogen *Streptococcus mutans*. PBS, Phosphate-buffered saline; L, Listerine Cool Mint; S, Scope Classic; G, Gum; PBOR, PHMB-botanical oral rinse and CBOR, CHX-botanical oral rinse. [a]$P \leq 0.05$, L, S, G, PBOR and CBOR *vs.* PBS (control); [b]$P < 0.05$, PBOR and CBOR *vs.* L; [c]$P < 0.05$, PBOR and CBOR *vs.* S; [d]$P < 0.05$, PBOR and CBOR *vs.* G; ns : Not significant.
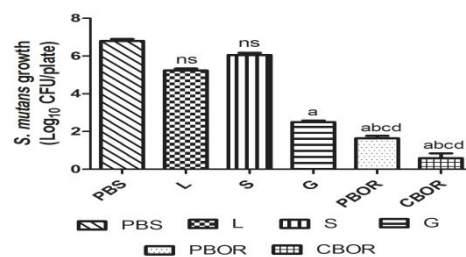
Figure 4

Figure 5. Antibacterial activity of oral rinses against biofilm forming VAP pathogens adhered on the artificial tooth surfaces. PBS, Phosphate-buffered saline; L, Listerine Cool Mint; S, Scope Classic; G, Gum; PBOR, PHMB-botanical oral rinse and CBOR, CHX-botanical oral rinse. [a]$P \leq 0.05$, L, S, G, PBOR and CBOR *vs.* PBS (control); [b]$P < 0.05$, PBOR and CBOR *vs.* L; [c]$P < 0.05$, PBOR and CBOR *vs.* S; [d]$P < 0.05$, PBOR and CBOR *vs.* G; ns : Not significant.
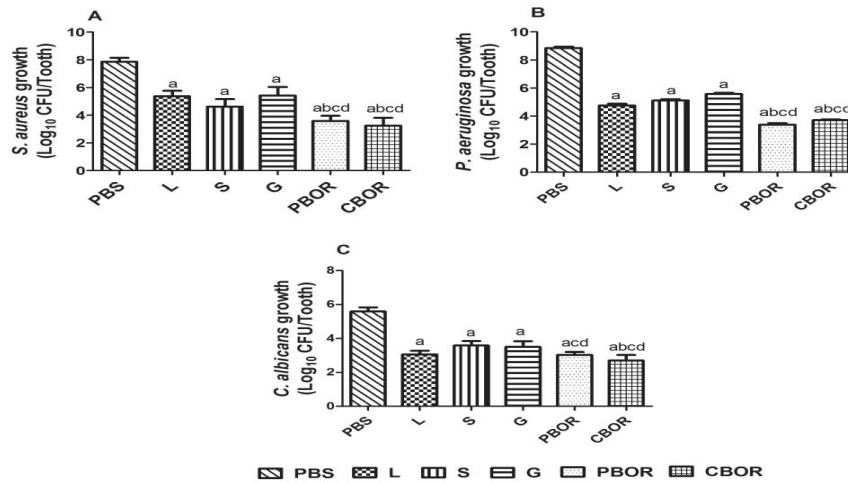
Figure 5

Figure 6. SEM images of *P. aeruginosa* biofilm on artificial teeth treated with oral rinses. (A) Phosphate-buffered saline [Control], (B) Listerine Cool Mint, (C) Scope Classic, (D) Gum, (E) PHMB-botanical oral rinse and (F) CHX-botanical oral rinse. Bacterial cells were arranged either as individual cells (white arrows) or as multicellular aggregates (black arrows). (G) inset indicates artificial teeth prior to bacterial inoculation and oral rinse treatment.

Figure 6