



Sports mouthguards: Contamination, roughness, and chlorhexidine for disinfection – A randomized clinical trial

Yuri Jivago Silva Ribeiro¹, Renata Zoraida Rizental Delgado¹, Francisco Wanderley Garcia Paula-Silva¹, Belen Rematal-Valdes², Magda Gomes Feres², Regina Guenka Palma-Dibb³, Juliana Jendiroba Faraoni³, Raquel Assed Bezerra Segato¹, Lea Assed Bezerra da Silva¹, Alexandra Mussolino de Queiroz¹, Paulo Nelson Filho¹.

The aim of the present *in vivo* study was to evaluate the bacterial contamination of sports mouthguards, surface roughness, and the efficacy of chlorhexidine gluconate spray in the disinfection of these devices. A randomized, blinded cross-over clinical trial was performed with twenty 9 to 13 years old children who practiced martial arts and participated in all phases of the study. They were instructed to wear mouthguards 3 alternated days a week for 1 hour and, after use, to spray sterile tap water or chlorhexidine 0.12%. The mouthguards were analyzed by MTT assay, Checkerboard DNA–DNA hybridization, and confocal laser microscopy prior and after use for 2 weeks. Data were analyzed by Wilcoxon and t-Student, and Pearson correlation tests, with 5% significance level. Were observed that mouthguards of the control group were more contaminated with cariogenic microorganisms than those of the chlorhexidine group ($p < 0.05$). The mouthguards use of spray of chlorhexidine reduced significantly the bacteria contamination compared with control group ($p = 0.007$). The surface roughness of the mouthguards increased significantly after use, irrespective of application of chlorhexidine spray. A moderate correlation ($r = 0.59$) was observed between surface roughness and the cariogenic microorganism's contamination only for control group. Sports mouthguards had intense microbial contamination and increased surface roughness after its use. The use of chlorhexidine spray was effective for reducing the mouthguards contamination used by children.

Introduction

One billion people worldwide have a history of dental trauma (1), demonstrating that its occurrence is extremely frequent. It is also known that orofacial injuries are closely linked to sports practice and their outcomes can have physical, psychological, and socioeconomic repercussions (2,3). Most sports present a high risk for traumatic injuries, especially those that may result in falls and collisions (3). American associations and academies recommend the use of mouthguards during sports to minimize the effect of trauma on deciduous and permanent dentition (4,5).

Sports mouthguards are made using silicone, ethylene vinyl acetate (EVA), and other porous polymers, which can be colonized by microorganisms (6). The oral cavity contains several niches with distinct microbial flora, including bacteria, fungi, and viruses, forming a complex community of microorganisms that can be associated with systemic diseases (7). According to this, sports mouthguards can be considered as additional sources of contamination and transmission of diseases since the presence of porous structure that can favor the accumulation of microorganisms (6,8). Clinicians have the responsibility to advise and perform the mouthguard care to improve the longevity (9). Storing the mouthguard in a dry and ventilated place associated with the hygiene after use can improve their durability (9).

To evaluate the microbial contamination of oral devices and to verify the effectiveness of different antiseptic solutions (10), microbiological culture techniques (11) and molecular biology techniques (10,12) have been used. However, the bacterial contamination profile of sports mouthguards, as well as the effectiveness of 0.12% chlorhexidine spray in reducing dental biofilm (13), has not been evaluated by cell viability enzymatic colorimetric assay (MTT) and Checkerboard DNA–DNA hybridization

¹ Department of Pediatric Dentistry, School of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil.

² Professor of Periodontology, Department of Periodontology – Division of Dentistry Research, University of Guarulhos, Guarulhos, São Paulo, Brazil

³ Department of Restorative Dentistry, School of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil.

