Int. J. Odontostomat.,
15(4):908-914, 2021.

Antibacterial Effect of Commercial Mouthwashes on *Streptococcus mutans*: An *in vitro* study

Efecto Antibacteriano de Colutorios Comerciales en la Proliferación de *Streptococcus mutans*: Un Estudio *in vitro*

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OPORTO, G. H.; RODRÍGUEZ-NIKLITSCHEK, C. & CHUHUAICURA, P. Antibacterial effect of commercial mouthwashes on Streptococcus mutans: An *in vitro* study. *Int. J. Odontostomat., 15(4)*:908-914, 2021.

ABSTRACT: The aim of this study was to compare the antibacterial effect of eight different commercial (MWs) on two *Streptococcus mutans* (SM) strains by using the agar well diffusion method. Eight commercial MWs were selected, all of them combined several ingredients in different concentrations, the main active ingredients were: Chlorhexidine gluconate, cetylpyridinium chloride, sodium fluoride, zinc lactate, vitamin B5 and super-oxidized water. The SM strains were extracted from Cultiloops® and incubated for 24 hours at 37 °C. The antimicrobial activity was evaluated using the agar well diffusion method. The inhibition zones were measured using an electronic digital caliper. The R© software was used to perform the statistical analysis using Kruskall-Wallis test and Dunn's multiple comparisons test. Seven commercial formulas demonstrated inhibitory effect over both SM strains. Only the MW containing super-oxidized water did not exhibit antibacterial activity. Higher inhibitory effect was observed in the chlorhexidine gluconate formula (27.38 ± 0.98 mm and 31.52 ± 0.64 mm). No statistically significant differences were observed when comparing formulas containing chlorhexidine gluconate in combination with other active ingredients. Seven MWs showed antibacterial activity except super-oxidized water formula. MWs containing chlorhexidine gluconate demonstrated the best effect against SM. However, no statistically significant differences were found when comparing formulas using exclusively chlorhexidine gluconate or combined with other antiseptics. Future research must be performed, focused on developing new MWs with similar antibacterial effects to chlorhexidine, but free of side effects, particularly in long-term treatments

KEY WORDS: mouthwash, mouthrinse, chlorhexidine gluconate, *Streptococcus mutans*, antibacterial, antimicrobial.

INTRODUCTION

The oral cavity is colonized by a taxonomically complex diversity of microorganisms (i.e. bacteria, fungi, protozoa and archaea) embedded in a matrix of polysaccharide material attached to tooth surfaces known as dental biofilm (Marsh, 2010; Ardizzoni *et al*., 2018). In a symbiotic environment, the oral microbiota cohabits with the host and has specific metabolic and immunological functions (Marsh). However, the presence of risk factors and diseases may break the equilibrium of the oral ecosystem, leading to a dysbiosis. (Franca *et al*., 2014; Kilian *et al*., 2016; Samaranayake & Matsubara, 2017).

Over the years, different hypotheses regarding dental caries etiology have been investigated (Aas *et al*., 2008; Fragkou *et al*., 2017) where studies support that dental biofilm formation has an important role in the development of this disease (Zanela *et al*., 2002). Frequent sugar exposure leads to modifications in the oral microbiota, providing the environmental conditions

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Recibido : 2021-04-15 Aceptado: 2021-08-28

to induce the dominance of acidogenic/aciduric groups of bacteria such as streptococci, lactobacilli and actinomyces (Aas *et al*.; Fragkou *et al*.). The perpetuation of the dysbiosis could cause tooth demineralization and dental caries.

Streptococcus mutans (SM) is a gram positive bacterium and one of the most common pathogens in cariogenic biofilm (Zanela *et al*.). Previous studies have demonstrated an association between SM and the initial phases of dental caries due to its early colonization of the tooth surface, sugar metabolism and polysaccharide production for bacterial adhesion (Zanela *et al*.; Baena-Monroy *et al*., 2005; Marsh). Among the caries preventive approaches, mechanical removal of biofilm is an important intervention to prevent the onset and progression of caries (Okada *et al*., 2005). However, in susceptible subjects with reported difficulties to perform a proper mechanical removal of dental biofilm, the use of chemotherapeutic agents is beneficial and highly recommended, because these agents can reach difficult-to-clean areas (interproximal surfaces) and could reduce the microorganisms growth (Quintas *et al*., 2015).

