A Novel Formulation Effective in Killing Oral Biofilm Bacteria

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Abstract

Objective: To determine if a novel formulation is effective in killing oral biofilm streptococci *in vitro* and *in vivo*. **Methods**: Efficacy of 0.5% levulinic acid and 0.05% sodium dodecyl sulfate (SDS) in killing *Streptococcus gordonii* CH1, *Streptococcus gordonii* DL1, *Streptococcus mitis* NCTC10712, *Streptococcus oralis* KS32AR, *Streptococcus mutans* BM71, and *Streptococcus mutans* GS5 in their biofilm form was measured *in vitro*, using microtiter plates and subsequent counts on Mitis-Salivarius agar plates. The safety and efficacy *in vivo* were evaluated using a mouse model. **Results**: Our anti-microbial formulation completely eliminated all the biofilm streptococcal species tested within 30 seconds (a reduction of 10^7 CFU/ml), whereas the positive control Listerine only demonstrated moderate reduction *in vitro*. Application of the formulation twice a day for 7 days in the murine oral cavity resulted in significantly more reduction of established *S. gordonii* DL1 oral biofilm bacteria than Listerine. The formulation did not cause any adverse effect in the murine oral cavity within a 2-week period. **Conclusions**: We have demonstrated that the novel mouth rinse exhibits high efficacy in killing oral bacteria in their biofilm forms, results in no adverse effect *in vivo*, and contains alcohol-free components.

Key words: Mouth rinse, biofilm, mouse, alcohol-free, safety

Introduction

Oral microorganisms form biofilms (dental plaque) on the surfaces of hard and soft tissues that, if left undisturbed, may initiate oral infectious diseases such as dental caries and periodontal diseases. Oral bacteria are not only implicated in oral infectious diseases, but also in systemic conditions such as cardiovascular disease, respiratory diseases and preterm birth (Clothier *et al.*, 2007; Friedewald *et al.*, 2009; Scannapieco, 1998).

There is a well-established relationship between dental plaque accumulation and the development of gingivitis (Loe *et al.*, 1965). Optimal plaque control is therefore essential in maintaining gingival health. Various antimicrobial mouth rinses have been utilized, in conjunction with brushing and flossing, in controlling plaque accumulation and gingivitis. Among commercially available mouth rinses, chlorhexidine has shown proven effectiveness in plaque control and gingivitis reduction (Loe and Schiott, 1970). However, chlorhexidine is not available over-the-counter in the United State of America and is associated with local adverse effects such as tooth staining and dysgeusia (Flotra *et al.*, 1971), which limits its long-term use. Listerine, containing essential oils and alcohol components, is another mouth rinse that exhibited long-term efficacy in controlling plaque and gingivitis (Ciancio *et al.*, 1995). However, up to 26.9% alcohol content in Listerine has raised concerns for potential adverse effects, including irritation and xerostomia (Gagari and Kabani, 1995). Therefore, alternative mouth rinses that offer efficacy, tolerability, and cost-effectiveness continue to be sought.

We have previously demonstrated that a chemical solution of 0.5% levulinic acid and 0.05 % sodium dodecyl sulfate (SDS) effectively kills *Escherichia coli* O157:H7 and *Salmonella typhimurium* (Zhao *et al.*, 2009, 2011). The components of this solution are individually designated by the U.S. Food and Drug Administration

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as generally recognized as safe for direct addition to food as a flavoring substance or adjunct. The objective of this study was to investigate the efficacy of this formulation in killing oral streptococcal biofilms, both *in vitro* and *in vivo*.

Materials and Methods

Bacterial strains and media

S. mutans BM71, *S. mutans* GS5, *S. gordonii* Challis, *S. gordonii* DH1, *S. mitis* 10712, and *S. oralis* KS32AR were maintained on tryptic soy agar (TSA, Difco, Detroit, MI) plates. Bacteria were routinely cultured in Todd Hewitt broth (THB, Difco).