Correspondence: Dra. Renata Zoraida Rizental Delgado; Department of Pediatric Dentistry, School of Dentistry of Ribeirão Preto, University of São Paulo – USP Avenida do Café s/n, Monte Alegre, 14040-904, Ribeirão Preto, SP, Brazil. Phone: +55-16-3315-4057. Fax: +55-16-3633-0999 / E-mail: renata.rizental@gmail.com

Key Words: sports mouthguards, microbiology, surface roughness, chlorhexidine, disinfection.

(CDDH). The aim of this randomized, blinded cross-over clinical trial was to evaluate the bacterial contamination and surface roughness of sports mouthguards before and after use, to verify the efficacy of 0.12% chlorhexidine gluconate spray used as a disinfection protocol, and correlate the microbial contamination with surface roughness. The null hypotheses were, 1) the use of 0.12% chlorhexidine gluconate spray would not decrease the cariogenic microorganisms' contamination of sport mouthguard after use; 2) the use of mouthguard would not alter its roughness.

Material and methods

This study was approved by the Research Ethics Committee involving human subjects (CAAE: 06252818.8.0000.5419), and informed consent was obtained from all participants. This clinical trial followed the CONSORT Group recommendations, and the trial was registered at the registered at the Brazilian Registry of Clinical Trials - (ReBEC No. RBR-23xz8s). The Declaration of Helsinki guidelines were followed in this investigation. In the present study, a sample calculation was performed for dependent samples, with G* Power Program (version 3.1.9.2, Heinrich-Heine University Düsseldorf, North Rhine-Westphalia, Germany). For the assessment of bacterial contamination, the sample calculation was based on data presented by Sifakakis *et al.* (14), and the surface roughness was based on data from a previous pilot study, both considering an alpha value of 5%, and a value of 80% for power of the test.

A randomized, blinded, cross-over clinical trial, twenty children who practiced martial arts in the Cava do Bosque Multisport Complex, Ribeirão Preto, São Paulo, Brazil, aged between 9 to 13 years old, of both gender were selected. The inclusion criteria were the absence of periodontal disease and cavitated carious lesions, good overall health, and no use of antibiotics and/or antimicrobial solutions for a minimum of 3 months. Users of fixed orthodontic appliances were excluded. The dental evaluation of the participants was carried out by anamnesis and clinical assessment performed with dental mirror nº 5 and Williams probe (Golgran, São Caetano do Sul, SP, Brazil), by the principal examiner previously trained and calibrated (Y.J.S.R). Using a simple randomization method with a random number table (15), an independent researcher who had no role in data collection (P.N.F) performed the random sequence of the participants in 2 groups, experimental and control, with 10 patients in each group (in a 1:1 ratio). Allocation concealment was achieved with numbered, opaque, sealed envelopes containing the treatment, which were opened moments before the intervention by the main operator (Y.J.S.R).

Sports mouthguards were made for the maxillary arch, composed of 2 vacuum-pressed 3 mm thick EVA plates (Bio-Art, São Carlos, SP, Brazil). One hour after total cooling, the excesses near of anatomical structures such as frenums, were removed through cuts with Iris scissors (Golgran, São Caetano do Sul, SP, Brazil). The occlusal surface adjustment was performed by heating this surface and requested the subjects to occlude under it, followed by polishing with abrasive burs from amalgam polishing kit (Kit 8089CA KG Sorensen, Cotia, SP, Brazil).

The patients received oral hygiene instructions and were instructed to brush their teeth and the appliance after use, 3 times a day, with a Colgate Professional® toothbrush (Colgate-Palmolive Indústria e Comércio Ltda, São Paulo, SP, Brazil) and Colgate Anticaries® Maximum Cavity Protection fluoride toothpaste (Colgate-Palmolive Indústria e Comércio Ltda, São Paulo, SP, Brazil), supplied by the researchers during the experimental period.

