**A combination of zinc and arginine disrupt the mechanical integrity of dental biofilms**

Erin S. Gloag a #\*, Yalda Khosravi a, James G. Masters b, Daniel J. Wozniak a,c, Carlo Amorin Daep b, Paul Stoodley a,d,e

a Department of Microbial Infection and Immunity, The Ohio State University, Columbus, OH, USA, 43210

b Colgate-Palmolive Technology Center, Piscataway, NJ, USA, 08854

c Department of Microbiology, The Ohio State University, Columbus, OH, USA, 43210

d Department of Orthopaedics, The Ohio State University, Columbus, OH, USA, 43210

e National Biofilm Innovation Centre (NBIC) and National Centre for Advanced Tribology at Southampton (nCATS), Mechanical Engineering, University of Southampton, Southampton, UK, SO17 1BJ

\* Present affiliation: Department of Biomedical Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Tech, Blacksburg, VA, USA, 24061

# Correspondence to: Erin S. Gloag

Center for One Health Research, 1410 Prices Fork Rd, Blacksburg, VA, 24061, USA

erinsg@vt.edu

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# Abstract

Mechanical cleaning remains the standard of care for maintaining oral hygiene. However, mechanical cleaning is often augmented with active therapeutics that further promote oral health. A dentifrice, consisting of the “Dual Zinc plus Arginine” (DZA) technology, was found to be effective at controlling bacteria using *in vitro* laboratory studies, translating to clinical efficacy to deliver plaque and gingivitis reduction benefits. Here we used biophysical analyses and confocal laser scanning microscopy, to understand how DZA dentifrice impacted the mechanical properties of dental plaque biofilms, with the objective of determining if changes to biofilm rheology enhances the removal of dental plaque. Using both uniaxial mechanical indentation and an adapted rotating-disc rheometry assay, it was found that DZA treatment compromised biofilm mechanical integrity, resulting in the biofilm being more susceptible to removal by shear forces, when compared to treatment with either arginine or zinc alone. Confocal laser scanning microscopy revealed that DZA treatment reduced the amount of extracellular polymeric slime within the biofilm, likely accounting for the reduced mechanical properties. We propose a model where arginine facilitates the entry of zinc into the biofilm, resulting in additive effects of the two activities towards dental plaque biofilms. Together, our results support the use of a dentifrice containing the Dual Zinc plus Arginine as part of daily oral hygiene regimens.

# Importance

Mechanical removal of dental plaque is augmented with therapeutic compounds to promote oral health. A dentifrice containing the ingredients zinc and arginine, has shown efficacy at reducing dental plaque both *in vitro* and *in vivo*. However, how these active compounds interact together to facilitate dental plaque removal is unclear. Here we use a combination of biophysical analyses and microscopy to demonstrate that combined treatment with zinc and arginine targets the matrix of dental plaque biofilms, which destabilizes the mechanical integrity of these microbial communities, making them more susceptible to removal by shear forces.

# Introduction

Routine daily oral hygiene is an effective method for preventing oral disease complimented by regular dentist visits. However, the benefits of mechanical cleaning alone by brushing, floss, picks or water jets can be improved by the addition of antimicrobial agents to daily dentifrices, for efficient dental plaque control. It has previously been shown that a combination of amino acids and salts of heavy metals, such as zinc and arginine, are effective at dental plaque removal (1-5).

Exogenous arginine has been shown to promote healthy plaque homeostasis, by maintaining a neutral pH in the dental plaque biofilm (6-8), and by promoting the growth of bacteria associated with a healthy dental plaque and preventing the out-growth of cariogenic organisms (9, 10). Furthermore, exogenous arginine can inhibit microbial coaggregation (11-13) and disrupt dental plaque biofilms (14-16). In support of this, we have previously demonstrated that arginine-treated *Streptococcus gordonii* biofilms detached from surfaces at lower external shear forces, compared to untreated biofilms (17). This indicated that arginine treatment weakened the mechanical integrity of the biofilm, making it more susceptible to removal by mechanical shear forces.