Biofilm formation

Streptococcal cells (10^5 CFU) at stationary phase were inoculated into 0.1 ml ¼ strength THB supplemented with 0.01% mucin (Sigma Chemical Co., St. Louis, MO) (Li *et al.*, 2001) in 96-well polystyrene microtiter plates to form biofilms. After 24 hours of aerobic incubation at 37^{0}C, the planktonic cells were discarded and the biofilms were carefully washed with 0.1% peptone in H_2O. The fluid in the wells was removed completely and killing assays were carried out as described below.

Killing assays

The solutions of 0.5% levulinic acid, 0.05 % SDS, or their combinations were assessed for their efficacy in killing oral streptococcal biofilm bacteria, with Listerine (one of the most popular mouth rinses sold in the United States, with essential oils as active ingredients and up to 26.9% alcohol content) as positive control and water as negative control. Test reagents and controls (20 µl/well) were added to streptococcal biofilms as described above and microtiter plates were incubated at room temperature or 37^{0}C for 0.5, 1.0, 5.0, or 10 minutes. At the end of incubation, 0.1% peptone in H_2O (100 µl/well) was added to stop the reaction. Biofilms in the wells were dispersed by sonication with a Brandson Sonifier 450 at Output 3 and Duty Cycle 3 for three times. The streptococcal cells were then plated on Mitis Salivarius (MS) agar plates (see below) and incubated for 36-48 h in anaerobic jars for cell counts.

Bacterial cell counts

Fifteen (15) µl/sample were dropped with multi-channel pipettes on vertically positioned square MS agar plates to screen the killing efficacy. We arbitrarily divided cell counts on the MS plates from – to ++++: –, no surviving colonies; ±, < 15 colonies; +, colony counts between 15-300; ++, colony counts between 300-1500; +++, colonies forming bacterial lawns, with individual colonies still distinguishable; ++++, bacterial lawns, individual colonies no longer distinguishable. We also determined the total cell count/well for bacterial lawns of +++ and ++++ using serial dilutions with 0.1% peptone in H_2O.

Mouse model

Specific-pathogen-free BALB/cByJ male mice (Jackson Laboratory, Bar Harbor, ME, USA) were maintained in the Laboratory Animal Facility of the University at Buffalo, State University of New York. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University at Buffalo. Six-week-old mice (3 mice per group) were treated with kanamycin (Sigma-Aldrich, Saint Louis, MO) at 1 mg/ml in water ad libitum for 7 days, followed by a three-day antibiotic-free period (Pathirana *et al.*, 2007). Mice were infected orally with micropipettes using 2 x 10^9 CFU of live *S. gordonii* DL1 twice a day for 5 days in 50 µl of PBS with 2% carboxymethyl cellulose (PBS-CMC). Mice were kept away from food and water for 1 h after each inoculation of bacteria. One day after the final intraoral bacterial inoculation, a combination of 0.5% levulinic acid and 0.05% SDS at pH 4.25 (test group), Listerine (positive control group), or water (negative control group) were applied intraorally (50 µl/mouse) with micropipettes twice/day for 7 days. Oral swabs were performed one day after the final application of reagents, using cotton applicators. Bacteria on the cotton applicators were smeared on MS agar plates. Blue colonies representing *S. gordonii* were counted after 36-48 h incubation at 37ºC in anaerobic jars.

The safety of the formulation was assessed using the same mouse strain, without oral streptococcal inoculation. A combination of 0.5% levulinic acid and 0.05% SDS at pH 4.25, Listerine, or water were applied intraorally (50 µl/mouse) with micropipettes twice/day for 14 days. One day after final applications, mice were sacrificed and oral mucosa samples (tip of the tongue and the lower lips) were sent for histological analysis.

Statistical analysis

Student's *t-test* was performed to determine significance. A difference was considered significant when a p value < 0.05 was obtained.