In the first stage, the patients used sports mouthguards every alternate day, 3 times a week for 1 hour/day, and were instructed to spray (about 2 mL) sterile tap water (control group) or 0.12 % chlorhexidine gluconate solution (experimental group) after brushing. After a 15 day wash-out period, patients received new appliances, and the patients who participated in the control group underwent the protocol of the experimental group and vice versa, totaling 40 sports mouthguards treated at the end of the study with sterile tap water (control group, n=20) and antimicrobial solution (experimental group, n=20). The patients were blinded to the solutions employed, and the disinfection of the devices was performed by a single operator (Y.J.S.R.).

The mouthguards were packed in 150 mL recipient containing 90 mL TE buffer solution (pH = 7.6) and 60 mL of 0.5 M NaOH and shaken in a Mixtron apparatus (Toptronix, São Paulo, SP, Brazil) at maximum speed for 30 seconds. The suspension containing the microorganisms was transferred to 50 mL tubes (stock solution) (Corning, São Paulo, SP, Brazil) and centrifuged at 4,000 rpm (Eppendorf Centrifuge - 5810R, São Paulo, SP, Brazil) for 12 min. The supernatant was discarded, and the pellet was suspended in 150 µL TE and 100 µL NaOH. Aliquots of 50 µL of this solution were separated for the MTT assay and 200 µL were frozen at 20°C for the CDDH assay.

The analysis of the cytotoxic effect of the solutions (sterile tap water and 0.12% chlorhexidine) for the disinfection of the sports mouthguards was carried out by an enzyme assay MTT [3-(4,5-

[Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. With a 50- μ L aliquot of the stock solution, dimethyl sulfoxide solvent (DMSO) and MTT were mixed at a rate of 5 mg/mL, according to previous study (16). These were vortexed for 1 min and stored at 37°C for 30 min. Samples were evaluated in Nanodrop (NanoDrop®ND-1000 UV-VIS Thermo Scientific, Waltham, MA, USA) spectrophotometer to perform the quantification of enzymatic inhibition at 570 nm wavelength (16). Absorbance values were analyzed by the percentage of cell viability, given by mitochondrial activity of bacterial cells, and compared after the use of the treatments (sterile tap water and chlorhexidine).

Analysis of the bacterial profile of the samples by the CDDH technique was performed according to the list of 4 strains of cariogenic bacteria (*Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus acidophilus*, and *Lactobacillus casei*). The strains were freeze-dried, purchased from the ATCC (American Type Culture Collection, Rockville, MD, USA), and the DNA probes were prepared containing all genomic DNA of these microorganisms according to a previously published study (10). The samples were boiled for 10 min to induce cell lysis and denaturation of the genetic material. DNA was fixed in individual strips on a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN, USA) using a minislot (Minislot 30 Immunetics, Cambridge, MA, USA). Genomic DNA probes were digoxigenin-labeled (Roche Applied Science, Indianapolis, IN, USA) and hybridized using a Miniblotter 45 apparatus (Immunetics, Cambridge, MA, USA). Bound probes were detected with a digoxigenin phosphate conjugated antibody (Roche Applied Science, Indianapolis, IN, USA). Following incubation in a solution containing the CDP-Star™ Substrate (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA), the membranes were placed in radiographic film autoradiography cassettes (X-Omat; Kodak, Rochester, NY, USA) developed for detection of chemiluminescence signals. Signals were visually assessed twice by a single calibrated examiner, comparing the standards of 10^5 and 10^6 bacterial cells in the samples, with controls, thus providing the approximate numerical value of bacterial cells per sample. Signal strength was assessed according to the following levels: 0 (not detected), 1×10^4 , 1×10^5 , 5×10^5 , and 1×10^6 .

The surface roughness of the sports mouthguards of both groups was evaluated at 2 moments, before and after use. The area chosen for the assessment of surface roughness in both groups was the labial surface of the maxillary right central incisor (0.5 mm^2), which received a perpendicular incidence of the laser beam. For the quantitative analysis of surface roughness, Ra measurement (μm) was used as a parameter, which corresponds to the arithmetic mean of the absolute values of the distance (peaks and valleys) of the roughness profile points, in relation to a midline within the measurement path, calculated using a laser confocal microscope (LEXT OLS4000® Olympus, Tokyo, Japan), coupled with the OLS4000 software (Olympus, Tokyo, Japan).