Exogenous zinc is bacteriostatic, by inhibiting bacterial metabolism (18-20). Furthermore, zinc inhibition of metabolic enzymes also reduces both acid and alkali production by cariogenic organisms, such as *Streptococcus mutans*, and oral streptococci including *Streptococcus rattus* and *Streptococcus salivarius*, respectively(19, 21). Consistent with this, exogenous zinc is able to maintain a neutral pH of dental plaque both *in vitro* and *in vivo* (22, 23). Exogenous zinc has also been proposed to reduce microbial colonization and plaque development by altering the bacterial cell surface, by either binding to surface adhesins or reducing the net negative charge of the cell (24). Finally, exogenous zinc can also potentiate the virulence of periodontal and gingivitis organisms, including *Porphyromonas gingivalis* and *Fusobacterium nucleatum* (18, 24, 25). However, the antimicrobial activity of zinc was reduced in biofilms compared to planktonic cultures, which was hypothesized to be due to the high cell density reducing zinc penetration into the biofilm (19, 23) explaining why the bacteriostatic action of zinc was most pronounced in the outer layer of dental plaque biofilms (26). Despite this, exogenous zinc was able to significantly reduce dental plaque formation both *in vitro* and *in vivo*, where an increased inhibition was observed in volunteers with high plaque levels, compared to those with low plaque levels (26-28).

Recent efforts have focused on the development of dentifrices with a combination of compounds that synergistically reduce dental plaque and maintain healthy homoeostasis. In line with this focus, a Dual Zinc plus Arginine (DZA) dentifrice has been developed, consisting of 0.96% zinc ions, as a combination of zinc oxide and zinc citrate, and 1.5% L-arginine (5). These two forms of zinc were selected to modulate the delivery of zinc within the oral cavity. Zinc citrate is water soluble and considered as an immediate source of zinc ions, whereas zinc oxide is insoluble in water and considered a slow-release source of zinc ions. L-arginine was found to increase the deposition of zinc on both oral surface mimetics and dental plaque biofilms (16). In line with this, dental plaque biofilms treated with DZA had both reduced metabolic activity, oxygen consumption and viability, compared to either untreated biofilms or biofilms treated with Dual Zinc (0.96% zinc ions; zinc oxide and zinc citrate) (16). Furthermore, trials where subjects used a DZA toothpaste as part of their daily oral hygiene regime showed reduced dental plaque and markers of oral disease, when compared to subjects that used a sodium fluoride control toothpaste (29, 30). Despite these observations, it is still unclear how DZA is impacting the structure and mechanical properties of dental plaque biofilms. Here we used confocal laser scanning microscopy, uniaxial mechanical indentation and a novel rotating-disc rheometry assay, to address this question.

# Results

## *Streptococcus gordonii* biofilms treated with Dual Zinc plus Arginine are more susceptible to mechanical removal.

An understanding of biofilm mechanics is important to the development and application of therapeutics, as these properties often define how a biofilm responds to chemical and mechanical forms of eradication. Furthermore, changing the biofilm mechanics is associated with improved penetration, and therefore enhanced action, of therapeutic compounds (31, 32). We therefore sought to understand how the DZA technology altered the mechanics of biofilms relevant to oral health. We recently adapted a novel rotating-disc rheology assay to analyze and quantify the detachment of arginine-treated *Streptococcus gordonii* biofilms (17). *S. gordonii* is a natural commensal of saliva-plaque biofilms, and is supportive of early colonizers of dental plaque (6, 33-35). We therefore used this assay to determine how treatment with DZA impacted *S. gordonii* biofilm mechanics and removal when exposed to shear stress.

*S. gordonii* biofilms were grown on coupons for 5 days, after which they were treated with either PBS (untreated control), Dual Zinc (0.96% zinc ions; zinc oxide and zinc citrate), or DZA (0.96% zinc ions and 1.5% L-arginine), for 2min. Biofilm-coated coupons were then attached to the rheometer and spun at increasing velocities from 0.1 – 300 rad·s-1 over 360 s, immersed in water. The resulting torque, a measurement of resistance to rotation of the coupon, was measured across the velocity range. This analysis revealed that treated *S. gordonii* biofilms displayed a reduced torque compared to untreated biofilms (Fig 1A-C). This indicates that coupons coated with treated *S. gordonii* biofilms had less resistance to rotation compared to untreated biofilms. Furthermore, depicting these data as the mean of individual replicates revealed that across the assayed velocity range, the torque of *S. gordonii* biofilms treated with DZA was lower, compared to both untreated and zinc treated biofilms (Fig 1D).

