ORAL MICROBIOLOGY – RESULTS

1) Submitted by:

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2) Project

Title of project	*In vitro* evaluation of antimicrobial activity of BlueM mouthwash: a pilot study
Aim	To evaluate the antibiofilm and antimicrobial effects of BlueM mouthwash against the caries pathogen *Streptococcus mutans*
Methodology	1) Bacterial cultures and growth conditions: *S. mutans* strains isolated from children with active caries were used in this study. *S. mutans* was cultivated in Brain Heart Infusion (BHI) broth at 37ºC in air with 5% CO_2. 2) Biofilms were developed in sterile microtiter plates. The growth of the biofilm was initiated by diluting overnight cultures into fresh ¼-BHI broth supplemented with glucose (20 mM). The biofilms were incubated at 37ºC in air with 5% CO_2 for 6 h (early biofilm), 1 day (intermediate biofilm), or 3 days (mature biofilm). For the development of mature biofilms, the planktonic phase was carefully removed after 24-h and 48-h and replaced with fresh ¼-BHI broth supplemented with glucose. 3) The minimal inhibitory concentration (MIC) test was performed according to the broth microdilution method using full BHI broth. Cell viability was assessed by counting colony forming unit (CFU) on replica agar plates. 4) Biofilms were washed with sterile saline to remove loosely bound cells. The biofilms were treated with 0.2 ml (10× MIC) of the test compound BlueM mouthwash (provided by HuberMed, Inc) for 60 s. Sterile saline and chlorhexidine were used as controls. Treated biofilms were then washed with sterile saline, collected by centrifugation, and resuspended in sterile saline. Cells were serially diluted and spot plated on BHI agar and the percentage of cell survival obtained after 60 sec of treatment was determined from plate counts. All experiments were performed in triplicate from three independent bacterial cultures. A p-value lower than 0.05 was considered significant.

3) Project results

A) Determination of the minimum inhibitory concentration (MIC) of BlueM mouthwash

S. mutans cells were cultivated overnight in BHI broth. Cells were diluted to the same density at 600 nm and used immediately to determine the MIC. BHI broth was used as medium and different concentrations (0; 0.01; 0.1; 1; 2; 4; 10% (vol/vol)) of BlueM mouthwash were tested in sterile 96-well microtiter plates. Chlorhexidine (0; 0.01; 1; 2; 4; 10 µg/ml (wt/vol)) was used as control. Plates were incubated overnight at 37°C in air with 5% CO_2. A microplate reader was used to determine the absorbance at 600 nm. The MIC was determined as the lowest concentration of BlueM mouthwash or chlorhexidine that inhibited the visible growth of *S. mutans* cells. All experiments were performed using three independent cultures of *S. mutans*. Our results showed that under our tested conditions, the MIC of BlueM mouthwash was 1%, while the MIC of chlorhexidine (control) was 10 µg/ml for *S. mutans* caries pathogens.

B) Antibiofilm activity of BlueM mouthwash

We assessed the *in vitro* susceptibility of *S. mutans* caries pathogens to BlueM mouthwash. In order to mimic the short exposure to a mouth rinse, biofilms of *S. mutans* (6-h-, 1-day-, and 3-day-old) were exposed for 60 sec. In order to kill biofilm cells, a concentration of 10 ×MIC is recommended. For the experiments involving biofilm cells, we then tested a concentration of 10% (vol/vol) and 100 µg/ml (wt/vol) of BlueM mouthwash and chlorhexidine, respectively. Exposure of biofilms to sterile saline was used as negative control. The results are presented at Figure 1 below.

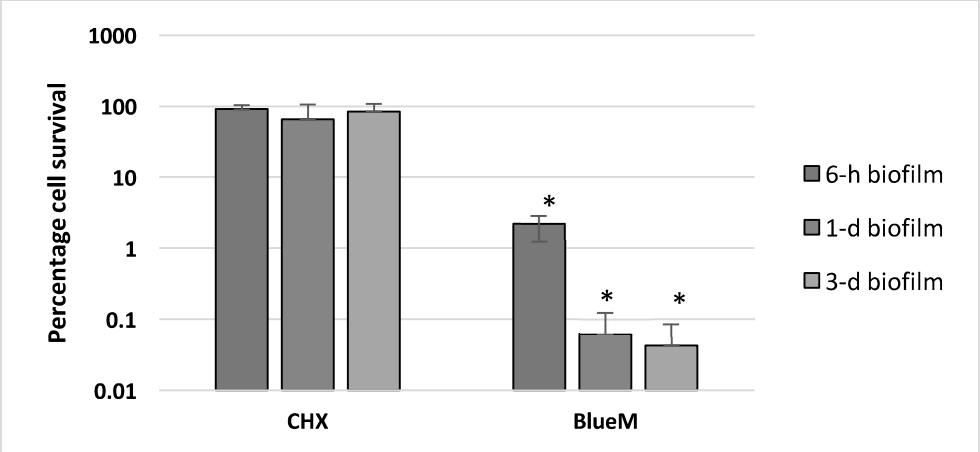

Figure 1. Antibiofilm activity of BlueM mouthwash against *S. mutans* caries pathogens. CHX: chlorhexidine; BlueM: BlueM mouthwash. Treatment (60 sec) with sterile saline was used to determine the viable biofilm cell number. The percentage of cell survival was determined by comparing the cell counts of treated-biofilms with that of saline controls. *, P value <0.05.

Our results showed that at a concentration of 10% (vol/vol), BlueM mouthwash was effective at reducing the viability of *S. mutans* biofilms by 98% (6-h-old biofilm), 99.9% (1-day-old biofilm), and 99.9% (3-day-old biofilm). In contrast, chlorhexidine treatment of 60 s at 10 ×MIC failed to reduce biofilm cell viability (Table 1).

Table 1. Killing of biofilm cells.

Biofilm	**Percentage of biofilm cell death (± SD)**		
	Saline (control)	**Chlorhexidine**	**BlueM**
6-h-old	0	10.13 (± 11.70)	97.80 (±0.59)
1-day-old	0	38.53 (± 33.37)	99.94 (± 0.07)
3-d-old	0	20.53 (± 21.29)	99.96 (± 0.04)

In conclusion, BlueM mouthwash solution is effective in killing unicellular (plantonkic) and multicellular (biofilm) of *S. mutans* caries pathogens, and more effective than chlorhexidine. Interestingly, BlueM is particularly effective at killing older thicker biofilms (3-d-old) that are usually less susceptible to antimicrobial treatments than younger biofilms.

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