The primary outcomes in this study were bacterial contamination and surface roughness of the sports mouthguards. The secondary outcome were the correlation in bacterial contamination and surface roughness.

Data distribution for continuous variables was analyzed using the Shapiro-Wilk normality test. The Wilcoxon test was used for CDDH and MTT assay, paired t-Student test was performed for confocal laser microscopy evaluation of surface roughness, and the Pearson correlation test was used to assess the correlation between the posttreatment surface roughness and microbial contamination, all at a significance level of 5%. GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA) was used for all comparisons.

Results

Twenty patients (100%) selected for the study participated in the study until its conclusion. Regarding the possible carryover effect between the control and experimental groups, it was found that the design was adequate, as there was no significant difference between the 2 stages of both groups ($p > 0.05$).

The use of 0.12% chlorhexidine spray after use of sports mouthguards reduced significantly the bacterial cell viability ($p = 0.007$) when compared with the control group that use sterile tap water spray (Figure 1).

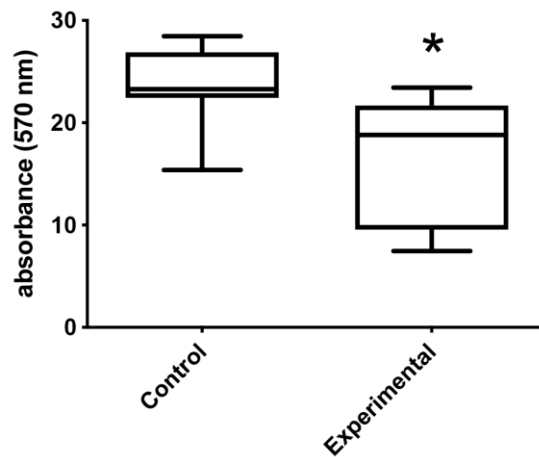


Figure 1 - Bacterial cell viability (absorbance values) in the sports mouthguards of the control (sterile tap water) and experimental (0.12% chlorhexidine gluconate) groups – Wilcoxon test ($p=0.007$).

From the CDDH technique, it was possible to prove that all sports mouthguards of both groups were colonized by the following microorganisms: *S. mutans*, *S. sobrinus*, *L. acidophilus*, and *L. casei*.

The median number of all cariogenic microorganisms found in sports mouthguards was statistically higher for the control group, when compared to the experimental group: *S. mutans* (Control group: 500000 [100000-500000]; Experimental group: 10000 [10000-10000]; $p < 0.0001$); *S. sobrinus* (Control group: 500000 [100000-500000]; Experimental group: 10000 [10000-100000]; $p = 0.0006$); *L. casei* (Control group: 500000 [100000-500000]; Experimental group: 10000 [10000-10000]; $p = 0.001$); and *L. acidophilus* (Control group: 500000 [100000- 500000; Experimental group: 10000 [10000-100000]; $p < 0.0001$). After using 0.12% chlorhexidine spray, it was observed that there was statistically significant reduction ($p < 0.05$) in the scores of all cariogenic microorganisms evaluated.

The use of sports mouthguards increased significantly their surface roughness ($p = 0.002$), irrespective of the decontamination protocol used, this can be seen in representative photomicrographs (Figure 2). No significant difference was found between surface roughness for control and experimental groups ($p = 0.92$).

