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Antibiofilm Activity of Zinc-Carbonate Hydroxyapatite Nanocrystals Against *Streptococcus mutans* and Mitis Group Streptococci

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Dental plaque is a complex multispecies biofilm consisting of a dense community of interacting bacteria ($\sim 10^{10}$ cells/mg) embedded in a self-produced polysaccharide matrix [1, 4]. Plaque control is critical for oral health, since plaque bacteria include pathogens involved in both dental caries and periodontal disease—the most prevalent human microbial diseases—in addition to a variety of opportunistic pathogens that cause endocarditis, bacteraemia and septicaemia [10].

Plaque formation follows a regimented pattern of colonisation steps that begin with specific adhesion of pioneer colonizers to the acquired pellicle on the tooth surface followed by adhesion of secondary colonizers through interbacterial coaggregation [6]. *Streptococcus mitis*, *Streptococcus gordonii*, *Streptococcus oralis* and *Streptococcus sanguinis,* all mitis group streptococci (MGS), are dominant pioneer species capable of irreversible attachment to the acquired pellicle by stereochemical interactions between bacterial adhesins and pellicle receptors [7]. MGS play a key role in early plaque formation and conversion to a community with the potential to cause caries and periodontal disease via interspecies coaggregation and selective recruitment of species [12]. Indeed, once MGS have adhered to the pellicle, they coaggregate in a specific manner with late colonizers—predominantly gram-negative anaerobic species involved in periodontitis such as *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella* spp. and *Treponema* spp.—as well as with cariogenic streptococci of the mutans group such as *S. mutans* and *S. sobrinus* [5]. Mutualistic relationships are established between MGS and late colonizers. One of the best characterised coaggregation systems is the one where *S. gordonii* and *S. oralis* coaggregate with *P. gingivalis*, *S. gordonii* also providing metabolic support for *P. gingivalis* [9]. A thick biofilm hosting a community of interacting microorganisms eventually forms [2], whose composition becomes stable over time. Inhibition of early biofilm formation by MGS can thus help prevent dental plaque development.

This study examines the anti-caries potential of nanocrystals of zinc-carbonate hydroxyapatite (Zn-CHA)—the active component of a recently investigated desensitizing toothpaste (BioRepair® Plus; Coswell S.p.A., Funo, Bologna, Italy) [8], by assessing whether they affect biofilm formation by MGS and *S. mutans* strains in polystyrene microtiter plates at concentrations devoid of antibacterial activity.

The streptococcal strains used in this study included: three strains from the American Type Culture Collection (ATCC) [*S. oralis* ATCC 10557 and *S. sanguinis* ATCC 10556 (both isolated from cases of endocarditis)], and [*S. mutans* ATCC 25175 (caries)] and two strains from the collection of the Department of Biomedical Sciences and Public Health of Polytechnic University of Marche [*S. gordonii* AN-10 and *S. mitis* AN-12 (dental plaque)]. Streptococci were routinely grown in blood agar base (Oxoid, Basingstoke, UK) supplemented with 5 % defibrinated sheep blood and Brain Heart (BH, Oxoid) broth at 37 °C in presence of 5 % CO_2.

Minimum inhibitory concentrations (MICs) were determined by the agar assay, according to the guide lines of the

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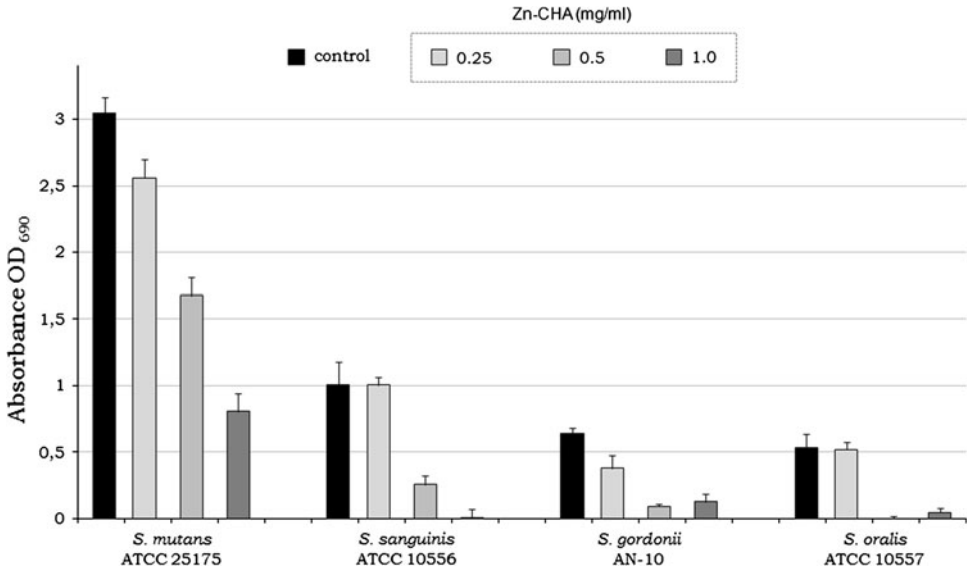