We previously observed that reductions in torque during the test correlated to detachment of biofilm-aggregates from the coupon surface (17). These biofilm detachment events were similarly observed here (Fig 1). To more clearly depict these events, a baseline correction was performed by which the data were linearly transformed and presented as the change in slope between measurements (i.e., an approximation of the first derivative), which enhanced the changes in torque, observed as negative slope values (Fig 2). This revealed that treatment of *S. gordonii* biofilms led to reductions in torque at lower velocity ranges compared to untreated biofilms (Fig 2). Moreover, changes in torque at lower velocities were observed for biofilms treated with DZA, compared to untreated and zinc treated biofilms (Fig 2; green bracket).

To quantify these differences the biofilm momentum coefficient was determined, which is an indication of the biofilm induced drag on rotation (17, 36, 37). Although there were no statistical differences between the biofilm momentum coefficient between treated and untreated biofilms, there was a general trend that both zinc, and DZA-treated *S. gordonii* biofilms had a reduced coefficient compared to untreated biofilms, more so for DZA-treated biofilms (Fig 3A; biofilm momentum coefficient mean ± SD: untreated 0.063 ± 0.021; Zn 0.053 ± 0.023; DZA 0.037 ± 0.023). This suggests that less drag was experienced by coupons coated with DZA-treated biofilms, which is associated with a less adhered biofilm (17, 36, 37). To look into this further, the area under the curve (AUC) of the torque – angular velocity curves were measured, which is an indication of the amount of energy required for the coupon to rotate. This analysis revealed that only DZA-treated biofilms had a significantly reduced AUC, compared to untreated biofilms (Fig 3B), corroborating the trends observed from the biofilm momentum coefficient analysis (Fig 3A). Together these data indicate that coupons coated with DZA-treated biofilms experienced less drag compared to untreated and zinc treated biofilms, suggestive of a less adherent and mechanically weaker biofilm.

To determine if these observations correlated to differences in biofilm-aggregate detachment, the angular velocity where the first reduction in torque occurred was converted to a shear stress, indicative of the initiation or critical biofilm detachment shear stress (Fig 3C). This revealed that reductions in torque, which correlate to biofilm-aggregate detachment, occurred at significantly lower shear stresses for both treated *S. gordonii* biofilms, compared to untreated biofilms (Fig 3C). Furthermore, DZA treated biofilms had significantly lower critical biofilm detachment shear stresses, compared to zinc treated biofilms (Fig 3C). This indicates that DZA-treated biofilms detached from the coupon surfaces at lower shear stresses, compared to untreated and zinc treated biofilms, suggesting greater susceptibility to removal by external shear forces.

## Combined treatment with Dual Zinc plus Arginine destabilizes the mechanical integrity of saliva-plaque biofilms.

Having demonstrated that treatment with DZA led to the mechanical destabilization of *S. gordonii* mono-species biofilms (Fig 1 – 3), we wanted to determine the effects of DZA treatment using a biofilm model that more closely mimics *in vivo* conditions.

To assess this, saliva-plaque biofilms were grown on hydroxyl-appetite (HA) discs for 5 days (38, 39). Biofilms were then treated with either solutions of PBS (untreated control), 1.5% arginine, Dual Zinc, or DZA for 2 min. The biofilm mechanical properties were then assessed using uniaxial mechanical indentation, where biofilms are compressed at a defined rate, and the resistance force is measured. The biofilm thickness was determined by an increase of force in resistance to compression. The treatment did not change the biofilm thickness (Fig 4A). The differences in the resulting stress-strain profiles revealed that all three treatments altered the biofilm mechanical properties, relative to the untreated control (Fig 4B). To therefore quantify the differences in the biofilm mechanical properties the Young’s modulus, a measure of the biofilms ability to resist compression and a measure of biofilm stiffness (31, 40) was determined. This revealed that biofilms treated with either arginine, zinc or DZA had a significantly lower Young’s modulus, compared to the untreated control (Fig 4C). This indicates that treatment with either solution destabilized the mechanical integrity of the saliva-plaque biofilm. While there was no statistically significant difference (P>0.05) between either treatments, there was a trend, where biofilms treated with arginine or zinc alone or DZA showed incremental decreases in the Young’s modulus, with biofilms treated with DZA displaying the lowest Young’s modulus (Young’s modulus mean ± SD: untreated 27.28 ± 8.68; arginine 15.38 ± 7.97; zinc 11.14 ± 5.02; DZA 9.29 ± 2.87). This suggests that treatment with DZA had the most impact on destabilizing the mechanical properties of saliva-plaque biofilms.