Mouthwashes (MWs) are chemical agents that inhibit the growth, colonization and metabolic activity of cariogenic microorganisms and are broadly used as a complementary method controlling dental biofilm (Singh *et al*., 2013). Among a wide range of active ingredients such as chlorhexidine gluconate (CHX), cetylpyridinium chloride (CPC) and sodium fluoride (NaF) are frequently used in the formulations and may contain different combinations and concentration of ingredients (Anita *et al*., 2015). Regardless of their components, most of MWs advertisements declare, among their properties, antiseptic effect. However, there are chemotherapeutic agents lacking sufficient scientific evidence to support their antibacterial properties. Based on the exposed antecedents, it is interesting to know the antimicrobial properties of the MWs available on the market. Thus, the purpose of this study was to evaluate the antimicrobial activity of eight commercial MWs against SM

MATERIAL AND METHOD

The Institutional Ethics Committee of the Universidad de La Frontera approved the study protocol (075_17) used in this research. Procedures were performed following the guidelines of the Clinical and Laboratory Standards Institute (Balouiri *et al*., 2016).

Bacterial strains and culture conditions. Two SM strains were used in this study (ATCC® 25175 / ATCC® 35668 Culti-loopsTM ,Thermo Fisher Scientific TM). Bacteria were re-hydrated with 500 μL of brain heart infusion broth. Then, 100 μL were extracted and deposited in a trypticase soy agar with 5 % sheep blood. The plate was then incubated anaerobically at 37 °C for 48 h using the BD GaspPakTM EZ container system. The colonies obtained were transferred to a tube containing tryptic soy broth and adjusted to 0.5 McFarland turbidity standard (equivalent to 1.5 x 10 8 CFU / mL) (Shafiee *et al*., 2016).

Selected Mouthwashes. Eight commercial MWs were selected and coded according to their active ingredients. Seven of them combined several ingredients in different concentrations (Table I): four MWs had CHX, five contained CPC, three contained NaF, two had zinc lactate (ZL) and one contained superoxidized water (SOW). Physiological saline solution was the negative control (NC).

Inhibition zone test. Antibacterial activity was assessed using the agar well diffusion method (Singh *et al*.). An inoculum of the tested microorganism adjusted to 0.5 McFarland turbidity standard was streaked over Müeller-Hinton agar plates with sterile laboratory swabs. Then, four equidistant wells with a diameter of 9.2 mm were made aseptically. Afterwards, a volume of 130 μL of each MW (Table I) was deposited into the well and incubated for 48 h at 37 °C in an anaerobic environment (Fig. 1). This procedure was

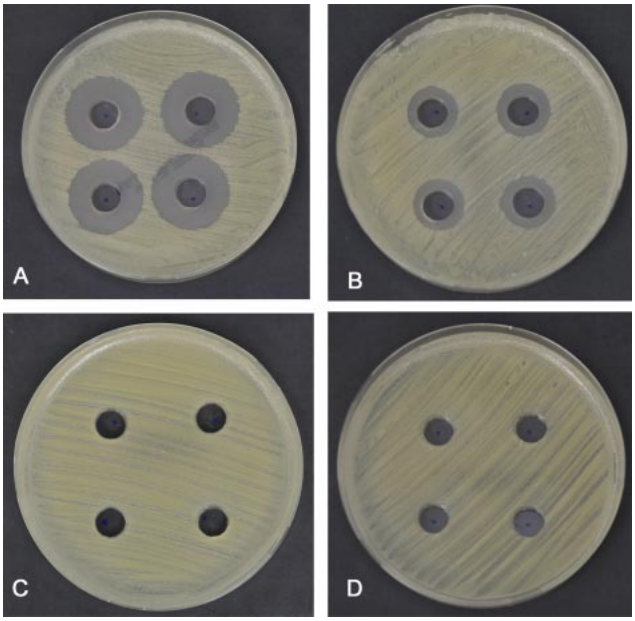

Fig. 1. Inhibition zones observed in agar plaques tested.A) CHX B) CPCNaF (a) C) SOW/NaCl D) NC.