Results

Efficacy of the formulation in killing biofilm oral streptococci in vitro

As shown in *Table 1*, our anti-microbial formulation (0.5% levulinic acid plus 0.05 % SDS) completely eliminated all the biofilm streptococcal species tested within 30 seconds (a reduction of 10^7 CFU/ml), whereas the positive control Listerine only demonstrated moderate reduction *in vitro*. Single chemical solutions of levulinic acid or SDS did not exhibit any killing effect, even at higher concentrations of 1% or 0.1 % (*Table 2*). Extending the incubation time

Table 1. Efficacy of the formulation in killing oral biofilm streptococci *in vitro*

	0.5% LA + 0.05% SDS	Listerine	0.1% peptone in H_2O
***S. mutans* BM71**	–*	+++	++++
***S. mutans* GS5**	–	+++	++++
***S. gordonii* CH1**	–	++	+++
***S. gordonii* DL1**	–	++	+++
***S. mitis* NCTC10712**	–	++	+++
***S. oralis* KS32AR**	–	±	+

Killing assays were carried out in duplicate and repeated at least three times. Data are from one of the experiments. *Colony counts are arbitrarily divided into – to ++++: -, no survival; ±, <15 colonies; +, colony counts between 15-300; ++, colony counts between 300-1500; +++, colonies forming bacterial lawns, with individual colonies still distinguishable; ++++, bacterial lawns, individual colonies no longer distinguishable. LA, levulinic acid; SDS, sodium dodecyl sulfate

Table 2. Titration of the formulation

Concentrations of LA; SDS	LA + SDS	LA Only	SDS Only
1%; 0.1%	–	++++	++++
0.5%; 0.05%	–	++++	++++
0.25%; 0.025%	–	++++	++++
0.1%; 0.01%	+++	++++	++++
0.05%; 0.005%	++++	++++	++++
0.025%; 0.0025%	++++	++++	++++
0%; 0%	++++	++++	++++

Levulinic acid or SDS alone or in combination were diluted in water to different concentrations and their efficacy in killing *S. mutans* BM71 biofilm cells was measured in duplicate and repeated at least three times. Data are from one of the experiments. Colony counts are expressed as – to ++++, as described in *Table 1*. LA, levulinic acid; SDS, sodium dodecyl sulfate

from 30 seconds to 1, 5, and 10 minutes exhibited the same killing efficacy for all the reagents tested (*Table 3*). The method was validated using serial dilutions; total bacterial counts/well were also assessed. There were 1.5-4.7 x 10^7 bacteria per well for ++++, 0.8-7.1 x 10^6 bacteria per well for +++, 0.4-1.6 x 10^4 bacteria per well for ++, and 0.1-2.4 x 10^3 bacteria per well for +. Listerine moderately reduced 2 to 3 log CFU/ml of oral streptococci.

The effectiveness of the reagents at lower concentrations in eliminating oral streptococci was assessed using the same methodology. A five-time and further dilutions ($\leqslant$ 0.1% levulinic acid plus 0.01% SDS) of the formulation resulted in minimum elimination of oral streptococci in their biofilm forms. However, a two-time dilution (0.25% levulinic acid plus 0.025 % SDS) exhibited efficacy similar to the original concentration (*Table 2*).

The original combination of the formulation has a pH of 3.06, which might be too low to be used as an everyday preventive product in oral homecare (although it can still be useful in the setting of a dental office). Further experiments were carried out to determine if elevating the pH of the formulation would affect its killing ability. The pH of different bottles of Listerine were measured and values ranged from 4.12 to 4.23. The formulation was neutralized with NaOH to a higher pH of 4.25 and it demonstrated a comparable killing effect to the formulation at pH 3.06 in 30 seconds (*Table 4*).