We were also interested in determining if the ability of DZA to destabilize the biofilm mechanical properties was maintained when developed into a dentifrice paste. Saliva-plaque biofilms were grown for 5 days and treated with either DZA paste, a commercially available stannous fluoride toothpaste, or PBS and analyzed by mechanical indentation as above. This revealed that biofilms treated with DZA dentifrice paste had a significantly lower Young’s modulus compared to untreated biofilm (Fig S1), similar to what we observed for treatment with the test solutions (Fig 4C). In contrast, biofilms treated with the stannous fluoride control paste had a significantly higher Young’s modulus, compared to both untreated biofilms and biofilms treated with DZA (Fig S1). This demonstrates that both the action of DZA on biofilms is maintained in a toothpaste formula and that the DZA technology may be more effective at reducing the mechanical integrity of saliva-plaque biofilms, compared to other commercially available antibacterial dentifrices.

## Combined treatment with Dual Zinc plus Arginine degrades the EPS of saliva-plaque biofilms.

Biofilm mechanics is a property associated with the collective behavior of the biofilm that is largely imparted by the EPS (31). Having observed that treatment with either compound did not result in changes to the biofilm thickness (Fig 4B), we therefore wanted to determine if treatment with DZA exhibited an impact on biofilm extracellular polymeric slime (EPS).

To assess this, 5 day saliva-plaque biofilms were treated with either PBS (untreated control), Dual Zinc, or DZA for 2 min. Treatment with arginine alone was omitted from this further analysis as it showed overall a higher Young’s modulus compared to the other two treatments (Fig 4C). Biofilms were then stained with Syto 9 to label the bacterial cells and calcofluor white to label the EPS, and imaged using confocal laser scanning microscopy (CLSM). This revealed that treatment with either zinc or DZA appeared to reduce the amount of calcofluor white labelled EPS within the biofilm (Fig 5A).

To quantify these changes, both Syto 9 and calcofluor white staining was analyzed using COMSTAT (41, 42). Consistent with the biofilm thickness analysis from the uniaxial indentation measurements (Fig 4B), either treatment did not significantly alter the biomass of cells, compared to the untreated control (Fig 5B). However, calcofluor white stained EPS revealed that treatment with either zinc, or DZA, showed a significant reduction in the volume of EPS within biofilms (Fig 5C). Furthermore, the roughness coefficient, a measurement of the variability in height and heterogeneity (41, 42), of calcofluor white-labelled EPS of treated biofilms was significantly higher compared to untreated controls (Fig 5D). This indicated that treatment with either zinc, or DZA, not only lead to an overall reduction in calcofluor white-labelled EPS, but also changed the distribution of EPS from a more homogenous layer in untreated biofilms, to a more heterogeneous punctate distribution in treated biofilms. Again, there was a trend that calcofluor white-labelled EPS of DZA treated biofilms had a higher roughness coefficient compared to treatment with zinc alone (Fig 5D). This suggests that DZA treatment led to a more uneven, heterogenous EPS distribution within the biofilm, compared to treatment with zinc, however this was not statistically significant.

# Discussion

Treating biofilms with DZA is effective at reducing dental plaque biofilms, both *in vitro* and *in vivo* (5, 16, 29, 30). However, it is unclear how DZA impacts the structure and mechanical properties of dental plaque biofilms. Here we demonstrate that DZA destabilizes the mechanical integrity of both *S. gordonii* mono-species biofilms (Fig 1 – 3) and dental plaque biofilms (Fig 4 and S1) by targeting the EPS (Fig 5). This correlated to biofilms being more susceptible to removal by shear forces (Fig 1 – 3). Furthermore, we observed that these effects of DZA appeared to be increased compared to either zinc-, or arginine-treatment alone. This suggests that there are synergistic or additive interactions between zinc and arginine that mediate the disruption of dental plaque biofilms.