Table I. Chemical composition of mouthwashes (MWs) tested.

MW Code	Compounds (Formula)	Concentration (%)
CHX	Chlorhexidine gluconate	0.12
CHX/NaF	Chlorhexidine gluconate	0.12
	Sodium Fluoride (NaF)	0.05
	Sodium saccharin	0.06
CHX/CPC/ZL	Chlorhexidine gluconate	0.05
	Cetylpyridinium chloride (CPC)	0.05
	Zinc lactate (ZL)	0.14
CHX/CPC	Chlorhexidine gluconate	0.12
	Cetylpyridinium chloride	0.05
VB5/CPC/ZL	Vitamin B5 (Panthenol) (VB5)	0.50
	Cetylpyridinium chloride	0.05
	Zinc lactate	0.14
CPC/NaF (a)	Permethol	0.10
	Cetylpyridinium chloride	0.075
	Sodium Fluoride	0.05
CPC/NaF (b)	Cetylpyridinium chloride	0.05
	Sodium Fluoride	0.05
SOW/NaCl	Super-oxidized water (SOW) (100 mL)	
	Sodium chloride (NaCl) > 1mg	0.001
	Hypochlorous acid (HOCl) > 0.8mg	0.0008
	Sodium hypochlorite (NaOCl) >0.2mg	0.0002
NC	Physiological saline solution	0.9

NC=Negative Control

performed in duplicate. Inhibition zones were measured in millimeters using an electronic digital caliper (RedLine® Tools, Plymouth, MN, USA).

Statistical analysis. The statistics software R (R Core Team, Vienna, Austria) was used for the statistical analysis, using the Kruskal-Wallis test and Dunn's multiple comparisons test (post hoc). p-values less than 0.05 were considered statistically significant.

RESULTS

Tested MWs exhibited an inhibitory effect over SM except SOW/NaCl (Table II). In fact, no statistically significant differences were found between SOW/NaCl and NC. In both SM strains, significant differences ($p < 0.0001$) were observed between CHX and CPC/NaF (a) as well as when comparing the inhibition zones of CHX vs. SOW/NaCl and CHX/CPC/ZL vs. SOW/NaCl. No significant differences were observed among CHX/NaF, CHX, CHX/CPC and CHX/CPC/ZL. The largest inhibition zone was found when using CHX in SM ATCC 35668 plates (Fig. 2), which was slightly higher than CHX/CPC, although no significant differences were found. Nevertheless, differences were detected when comparing CHX vs. vitamin B5 (VB5)-CPC-ZL ($p < 0.0001$).

DISCUSSION

Today practitioners and patients have a wide array of MWs with different active ingredients and concentrations. Among them, CHX is considered the gold standard due to its proven antibacterial efficacy (Tang *et al*., 2015). Its cationic nature is included among the molecular CHX properties, which allows binding to the bacterial wall. Moreover, depending on the therapeutic indication, CHX MWs could be used at different concentrations as a bacteriostatic or bactericidal agent (Gunsolley, 2006). The results of this research showed that larger inhibition zones were found in MWs containing CHX as the active ingredient, even combined with other compounds such as CPC, NaF or ZL. These results support previous reports regarding its efficacy (Coelho *et al*., 2017). However, side effects such as tooth discoloration, alteration in taste perception and oral mucosal erosion have been reported, especially in its long-term use (Tsourounakis *et al*., 2013; Coelho *et al*.). Thus, patients should avoid using it as a MW for longer than four weeks. Some *in vitro* studies also reported that may be potentially toxic to the host's cells (de Oliveira *et al*., 2018).

CPC is a quaternary ammonium compound with broad-spectrum antibacterial activity, which disrupts the

Table II. Streptococcus mutans (SM) strains - mean values of inhibition zones in millimeters (mm) and statistical difference.

