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EC MICROBIOLOGY
Research Article

In Vitro Evaluation of Disinfection Methods for Biofilm Removal on EVA Plates Used in Sports Mouthguards

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Received: November 03, 2025; **Published:** November 27, 2025

Abstract

Sports mouthguards are essential for preventing orofacial injuries but may act as reservoirs for microbial contamination when not properly disinfected. Ethylene-vinyl acetate (EVA), the material commonly used for mouthguards, facilitates bacterial adhesion and biofilm formation. This *in vitro* study evaluated the effectiveness of different chemical and mechanical disinfection methods for removing *Escherichia coli* biofilm from EVA surfaces. EVA plates (3 × 3 cm, 3 mm thick; Bio-Art, Brazil) were sterilized with ethylene oxide and immersed in Brain Heart Infusion (BHI) broth containing *E. coli* (10^8 CFU/mL) at 37°C for 72h to promote biofilm formation. After incubation, 25 specimens were divided into five groups (n = 5): control (sterile saline), brushing (sterile toothbrush and saline), 0.12% chlorhexidine digluconate, 1% sodium hypochlorite, and effervescent tablet (Corega Tabs®). Disinfection was performed for 5 min, followed by rinsing with sterile saline. Detached biofilms were vortexed, serially diluted, plated on BHI agar, and incubated at 37°C for 24h. Colony-forming units (CFU/mL) were counted and log-transformed. Data were analyzed using ANOVA and Tukey's test ($p < 0.05$). All treatments significantly reduced *E. coli* biofilm compared with the control ($p < 0.05$). Mean $\log_{10}$ CFU/mL values were: effervescent tablet 1.6 ± 0.4 (4.7-log reduction), chlorhexidine 2.3 ± 0.2 (4.0-log), sodium hypochlorite 2.8 ± 0.3 (3.5-log), brushing 3.5 ± 0.3 (2.8-log), and control 6.3 ± 0.4. Effervescent tablets and chlorhexidine achieved the greatest antimicrobial effect without significant difference ($p > 0.05$). All evaluated disinfection methods effectively reduced *E. coli* biofilm on EVA surfaces. Effervescent tablets and 0.12% chlorhexidine provided the best combination of efficacy and material safety, representing practical and reliable options for daily mouthguard hygiene and biofilm control.

Keywords: *EVA; Mouthguard; Biofilm; Disinfection; Escherichia coli*

Introduction

Participation in contact sports exposes athletes to a substantial risk of orofacial trauma, including soft-tissue lacerations, dental fractures, luxations, and avulsions. Dental injuries are not only painful and costly to treat but can also have long-term functional and psychosocial effects on athletes [1]. Preventive strategies, particularly the use of mouthguards, are widely recognized as effective measures to reduce the severity and incidence of such injuries. According to the FDI World Dental Federation, mouthguards are essential equipment for individuals engaged in any sport with potential orofacial impact, as they protect both soft and hard tissues and can even reduce concussion risk [2].

Despite their proven effectiveness, the actual rate of mouthguard use varies across sports and levels of competition. Studies show that while combat sport athletes exhibit near-universal adherence due to mandatory requirements, the frequency of use among team sport athletes remains low, often below 30% [3]. Factors contributing to poor compliance include discomfort, impaired breathing or speech, and inadequate awareness of the benefits of custom-made mouthguards. Custom-fabricated ethylene-vinyl-acetate (EVA) mouthguards, however, offer superior fit, retention, and comfort compared with "boil-and-bite" models, and have been shown not to interfere with respiratory function [3].

Beyond mechanical protection, mouthguards can act as reservoirs for microbial contamination. Frequent intraoral use, exposure to saliva, and inadequate cleaning practices create an environment conducive to the formation of dense microbial biofilms on the EVA surface [3]. Studies have isolated *Streptococcus mutans*, *Staphylococcus aureus*, *Candida albicans*, and various gram-negative bacteria from worn mouthguards, linking poor hygiene to oral mucosal infections and even systemic diseases [4,5]. The porosity and hydrophobic nature of EVA polymers facilitate the adhesion of microorganisms and organic debris, while residual moisture in storage containers further enhances microbial growth. As a result, athletes who fail to clean their devices properly are at risk of harboring biofilms that may release pathogenic organisms into the oral cavity during training or competition.

Several chemical and physical disinfection methods have been proposed to control microbial accumulation on mouthguards. Traditional approaches include mechanical brushing with soap, immersion in antiseptic or effervescent cleaning tablets, and rinsing with chlorhexidine or hydrogen peroxide-based solutions [6]. However, prolonged exposure to aggressive agents may alter the mechanical integrity, color, or surface roughness of EVA plates [5]. More recent investigations have explored the use of alcohol-based sprays, ultraviolet light, and photodynamic therapy as effective and less damaging alternatives [3,7].