Table 3. Time course

Minutes	LA + SDS	LA Only	SDS Only	Listerine	0.1% Peptone
0.5	–	++++	++++	+++	++++
1	–	++++	++++	+++	++++
5	–	++++	++++	+++	++++
10	–	++++	++++	+++	++++

The efficacy of the formulation in killing *S. mutans* BM71 biofilm cells compared to solutions of LA (0.5%), SDS (0.05%), Listerine, or 0.1% peptone at different times was assessed. Experiments were carried out in duplicate and repeated at least three times. Data are from one of the experiments. Colony counts are expressed as – to ++++, as described in *Table 1*. LA, levulinic acid; SDS, sodium dodecyl sulfate

Table 4. Efficacy of the formulation at different pH levels

Formulation pH	Colony Counts
3.06	–
4.25	–
4.5	+
5.0	+++
6.0	+++
7.0	+++

The efficacy of the formulation in killing *S. mutans* BM71 biofilm cells at different pH levels was assessed. Experiments were carried out in duplicate and repeated at least three times. Data are from one of the experiments. Colony counts are expressed as – to ++++, as described in *Table 1*.

Efficacy of the formulation in eliminating S. gordonii in a mouse model

Efficacy of the formulation was tested in a mouse model. As shown in *Table 5*, the application of the formulation at pH 4.25 twice a day for 7 days significantly reduced the numbers of *S. gordonii* in the oral cavity (p value = 0.042) and was more effective in eliminating *S. gordonii* DL1 oral biofilm bacteria than was Listerine.

Safety in the oral cavity

The safety of the formulation in the oral cavity was determined using histological analysis. The formulation was applied to the oral cavity of mice twice daily for 14 days. No adverse effect such as redness or ulceration was found on mouse oral tissue at the end of the experiment. There was no difference in histology among the formulation experimental group, Listerine group, and water control group.

Discussion

Oral bacteria sequentially form dental plaque on freshly cleaned tooth surfaces and oral streptococci dominate as early colonizers (Diaz *et al.*, 2006) that may provide attachment sites and lower oxygen tension for late colonizers. Bacteria in the biofilm form exhibit increased resistance to antimicrobial agents (Gilbert *et al.*, 1997; Wilson, 1996) than their planktonic counterparts. Therefore, to test the efficacy of a potential mouth rinse *in vitro*, bacteria should be cultured in their biofilm forms, instead of as planktonic cells. Taking advantage of the fact that the viability of oral streptococci is not affected by sonication (Wang *et al.*, 2011), we developed a methodology to effectively test the efficacy of our formulation in killing oral streptococci. Biofilms of oral streptococci were formed in 96-well microtiter plates and bacteria in the wells after sonication were dropped directly onto square MS agar plates using eight-channel multi-pipettes. This *in vitro* testing system, to our knowledge,

Table 5. Efficacy of the formulation in murine oral cavity

Reagents	Number of Colonies
0.5% LA + 0.05% SDS (pH 4.25)	241.0 ± 116.0*
Listerine (pH 4.13)	396.7 ± 89.8
H_2O	742.0 ± 287.5

*Mean ± SD from 3 mice per group. LA, levulinic acid; SDS, sodium dodecyl sulfate

has not been reported before. The method was validated by parallel serial dilutions and colony counts on round MS plates.

Our methodology of assessing the efficacy of a formulation in killing streptococci in their biofilm forms has a distinct advantage over the traditional method of serial dilutions and plating on agars: A very short operation time is required when plating treated bacteria from 96-well microtiter plates using multi-channel pipettes, after the disruption of biofilms. This is important for testing antimicrobial effectiveness against bacteria in biofilm forms, since bacteria become planktonic after sonication and planktonic bacteria are much more susceptible (up to 1000 times) to antimicrobial agents than those in biofilm conditions (Gilbert *et al.*, 1997; Wilson, 1996). Even after multiple dilutions, antimicrobial agents could be detrimental to planktonic bacteria, whereas they show no effect on biofilm bacteria. We understand that a scale of "-" to "++++" does not represent exact cell counts. However, up to 4.7 x 10^7 streptococcal biofilm cells/well were present without antimicrobial treatment (represented by a scale of ++++, i.e., colonies forming bacterial lawns and individual colonies not distinguishable in our *in vitro* system), by using traditional cell counts. Two to three log reduction of bacteria (within a range of 10^5 to 10^6 surviving bacteria/well) is presented as +++ (colonies forming bacterial lawns, with individual colonies still distinguishable) and a 4-, 5-, and 6-log reduction (within the range of 10^4, 10^3, and 10^2 surviving bacteria/well, respectively) for ++, +, and ±, respectively. We would also like to emphasize that the efficacy of the formulation so tested represents its effectiveness in killing oral biofilm streptococci in this *in vitro* model, not in the oral cavity.