MW	SM	Statistical difference	SM	Statistical difference
Code	ATCC 25175	(p < 0.0001)	ATCC 35668	(p < 0.0001)
CHX/NaF	24.50 ±1.19	_ SOW/NaCl _ NC	25.36 ± 0.55	_ SOW/NaCl _ NC
CHX	27.38 ± 0.98	_ CPC/NaF (a) _ SOW/NaCl _ NC	31.52 ± 0.64	_ VB5_CPC_ZL _ CPC/NaF (a) _ SOW/NaCl _ NC
CHX/CPC/ZL	23.13 ±0.64	_ SOW/NaCl _ NC	24.93 ± 1.07	_ SOW/NaCl _ NC
CHX/CPC	27.38 ±0.91	_ CPC/NaF (a) _ SOW/NaCl _ NC	30.31 ± 1,01	_ CPC/NaF (a) _ SOW/NaCl _ NC
VB5/CPC/ZL	18.25 ±0.88		16.27 ± 0.55	
CPC/NaF (a)	16.50 ±0.53		15.29 ± 0.30	
CPC/NaF (b)	18.38 ±0.91		16.52 ± 0.35	
SOW/NaCl	9.2		9.2	
NC	9.2		9.2	

CHX= Chlorhexidine gluconate, CHX/NaF= Chlorhexidine gluconate + sodium fluoride, CHX/CPC= Chlorhexidine gluconate + cetylpyridinium chloride, CHX/CPC/ZL= Chlorhexidine gluconate + cetylpyridinium chloride + zinc lactate, VB5/CPC/ZL = Vitamin B5 (Panthenol) + cetylpyridinium chloride + zinc lactate, CPC/NaF (a) = Cetylpyridinium chloride (0.075 %) + sodium fluoride CPC/NaF (b) = Cetylpyridinium chloride (0.05 %) + sodium fluoride SOW/NaCl= Super-oxidided water + sodium chloride. NC=Negative Control

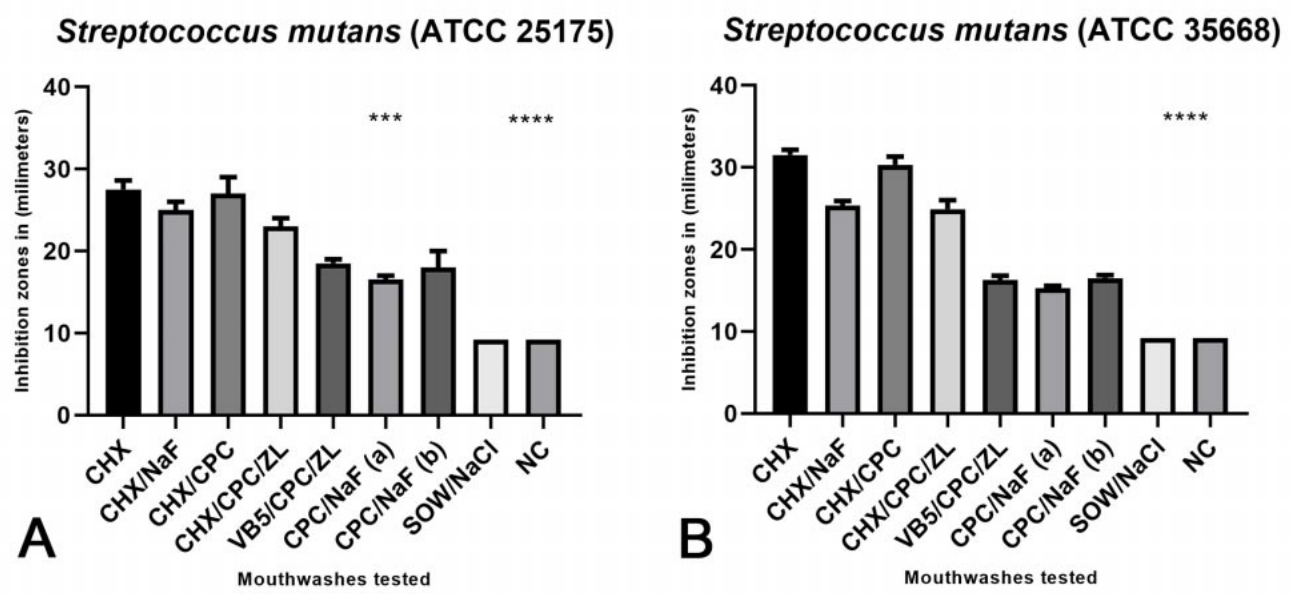

Fig. 2. Inhibition zones (measured in millimeters) in both Streptococcus mutans (SM) strains. A) SM ATCC® 25175 B) SM ATCC® 35668.