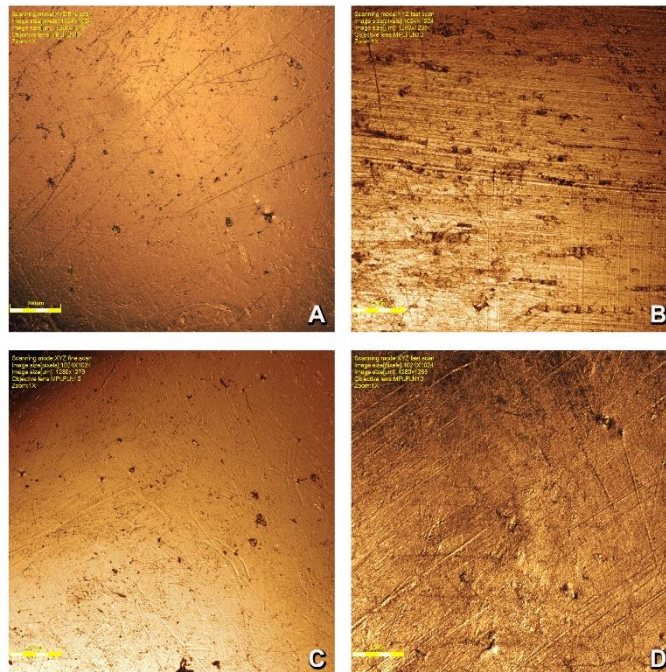


Figure 2 - Representative photomicrographs of the surface of sports mouthguards used by children. Control group (sterile tap water): pretreatment (A) and posttreatment (B). Experimental group (0.12% chlorhexidine gluconate): pretreatment (C) and posttreatment (D) (Bar: 600µm) – Paired t-Student test ($p<0.05$).

Moderate correlation ($r = 0.59$) was found between surface roughness and microbial contamination by cariogenic microorganisms for the control group, whereas no correlation ($r = -0.03$) was found for experimental group (Figure 3).

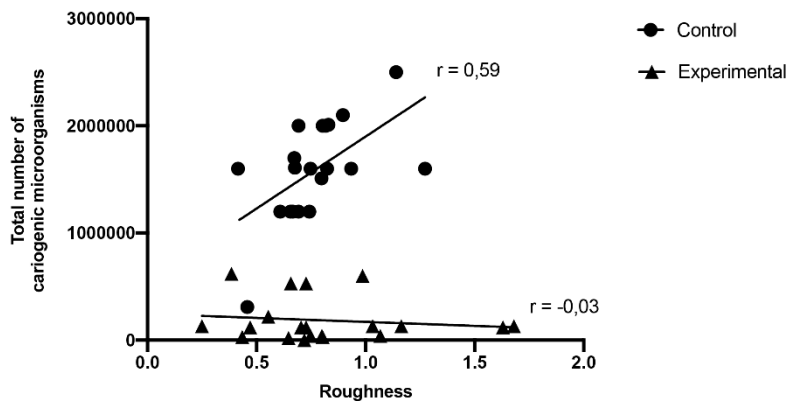


Figure 3 - Correlation between surface roughness after treatment with sterile tap water (control) or 0.12% chlorhexidine gluconate (experimental) and the total number of cariogenic microorganisms detected by Checkerboard DNA-DNA Hybridization technique in mouthguards used by children - Pearson correlation test.

Discussion

Cell viability analysis using the MTT assay is useful for verifying the cytotoxic effects of dental materials and substances (16,17). In a recent study, the authors verified through MTT assay that daily immersion of acrylic bases of biofilm-contaminated total dentures in 2% chlorhexidine gluconate solution caused antimicrobial and cytotoxic effect (18). Our findings demonstrate similar results in mouthguards, differing only in the concentration of chlorhexidine solution used and in the form of application. This difference can be attributed to the cytotoxic effect of chlorhexidine, targeting microorganisms.

In the present study, CDDH technique showed that all 4 probes of cariogenic microorganisms (*S. mutans*, *S. sobrinus*, *L. acidophilus*, and *L. casei*) occurred on the mouthguard surfaces in 100% of the devices (control and experimental group). Studies shows that *S. mutans* and *S. sobrinus* play a central role in the onset of dental caries, which is considered biofilm-sugar dependent (19,20,21). Individuals presenting mainly these 2 species of microorganisms are more likely to develop carious lesions compared to individuals who exhibit only *S. mutans* (20). Our findings showed that *S. sobrinus* and *S. mutans*, were densely colonizing all (100%) mouthguards in the control group.