We have previously demonstrated that the formulation effectively kills *E. coli* O157:H7 and *S. typhimurium* (Zhao *et al.*, 2009, 2011). In this study, we investigated the efficacy of the formulation in killing oral streptococci. The formulation is composed of two ingredients: levulinic acid and SDS. Levulinic acid, or 4-oxopentanoic acid, is an organic compound that is widely used as a cosmetic and flavor agent. It is also utilized as a cigarette additive to desensitize the upper respiratory tract, which can mask the irritation caused by smoke and increase the potential for cigarette smoke to be inhaled deeper into the lungs (Keithly *et al.*, 2005). SDS is an anionic surfactant used in many cleaning and hygiene products, including toothpastes. Levulinic acid or SDS alone (when used as a single agent in our *in vitro* testing system) had limited antimicrobial effects (*Tables 2 and 3*). However, a combination of levulinic acid and SDS, even at a lower concentration (0.25% and 0.025%, respectively) than the formulation used (0.5% and 0.05%. respectively), completely eliminated biofilm streptococci *in vitro* (*Table 2*). The mechanism of killing oral streptococci by this formulation has not been determined in this study, which merits further investigation.

Our formulation exhibits other desirable properties besides its high efficacy in eliminating oral biofilm streptococci within 30 seconds. First, it is user friendly. Levulinic acid has a desensitizing effect (Keithly *et al.*, 2005) and has been utilized in cosmetic products. Therefore, it will not irritate the soft tissue in the oral cavity, which is especially useful for children and the elderly population, whereas the alcohol component in some mouth rinses such as Listerine could irritate oral mucosa. Second, it is cost-effective: levulinic acid is produced from cellulose-containing waste materials and thereby costs very little in mass production, whereas the essential oil and alcohol components in Listerine are much more expensive. Third, the formulation is readily soluble in water, a property that allows it to be used in different formats such as solutions, pastes, gels, varnishes, and local delivery devices. Fourth, no obvious adverse side effects such as edema or ulceration were observed in our 2-week *in vivo* study, and, in addition, both levulinic acid (FDA 2008, 21 CFR, 172.515) and SDS (FDA 2007, 21 CFR, 172.822) were individually designated by the U.S. Food and Drug Administration as generally recognized as safe for direct addition to food as a flavoring substance or adjunct.

We have tested another organic acid, lactic acid, for its efficacy in eliminating oral streptococcal biofilms. Lactic acid demonstrated the same efficacy as levulinic acid when combined with SDS. The formulation of

0.5% lactic acid plus 0.05% SDS resulted in complete elimination of *S. mutans* BM71 biofilm cells in our *in vitro* model.

We have also tested another popular detergent in toothpastes, $NaHCO_3$ (baking soda), instead of SDS, in the formulation. The combination of levulinic acid and baking soda did not exhibit any antimicrobial effect (++++, representing 100% survival of biofilm bacteria, relative to no-treatment controls) as levulinic acid plus SDS did (-, representing 0% survival of biofilm bacteria) in our *in vitro* model.

In conclusion, we have demonstrated that the formulation of 0.5% levulinic acid plus 0.05% SDS is an attractive alternative mouth rinse that exhibits excellent efficacy in eliminating oral streptococcal biofilms both *in vitro* and *in vivo*. No adverse effects were detected in the murine oral cavity within a 2-week period when used as a mouthwash.