permeability of the cell membrane, inducing leakage of intracellular components until cell death (Watanabe *et al*., 2015). Yang *et al*. (2015) evaluated the effect of commercial MWs on the reduction of SM colonies, and a significant antibacterial activity of CPC 0.05 % was determined. Other studies havereported that short-term treatments with various concentrations of CPC (0.025 % – 0.1 %) could reduce the cariogenic biofilm accumulation, especially in the early stages of SM biofilm formation. However, when comparing the antimicrobial effect of CHX 0.2 % and CPC 0.05 % in mature SM biofilm, it was found that CHX was more effective at reducing the colony forming units of SM (Pandit *et al*., 2015). Five MWs formulas containing CPC were tested, including concentrations between 0.075 % and 0.05 %. The best performance of CPC

was detected when it was used in combination with CHX (inhibition zones were 27.38 ±0.9 mm over ATCC® 25175 and 30.31 ± 1.01mm over ATCC® 35668) in both SM strains. Furthermore, CHX/CPC also showed better results (27.38 ±0.91 and 30.31 ± 1.01) than CPC/NaF (a) (16.50 ±0.53 and 15.29 ± 0.30), an effect which may be attributed to differences in their composition (CHX effect). The meta-analysis carried out by Gunsolley found a significant antiplaque effect of CHX 0.12 %. However, products containing a varied concentration of CPC (4.5 – 7 %) showed inconsistent results and significant heterogeneity among studies (Gunsolley).

Fluoride MWs have been used in preventive treatments against dental caries in children for decades and their effectiveness has been extensively investigated and proven (Ripa, 1991; Marinho *et al*., 2016). NaF is the most common fluoride salt, and it is combined with CHX or CPC to improve its antibacterial activity. However, results on this issue are inconclusive. Latimer *et al*. (2015) compared the effect of CPC 0.075 % and CPC 0.075 % combined with NaF. In this study, no influence was identified on the antibacterial and anti-biofilm potency of CPC containing NaF. On the other hand, Yang *et al*. observed significant antibacterial effects in MWs formulas that combined 0.02 % NaF and 0.05 % CPC. In our study, three MWs containing NaF 0.05 % were included, and even though differences in inhibition zones were observed (higher for CHX/NaF and lowest for CPC/NaF (a), Table II), these differences were not statistically significant in the antibacterial effect.

ZL has been used in oral hygiene products due to its antimicrobial activity and for reducing volatile sulfur compounds that could be associated with halitosis. Srisilapanan *et al*. (2019) compared the effect of 0.14 % of ZL against a placebo to reduce oral malodor. The results suggest that zinc salts have a potential effect in reducing malodor (Srisilapanan *et al*.). Yet the scientific literature is inconclusive regarding the long-term effect of these products on oral halitosis (Roldán *et al*., 2003). In our study, two MWs containing ZL were compared (VB5/CPC/ZL and CHX/CPC/ZL); however, no statistical significance were exhibited between them in their antibacterial effect.

Reactive species such as SOW have also been studied for antimicrobial activity. It is obtained by exposing sodium chloride (NaCl) through a semipermeable membrane and finally, producing oxychlorine ions using electrolysis. The formula SOW/NaCl is defined as a broad spectrum antimicrobial that denatures the microbial wall proteins with no harm to human cells (Yahagi *et al*., 2000). The use of SOW is indicated in infectious disease treatment, including skin abnormalities or ulcers, and may be associated with the wound healing process (Yahagi *et al*.). In dentistry, it has been used in endodontics, for root canal irrigation (Shimizu *et al*., 1994), in periodontics, for irrigation of the gingival pocket and in treatment of oropharyngeal lesions (Hata *et al*., 1996). According to our results, the formula SOW/NaCl was the only MW tested that did not exhibit any antibacterial effect against SM. In fact, no statistical difference was found when comparing SOW/NaCl and negative control (NC) of saline solution in the two SM strains. Some studies have described the antimicrobial properties of electrolyzed water as fluctuating depending on the oxidation-reduction potential, pH solution (neutral, acidic or alkaline) and chlorine concentration (Len *et al*., 2000; Landa-Solis *et al*., 2005). This background may explain the variability in the behavior of the SOW, especially in the antibacterial effect on oral bacteria. Although this *in vitro* study has limitations, the results support that CHX had the best antibacterial effect alone or in combination to other active ingredients. However, clinical studies are needed to determine its effect in oral biofilm.