We propose the following model for potential interactions between arginine and zinc (Fig 6). There is evidence to suggest that exogenous arginine is able to penetrate bacterial biofilms (43), and disrupt cell-cell and EPS interactions within the biofilm (10, 15). We therefore propose that arginine is able to penetrate the dental plaque biofilm, targeting interactions within the EPS, destabilizing the mechanical structure of the biofilm (Fig 6). This is consistent with the reduced Young’s modulus observed for arginine-treated saliva-plaque biofilms compared to untreated biofilms (Fig 4C). Furthermore, we have previously reported that arginine-treated *S. gordonii* biofilms are more susceptible to removal by shear forces, due to the weakened mechanical structure (Fig 6; (17)). Here we also demonstrate that treatment with zinc alone is able to weaken the mechanical structure of dental plaque biofilms (Fig 4 and S1), by targeting the biofilm EPS (Fig 5). Similarly, zinc has been shown to inhibit surface attachment and EPS production of *Streptococcus mutans* (44). However, the antimicrobial effects of zinc are reduced in biofilms compared to planktonic cultures, and it has been suggested that zinc is unable to penetrate the biofilm (19, 23, 26). We therefore propose that zinc is able to target the dental plaque biofilm EPS, possibly through cationic interactions, however this action is limited to the periphery of the biofilm (Fig 6). We predict that zinc treatment weakens the mechanical structure of the biofilm to a similar extent as arginine treatment, consistent with the Young’s modulus of zinc or arginine treated saliva-plaque biofilms being similar (Fig 4C). For treatment with DZA, we predict that arginine first penetrates and structurally weakens the biofilm, through disruption of the biofilm EPS. Loosening the biofilm structure then permits zinc to gain entry deeper into the biofilm, extending the effects of zinc to deeper regions of the biofilm (Fig 6). This results in further weakening of the biofilm mechanical structure and increased susceptibility of the biofilm to removal by shear forces, compared to treatment with either arginine or zinc alone. Supporting this model, Matrix-assisted laser desorption /ionization-time of flight (MALDI-TOF) mass spectrometry (MS) analysis revealed that saliva-plaque biofilms treated with a DZA dentifrice had increased penetration and retention of zinc compared to treatment with Dual Zinc alone (16). This model currently only takes into consideration the physical effects over the short time scales (2 min) analyzed here.

To determine if the mechanical destabilization of dental plaque biofilms was due to synergistic or additive interactions between zinc and arginine, we applied a modified fractional inhibitory concentration (FIC) index, referred to here as the interaction index. The FIC index is traditionally used to determine whether the interactions between antimicrobials are synergistic, additive or antagonistic when used in combination (45, 46). By substituting the minimum inhibitory concentrations for the Young’s modulus, according to equations 2 – 4 we obtain an interaction index of 1.87 ± 0.95 (mean ± SD) for DZA, suggestive of additive interactions (45, 46). Similarly, when the Young’s modulus of arginine-treated and zinc-treated saliva-plaque biofilms are subtracted, we obtain a value (4.95 ± 3.30) more similar to the Young’s modulus of DZA-treated biofilms (9.29 ± 2.87), compared to when moduli are divided (1.51 ± 0.35). This is further supportive that the interactions between zinc and arginine are additive. This suggests that across the short time scales analyzed here (2 min), both arginine and zinc have a similar target, presumably EPS (Fig 5), that is responsible for the mechanical destabilization of the biofilm. However, across longer time scales with repeat treatments, it is likely that the antimicrobial effects of zinc, and the metabolic effects of arginine will influence these interactions, potentially shifting them to being more synergic. Therefore, future studies that focus of these interactions, across longer time scales, are warranted. Furthermore, this study focused on maintenance of oral health hygiene, rather than the removal of pathogenic oral biofilms. Therefore, biofilms formed by oral commensals (*S. gordonii*) and saliva-plaque biofilms grown from healthy donors were only examined here. Future studies that focus on the cariogenic oral biofilm pathogen *S. mutans*, grown in the presence of sucrose are also warranted, to determine if these interactions between zinc and arginine are maintained in treatment of a cariogenic biofilm which includes a sticky glucan EPS matrix and acidic environment.