Fig. 1 Inhibitory activity of Zn-CHA on early biofilm development

Clinical Laboratory Standard Institute for streptococci [2]. MIC values ranged from 2.0 mg/ml (*S. sanguinis* ATCC 10556 and *S. mitis* AN-12) to 60 mg/ml (*S. mutans* ATCC 25175); intermediate MIC values were observed with *S. gordonii* AN-10 (4 mg/ml), and *S. oralis* ATCC 10557 (30 mg/ml).

Biofilm was studied by the microtiter plate assay as described previously [11]. Briefly, overnight streptococcal cultures were transferred to pre-warmed BH broth and grown at 37 °C in a 5 % CO_2, aerobic atmosphere to an OD600 of 0.5. The cultures were diluted 1:100 in fresh BH medium supplemented with 0.2 % sucrose: 200 µl aliquots of cell suspension were then inoculated, at least in triplicate, into saliva-conditioned microtiter plates. Plates were incubated at 37 °C in 5 % CO_2 atmosphere for 18 h. After growth, plates were gently washed three times with PBS, blotted on paper towels and air dried. The adherent bacteria were stained with 100 µl of 0.01 % crystal violet for 15 min at room temperature and then the plates were slowly immersed in water twice to rinse the wells. The bound dye was extracted from the stained cells by adding 200 µl of ethanol–acetone (8:2). Biofilm formation was then quantified by measuring the absorbance of the solution at 690 nm (Multiskan Ascent®, Thermo). All assays were carried out in triplicate in two independent experiments. Strains that gave OD readings below 0.061 (mean ± 3 standard deviations of the blank) were classified as non-biofilm formers. All strains but one (*S. mitis* AN-12) were able to form stable biofilms; *S. mutans* ATCC 25175 being the strongest producer (Fig. 1).

The inhibitory activity of Zn-CHA on early biofilm development was evaluated in biofilm producers. Briefly, after the cultures reached an OD600 of 0.5 they were diluted 1:100 in fresh BH medium supplemented with 0.2 % sucrose containing Zn-CHA at different sub-MICs; 200 µl aliquots of the different cell suspensions were then inoculated at least in triplicate into saliva-conditioned microtiter plates. Student's *t* test was applied to assess the difference in adsorption efficiency of treated and untreated samples, which were analysed on the same day. Results were considered significant for $P \leq 0.05$. The inhibition experiment data refer to concentrations of 0.25, 0.5 and 1.0 mg/ml (Fig. 1). Zn-CHA concentrations >1 mg/ml formed a precipitate that hampered the spectrophotometric reading; concentrations <0.25 mg/ml did not significantly affect biofilm development. Zn-CHA showed a concentration-dependent inhibitory activity in the range considered. The maximum inhibition observed was 75 % for *S. mutans* ATCC 25175, 80 % for *S. gordonii* AN-10 and >99 % for *S. oralis* ATCC 10557 and *S. sanguinis* ATCC 10556.

Biofilm inhibition studies demonstrated that sub-MIC concentrations exerted a significant inhibitory effect against all strains in the early phase of biofilm development, including the strong biofilm-producer *S. mutans* ATCC 25175. Remarkably *S. mutans* inhibition was obtained at Zn-CHA concentrations up to 60 times less than the MIC, suggesting a specific antibiofilm activity. This is the first microbiological report documenting the anti-caries potential of the active ingredient of a toothpaste based on Zn-CHA microaggregation. Our findings are in line with a recent clinical study [3] demonstrating that Zn-CHA is effective against *S. mutans* biofilm.

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Conflict of interest All authors declare that they have no conflict of interest.

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