Microbiological studies performed in orthodontics using different microbiological techniques have demonstrated that chlorhexidine is a solution that can be used to reduce the microorganisms contamination in comparison to the control group, where was made only the tooth brushing (9,10,12). This data shows that only the tooth brushing procedure would not be enough to decontaminate the mouthguard, as can be seen in our findings.

Chlorhexidine is the antimicrobial agent that most meets the needs of the population expectations of an efficient bactericidal agent (9,10). Our results showed that the use of 0.12% chlorhexidine gluconate as a spray was effective in reducing the levels of all cariogenic microorganisms evaluated, this finding confirm our initial hypothesis and corroborate with the results revealed by other researchers (9,10). Furthermore, each patient participated in both groups – control and experimental, at different times, thus, the patient's oral microbiome not interfering in these results.

The results of this study showed that after being used, the sports mouthguards became rougher. However, when comparing the surface roughness values of the control and experimental groups, no significant difference was observed. In the present study, the factors that may have influenced increase in surface roughness of used sports mouthguards made from EVA plates are friction and abrasion caused by use and mechanical biofilm removal method (tooth brushing). This statement is supported by another study, which evaluated the effect of different total denture cleaning methods on surface roughness (22). Regarding the increase in surface roughness after use, it should also be noted that the abrasive effect

caused by tooth brushing (23), may be influenced by various factors, such as brushing technique, strength, duration, and frequency, and abrasiveness of the toothpaste caused by the movement of toothpaste on the tooth surface (22,23). Although in the present study the toothbrush and toothpaste were standardized, the children brushed the mouthguard after use on alternate days, 3 times a week, which when combined with the use caused the increased roughness observed.

Another factor that could alter the roughness of sports mouthguards is the type of disinfection solution employed. In previous studies evaluating roughness after disinfection of denture bases, it was shown that sodium hypochlorite and chlorhexidine solutions did not cause changes in surface roughness (24, 25). Our results are in agreement with these authors since sterile tap water and 0.12% chlorhexidine gluconate showed no significant difference after treatment. Analysis of the correlation between posttreatment surface roughness and microbial contamination showed that, in the control group, the correlation was positive and moderate ($r = 0.59$). These data demonstrate that the higher the roughness, the greater the number of cariogenic microorganisms. Indeed, after sports mouthguard's routine use, possibly the roughness increases, favoring the retention of microorganisms justifying the need for disinfection. In the experimental group, where chlorhexidine was used as a disinfection method, the correlation was negative and very weak ($r = -0.03$), indicating that even in the roughest mouthguards, the amount of microorganisms cariogenic agents was lower.

The limitations found in the present study was for the Checkerboard DNA-DNA hybridization technique, due the reading, that is performed only in the presence of about 10,000 cells per milliliter of sample. Another limitation, was that for the cell viability test, no studies were found in the literature on the cytotoxic potential of the antimicrobial agent incorporated into the removable mouthguards. Owing this, only indirect comparisons with the results obtained in this study could be made. Further studies are needed to evaluate the disinfection protocol for sports mouthguards in children, with different antimicrobial agents, microorganisms and types of mouthguards. It is also essential to conduct more *in vitro* investigations regarding the virucidal effect of a large variety of cleansers and to evaluate their possible effects on viruses like SARS-CoV-2 because of the current emergence of infections that have become a global health concern.

Our results shows that after use by children, sports mouthguards present intense contamination and greater surface roughness, and chlorhexidine gluconate (0.12%) spray was effective in reducing this contamination.