CONCLUSION

According to the results, only the MW based on SOW/NaCl did not show any antibacterial effect, whereas the formulas containing CHX had the strongest effect. Interestingly, no statistically significant differences were found when comparing MWs using exclusively CHX orCHX combined with other antiseptics. To date, CHX remains the most effective chemotherapeutic agent used against SM, but still remains unknown the effect of its long-term use in microbial cells and oral tissues. Thus, an alternative MW with similar antibacterial effect but with no side effects is needed, particularly for use in long-term therapies.

ACKNOWLEDGMENTS

PC would like to thank the National Agency for Research and Development (ANID) / Scholarship Program / BECA MAGISTER NACIONAL/2018-22182175. Also, this study was partially supported by

the Dirección de Investigación (DIUFRO), Universidad de La Frontera, Temuco, Chile (DI19-0040, DI17-0101 and DI20-0152) and the Dirección de Investigación y Creación Artística, Universidad Mayor, Chile (Fondo Start Up-2017 (OI-101166)).

OPORTO, G. H.; RODRÍGUEZ-NIKLITSCHEK, C. & CHUHUAICURA, P. Efecto antibacteriano de colutorios comerciales en la proliferación de *Streptococcus mutans*: Un estudio *in vitro*. *Int. J. Odontostomat., 15(4)*:908-914, 2021.

RESUMEN: El objetivo de este estudio fue comparar el efecto antibacteriano de ocho colutorios comerciales en la proliferación de dos cepas de *Streptococcus mutans* (SM) mediante el método de difusión de pozos de agar. Se seleccionaron ocho colutorios comerciales, todos ellos combinados con varios ingredientes en diferentes concentraciones, los principales ingredientes activos fueron: gluconato de clorhexidina, cloruro de cetilpiridinio, fluoruro de sodio, lactato de zinc, vitamina B5 y agua superoxidada. Las cepas SM se extrajeron de Cultiloops® y se incubaron durante 24 horas a 37 ° C. La actividad antimicrobiana se evaluó mediante el método de difusión de placa de agar. Las zonas de inhibición se midieron utilizando un calibre digital electrónico. Se utilizó el software R © para realizar el análisis estadístico mediante la prueba de Kruskall-Wallis y la prueba de comparaciones múltiples de Dunn. Siete fórmulas comerciales demostraron efecto inhibidor sobre ambas cepas SM. Solo el colutorio que contenía agua superoxidada no mostró actividad antibacteriana. Se observó un mayor efecto inhibidor en las fórmulas congluconato de clorhexidina (27,38 ± 0,98 mm y 31,52 ± 0,64 mm). No se observaron diferencias estadísticamente significativas al comparar fórmulas que contienen gluconato de clorhexidina en combinación con otros ingredientes activos. Siete MW mostraron actividad antibacteriana excepto la fórmula de agua superoxidada. Los colutorios que contienen gluconato de clorhexidina mostraron el mejor efecto contra SM. Sin embargo, no se encontraron diferencias estadísticamente significativas al comparar fórmulas que combinaron con otros principios activos. Se deben realizar investigaciones, enfocadas en el desarrollo de nuevos colutorios con efectos antibacterianos similares a la clorhexidina, pero libres de efectos secundarios, particularmente en tratamientos a largo plazo.

PALABRAS CLAVE: enjuague bucal, enjuague bucal, gluconato de clorhexidina, ***Streptococcus mutans*****, antibacteriano, antimicrobiano.**

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