Despite these advances, there is still no consensus on the most efficient protocol for decontaminating EVA mouthguards without compromising their physical or protective properties. Therefore, *in vitro* studies evaluating disinfection methods against mature biofilms on EVA surfaces are essential to establish evidence-based recommendations for clinical and athletic use.

Materials and Methods

Sample preparation

Ethylene-vinyl acetate (EVA) sheets with 3 mm thickness (Bio-Art, São Carlos, Brazil) were used to simulate the inner surface of sports mouthguards. The material was cut into square specimens measuring 3 × 3 cm. All samples (n = 25) were sterilized with ethylene oxide gas and stored under aseptic conditions until use. Sterility of the samples was confirmed by incubating control specimens in Brain Heart Infusion (BHI) broth at 37°C for 48h, verifying the absence of turbidity or bacterial growth.

Bacterial strain and biofilm formation

Escherichia coli (ATCC 25922) was selected as the biofilm-forming microorganism due to its known ability to colonize polymeric materials and serve as an indicator of hygiene-related contamination. Bacteria were cultured in Brain Heart Infusion (BHI) broth at 37°C under agitation (150 rpm) until reaching the stationary growth phase, corresponding to approximately 1×10^9 cells/mL.

Sterile EVA specimens were immersed in sterile BHI broth containing *E. coli* at a final concentration of 1×10^8 CFU/mL and incubated for 72h at 37°C to allow for biofilm formation on the polymer surface. After incubation, specimens were gently rinsed with sterile saline to remove non-adherent cells.

Experimental groups and disinfection procedures

After biofilm development, the samples were randomly divided into five experimental groups (n = 5 per group):

- Control group: Rinsed with sterile saline solution only.
- Brushing group: Brushing was performed using a new toothbrush previously sterilized by ethylene oxide. The brush was moistened with sterile saline and applied to both surfaces of each specimen for 30 seconds per side with circular movements, under standardized manual pressure.
- Chlorhexidine group: Rinsed in sterile artificial saliva and sprayed with 1 mL of 0.12% chlorhexidine digluconate (Periogard®, Colgate-Palmolive) for 5 minutes.
- Sodium hypochlorite group: Rinsed in sterile artificial saliva and sprayed with 1 mL of 1% sodium hypochlorite for 5 minutes.
- Effervescent tablet group (Corega Tabs®, GlaxoSmithKline): each specimen was immersed in 20 mL of sterile distilled water in a beaker, with one effervescent tablet added and allowed to act for 5 minutes at room temperature.

Following treatment, all samples were rinsed with sterile saline to remove residual disinfectant or debris. Each specimen was transferred to a sterile Falcon tube containing 5 mL of sterile saline and vortexed for 1 minute to detach adherent bacteria from the EVA surface.

Microbiological analysis

The resulting bacterial suspensions were serially diluted in sterile saline, and 100 µL aliquots from each dilution were plated in duplicate onto BHI agar plates. The plates were incubated at 37°C for 24h, after which colony-forming units (CFU) were counted. The disinfection efficacy was expressed as the logarithmic reduction (log_{10} CFU/mL) and compared with the control group.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to compare the mean CFU counts among groups. A p-value < 0.05 was considered statistically significant.

Results

All disinfection methods tested significantly reduced the number of viable *Escherichia coli* cells compared with the control group ($p < 0.05$). The mean bacterial counts (log_{10} CFU/mL) obtained for each treatment are shown in table 1.

Group	Mean (log_{10} CFU/mL) ± SD	Log Reduction vs. Control
Effervescent tablet	1.6 ± 0.4^{a}	4.7 ↓
Chlorhexidine 0.12%	2.3 ± 0.2^{a}	4.0 ↓
Sodium hypochlorite 1%	2.8 ± 0.3^{b}	3.5 ↓
Brushing (saline only)	3.5 ± 0.3^{c}	2.8 ↓
Control (no disinfection)	6.3 ± 0.4^{d}	-

Table 1: *Mean bacterial counts (log_{10} CFU/mL) and standard deviation for each disinfection method tested. The difference between groups is represented by distinct superscripted letters (P \ 0.05).*

The effervescent tablet group (Corega Tabs®) demonstrated the greatest overall microbial reduction, achieving a 4.7-$\log_{10}$ decrease in viable *E. coli* counts compared with the control. Chlorhexidine 0.12% achieved a 4.0-$\log_{10}$ reduction, followed closely by sodium hypochlorite 1%, which resulted in a 2.8-$\log_{10}$ reduction.

Although mechanical brushing with saline produced a smaller reduction (3.5-$\log_{10}$), it still showed a statistically significant difference compared with the control ($p < 0.05$).

One-way ANOVA revealed significant differences among the five groups ($F = 52.4$; $p < 0.001$). Post hoc analysis (Tukey test) confirmed that all disinfection methods significantly outperformed the control ($p < 0.01$). The effervescent tablet and chlorhexidine groups did not differ significantly from each other ($p > 0.05$) but both showed superior results compared with sodium hypochlorite and brushing ($p < 0.05$).