Importantly, long term (6 months) clinical trials of a dentifrice containing DZA did not report any adverse side effects among the 100 participants (47). Similarly, long term studies (> 1 year) of zinc supplemented dentifrices reported no significant changes is oral microbial ecology or in antimicrobial resistant bacteria (48, 49). Our results and proposed model suggest that targeting the dental plaque biofilm mechanics is a strategy to enhance the action of actives against bacteria within dentifrices. Our results are also supported by similar observations, where arginine increased the susceptibility of saliva-plaque biofilms to cetylpyridinium chloride (15), *Streptococcus pyogenes* biofilms to penicillin (50), and *Pseudomonas aeruginosa* biofilms to tobramycin and ciprofloxacin (43). Our results therefore support the growing evidence that arginine has the potential to be used as an antimicrobial adjuvant. Finally, zinc oxide inhibits biofilm formation of *P. aeruginosa*, *Chromobacterium violaceum* (51) and *Candida albicans* (52). This suggests that DZA has the potential to be used across diverse clinical applications for the treatment of biofilm-associated infections, in addition to maintenance of oral health and hygiene.

# Materials and Methods

## Preparation of treatment formulations

Test solutions consisted of 1.5% arginine, Dual Zinc (0.96% total zinc ions; zinc citrate and zinc oxide), or Dual Zinc plus Arginine (0.96% zinc ions; zinc oxide and zinc citrate, and 1.5% L-arginine). Solutions were adjusted to a final pH of 7.0. In toothpaste-treated biofilm experiments, the biofilms were treated with either commercially available stannous fluoride 0.0454 % based active agent, stabilized with sodium hexametaphosphate (Procter and Gamble, UK) or Colgate Total containing Dual Zinc plus Arginine and (Colgate-Palmolive, UK).

## Saliva-plaque inoculum collection

Saliva-plaque was collected from healthy individuals according to protocols in the approved OSU IRB (#2017H0016, 03/22/2017) (39). Collected samples were pooled and separated into 1mL aliquots supplemented with 30% glycerol. Samples were stored at -80°C prior to experimentation.

## Biofilm growth

### *S. gordonii* biofilms

*S. gordonii* wild type strain DL1 was used in this study. Biofilms were grown as previously described (17). Briefly, sterile 40mm diameter coupons were submerged in 40mL brain heart infusion media, supplemented with 0.5% sucrose, and inoculated with 400μL of overnight *S. gordonii* culture. Coupons were incubated in a humidified chamber at 37°C with 5% CO2, on an orbital shaker at 150rpm. The growth media was replaced every 24h. Biofilms were grown for 5 days.

### *In vitro* saliva-plaque biofilms

Sterile hydroxyl-appetite (HA) discs were transferred to a 24-well plate, one disc per well. 900μL of McBain media (2g/L peptone, 2g/L trypticase peptone, 1g/L yeast extract, 0.35g/L NaCl, 0.2g/L KCl, 0.2g/L CaCl2, 2.5g/L mucin, 50mM PIPES buffer, 5μg/mL hemin, 1μg/mL vitamin K) was added to each well and inoculated will 100μL of pooled saliva-plaque. The plate was incubated in a humidified chamber at 37°C with 5% CO2, on an orbital shaker at 150rpm. Every 24h the growth media was replaced with 1mL fresh McBain media. Biofilms were grown for 5 days. This model is supportive of the growth of early colonizer aerobes and late colonizer anaerobes within the saliva-plaque biofilm (38, 39).

## Biofilm treatment

### Treatment with solutions

After 5 days of growth, *S. gordonii* biofilm coated coupons were transferred to a dish containing 60mL of either PBS (untreated control), Dual Zinc (0.96% zinc ions) or DZA. Biofilms were treated for 2min at 37°C with 5% CO2, shaking at 150rpm. Biofilms were then washed twice in PBS, before being transferred into 60mL PBS until analysis.

Saliva-plaque biofilm coated HA dics were treated as above except biofilms were transferred to a new 24-well plate, with each well containing 1mL of either PBS (untreated control), 1.5% arginine, Dual Zinc, DZA. After treatment biofilms were washed twice in PBS, before being transferred into 1mL PBS until analysis.