Acknowledgements

This research study was supported by a postgraduate scholarship and grant from CNPq (Brazilian National Council for Scientific and Technological Development - #130175/2019-6). The authors are grateful to Izilvânia Maroly Quindere Barreto from University of Guarulhos, for helpful assistance during CDDH technique performance, and to Nilza Letícia Magalhães from School of Dentistry of Ribeirão Preto, for helpful assistance during MTT assay and Confocal Laser Scanning Microscopy technique performance.

Resumo

O objetivo do presente estudo *in vivo* foi avaliar a contaminação bacteriana de protetores bucais esportivos, a rugosidade da superfície e a eficácia do spray de gluconato de clorexidina na desinfecção desses dispositivos. Um ensaio clínico randomizado, cego, cruzado foi realizado com vinte crianças de 9 a 13 anos, que praticavam artes marciais, participaram de todas as fases do estudo. As crianças foram orientadas a usar o protetor bucal por 3 dias alternados durante 1 hora e, após o uso, borrifar água de torneira estéril ou clorexidina 0,12%. Os protetores foram analisados por ensaio MTT, Hibridização DNA-DNA e microscopia confocal a laser antes e após o uso por 2 semanas. Os dados foram analisados pelos teste de Wilcoxon, teste t de Student, e correlação de Pearson, com nível de significância de 5%. Observou-se que os protetores bucais do grupo controle estavam mais contaminados com microrganismos cariogênicos do que os do grupo experimental (clorexidina) ($p < 0,05$). O uso de protetores bucais com spray de clorexidina reduziu significativamente a contaminação bacteriana em relação ao grupo controle ($p = 0,007$). A rugosidade da superfície dos protetores bucais aumentou significativamente após o uso, independentemente da aplicação de spray de clorexidina. Uma correlação moderada ($r = 0,59$) foi observada entre a rugosidade da superfície e a contaminação do micro-organismo apenas para o grupo controle. Os protetores bucais esportivos apresentam intensa contaminação microbiana e aumento da rugosidade superficial após o uso. O uso de spray de clorexidina foi eficaz para reduzir a contaminação dos protetores bucais usados por crianças.