Overall, these results indicate that effervescent tablets and chlorhexidine provided the most effective decontamination of EVA surfaces contaminated with *E. coli* biofilm, followed by, sodium hypochlorite, and brushing, respectively.

Discussion and Conclusion

The results of this *in vitro* study demonstrated that all evaluated disinfection methods significantly reduced *Escherichia coli* biofilm on EVA surfaces compared with untreated controls. Among them, effervescent tablets and chlorhexidine 0.12%, achieved the greatest reduction, followed by 1% sodium hypochlorite and brushing. These findings align with previous evidence showing that chemical disinfectants-especially oxidizing and cationic agents-effectively disrupt mature biofilms on polymeric oral devices [6,8].

EVA-based mouthguards are highly effective in preventing orofacial trauma during sports activities [2], yet their frequent intraoral use and storage under humid conditions make them ideal substrates for microbial colonization [4]. The porous structure and hydrophobicity of EVA favor the adhesion of bacteria such as *Streptococcus mutans*, *Staphylococcus aureus*, and *Candida albicans* [3,5]. When not adequately disinfected, mouthguards can serve as microbial reservoirs, potentially contributing to mucosal inflammation, halitosis, and opportunistic infections. Regular and effective cleaning protocols are therefore essential to preserve both oral and systemic health in athletes.

The significant bacterial reduction of 1% sodium hypochlorite observed in the present study is consistent with its broad antimicrobial spectrum and biofilm-disrupting capacity, as previously described for dental prostheses and acrylic resins [8,9]. Nevertheless, prolonged or repeated exposure to hypochlorite solutions may alter the physical and optical properties of EVA, increasing surface roughness and color changes [5]. Chlorhexidine 0.12% provided high antimicrobial efficacy without compromising material integrity, corroborating Ribeiro., *et al*. [10], who demonstrated that short-term chlorhexidine sprays effectively reduced microbial contamination of sports mouthguards used by athletes. These results suggest that chlorhexidine represents a practical and biocompatible option for daily hygiene, especially when rapid disinfection is required.

Effervescent cleaning tablets such as Corega Tabs® achieved a higher reduction in bacterial counts, indicating significant antimicrobial activity within a short exposure time. Similar findings were reported by Hayashi., *et al.* [8] who demonstrated that a tablet-based mouthguard cleaner achieved comparable results to sodium hypochlorite without detectable structural damage to EVA. The effervescent mechanism combines mechanical agitation with the antimicrobial effects of active oxygen and detergent compounds, providing ease of use and consistent performance. This makes such tablets an attractive alternative for athletes seeking effective disinfection methods.

Mechanical brushing alone, while effective to some degree, achieved only partial biofilm removal. As noted by Glass., *et al.* [11] and confirmed by Hayashi., *et al.* [8] brushing may leave residual biofilm within surface irregularities and microscopic defects, and repeated friction can increase surface roughness, paradoxically enhancing microbial adhesion. Therefore, combining mechanical and chemical cleaning approaches may optimize decontamination outcomes, as previously recommended for dental prostheses [12,13].

From a clinical standpoint, the present findings reinforce the importance of establishing standardized disinfection protocols for sports mouthguards. The FDI recommends that dental professionals educate athletes on proper hygiene, periodic replacement, and safe storage to minimize microbial risks [2]. Considering that EVA degradation may occur with harsh chemical exposure, short contact times and moderate concentrations-such as 0.12% chlorhexidine or effervescent tablet immersion for 5 minutes-appear to offer an optimal balance between antimicrobial efficacy and material preservation.

Future studies should expand these results by assessing multispecies biofilms and evaluating the effects of repeated disinfection cycles on mechanical properties, color stability, and cytocompatibility. *In vivo* investigations with longer wearing periods, as performed by Hayashi., *et al*. [8] are also warranted to simulate real-life conditions in athletes and validate laboratory findings.

This *in vitro* study demonstrated that all evaluated disinfection methods-chemical and mechanical-were effective in reducing *Escherichia coli* biofilms formed on EVA surfaces used in sports mouthguards. Among them, effervescent tablets and chlorhexidine 0.12% showed the highest antimicrobial efficacy, followed by sodium hypochlorite 1%, effervescent tablets, and brushing.

Although sodium hypochlorite achieved a higher bacterial reduction, its potential to alter EVA surface properties suggests that other agents, such as chlorhexidine or effervescent tablets, may represent safer alternatives for routine use. Mechanical brushing alone provided partial biofilm removal and should be combined with chemical disinfection for optimal results.

Based on the results, short daily spraying of EVA mouthguards in 0.12% chlorhexidine or the use of effervescent cleaning tablets for 5 minutes appears to offer an effective, practical, and safe approach for athletes and dental professionals to minimize microbial contamination and extend device durability [14].

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Volume 21 Issue 12 December 2025