### Treatment with pastes

1 g of DZA paste was dissolved in 4mL sterile water (1:5 vol/vol dilution). 1g of a commercially available stannous fluoride toothpaste was also dissolved in 4mL sterile water, and used as a control paste treatment. The foam was allowed to settle before 1mL of paste suspension was transferred to wells of 24-well plate. HA discs coated with 5 day saliva-plaque biofilms were transferred into wells containing paste suspension, or sterile water (untreated control). Biofilms were treated for 2min at 37°C with 5% CO2, shaking at 150rpm. Biofilms were then washed twice in PBS, before being transferred into 1mL PBS until analysis.

## Rheometry analysis

### Adapted rotating-disc rheology

*S. gordonii* biofilms were analyzed using an adapted rotating-disc rheology assay as previously described (17). Briefly, biofilm coated coupons were attached to the shaft of a Discovery Hybrid Rheometer-2 (DHR-2) (TA Instruments). Biofilms were immersed in a 15 x 15cm square clear acrylic container filled with 2.8L reverse osmosis water, with the gap distance set to 3.5cm. Immersed coupons were spun at an angular velocity (ω) range of 0.1 – 300rad⋅s-1, incrementing the speed across 360s. For each biofilm treatment (untreated, Dual Zinc, or DZA), 3 biological replicates were performed, each with duplicate biofilms (total of 6 biofilms analyzed per treatment group).

The torque – angular velocity curves were linearized and transformed to more clearly depict the data as previously described (17). The fouling coefficient and conversion of torque to shear stress was calculated as previously described (17). The area under the curve (AUC) of the torque – angular velocity curves was determined using the analysis function within GraphPad Prism v8.

### Uniaxial mechanical indentation

Biofilms were analyzed on a DHR-2, with Peltier plate connected to a heat exchanger (TA Instruments). Biofilms were analyzed submerged in PBS. Indentation measurements were performed at 25°C, using the 8mm Smart Swap geometry with an approach rate of 1μm/s, and a termination detection at 15N. Contact with the biofilm was determined where the force began to steadily increase. Young’s modulus (*E*) was calculated using the force-displacement relationship previously described (40):

where the slope is of the force-displacement curve (N/m), *r* is the radius of the probe (*r* = 0.004m) and *v* is the assumed Poisson’s ratio of a biofilm (*v* = 0.5) (53). The slope of the force-displacement curve was measured at the region corresponding to 0-20% strain. 4 biological replicates, each with 3 technical replicates were performed for each treatment, for both solutions and paste suspensions (total of 12 biofilms analyzed for each treatment group).

To determine if the combined actions of DZA to the biofilm mechanical properties were synergistic or additive, we used the fractional inhibitory concentration (FIC) index, referred to here as the interaction index (IAI), that has traditionally been used to determine the interactions between multiple antimicrobials (45, 46). However, here we substituted the minimum inhibitory concentration for the Young’s modulus, according to equation 2 – 4.

where IAI ≤ 0.5 is indicative of synergism, IAI > 0.5 – 4 of additive and IAI ≥ 4 of antagonism.

## Confocal laser scanning microscopy

For confocal laser scanning microscopy (CLSM), treated 5 day saliva-plaque biofilms were transferred to 1mL 4% paraformaldehyde and fixed at room temperature for 1h. Biofilms were then transferred to 1mL PBS supplemented with 5μM Syto-9 and 10μg/mL calcofluor white and incubated for 1h at room temperature. Biofilms were washed twice in PBS and transferred to fresh PBS until imaging. Biofilms were imaged on an Olympus FV1000 Multiphoton microscope, in single photon mode, fitted with a 25x water immersion objective. Images were processed using the Imaris software and analyzed using COMSTAT-2 (41, 42). For each biofilm treatment (untreated, Dual Zinc, or DZA), 3 biological replicates were imaged, with 4 images taken per biofilm.

**Statistical analysis**

Statistical analysis was performed using a one-way ANOVA with a Tukey’s post-hoc test. Analyses were performed using GraphPad Prism v8 (Graphpad Software). Statistical significance was determined using a p-value <0.05.