References

Ciancio, S.G., Lauciello, F., Shibly, O., Vitello, M., and Mather, M. The effect of an antiseptic mouth rinse on implant maintenance: plaque and peri-implant gingival tissues. *Journal of Periodontology* 1995; **66**:962-965.

Clothier, B., Stringer, M., and Jeffcoat, M.K. Periodontal disease and pregnancy outcomes: exposure, risk and intervention. *Best Practice & Research Clinical Obstetrics & Gynaecology* 2007; **21**:451-466.

Diaz, P.I., Chalmers, N.I., Rickard, A.H., Kong, C., Milburn, C.L., Palmer, R.J., Jr., and Kolenbrander, P.E. Molecular characterization of subject-specific oral microflora during initial colonization of enamel. *Applied and Environmental Microbiology* 2006; **72**:2837-2848.

Flotra, L., Gjermo, P., Rolla, G., and Waerhaug, J. Side effects of chlorhexidine mouth washes. *Scandinavian Journal of Dental Research* 1971; **79**:119-125.

Friedewald, V.E., Kornman, K.S., Beck, J.D., Genco, R., Goldfine, A., Libby, P., Offenbacher, S., Ridker, P.M., Van Dyke, T.E., and Roberts, W.C. The American Journal of Cardiology and Journal of Periodontology editors' consensus: periodontitis and atherosclerotic cardiovascular disease. *Journal of Periodontology* 2009; **80**:1021-1032.

Gagari, E., and Kabani, S. Adverse effects of mouthwash use. A review. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* 1995; **80**:432-439.

Gilbert, P., Das, J., and Foley, I. Biofilm susceptibility to antimicrobials. *Advances in Dental Research* 1997; **11**:160-167.

Keithly, L., Ferris Wayne, G., Cullen, D.M., Connolly, G.N. Industry research on the use and effects of levulinic acid: a case study in cigarette additives. *Nicotine Tob Res.* 2005; **7**:761–771.

Li, Y.H., Lau, P.C., Lee, J.H., Ellen, R.P., and Cvitkovitch, D.G. Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *Journal of Bacteriology* 2001; **183**:897-908.

Loe, H., and Schiott, C.R. The effect of mouth rinses and topical application of chlorhexidine on the development of dental plaque and gingivitis in man. *J Periodontal Res* 1970; **5**:79-83.

Loe, H., Theilade, E., and Jensen, S.B. Experimental gingivitis in man. *Journal of Periodontology* 1965; **36**:177-187.

Pathirana, R.D., O'Brien-Simpson, N.M., Brammar, G.C., Slakeski, N., and Reynolds, E.C. Kgp and RgpB, but not RgpA, are important for *Porphyromonas gingivalis* virulence in the murine periodontitis model. *Infection and Immunity* 2007; **75**:1436-1442.

Scannapieco, F.A. Position paper of The American Academy of Periodontology: periodontal disease as a potential risk factor for systemic diseases. *Journal of Periodontology* 1998; **69**:841-850.

Wang, B.Y., Deutch, A., Hong, J., and Kuramitsu, H.K. Proteases of an early colonizer can hinder *Streptococcus mutans* colonization *in vitro*. *Journal of Dental Research* 2011; **90**:501-505.

Wilson, M. Susceptibility of oral bacterial biofilms to antimicrobial agents. *Journal of Medical Microbiology* 1996; **44**:79-87.

Zhao, T., Zhao, P., and Doyle, M.P. Inactivation of *Salmonella* and *Escherichia coli* O157:H7 on lettuce and poultry skin by combinations of levulinic acid and sodium dodecyl sulfate. *Journal of Food Protection* 2009; **72**:928-936.

Zhao, T., Zhao, P., Cannon, J.L., and Doyle, M.P. Inactivation of salmonella in biofilms and on chicken cages and preharvest poultry by levulinic acid and sodium dodecyl sulfate. *Journal of Food Protection* 2011; **12**: 2024–2030.