References

- 1- Richards D. One billion people have experienced a traumatic dental injury. *Evid Based Dent* 2018;19(2):34-35.
- 2- Deogade SC, Dube G, Sumathi K, Dube P, Katare U, Katare D. Sports dentistry and mouthguards. *Brit J Med Med Res* 2016;11(6):1-10.
- 3- Newsome PR, Tran DC, Cooke MS. The role of the mouthguard in the prevention of sports-related dental injuries: A review. *Int J Paediatr Dent* 2001;11:396-404.
- 4- American Academy of Pediatric Dentistry. Policy on Prevention of Sports-related Orofacial Injuries. *Review Council* 2018/2019;40(6):86-91.
- 5- American Dental Association. Council on Access, Prevention, and Inter-professional Relations and Council on Scientific Affairs. Using mouthguards to reduce the incidence and severity of sports-related oral injuries. *J Am Dent Assoc* 2006;137(12):1712-1720.
- 6- Glass RT, Conrad RS, Köhler GA, Warren AJ, Bullard JW. Microbiota found in protective athletic mouthguards. *Sports Health* 2011;3(3):244-248.
- 7- Arweiler NB, Netuschil L. The Oral Microbiota. *Adv Exp Med Biol* 2016;902:45-60.
- 8- Namba EL, Strapasson A, Jarzynski J, Baratto SP, Tomazinho PH. Sanitization of sports mouthguards. *RSBO* 2013;10(1):72-5.
- 9- D'Ercole S, Tieri M, Fulco D, Martinelli D, Tripodi D. The use of chlorhexidine in mouthguards. *J Biol Regul Homeost Agents* 2017;31(2): 487-493.
- 10- Nelson-Filho P, Olmedo LY, Andrucio MC, Saraiva M da C, Matsumoto MA, de Queiroz AM, da Silva RA, da Silva LA. Use of the checkerboard DNA-DNA hybridization technique for in vivo detection of cariogenic microorganisms on metallic brackets, with or without use of an antimicrobial agent. *J Dent* 2011;39(7):513-7.
- 11- D'Ercole S, Tieri M, Martinelli D, Ciaravino C, Fulco D, Tripodi D. Microbial Contamination and Disinfection of Sport Mouthguard: In Vitro Study. *Curr Microbiol* 2020;77(2):246-253.
- 12- Nelson-Filho P, Carpio-Horta KO, Andrucio MC, Feres M, Bezerra da Silva RA, Garcia Paula-Silva FW, Romano FL. Molecular detection of *Aggregatibacter actinomycetemcomitans* on metallic brackets by the checkerboard DNA-DNA hybridization technique. *Am J Orthod Dentofacial Orthop* 2012;142(4):481-6.
- 13- Moshrefi A. Chlorhexidine. *J West Soc Periodontol Periodontol Abstr* 2002;50(1):5-9.
- 14- Sifakakis I, Papaioannou W, Papadimitriou A, Kloukos D, Papageorgiou SN, Eliades T. Salivary levels of cariogenic bacterial species during orthodontic treatment with thermoplastic aligners or fixed appliances: a prospective cohort study. *Progr Orthod* 2018;19(25):1-9.
- 15- Petrie A, Bulman JS, Osborn JF. Further statistics in dentistry. Part 3: Clinical trials 1. *Br Dent J* 2002;193(9):495-8.
- 16- Grela E, Kozłowska J, Grabowiecka A. Current methodology of MTT assay in bacteria - A review. *Acta Histochem* 2018;120(4):303-311.
- 17- Maquera-Huacho PM, Tonon CC, Correia MF, Francisoni RS, Bordini EAF, Marcantonio E, Spolidorio DMP. In vitro antibacterial and cytotoxic activities of carvacrol and terpinen-4-ol against biofilm formation on titanium implant surfaces. *Biofouling* 2018;34(6):699-709.
- 18- Procópio ALF, da Silva RA, Maciel JG, Sugio CYC, Soares S, Urban VM, Neppelenbroek KH. Antimicrobial and cytotoxic effects of denture base acrylic resin impregnated with cleaning agents after long-term immersion. *Toxicol In Vitro* 2018;52:8-13.
- 19- Mosaddad SA, Tahmasebi E, Yazdani A, Rezvani MB, Seifalian A, Yazdani M, Tebyanian H. Oral microbial biofilms: an update. *Eur J Clin Microbiol Infect Dis* 2019;38(11):2005-2019.
- 20- Seki M, Yamashita Y, Shibata Y, Torigoe H, Tsuda H, Maeno M. Effect of mixed mutans streptococci colonization on caries development. *Oral Microbiol Immunol* 2006;21(1):47-52.
- 21- Tanzer JM, Livingston J, Thompson AM. The microbiology of primary dental caries in humans. *J Dent Educ* 2001;65(10):1028-1037.
- 22- Panariello BHD, Izumida FE, Moffa EB, Pavarina AC, Jorge JH, Giampaolo ET. Effects of short-term immersion and brushing with different denture cleansers on the roughness, hardness, and color of two types of acrylic resin. *Am J Dent* 2015;28(3):150-156.
- 23- Wiegand A, Schlueter N. The role of oral hygiene: does toothbrushing harm? *Monogr Oral Sci* 2014;25:215-219.
- 24- Azevedo A, Machado AL, Vergani CE, Giampaolo ET, Pavarina AC, Magnani R. Effect of disinfectants on the hardness and roughness of reline acrylic resins. *J Prosthodont* 2006;15(4):235-242.

25- Mohammed MR. The effect of different disinfectant solutions on the surface roughness of heat cure acrylic resin in comparison to visible light cure acrylic resin denture base. J Bagh College Dent 2011;23(4):31-35.

Received: 30/05/2021
Accepted: 08/11/2021