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# Figure legends

**Figure 1: *S. gordonii* biofilms treated with Dual Zinc plus Arginine display reduced torque when analyzed by adapted rotating-disc rheology.** *S. gordonii* biofilms were grown for 5 days and were treated with either **(A)** PBS (untreated), **(B)** 0.96% zinc (Zn), or **(C)** DZA for 2 min. Biofilms were analyzed by adapted rotating-disc rheology. Data is presented as torque – angular velocity curves of individual replicate biofilms, separated per treatment group for clarity. **(D)** Data from **(A - C)** presented as the mean ± 95% confidence interval of combined replicates for direct comparison across treatments. 3 biological replicates were performed, each with duplicate biofilms.

**Figure 2: Transformed linearized analysis reveals early attachment events for Dual Zinc plus Arginine treated *S. gordonii* biofilms.** Transformed linearized analysis of data depicted in Figure 3 for **(A)** untreated and **(B)** zinc (Zn) and **(C)** Dual Zinc plus Arginine (DZA) treated *S. gordonii* biofilms. Data is presented as mean ± 95% confidence interval. Green brackets indicate regions where changes in torque, depicted here as negative slope values, were observed for DZA treated biofilms, but not for untreated or zinc treated biofilms. 3 biological replicates were performed, each with duplicate biofilms.

**Figure 3: *S. gordonii* biofilms treated with Dual Zinc plus Arginine are more easily detached from surfaces.** From the rotating-disc rheology analysis depicted in Fig 4, the **(A)** biofilm momentum coefficient, **(B)** area under the curve (AUC) and **(C)** initiation of detachment shear stress, that corresponded to the first decrease in torque was determined.Data is depicted as box and whisker plots of 3 biological replicates, each with duplicate biofilms. \* p-value <0.05; ns indicates not significant. Statistical comparisons are to the untreated control, unless were indicated in the graph.

**Figure 4: Treatment of *in vitro* saliva-plaque biofilms with Dual Zinc plus Arginine reduces the biofilm Young’s modulus.** Saliva-plaque biofilms were grown for 5 days and treated with solutions of 1.5% arginine (Arg), 0.96% zinc (Zn), DZA, or PBS (untreated control) for 2 min. Treated biofilms were then analyzed using uniaxial mechanical indentation. **(A)** Biofilm thickness was determined from this analysis. Data presented as a box and whisker plot. **(B)** Lower linear portion of stress-strain curves of saliva-plaque biofilms. Data presented as mean ± SD. **(C)** From this analysis the Young’s modulus (stiffness) was also determined. Data presented as a box and whisker plot. 4 biological replicates, each with 3 technical replicates. \* p-value <0.05; ns indicates not significant. Statistical comparisons are to the untreated control.

**Figure 5: Treatment of saliva-plaque biofilms with Dual Zinc plus Arginine targets the biofilm EPS. (A)** Representative images of 5 day saliva-plaque biofilms either untreated (left panel) or treated with 0.96% zinc (Zn; middle panel) or DZA; (right panel) for 2 min. Biofilms were stained with Syto 9 (magenta) and calcofluor white (yellow) to label the bacterial cells and the EPS respectively. Scale bar indicates 100 m. COMSTAT image analysis was performed to quantitate the **(B)** biomass of Syto 9 stained cells and the **(C)** biomassand **(D)** roughness coefficient of  calcofluor white labelled EPS. Data presented as a box and whisker plot of 3 biological replicates, with 4 images per biofilm. \* p-value <0.05; ns indicates not significant. Statistical comparisons are to the untreated control.

**Figure 6: Proposed model for the effect of Dual Zinc plus Arginine treatment on biofilms.** Dental-plaque biofilms are polymicrobial with an EPS comprising exopolysaccharides and proteins, lending wild type mechanical properties to the biofilm (left). Treatment with either arginine (middle left) or zinc (middle right) alone destabilizes interactions between the EPS, leading to a comparable reduction in the biofilm mechanical properties between either treatment. When treated with Dual Zinc plus Arginine, arginine penetrates the biofilm, destabilizing the EPS, allowing zinc to gain entry further into the biofilm. Subsequently the actions of zinc are no longer localized to the biofilm periphery, amplifying the destabilization to the EPS, and reduction in the mechanical properties. These combined actions of DZA result in dental plaque-biofilms being more susceptible to removal by mechanical shear (right). Figure created in BioRender.com